Recommendations for the prevention and treatment of haemolytic disease of the foetus and newborn

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Introduction

The publication of the second edition of the "*Recommendations for the prevention and treatment of haemolytic disease of the foetus and newborn*" is the result of collaboration between the Italian Society of Transfusion Medicine and Immunohaematology (SIMTI, *Società Italiana di Medicina Trasfusionale e Immunoematologia*) and the Italian Society of Gynaecology and Obstetrics (SIGO, *Società Italiana di Ginecologia e Ostetricia*).

The recommendations published in 2006[1] have been revised in the light of current scientific evidence: the immunohaematological and instrumental investigations that should be performed in the antenatal and perinatal periods, the immunoprophylaxis (IP) to prevent the haemolytic disease of the foetus and newborn (HDFN due to RhD incompatibility and the treatment to use if HDFN develops are described.

The recommendations are focused on the prevention and management of HDFN, in particular that one due to RhD incompatibility, the most serious form of this condition. Although IP has dramatically reduced the number of cases of HDFN, this disease continues to occur and engage specialists in Transfusion Medicine, Obstetrics and Neonatology. The recommendations are aimed at Transfusion Structures (TS) and all public facilities pertaining to Mother and Child Departments, Family Planning Clinics and private structures managing pregnancies, including those in which the woman gives birth at home. The prevention of HDFN must be guaranteed, through organisational models adapted to local circumstances, to all pregnant women for whom it is deemed necessary and the women must also be ensured adequate information.

Besides HDFN due to RhD incompatibility, the recommendations also cover less frequent forms of the disease, caused by immunisation to other blood group antigens, and by ABO incompatibility, which is a more frequent laboratory finding, although of less importance from a clinical point of view.

These recommendations will be periodically reviewed in the light of evolving scientific knowledge, technology and clinical practice. They were developed on the basis of an analysis of current scientific literature (identified through bibliographic searches of Medline/PubMed and Ovid databases) and were submitted to the consensus of experts from SIMTI and SIGO. Protocols jointly agreed upon by the Transfusion Medicine and Immunohaematology Services (SIMT, *Servizio di Immunoematologia e Medicina Trasfusionale*) and Obstetricians-Gynaecologists working in the same territory, including at a regional level, should be drawn up to promote compliance among pregnant women.

Purpose of the recommendations

The purpose of this document is to give correct guidance on the management and prevention of HDFN with the aim of promoting homogeneous practices throughout Italy, ensuring a minimum common denominator of quality that can be achieved in all health care structures[2] used by pregnant women or females of childbearing potential*.

The dual value of these recommendations is that besides being a technical and scientific support for doctors making clinical decisions regarding the management of HDFN, they also provide updates on the risks associated with immunisation in females of childbearing potential.

The recommendations are not intended in any way to replace either the physician's clinical evaluation of individual cases or the doctor's personal experience; they are, rather, a reference tool that can also be used to check the correctness of treatment. The final decision on a given treatment must always be taken by the doctor in the light of the clinical picture and resources available; however, substantial deviations from these recommendations should be documented and justified in the patient's clinical records. For this purpose specific indicators for monitoring and evaluation have been identified to use in clinical audits.

* The term "females of childbearing potential" means patients (from the age of 4-6 months until menopause) who could be or become pregnant and, therefore, be at risk of HDFN.

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Expected benefits

The expected benefits of the dissemination of these recommendations for the prevention and management of HDFN are as follows:

- a decrease in the incidence of HDFN;
- a decrease in the incidence of alloimmunisation;
- an increase in appropriate clinical use of blood components in the foetal and neonatal periods;
- an increase in the appropriate clinical use of blood components in females of childbearing potential;
- an increase in the appropriate clinical use and dosages of anti-D immunoglobulin (Ig);
- greater involvement of patients in decisions related to the prevention and management of HDFN.

Intended users of the recommendations

Doctors and healthcare workers involved in the prevention, diagnosis and treatment of HDFN.

Applicability

These recommendations are applicable to females of childbearing potential, pregnant women at risk of HDFN and foetuses/neonates affected by haemolytic disease caused by materno-foetal alloimmunisation.

