Abstract
Morbidity and mortality due to Rhesus antibodies in pregnant women's serum have steadily declined because of various factors which include, implementation of routine antenatal anti-D prophylaxis and development of non-invasive investigations for monitoring Rhesus affected pregnancies. Because at present this condition is so rare, any case of red-cell alloimmunization should be managed in liaison with a specialist in foetal medicine. Unlike the first immunized pregnancy, maternal antibody titres are not predictive of foetal risk in any subsequent pregnancies. Serial peak middle cerebral artery velocities using Doppler can be used in these pregnancies to detect foetal anaemia. Foetal blood type can now be determined by new techniques to detect free foetal DNA in maternal plasma. In selected cases depending on the gestational age of foetus intrauterine transfusion is necessary through ultrasound directed puncture of the umbilical cord with the direct intravascular infusion of red blood cells. Perinatal survival rates of more than 90% have been reported.

Keywords anti-D immunoglobulin; anti-D prophylaxis; foetal DNA in maternal blood; haemolytic disease of the newborn; peak middle cerebral artery velocity; red-cell antibodies; Rhesus antigen

Historical perspective
It has been suggested that the marriage of Catherine of Aragon to Henry VIII resulted in some of the most notable cases of HDN. In 1609 a French midwife named Louise Bourgeois was the first to report the death of twins, one oedematous and died immediately after birth and the second died a few days later following development of severe jaundice (icterus gravid). It was not until the twentieth century that oedema (hydroptic foetalis), jaundice (icterus gravid neonatorum), presence of immature nucleated red blood cells in foetal circulation (erythroblastosis foetalis) and severe anaemia of the newborn were recognized as manifestations of the same disease characterized by haemolysis in the foetus or newborn.

In 1939 Levine and Stetson discovered a red-cell agglutinogen following an unusual transfusion reaction in a Group O woman shortly after delivery of a stillborn foetus with erythroblastosis when she was transfused with her husband’s Group O blood. In 1940, Landsteiner and Weiner using Rhesus monkeys discovered the Rh antigen. They demonstrated that Rhesus monkey red-cell antiseraum agglutinated red blood cells from 85% of human subjects, but not in the 15% who lacked the antigen on their red cells. Dr Bruce Chown, in 1953, showed that the cause of Rh immunization was the passage of foetal Rh (D)-positive red cells into the maternal circulation. Kleihauer-Betke measurements indicated that 75% of women have such haemorrhages. More sensitive detection measures indicated that such haemorrhages are universal.

Initially Rh antibodies were detected by agglutination of red cells in saline, but these were IgM (19S) antibodies which do not cross the placenta and therefore are not implicated in HDN. Coombs et al (1945) developed a test using rabbit anti-IgG antibodies to agglutinate IgG-sensitized red cells. This indirect anti-globulin test (IAT) remains of great importance in the detection of clinically significant IgG (7S) antibodies in maternal sera because this class of antibody is actively transported across the placenta and is the causative immunoglobulin in HDN.

During 1950s it was shown that IgG molecule consists of an antigen-binding fragment (Fab) and a crystallizable (Fc) fragment (Porter 1959). In 1965, Archer showed that antibodies were bi-functional molecules and that antibody adherence to phagocytic cell Fc receptors was an essential step in the immune destruction of red cells. This also provided evidence for the role of macrophages in cell destruction.

In the early 1960s in Liverpool, Drs. Ronald Finn, Cyril Astley Clarke and colleagues noted, as had Levine previously, the protective influence of Group A and B haemagglutinins in Group O, Rh-negative mothers of Group A or B, Rh-positive infants. Using the Kleihauer-Betke technique, they showed that foetal erythrocytes enter maternal circulation chiefly during labour rather than throughout pregnancy. These workers used gammaglobulin concentrate of Rh serum in a series of well-controlled studies and showed that immunization at or after delivery of an Rh-negative woman ought to be preventable.

Rhesus antigens
The most widely used nomenclature for the Rh system was devised by Fisher and Race. Three pairs of antigens Dd,Cc and Ee are normally present and the genes which determine these antigens are inherited as two haplotypes consisting of three alleles, one haplotype being inherited from each parent. Of the approximately 45 Rh antigens, D is the major cause of Rh incompatibility. It is estimated that 15–17% of whites, 5–7% of blacks and about 2% Indo-Eurasians do not express the D antigen and so are called Rh negative. The D antigen and anti-d has never been isolated but it is included in the nomenclature for completeness. In Table 1 the symbols R and r indicate the presence or absence of the D antigen on the red cell.

Pathophysiology of haemolytic disease of the newborn
Microscopic fetomaternal haemorrhages in pregnancy have been documented to be virtually universal using flow cytometry
methods. Occurrence of HDN as a result of red blood cell alloimmunization secondary to pregnancy involves three key stages. First, a paternally derived red blood cell antigen foreign to the mother must be inherited by the foetus. Second, the volume of foetal red cells that gain access to the maternal circulation must be sufficient to stimulate an immune response in the particular individual. Finally, maternal antibodies to foetal red cells must gain transplacental access and cause immune destruction of sensitized red cells by Fc receptor-bearing effector cells in the foetus and neonate.