Methodology of the Working Group and grades of recommendation

The process of developing these recommendations, in accordance with the indications contained in the methodology manual of the National Guidelines Programme[3], was based on systematic reviews of the literature and updating of already existing recommendations on the subject. For most of the recommendations there is an explicit evaluation of the quality of the proof leading to the recommendation and the strength with which the recommendation is made. In the absence of clear proof, the recommendations are based on a consensus of published opinions of experts and that of the Working Group.

The methodology used to derive the grades of recommendation was based on that used by the Grading of Recommendations Assessment, Development and Evaluation (GRADE) Working Group[4-6]. According to the GRADE system, recommendations are classified by grades, expressed in Arabic numbers (1, 2), depending on their strength, and by letters (A, B, C) depending on the quality and type of evidence provided by the studies on which the recommendations are based.

In detail:

- *Grade 1*: the authors are confident that the benefits for health clearly outweigh the undesirable effects, in terms of both risk and economic cost. This is, therefore, a strong recommendation.
- *Grade 2*: the authors are less certain and the difference between desirable and undesirable effects is less clear. This is, therefore, a weak recommendation.

As to the quality and type of evidence provided by the studies in support of the recommendations, there are three levels of classification:

- *Grade A: high level of evidence.*
  The evidence derives from the analysis of numerous, substantial randomised studies without major limitations. It is unlikely that further research would alter the conclusions reached by these studies.
- *Grade B: moderate level of evidence.*
  The evidence is derived from randomised clinical trials but with important limitations (for example, inconsistent results, wide confidence intervals, methodological problems). Grade B is also attributed to recommendations derived from strong evidence collected in observational studies or case series (for example, treatment effects or the demonstration of a dose-response effect). Further research could change the conclusions of these studies.
- *Grade C: low or very low level of evidence.*
  The evidence is derived from an analysis of observational clinical studies with less consistent results or from the clinical experience/opinions of experts. Further research is required to consolidate or change the conclusions presented.

Generally speaking, it can be assumed that for all recommendations other than Grade 1A the authors recognise that other interpretations of the available evidence and other "clinical policies" are reasonable.

The conventional classification of evidence is based on mathematical and statistical criteria, with the "strength" of the evidence being assigned, in order, to: meta-analyses, randomised controlled trials, retrospective analyses, prospective follow-ups, cross-sectional population studies, reviews, anecdotal reports. This is correct as far as regards strictly clinical studies, especially if they are investigations of therapies and focused on objective evaluations of outcome.

Nevertheless, the recommendations in some fields are weak; in contrast, in other areas the availability of clinical studies carried out with rigorous methodology in large groups of subjects has enabled specific recommendations to be made with more confidence.

It was not always possible to use aggregate data from meta-analyses: these variables increase the margins for individual decisions by each doctor and for each patient.

As to transfusion support for HDFN in the antenatal and postnatal periods (intrauterine transfusion, exchange transfusion [ET], neonatal transfusion), the fundamental principles taken from the "*Recommendations for transfusion therapy in*

neonatology"[7] and subsequent amendments are reported in the appendix.

The appendix also contains some recommendations to be followed in order to avoid the risk of immunisation when transfusing females of childbearing potential, a summary of the investigations to carry out during pregnancy and the puerperium to enable the correct prevention of HDFN, and a flow-chart describing the immunohaematological monitoring of women during pregnancy and at delivery. Finally, the recommendations are summarised and reported with their classification at the end of the appendix.

Each member of the Working Group has signed a statement, which conforms with the one adopted by the National Guidelines Programme, declaring that they have no conflicts of interest[3].

Haemolytic disease of the foetus and newborn due to maternal-foetal RhD incompatibility

The anti-D alloantibody is the antibody most frequently responsible for HDFN[8,9]. Before the introduction of anti-D IP, HDFN secondary to anti-D immunisation affected 1% of neonates and was the cause of death of one in every 2,200 babies born[10]. Although the introduction of post-partum IP in RhD negative pregnant women drastically reduced the incidence of cases of HDFN[11], HDFN due to anti-D continues to occur in 0.4 of every 1,000 births[12-13] and red blood cell alloimmunisation still remains the most common cause of foetal anaemia[14]. There are various reasons for the continued occurrence of this disease: (i) the possible development of anti-D immunisation during a pregnancy as a result of an occult foetal-maternal haemorrhage (FMH), usually after the 28th week of gestation, which affects about 1% of RhD negative mothers of a RhD positive foetus[15]; (ii) lack of administration of IP; (iii) ineffective IP because the amount administered was not sufficient for the volume of the FMH; (iv) possible errors in the typing of the pregnant woman, puerpera or neonate; and (v) possible errors in the transfusion treatment of females of childbearing potential (transfusion of red blood cell concentrates with mismatched RhD antigen).

The fundamental cause of HDFN is the reaction between class IgG maternal antibodies and antigens on foetal red blood cells, leading to the destruction of these cells, mainly in the spleen.

HDFN rarely occurs during a first pregnancy, unless the mother has been previously sensitised by transfusions. Usually, during the first pregnancy primary immunisation takes place; this immunisation is characterised by the production of a small amount of IgM antibodies, immunoglobulins which do not cross the placenta. In subsequent pregnancies, and after further exposure to the antigen, as a result of the secondary immunisation, IgG antibodies, which can cross the placenta and cause haemolysis, are produced. The immune response depends on the entity of the FMH, the number of immunising events and the capacity of the woman's response. ABO incompatibility between mother and foetus partially protects against immunisation.

In the natural history of HDFN, without any kind of intervention, in 50% of cases the foetus has only mild signs of the disease and recovers without any treatment; in 25% of cases the foetus develops haemolysis and kernicterus, if not treated adequately at birth; and in the remaining 20-25% of cases, HDFN due to anti-D may present in its most severe form (hydrops foetalis and death) before the 34th week of gestation[16].

However, with the improvement of maternal and foetal monitoring and the current possibility of *in utero* treatment, the incidence of severe cases (hydrops and death) has now been reduced to about 10%[17].

Haemolytic disease of the foetus and newborn due to incompatibility for other red blood cell antigens

Besides the RhD antigen, other antigens belonging to the Rh system and other known blood group systems (with the possible exclusion of those of the Lewis, Chido and Rodgers, and Knops systems and of the I/i collection) can also induce the production of IgG antibodies and, therefore, provoke HDFN if a person lacking an antigen comes into contact with that antigen as a result of a pregnancy or transfusion. As a general rule, the forms of HDFN not due to RhD incompatibility are clinically benign, such that only 10% of them are clinically severe enough to require transfusion therapy; nevertheless, there are descriptions of fatal cases in the literature[18].

The order of frequency of HDFN, after the forms due to RhD incompatibility and ABO incompatibility, are those caused by incompatibility for the c antigen (r'), the Kell antigen (K1), the C antigen and the antigens of the Duffy system[19-20]. Still in strict order of frequency, there are the forms of HDFN due to incompatibility for antigens of the Kidd, MNS, and Dombrock systems and others, which are all very rare. Anti-Cw, -Fyb, -Jka, -Jkb, -Jk3, -S, and -s usually only cause a positive direct antiglobulin test (DAT) in the neonate and treatment, if necessary is almost always limited to phototherapy[21].

Anti-M, which may also be of the IgG class, rarely cause HDFN. The same applies for warm autoantibodies. Antibodies such as anti-I, -P, -Lea and -Leb can be ignored because the corresponding antigens are scarcely present at birth.

Various studies[22-25] have shown that HDFN caused by anti-K differs from that due to anti-D in a number of ways. In women with anti-K, the obstetric history is not

usually predictive of the severity of the disease; there is only a weak correlation between antibody titre and the severity of the disease, haemolysis and the consequent hyperbilirubinaemia are not dominant features of the disease and the suppression of foetal erythropoiesis, rather than haemolysis, is the most important pathogenic mechanism in causing foetal anaemia. Pregnancies in which anti-K maternal-foetal alloimmunisation has occurred, even when the antibody titre is low (1:8 or greater), must, therefore, be considered at risk, given the severity of the foetal and/or neonatal clinical manifestations.