The D antigen is a high-incidence and strongly immunogenic antigen, 50 times more so than the other Rh antigens. In Rh and other forms of HDN, the antibody is produced as a result of sensitization by pregnancy or transfusion. A series of small immunizing doses are more likely to initiate antibody production than a single dose. Sensitization can occur throughout pregnancy and the risk of transplacental FMH increases as the pregnancy progresses, being 3% in the first trimester, 43% in the second and 64% in the third trimester. The primary response is usually weak and often IgM antibodies, which do not cross the placenta; thereafter IgG antibodies, which are capable of transplacental passage, are produced.

The following factors influence the immune response to Rhesus positive cells:

1. If an Rh-positive foetus is ABO compatible with its mother the risk of Rh immunization is 16% and if ABO incompatible the risk is 1.5–2%. Concurrent ABO incompatibility offers the mother protection against immunization presumably because leaked foetal red cells are promptly coated by circulating isohaemagglutinins (IgM) and probably also by complement, and then removed from the circulation by the mononuclear phagocyte system (MPS), mainly in the liver, which is less immunoresponsive than the spleen and therefore is less likely to stimulate antibody production.

2. The likelihood of anti-D appearing in the maternal circulation depends on the size of transplacental FMH and the Rh phenotype of the foetal blood. Although the average FMH occurring at delivery is less than 1 ml of whole blood, approximately 50% of all women with ABO-compatible pregnancies have detectable circulating foetal red cells. By the end of the third trimester, anti-D may be detected in sera from less than 1% of D-negative women bearing D-positive foetuses. After the birth of a first D-positive infant to a D-negative mother, the chance of maternal anti-D formation can be related to the number of foetal red cells demonstrable in the mother’s circulation at the time of delivery. When no foetal cells are detectable, anti-D is found in only about 3% of cases, whereas when the amount is 0.1 ml or more anti-D is found in about 31% of cases. In the absence of Rh prophylaxis, about 16% of Rh-negative women will become immunized as a result of their first Rh-positive ABO-compatible pregnancy. Of those women who become immune, about half have a detectable anti-D about 6 months after delivery, and half mount a brisk secondary response in a subsequent Rh-positive pregnancy, indicating that primary immunization had occurred.

3. Infants with R2 phenotype are more effective in sensitizing their mothers to RhD than are infants of other phenotypes, since the R2 phenotype expresses most D antigen.

4. The pregnant mother’s immune responsiveness influences the immune response to RhD-positive cells. Some women produce potent anti-D in a first pregnancy sufficient to cause severe haemolytic disease but usually no first child of an RhD-negative woman will be affected, unless the mother has been sensitized as a result of a prior miscarriage or abortion or, rarely, by a sensitizing event earlier in the pregnancy.

Incidence and epidemiology

The introduction of prophylactic anti-D immunoglobulin in the late 1960s reduced the number of deaths from RhD haemolytic disease of the newborn from 18.4/100,000 live births in 1977 to 1.3/100,000 in 1992. Despite this, anti-D remains the commonest cause of morbidity and mortality due to haemolytic disease, although its prevalence in relation to other red-cell antibodies has declined significantly since prophylaxis was introduced. Most commonly implicated non-D antibodies are anti-c, anti-C, anti-E and those of the Kell blood group system.

There are about 105,000 births to RhD-negative women each year in England and Wales. This constitutes some 17% of all births. Of these babies, about 59% or 62,000 are RhD positive. This represents about 10% of all births each year in England and Wales. Before immunoprophylaxis became available, the frequency of HDN was 1% of all births and HDN was responsible for the death of one baby in every 2200 births. Anti-D prophylaxis (mostly administered postnatally) and advances in neonatal care have reduced the frequency of HDN by almost a factor of 10 to 1 in 21,000 births. In England and Wales about 500 foetuses develop haemolytic disease each year, resulting in the loss of 20 foetuses before 28 weeks’ gestation, death of 25–30 babies due to HDN and further 45 foetuses being affected with developmental problems.

Prevention of red-cell (Rhesus D) alloimmunization

The correct administration of anti-D immune globulin dramatically reduces the rate of alloimmunization. Initial studies proved that the postpartum administration of a single dose of anti-D immune globulin to susceptible RhD-negative women within 72 h of delivery reduced the alloimmunization rate by 90%. The British Committee for Standards in Haematology recommends

<table>
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<tr>
<th>Phenotype</th>
<th>Assumed genotype</th>
<th>Frequency of phenotype (%)</th>
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<tr>
<td>R1r</td>
<td>Cde/cde</td>
<td>33.9</td>
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<td>cDE/cde</td>
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<tr>
<td>R2r</td>
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</tr>
<tr>
<td>rr</td>
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<td>15.1</td>
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Table 1
that RhD-negative mothers delivering RhD-positive infants undergo a test to screen for fetomaternal haemorrhage in excess of the amount covered by the standard dose of anti-D immune globulin. This test will determine if additional anti-D immune globulin is necessary.

It was subsequently recognized that third-trimester antenatal alloimmunization posed a significant problem. In the United Kingdom, the National Institute of Clinical Excellence (NICE, May 2002) now recommends antenatal prophylaxis at 28 and 34 weeks of gestation and the dose for each antenatal administration as well as the dose given after delivery is 500 IU. It has also been shown experimentally that one prophylactic dose of 300 µg (1500 IU) of anti-D immune globulin can prevent RhD alloimmunization after an exposure to up to 30 ml of RhD-positive blood or 15 ml of foetal cells. In its guidance NICE indicates that one dose of 1500 IU at 28 weeks appears to be as effective as two doses as mentioned above. Considering cost, manpower resource factors and patient convenience there has been some debate on the optimum dosage regimen to be used for routine antenatal prophylaxis and many Trust hospitals now prefer to use the single prophylactic dose between 28 and 30 weeks.

In spite of recommendations for immunoprophylaxis some susceptible RhD-negative women still become alloimmunized. There are two primary reasons for the continuing problem. One reason is failure to implement prophylaxis protocols, resulting in preventable RhD alloimmunizations. One of two studies from the UK reported that only 59% of 900 RhD-negative women received recommended prophylaxis after potentially alloimmunizing clinical events while the other showed that 16% of 63 cases of RhD alloimmunization occurred because of similar reason.

Most women who become alloimmunized do so as a result of FMH of less than 0.1 ml. Several first and second trimester clinical events may cause RhD alloimmunization. Therapeutic and spontaneous abortions are associated respectively with a 4–5% and a 1.5–2% risk of alloimmunization in susceptible women. Ectopic pregnancy is also associated with alloimmunization in susceptible women. Although threatened abortion is an infrequent cause, 10% of women with threatened abortion show evidence of FMH. Clinical procedures which may breach the integrity of the choriodecidual space, also may cause RhD alloimmunization. Chorionic villus sampling is associated with a 14% risk of FMH of more than 0.6 ml, and amniocentesis is associated with a 7–15% risk of FMH, even if the placenta is not traversed. Likewise, cordocentesis and other percutaneous foetal procedures pose a risk for FMH. External cephalic version whether or not it is successful, results in FMH in 2–6% cases.

Preventable RhD alloimmunization occurs in susceptible RhD-negative women for the following three reasons:
1. Failure to administer antenatal doses of anti-D immune globulin at 28 and 34 weeks gestation.
2. Failure to recognize clinical events that place patients at risk for alloimmunization and failure to administer anti-D immune globulin appropriately.
3. Failure to administer or untimely administration of anti-D immune globulin to postnatal women who have given birth to an Rh-D positive or untyped foetus. The second reason for the continuing problem of RhD alloimmunization is the small rate (0.1–0.2%) of spontaneous immunization despite the recommended prophylaxis protocol.

RhD monoclonal antibodies

Anti-D has usually been derived from serum of hyper immunized Rh-negative individuals but there are possibilities of transmitting west Nile virus, Creutzfeldt-Jakob disease, etc. According to one of the manufacturers, BPL, the chance of contamination with a known virus is in the order of 1 in 10,000 billion doses. The human plasma derived anti-D is polyclonal and it is composed of IgG1 and IgG3 subclasses. The first monoclonal anti-D antibodies were produced by Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines but regulatory authorities were reluctant to sanction the use of these antibodies as there is a rare chance of infectious EBV in the purified product. Another approach had been to fuse B cells or B-lymphoblastoid cell lines with murine myeloma cells to produce heterohybridoma cell lines.

The efficiency of an antibody is essentially dependent on posttranslational modifications (PTM) and each production system offers different challenge because PTM show species, tissue, and site specificity. Several monoclonal anti-D are not as effective as polyclonal anti-D and it is probably due to unusual oligosaccharides on monoclonal anti-D affecting binding Fc receptors. Recent research shows that the binding to FcyRIII and subsequent activation of effector cells (natural killer cells or macrophages) is an important criterion to select efficient monoclonal antibodies. Recombinant anti-D has been manufactured by phage display and antibody engineering. Encouraging results have been been reported from phase I clinical trials for recombinant antibodies. On the other hand mutant recombinant anti-D has been shown to inhibit haemolysis of RhD-positive cells in vitro by working as blocking antibodies. If this technology is developed it will be an alternative to intrauterine transfusion.

Non-Rhesus D alloimmunization

Antenatal screening programmes detect clinically significant antibodies in 0.24–1% of pregnant women. Although the Rh antibody (anti-D) was, and still is, the most common cause of severe HDN, as Rh immunization decreases due to Rh prevention programmes, other alloimmune antibodies have become more important as a cause. Prophylactic immune globulin is not available to prevent these cases. Table 2 shows seroprevalence of red-cell antibodies in four studies.