The recent increase in migration to Italy has led to the diagnosis of other forms of HDFN due to antigens rarely observed in the Italian population. The search for irregular antibodies in these forms of HDFN is often falsely negative because of the lack of the relevant antigens in the test red cell panels commonly used, which are prepared with red blood cells from Caucasians. In these cases, the alloantibody involved can be detected and identified by using the father's red cells (if ABO compatible with the mother' ones), or, after delivery, the neonate's cells. The protocols regarding investigations to carry out during pregnancy and in the perinatal and postnatal periods, as well as the treatment, are not different from those recommended for HDFN due to RhD incompatibility, to which the reader is referred.

Once an antibody specificity has been identified, the test red cells to use in controls, in determining the titre and in studies of the eluate of neonatal erythrocytes must express the antigen in question. In contrast, the red cells to use for a possible ET or for transfusion into the neonate must not carry the antigen involved.

Haemolytic disease of the foetus and newborn due to maternal-foetal ABO incompatibility

HDFN due to ABO incompatibility is currently the most common neonatal haemolytic disease in the western world; indeed, in 15-20% of pregnancies in the white population there is incompatibility between a group O mother and a group A or B child; in 10% of these pregnancies, HDFN develops as a result of destruction of the foetal red blood cells, caused by IgG class anti-A and/or anti-B antibodies in the maternal serum. The mother-child serological combination in which a clinically relevant ABO HDFN develops most readily is a group O mother and a group A neonate.

However, only in about 1.5-2% of cases does the haemolytic disease require transfusion support[26,27]. There are various reasons for the prevailing modest clinical expression of HDFN due to ABO incompatibility:

- the expression of A and B antigens on foetal and neonatal red blood cells is low;
- the A and B substances, ubiquitously present on endothelial and epithelial cells, including placental ones, adsorb some of the maternal IgG that crosses the placenta;
- anti-A and anti-B IgG are predominantly IgG_2, a subclass of Ig with a lesser capacity to cross the placental barrier actively.

Nevertheless, there are occasional reports in the literature of severe cases of haemolytic disease that have required ET and complex management[28,29].

The incidence of HDFN due to ABO incompatibility is higher in African and Arab populations because of the more frequent expression of A and B genes in these populations. Given the migratory phenomena involving Italy (the 2013 CEDAP report [analysis of Birth Support Certificates] described that, in 2010, 18.3% of births were to women of non-Italian citizenship, with the peak being 28% in the region of Emilia Romagna), it can be predicted that the incidence of this type of HDFN will increase in the future[30].

The incidence of HDFN due to ABO incompatibility is the same in first pregnancies as it is in subsequent pregnancies; the disease is, therefore, neither preventable nor predictable.

The search for anti-A and/or anti-B IgG during a pregnancy is of little use for predicting the development of ABO HDFN in the unborn child. In fact, most pregnant women, especially those with group O blood, have anti-A and/or anti-B (and anti-A,B) IgG in their serum, whereas relatively few neonates are affected by haemolytic disease, particularly clinically important forms.

Investigations during pregnancy to prevent and manage haemolytic disease of the foetus and newborn

Immunohaematological tests to perform in all women (Table I)

- ABO blood group and RhD factor must be determined in all pregnant women, preferably within the first trimester of pregnancy. The tests must be performed in a TS using validated methods[9,31-32].
- Samples of blood from pregnant women must carry the surname, name and date of birth of the patient and the signature of the person who took the sample[33,34]. The patient's personal data must be transcribed in the presence of the patient herself, who must confirm the data.
- Two different monoclonal anti-D reagents, which must not recognise the D^{VI} variant of the RhD antigen, must be used to determine the RhD type[32,33]. Determination of weak D antigen is not recommended since this is not useful and could lead to a dangerous omission of IP in the absence of in depth investigations, which cannot be carried out in all immunohaematology laboratories.
- All pregnant RhD negative women should be given an appropriate certificate stating their RhD group and the indication for IP with anti-D Ig.
- At the same time as typing a woman's blood group in the first trimester, a search for irregular antibodies

to red blood cells must be carried out in the plasma/serum of the pregnant woman using an indirect antiglobulin test (IAT), with a validated method, capable of picking up all clinically significant antibodies.