Besides Rhesus D, more than 43 other red blood cell antigens have been implicated in HDN. The antigen–antibody interactions that produce severe foetal disease necessitating intrauterine transfusion are, however, limited in number. In most cases non-D antibodies are of low titre and of no clinical significance, and when HDN does occur, it is usually less severe than with anti-D. However, foetal death has been reported due to anti-c and anti-Kell. Isolated cases of HDN due to anti-E, anti-c, anti-k (Kell system) and anti-Fy² (Duffy system) and rare cases of severe foetal anaemia due to Kidd (JkA, JkB), MNS system (M, N, S, s), Lutheran (Lu⁰, Lu¹), Diego (Di⁰, Di¹) have been reported. Anti-c, anti-Kell and anti-E may cause haemolytic disease of the newborn as severe as that seen in anti-D haemolytic disease of the newborn. Often D antibody is found in conjunction with other Rhesus antibodies (c, C, E, e) of weaker titre.

In a series of 1022 cases of non-RhD alloimmunization between 1962 and 1988 reported from Manitoba, Canada, only anti-c was associated with severe HDN that ended in a hydropic
stillbirth or necessitated intrauterine transfusion. Anti-c resulted in twofold and sevenfold greater incidence of haemolytic disease compared with anti-K and anti-E antibodies, respectively. Anti-c and anti-K antibodies were equally likely to be associated with the need for neonatal exchange transfusion or phototherapy; anti-E was half as likely to require neonatal treatment. In a series of 258 pregnancies managed with intrauterine transfusion at a single national referral centre in the Netherlands from 1988 to 2001, 85% of cases involved RhD alloimmunization. 10% of cases involved anti-K and 3.5% involved anti-c; anti-E, anti-e and anti-Fy were each associated with a single case.

Kell antibody
The Kell blood group comprises more than 20 antigens, of which Kell (K or KEL1) and Cellano (k or KEL2) are the strongest immunogens. Ninety-one percent of the population are Kell negative (i.e. kk). The rest are Kell positive having the genotype KK or Kk. The development of anti-K antibodies is primarily a result of prior maternal transfusion, and only a few cases occur following a previous pregnancy or are present naturally.

Kell haemolytic disease accounts for 10% of the cases of antibody mediated severe foetal anaemia. The reason that Kell sensitization is uncommon is that while 9% of the population has at least one Kell (K) antigen allele, only 5% of the 91% who are Kell negative will develop anti-K antibodies after incompatible transfusion. This is because partners of Kell negative women are likely to be Kell positive in 10% of pregnancies and only half of these pregnancies are likely to be incompatible because of paternal heterozygosity. Published results on the outcome of maternal Kell alloimmunization indicate that between 2.5 and 10% of Kell-immunized pregnancies end in the delivery of affected infants with about half the infants requiring intervention.

Anti-Kell antibodies appear to cause significant suppression of erythropoiesis rather than red-cell destruction and unlike Rhesus disease the outcome is not affected by previous obstetric history. Maternal Kell antibody levels and amniotic fluid spectrophotometric estimation (OD450) do not correlate with foetal anaemia. Use of peak middle cerebral artery (MCA) systolic velocity has proved to be successful for the detection of foetal anaemia in cases of Kell alloimmunization. In one series of 27 pregnancies complicated by Kell alloimmunization a peak MCA of greater than 1.5 MoM yielded an 89% sensitivity and specificity (false positive rate 1%) for detecting the subgroup with anaemia. Exclusion of foetuses with ultrasound evidence of hydrops did not change the sensitivity and specificity of detection of severe anaemia.

Multiple maternal antibodies
Despite extensive information on management of pregnancies complicated by Rhesus isoimmunization from RhD, little information exists about the management of obstetric patients with

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**Table 2**

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<td>159 (19.0%)</td>
<td>158 (12.9%)</td>
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<td>C</td>
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<td>28 (11.5%)**</td>
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<td>Lea b</td>
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<td>I</td>
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<tr>
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<td>Diego</td>
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<tr>
<td>Others</td>
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<td>Total antibodies</td>
<td>550</td>
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<td>37,506</td>
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<td>110,765</td>
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Population distribution: Caucasian 70%, black 20%, and other 10%.
* Includes Cw.
** Includes C and E.
multiple maternal red-cell antibodies. Pregnancies complicated by more than one antibody may suggest a worse clinical scenario. The management of these cases has typically been equivalent to that of Rh-isoinimmunization. Some studies have reported that presence of multiple maternal antibodies (anti-C + anti-D, Anti-E + anti-c) appear to have more severe disease than those with only anti-D. However, there is no large scale study result to confirm this, nor any guidelines for appropriate management for pregnancies complicated by more than one red blood cell antibody.

**ABO haemolytic disease**

ABO haemolytic disease is estimated to occur in about 2–3% of all births but only in 1 in 3000 births does severe HDN occur. Mild cases not requiring exchange transfusion are identified as 1 in 150 births. Less than 5% of affected newborns require phototherapy and only in very rare cases is exchange transfusion required.

ABO haemolytic disease of the newborn is limited to mothers with blood group type O whose babies are Group A or B. One of the most important reasons for low incidence and severity of ABO HDN despite considerable foeto-maternal ABO incompatibility is that most anti-A and anti-B antibodies are of the IgM type and do not cross the placenta. Unlike Rh disease, ABO haemolytic disease of the newborn occurs with the same frequency in the first as in subsequent pregnancies, since maternal anti-A and anti-B antibodies are present normally probably secondary to sensitization against A or B substances in food or bacteria.

**Management of red-cell alloimmunization**

Because of the rarity of significant alloimmunization, all such cases should be managed in close liaison with the regional referral centre so that continuity of care is maintained in case transfer of patient to regional centre becomes necessary.

**Obstetric history**

Information regarding prior blood transfusion is important in sensitization to antibodies other than D, particularly Kell alloimmunization, however, the exact events and outcome of prior pregnancies are very important in the evaluation of an alloimmunized pregnancy. In Rh alloimmunization the severity of HDN is likely to remain the same or worsen in subsequent affected pregnancies. History of early foetal death or hydrops is ominous, as hydrops recurs in 90% of affected pregnancies, often at an earlier gestation. Neonatal jaundice due to haemolysis is also likely to recur to the same degree of severity in subsequent affected pregnancies. Before determining paternal blood group genotype it is essential to establish paternity for each pregnancy because the foetus is at risk only if the father is positive for the antigen in question. If the father is homozygous RhD positive then he can only have RhD-positive children, whereas if he is heterozygous (Dd) RhD positive and his wife is RhD negative, there is a 50:50 chance of his fathering an RhD-negative baby who will not be affected.

Following questions are helpful in eliciting previous history when hospital records are not available:

1. Did you have any invasive tests because of antibodies in your previous pregnancy? If yes, what type and at which gestation?
2. Was your labour induced because of antibodies? If yes, at which gestation?
3. Did any of your babies need transfusion after birth?
4. Did your baby need phototherapy (’under blue light’) after birth?

As a rule, previous history of perinatal loss related to red-cell antibodies, a previous need for intrauterine transfusion or neonatal exchange transfusion should indicate referral to tertiary referral centre with experience in management of the severely alloimmunized pregnancy. In such cases maternal titres are not predictive of the degree of foetal anaemia and invasive tests should be considered if the foetus is thought to be at risk.

**Paternal blood group**

It is important to establish paternal blood group genotype because the foetus is at risk only if the father is positive for the antigen in question. If the father is homozygous RhD positive then he can only have RhD-positive children, whereas if he is heterozygous (Dd) RhD positive and his wife is RhD negative, there is a 1 in 2 chance of his fathering an RhD-negative baby who will not be affected. Unfortunately the issues related to the determination of paternity have important ethical dimension that should not be underestimated. Moreover, in the UK an increasing number of pregnancies are from single parent families or same female sex families and father’s blood is simply not available for testing. If father’s blood is available and consent can be obtained, testing should be done as early in pregnancy as possible. In cases of heterozygous father and significant level of maternal antibody, where facility for detection and identification of foetal DNA in maternal blood is not available, consideration should be given to foetal genotype testing by chorion villus sampling or amniocentesis. Obviously such decision needs to be taken depending upon the balance of risk (of miscarriage and further sensitization from invasive testing) and benefit of knowing foetal genotype before foetus becomes affected.

**Prenatal diagnosis of foetal RhD status**

One of the most important advances in recent years in the antenatal management of red-cell alloimmunization has been the introduction of molecular methods of blood grouping from genomic DNA. Presence of free foetal DNA in maternal plasma and serum was first demonstrated in 1997 by Lo et al. It was demonstrated by this group of researchers that foetal DNA could be detected from even a very small sample (10 μl) of maternal blood. The RhD gene, cloned in 1992 is absent, or non-functional in RhD-negative individuals. In RhD-positive individuals polymerase chain reaction (PCR) could be utilized to identify the RhD DNA sequence and this technique can be used to detect RhD-positive foetal cells in blood sample of an RhD-negative mother.

This non-invasive technique is useful in the management RhD sensitized Rhesus negative pregnant women whose partners are heterozygous for the RhD gene because no further testing or therapeutic procedures would be necessary if the foetus is found to be RhD negative. It avoids the risks associated with amniocentesis and chorionic villus sampling. Another important advantage of this technique is that it avoids immunogenic sensitization as a result of fetomaternatal haemorrhage associated with invasive procedures. Wider use of this technique would also allow selection of those women who have RhD-positive foetuses and thus need to receive antenatal anti-D prophylaxis.