- The requests accompanying the samples must carry information on the state of the pregnancy and any anti-D IP performed in the preceding 6 months.
- It is suggested that an anti-IgG antiserum antiglobulin is used for the IAT. A "broad spectrum" antiserum antiglobulin is acceptable provided that, if the result is positive, the test is repeated only with anti-IgG antiserum antiglobulin or with methods suitable for revealing the clinical significance of the antibody detected.
- When antibodies are detected, the immunohaematological report must contain information on the clinical relevance of the result and whether further investigations are necessary.
- The red blood cell panels used for antibody screening and detection must conform with the indications of the SIMTI Standards[33], although it is not considered necessary to test red blood cells that express low frequency antigens such as Cw, Kpa and Lua[35]. The routine use of techniques involving enzyme-treated red blood cells is not advised[36,37], since they could show antibodies of no significance for HDFN (cold autoantibodies, anti-Lewis, anti-P, etc.).
- If the search for antibodies is positive, in order to evaluate the risk of HDFN, the specificity, titre and origin of the antibodies must be determined, with a careful immunohaematological and obstetric history from the woman. In this phase it can also be useful to determine the IgG subclasses present to assess the real risk of haemolysis as precisely as possible.
- The search for and monitoring of anti-A and anti-B immune antibodies in pregnant women are not recommended, because their presence does not predict the development of ABO HDFN and does not cause problems to the foetus *in utero*[38].
- At 28 weeks of gestation, the IAT must be repeated in all women[29,39], regardless of their RhD status. If no further blood group typing results are available in the archives of the TS in addition to the one performed during the first trimester, it is suggested that, besides the IAT, ABO and RhD groups are checked again[37].
- In RhD negative women who undergo antenatal prophylaxis at 28 weeks of gestation, the IAT must be performed before the IP is administered.
- Searches for other antibodies should not be carried out routinely if the result of the IAT at 28 weeks of gestation is negative. Irregular antibodies detected only in the third trimester of pregnancy do not usually cause HDFN[40,41].
- In women with antibodies that are not clinically significant, it is recommended that red blood cell antibodies are identified again at 24 and 34 weeks of gestation, if the woman is RhD negative, and at 34 weeks of gestation, if the woman is RhD positive[29].

Table I - Recommendations on immunohaematological tests to perform in all women.

Rec. n.	Recommendation	GoR
1	It is **recommended** that the ABO group and RhD factor are determined and a search for irregular antibodies is carried out with an IAT in all pregnant women, independently of their RhD status, within the first trimester of pregnancy in a Transfusion Structure.	***1B***
2	It is **suggested** that the samples for immunohaematological investigations are identified as samples for pre-transfusion tests and carry the surname, name and date of birth of the patient and the signature of the person who took the sample.	***2C***
3	It is **suggested** that all pregnant women are notified of their RhD status because of the possible need for prophylaxis with anti-D Ig.	***2B***
4	It is **suggested** that the search for irregular antibodies is repeated in all pregnant women at 28 weeks of gestation, regardless of their RhD status. In RhD negative women receiving antenatal prophylaxis at 28 weeks of gestation, the IAT should be performed before the IP is administered.	***2B***
5	If the search for antibodies is positive, for the purpose of evaluating the risk of HDFN, it is **suggested** that the specificity, titre and origin of the antibodies are determined and that a careful immunohaematological and obstetric history of the woman is taken.	***2B***
6	It is **suggested** that RhD typing and screening and identification of irregular antibodies is performed using methods in line with those set out in the SIMTI Standards.	***2C***
7	It is **recommended** that anti-A and anti-B immune antibodies are not searched for or monitored in pregnant women.	***2B***

Immunohaematological investigations to perform in the case of positive antibodies (Table II)

- In the presence of clinically significant antibodies, the pregnancy should be followed in a centre with monitoring protocols jointly agreed by immunohaematologists and obstetricians.
- Following the identification of an antibody that is clinically significant with regards to HDFN, the next step is to establish whether the father of the unborn baby has the corresponding antigen and whether he is a heterozygote or homozygote[42,43]. Therefore, if the couple agrees, it is suggested that

the partner's ABO and RhD groups are determined and that he is phenotyped for Rh and other red blood cell antigens, in the event that there are clinically significant alloantibodies to these antigens. If the father is a homozygote for the corresponding antigen and paternity is certain, the foetus is definitely at risk of HDFN.