The quantity of foetal DNA in maternal plasma increases throughout the period of gestation. Even though false negative
results have been reported on samples taken in the first trimester, RhD PCR results are reliable from beginning of second trimester onwards. Most of the white Caucasian RhD-negative patients have deletion of gene on both copies of their chromosome 1 but 82% of RhD-negative black Africans have RhD non-functional variants such as RhD pseudo gene or CE-D hybrid gene. These RhD variants could result in false positive results but PCR primer and probe combinations have been developed to recognize each of these variants.

The Bristol Institute for transfusion sciences and the international blood group reference laboratory (website: http://ibgrl.blood.co.uk/) perform prenatal diagnosis for the blood group antigens, RhD, Kell, Rhc and RhE. When a test becomes negative for the presence of RhD, the possibilities are that either the foetus is Rhesus negative or that there is no foetal DNA in the maternal blood sample. In such circumstances testing for the presence of SRY gene in the foetal cells in maternal plasma can confirm the presence of a male foetus and hence the presence of foetal DNA in the sample. A negative test result and the absence of SRY gene can occur in the presence of a female foetus with RhD-negative blood group and such result can potentially complicate the diagnosis, but one useful option is to repeat the test few weeks later. The last option would be to do the genetic test for RhD detection on amniocytes obtained following amniocentesis but practically this option is rarely needed.

Maternal serology

The aims of prenatal serological testing are to identify Rh-negative women who will benefit from anti-D immunoglobulin prophylaxis, to detect maternal alloimmunization and to ascertain risk to the foetus from alloimmune haemolytic disease. All pregnant women should be tested for ABO, RhD typing and for irregular serum antibodies at the initial antenatal visit preferably in the first trimester and repeated at 28 weeks’ gestation (prior to administration of prophylactic anti-D).

Conventionally, an indirect antiglobulin test (IAT) is performed with untreated red cells in order to detect clinically important IgG antibodies which may cross the placenta. Often an additional test is carried out using enzyme modified red cells, which facilitates early detection of low levels of antibodies some of which may be clinically important. When clinically significant alloantibodies are detected, quantification (in our laboratory) is usually performed by an auto-analyzer technique (for anti-D and anti-c) or by IAT titration usually reported as a titre score. The human antiglobulin titre (indirect Coombs) is used to determine the degree of alloimmunization. By convention IAT titre values are reported as the integer of the greatest tube dilution with a positive agglutination reaction (i.e. a titre of 16 is equivalent to a dilution of 1:16). Old samples should be stored frozen and tested in parallel with the current sample using the same techniques.

A critical antibody titre is defined as the titre associated with a significant risk for foetal anaemia. This titre will vary with institutions based on the correlation with clinical outcome of HDN. However, there are conflicting views regarding the existence of a critical titre of antibodies. The absolute level of antibody is not as important as the trend, with a rising level requiring more frequent monitoring especially if there is a history of previous HDN. Significant HDN due to anti-D is highly unlikely below a concentration of 4 IU/ml. Level above 15 IU/ml should be treated as high risk for severe HDN until proven otherwise by clinical investigations. Where quantification is not routine, antibody titration is performed using the IAT with different laboratories establishing critical titres for RhD antibody, varying from 1:8 to 1:32. Close monitoring is usually indicated if titre is more than 1/8.

Where more than one antibody is detected, maternal titres should be followed closely at intervals of 2–4 weeks. If titres significantly increase or become greater than 1:16 for any of the antibodies that cause haemolysis, intensive foetal surveillance should be offered. At titres above 1:16, the decision of when to proceed with invasive testing should be made based on clinical situation and obstetric history. Considering the classic study by Allen et al which showed no risk to the foetus when maternal titre was <1:32 and given that interlaboratory variability exists, invasive testing is usually not recommended if titre is <1:16.

Previously affected foetus or infant

Previous history of perinatal loss related to haemolytic disease of the newborn, a previous need for intrauterine transfusion or neonatal exchange transfusion should indicate referral to a perinatal centre with experience in the management of the severely alloimmunized pregnant. In such cases maternal titres are not predictive of the degree of foetal anaemia and amniocentesis should be considered if the foetus is thought to be at risk.

Amniotic fluid spectrophotometry

In most developed countries and many developing countries this technique has now been superseded by antenatal foetal Doppler ultrasound for assessment and monitoring. This newer technique is detailed in a later section of this article. However for the sake of historical interest and for the benefit of all interested readers we wish to briefly mention in this section, the use of spectrophotometry.

When maternal blood tests show a critical titre of antibody as mentioned above or when indicated by clinical circumstances and past history, amniotic fluid bilirubin estimation used to be considered. The method of doing this was first introduced by Liley in 1961, when he obtained amniotic fluid from 101 Rh-sensitized pregnant women between 27 and 41 weeks’ gestation and spectrophotometrically analyzed it for bilirubin at an optical density of 450 nm. The optical density reading at 450 nm was subtracted from an arbitrary line drawn between 375 and 525 nm to derive a OD450 value. Contamination of amniotic fluid by meconium and by erythrocytes and their porphyrin breakdown products can significantly alter spectrophotometric analysis at 450 nm, but these problems can be largely overcome by chloroform extraction of the amniotic fluid. Liley used his data to delineate three zones related to gestational age. The OD450 values in the lower zone (zone 1) indicated a foetus with mild or no haemolytic disease while those in the upper zone (zone 3) indicated severe haemolytic disease with foetal death probable within 7–10 days.