- In the past, serological tests were used for the study of paternal zygosity, but once the Rh phenotype was determined, heterozygosity of the RhD antigen could only be evaluated through the analysis of tables of frequency, with often inaccurate results. More recently polymerase chain reaction (PCR) techniques have been developed for the determination of RhD zygosity[44].
- PCR methods are not necessary to determine paternal zygosity for the other antigens that are always expressed (systems with co-dominant alleles) and in these cases serological methods can be used to determine the paternal genotype.
- If the father is heterozygous for the antigen against which the identified antibody is targeted and the woman is undergoing invasive antenatal diagnosis for other indications, it is suggested that the genotype of the foetus is determined by PCR analysis carried out on samples of foetal material collected by amniocentesis, from chorionic villi or by cordocentesis[45]. Unfortunately, however, these invasive techniques increase the risk of spontaneous abortion and can raise the levels of antibodies and should not, therefore, be used unless there are other reasons for performing them.
- The foetal RhD genotype can also be determined directly on samples of maternal plasma between the end of the first and the beginning of the second trimester of pregnancy[46]. Genomic identification of the RhD characteristics of the foetus is, currently, the standard of management in women with anti-D alloimmunisation in many European countries. However, it is not free of drawbacks and/or errors, and must be carried out in centres that have validated the procedures. The reported sensitivity and specificity of these PCR DNA tests are 98.7 and 100%, respectively, with a low percentage of false negatives (1-3%)[47,48].
- DNA probes for the RhD gene are currently available in Italy, but some experience is also reported with probes for the genes of the following antigens: c, e, C, E, K, k, Fya, Fyb, Jka, Jkb, M, S, s[49,50].
- In RhD negative women anti-D Ig administered passively after potentially immunising events or at 28 weeks of gestation for antenatal prophylaxis are detectable in the circulation for at least 3 months, depending on the dose administered and the sensitivity of the test. The anti-D Ig administered for IP cannot be differentiated from the low levels of active natural immune anti-D Ig[51], but the levels of anti-D Ig introduced with IP decrease, whereas the levels of natural immune anti-D remain stable or increase if the antigenic stimulus persists.
- If no IP has been given or there is no information on IP, the anti-D found must be carefully monitored. If the anti-D titre tends to decrease, undocumented IP should be suspected, whereas if the titres are stable or increase, immune anti-D must be suspected.

Table II - Recommendations on immunohaematological investigations to perform in the case of positive antibodies.

Rec. n.	Recommendation	GoR
8	If a woman has alloantibodies against clinically significant antigens for HDFN, it is **suggested** that, if the couple agrees, the red cell antigens against which the antibodies are targeted are determined in the woman's partner, in order to assess the risk of HDFN.	*2C*
9	In a woman undergoing invasive antenatal diagnosis for other reasons, it is **suggested** that the foetal genotype is determined by PCR analysis of a sample of foetal material obtained by amniocentesis, from chorionic villi or by cordocentesis if the father is heterozygous for the antigen against which the identified antibody is targeted. The foetal RhD genotype can also be determined directly on a sample of maternal plasma between the end of the first trimester of pregnancy and the beginning of the second.	*2C*

Antibody titration (Table III)

Titration is the simplest and most commonly used laboratory method to evaluate the strength of an antibody. Titration of anti-D or other antibodies that are clinically significant for HDFN helps the clinician to make decisions, for example, on when to start monitoring a foetus with the techniques available. The anti-D titre is not, however, strictly related to the onset of HDFN; fast increases in the antibody titre are more significant.

- Once an antibody has been identified and recognised as a potential cause of HDFN, its titre must be determined using a standardised technique.
- If a pregnant woman has anti-D antibodies, or other antibodies that are clinically significant with regards to HDFN, the antibody titre must be determined every 4 weeks until the 18th week of gestation and then every 2 to 4 weeks, depending on the clinical relevance and the values found. A fast increase in the titre necessitates more frequent immunohaematological monitoring and closer maternal-foetal surveillance.