In order to decide the optimum time of intervention (delivery or in-utero transfusion), Whitfield et al introduced the concept of an action line based on the presence, severity and likely further trend of haemolysis in the foetus as indicated
by the trend of amniotic fluid bilirubin concentration. For a detailed information on this method, interested readers should refer to the relevant publication as referenced in the paragraph above.

There are no reliable data concerning the optimal frequency for repeated sampling. In general, amniocentesis is repeated every 1–4 weeks if the ∆OD450 measurement is in zone 2 (middle zone) and every 3–4 weeks if it has dropped into zone 1.

As neonatal survival at early gestational ages improved, “modified” Liley curves were created by extrapolating the Liley zones backward to assess ∆OD450 values prior to 27 weeks of gestation. Some workers, using modified Liley curve, found that 70% of anaemic foetuses in their group of 59 Rh-sensitized pregnancies had ∆OD450 values in zone 2 and would therefore, have been misdiagnosed as being only moderately affected. This report led many centres to lose faith in amniocentesis as a useful tool for predicting foetal disease and gave rise to increasing reliance on percutaneous umbilical blood sampling (PUBS) as the primary tool for foetal surveillance. However, in 1993, Queenan et al published normal ∆OD450 values between 14 and 40 weeks’ gestation based on 520 unaffected pregnancies and also proposed four management zones for following alloimmunized pregnancies. They showed that regardless of Rh erythroblastosis fetalis severity the midtrimester amniotic fluid ∆OD450 may be low initially and therefore serial values were critical for determining foetal condition. However the management based on this work has not gained wider acceptance in the UK, because of anecdotal evidence of high number of false positive results, particularly in early gestation.

Transfusion volumes usually range between 30 and 100 ml and a final target haematocrit of 40–50% is used with an anticipated decline in haematocrit of approximately 1% per day. After a viable gestational age is attained, performing the transfusion in immediate proximity to the delivery suite appears prudent so that operative delivery can be undertaken if foetal distress should occur.

In an extremely anaemic foetus the initial haematocrit should not be increased by more than fourfold to allow the foetal cardiovascular system to compensate for the acute change in viscosity. In such cases a repeat procedure may be undertaken 48 h later to normalize the foetal haematocrit, but it may be too risky to transfuse so soon and many centres prefer to wait for a week before further transfusion. Hydrops will usually reverse rapidly after one or two intravascular transfusions; placentomegaly is the last feature of the hydropic state to reverse.

**Intraperitoneal transfusion (IPT)**

In this technique donor red cells are placed in the foetal peritoneal cavity and they are absorbed via subdiaphragmatic lymphatics and thoracic duct into the foetal circulation. This was superseded by intravascular intrauterine transfusion because of
several disadvantages of IPT like: unsuitable for hydropic foetus, lack of pre- and post-transfusion levels, increased intraperitoneal pressure compromising the venous return. However IPT is ideal when treatment becomes essential at very early gestations (<18 weeks) because intravascular access is technically difficult. Combined intravascular and intraperitoneal transfusion is useful in prolonging the interval between the transfusion but most centres use intravascular transfusion alone to avoid the need for two punctures and to shorten the procedure time.

Real-time ultrasonography

Ultrasoundography is non-invasive, can be performed serially, and may be combined with other diagnostic studies to assess the foetal condition, to estimate the need for further invasive management. It should be employed early in pregnancy to establish correct gestational age because this parameter becomes important in determining such normative laboratory values as foeto-placental blood volume or amniotic fluid bilirubin OD450 levels. A variety of ultrasonographic parameters have been used in an attempt to determine when foetal anaemia is present. These have included placental thickness, umbilical vein diameter, hepatic length, splenic perimeter and polyhydramnios. The earliest sonographic signs of cardiac decompensation are a small pericardial effusion and dilatation of the cardiac chambers. Late findings include pleural effusion and scalp oedema. Foetal hydrops is usually heralded by the onset of ascites and should be considered end-stage haemolytic disease because the foetal haemoglobin is often one-third of normal or less in these situations. Most of these features have not proven reliable in clinical practice.

The major breakthrough in the monitoring of Rhesus disease came with the introduction of Doppler velocity of blood flow in the middle cerebral artery (MCA) of the foetus (Figure 2).

Several recent studies have reported Doppler assessment of peak systolic velocity (PSV) in the foetal middle cerebral artery to be accurate in the determination of foetal anaemia. Mari et al carried out a multi-centre prospective study of 110 consecutive pregnant women referred for red-cell alloimmunization, which included immunization due to Rhesus non-D, Kell and other irregular red-cell antibodies. To identify the foetuses with anaemia, haemoglobin concentrations in 265 normal foetuses were compared with 111 foetuses that underwent cordocentesis.

Figure 3 shows that reference range in the normal foetuses was between 0.84 and 1.16 times the median (corresponding to the 5th and 95th percentiles). Values for the 111 foetuses that underwent cordocentesis are plotted individually. Solid circles indicate foetuses with hydrops.

The sensitivity of the PSV for prediction of moderate anaemia and severe anaemia (haemoglobin concentration of <0.55 times the median) in the foetuses without hydrops was 100% (95% confidence interval 86–100) with a false positive rate of 12%.
Serial antibody measurement is performed every 4 weeks before 28 weeks and 2 weeks after 28 weeks. Some minor antibodies can be repeated every 4 weeks. Multiple antibodies should be repeated after a fortnight.

If father is heterozygous or homozygous and maternal blood results did not reveal foetal genotype, then foetal testing for genotype probably not justified.

**Timing of delivery:** Good quality evidence is not available to influence decision on this issue. We induce around 38–39 weeks’ gestation.

**Antibodies > 4 IU/ml or > 1:64**

All high-risk cases (previous loss, rising antibodies) should fall in this group. All such cases should be referred to, or discussed with tertiary centre. Women with hydrops in previous pregnancy should be seen as early as 14/16 weeks. As invasive testing is likely the plan is to minimize the number of procedures and do them as late as possible. As a rule MCA Doppler to estimate peak systolic velocity should start around 18 weeks and usually continued fortnightly. If antibody levels rise then Doppler should be considered weekly or even twice per week. Timing of repeat Doppler depends on the history, antibody levels and MCA values. Stable MCA below 1.29 MoM is reassuring. If antibodies are high, but stable and MCA persistently normal, foetal genotyping should be considered. Negative result will avoid further visits to tertiary centre, but this procedure has to be set against risk of further sensitization if foetus positive.

When MCA peak systolic velocity reaches 1.5 MoM before 32 weeks most modern centres are likely to opt for foetal blood sampling to detect foetal haemoglobin level. Our view is that each foetal blood sampling procedure should be organized and planned meticulously with prior administration of antenatal corticosteroids, availability of blood for transfusion should it become necessary and operating theatre maintained on stand by (after 24 weeks’ gestation) if urgent delivery is needed. In some instances, it may be appropriate to perform amniotic fluid spectrophotometry first, particularly at later gestations when, in our experience, false positive MCA peak systolic velocity results are more common.

As a rule this group of women will be delivered between 36 and 38 weeks and the timing of invasive procedures often reflects that. In the past the last episode of in-utero transfusion used to be scheduled around 32 weeks, but with more experience and better results the upper cut-off point seems to be shifting towards later gestations, particularly when access is thought to be easier (example, anterior placenta).

Some units have attempted to modify the disease using high dose intravenous gammaglobulins (IVIG), plasmapheresis or steroids as adjunct to intravascular transfusion in severe haemolytic disease where treatment is required prior to 20 weeks, or even before pregnancy. The mechanism of action could be down regulation of maternal immune system or antagonistic action in the foetal reticular endothelial system.

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**Expected peak systolic velocity of systolic blood flow in the middle cerebral artery as a function of gestational age**

<table>
<thead>
<tr>
<th>Week of gestation</th>
<th>Multiples of median PSV</th>
<th>PSV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>1.00 (median)</td>
<td>1.29 (cm/s)</td>
</tr>
<tr>
<td>20</td>
<td>23.2</td>
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</tr>
<tr>
<td>40</td>
<td>58.7</td>
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</tr>
</tbody>
</table>

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**Table 3**

The positive and negative predictive values were 65% and 100%, respectively.

The threshold values for peak systolic velocity in the middle cerebral artery at different gestational ages are shown in Table 3. All of the foetuses with moderate or severe anaemia had PSV values above 1.50 times the median.

In another recent publication of a multi-centre prospective study of 124 consecutive women carrying 125 foetuses, similar conclusions were drawn. Even when amniocentesis for ΔOD450 or foetal blood sampling is performed, middle cerebral artery PSV evaluation can reduce the number of invasive procedures by at least two-thirds. To minimize the probability of false negative cases, they suggested Doppler measurements to be repeated every seven days. In their study, when gestational age was greater than 35 weeks, labour was induced, indicating that this non-invasive method was not useful after 35 weeks.

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**Management protocol**

**Stable antibodies < 4 IU/ml or < 1:64**

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**Further reading**


**Practice points**

- The major breakthrough in the monitoring of Rhesus disease came with the introduction of Doppler velocity of blood flow in the middle cerebral artery of the foetus.
- Routine antenatal anti-D prophylaxis would reduce the deaths caused by haemolytic disease of newborn.
- RhD monoclonal anti-D would eliminate risk of viral disease transmission via plasma derived anti-D products.
- Prenatal diagnosis of foetal RhD status would reduce the need for invasive procedures and the number of antenatal anti-D prophylaxis required.