- It is suggested that D-heterozygous (R1r, R2r) red blood cells are used for the anti-D titration, since these have the same antigenic profile as that of the red blood cells from a heterozygous foetus[52,53]; it is, however, recommended that test red blood cells of the same D zygosity are always used in the comparative titrations performed in the following weeks.
- The critical value of the antibody titre is that associated with a significant risk of hydrops foetalis. For anti-D antibody, if the titration is performed with an IAT in physiological saline (without additives), with an incubation of 60 minutes at 37 °C (standard technique), using anti-IgG, the critical titre is 1:32.
- If other techniques are used, each laboratory must validate the methods involved and establish the critical value for each method adopted.
- The titration must be performed on samples of serum or plasma and in subsequent controls the same type of biological material must be used: the results should always be compared with those of the previously tested samples (kept frozen), using the same test red blood cells with the same antigen expression in order to determine correctly any changes in antibody titre occurring during pregnancy.
- To ensure that results from different laboratories can be compared, each laboratory must indicate on its report the method used for the titration and the specific critical level.

Table III - Recommendations on antibody titration.

Rec. n.	Recommendation	GoR
10	In order to perform anti-D titration, it is **suggested** that D-heterozygous (R1r, R2r) red blood cells are used, because these are best able to demonstrate foetal antigen expression. In any case, the same test red blood cells with the same zygosity must always be used in order to be able to interpret the trend in titres correctly.	***2B***
11	If a pregnant woman has anti-D antibodies, or other antibodies that are clinically significant with regards to HDFN, it is **suggested** that the antibody titre is measured every 4 weeks until the 18th week of gestation and then every 2-4 weeks. The results of the titration must be compared with those obtained in the previously tested samples, using test red blood cells with the same antigen expression.	***2C***

Instrumental investigations and foetal monitoring (Table IV)

- If the immunohaematology laboratory reports that a specific critical level has been reached or that there is a rapid increase in the antibody titre, close monitoring of HDFN must be established with non-invasive methods, such as ultrasonography and/or Doppler velocimetry.
- The test of choice for evaluating the degree of foetal anaemia, before the manifestations of hydrops foetalis, is the determination of the peak systolic flow velocity in the middle cerebral artery (MCA-PSV) with Doppler ultrasound[54-56]. This technique is now recognised as the most effective for non-invasively identifying moderate-severe anaemia and has definitively replaced spectrophotometric analysis of the amniotic fluid, used in the past to determine bilirubin levels[57,58].

The methods of maternal-foetal monitoring, used in different periods of a pregnancy, are indicated below[10,54,59].

- *Before 18 weeks of gestation:* ultrasound dating of the pregnancy and ultrasonographic evaluation of the presence/absence of ascites/hydrops foetalis and foetal heart beat every 4 weeks.
- *Between 18^{+0} and 25^{+6} weeks of gestation***:** ultrasound and Doppler-velocimetric evaluation of indirect signs of foetal anaemia at intervals of 1-3 weeks, depending on the severity of the case. Basically, two parameters are evaluated: presence/absence of ascites/hydrops foetalis and the MCA-PSV. If the values of this latter are found, and confirmed, to be more than 1.5 times higher than the median value for gestational age, the foetal blood count is evaluated by cordocentesis.
- *Between 26^{+0} and 34^{+6} weeks of gestation:* ultrasound and Doppler-velocimetric evaluation of indirect signs of foetal anaemia every 1-2 weeks, depending on the severity of the case, using the above described criteria. If ultrasonographic signs of foetal anaemia are found, the foetal blood count is evaluated by cordocentesis.
- *After 35^{+0} weeks of gestation:* ultrasound evaluation of indirect signs of foetal anaemia every 4-10 days, depending on the severity of the case, using the above described criteria. After 35 weeks of gestation, the reliability of the MCA-PSV test alone is limited; greater attention should, therefore, be given to any appearance of ascitic fluid levels, and cardiotocographic monitoring, which should be performed at least weekly. If one or more of the parameters are abnormal, the delivery should be induced, following prophylaxis against respiratory distress syndrome.
- If the parameters remain within the normal range, it is advisable that the delivery takes place between 38^{+0} and 38^{+6} weeks, in any case. The method of delivery must be chosen exclusively on the basis of obstetric considerations; the presence of alloimmunisation is not a contraindication to vaginal delivery.

Table IV - Recommendations on instrumental investigations and foetal monitoring.

Rec. n.	Recommendation	GoR
12	In the case of risk of HDFN, it is **recommended** that the foetus is monitored with non-invasive methods, such as ultrasonography and/or Doppler velocimetry. The parameters to evaluate are the presence/absence of ascites/hydrops foetalis and the MCA-PSV.	*1B*

Postnatal immunohaematological investigations

Tests to perform in all neonates (Table V)

- It is suggested that a DAT is performed on the cord blood of all neonates.
- The isolated presence of a positive DAT does not allow the diagnosis of HDFN to be made. However, in the presence of a positive DAT, the neonate's levels of haemoglobin and bilirubin must be monitored in order to diagnose or exclude HDFN.
- In the event that the DAT is positive and the neonate shows signs and symptoms of HDFN, the antibody must be eluted from cord red blood cells in order to confirm the antibody specificity. In suspected cases of HDFN, the cord blood red cells must be tested for the corresponding antigen.
- There are numerous elution methods: in the case of anti-D antibodies, it is recommended that a technique sufficiently specific for the elution of anti-Rh antibodies is used.
- If an anti-D is eluted, consideration should be given to whether the mother has previously been administered antenatal IP. It has been demonstrated that following systemic antenatal IP, the anti-D Ig can cross the placenta, reach the foetal circulation and bind to RhD positive foetal red blood cells. These anti-D do not cause the destruction of foetal or neonatal red cells[60].
- In neonates of RhD negative mothers, the RhD phenotype should be determined on the same sample of cord blood with methods that can also demonstrate the weak D phenotype, and at least its most common variant, D^{VI} [33].

Table V - Recommendations on tests to perform in all neonates.

Rec. n.	Recommendation	GoR
13	It is **suggested** that a DAT is carried out on a sample of cord blood of all neonates. In the event of a positive DAT and in the presence of clinical signs of HDFN, it is suggested that the antibody is eluted from the cord blood red cells, in order to confirm the antibody's specificity.	*2C*
14	In neonates of RhD negative mothers, it is **suggested** that the same sample of cord blood used for the DAT is used to determine the RhD phenotype, with methods capable of also detecting the weak D phenotype and at least its most common variant, D^{VI}.	*2C*

Investigations to perform at birth in the case of suspected haemolytic disease of the foetus and newborn due to maternal-foetal ABO incompatibility (Table VI)

- When the mother is group O and there is laboratory (DAT positive) or clinical (jaundice) evidence of neonatal haemolysis, in the absence of known causes, ABO/RhD blood group typing should be performed on cord blood cells and anti-A and/or anti-B IgG should be searched for in the maternal serum and titrated. The diagnosis of ABO haemolytic disease of the newborn is essentially clinical: in fact, very often, despite ABO incompatibility and anti-A and/or anti-B IgG in the mother's serum, the DAT is negative or inconclusive[61,62].
- The search for and titration of IgG class immune anti-A or anti-B antibodies must be performed by an IAT after having cleaved the anti-A and/or anti-B isoagglutinins present in the maternal serum with reducing substances such as 2-mercaptoethanol (2-ME) or dithiothreitol (DTT), or using other commercially available neutralising substances.
- If the cord red blood cells are DAT positive, it is suggested that the IgG (anti-A and/or anti-B) attached to the neonatal erythrocytes are eluted. The best technique for eluting anti-A and anti-B IgG is rapid freeze-thawing, developed by Lui and described by Feng *et al*[63]; another method, which is quick and gives useful results, is heat elution[64].

Table VI - Recommendations on investigations to perform at birth in the case of suspected haemolytic disease of the foetus and newborn due to maternal-foetal ABO incompatibility.

Rec. n.	Recommendation	GoR
15	In the presence of laboratory evidence (positive DAT) or clinical evidence (jaundice) of neonatal haemolysis, when the mother is group O, it is **suggested** that ABO/RhD blood group typing is performed on cord blood, anti-A and/or anti-B IgG are detected and titrated in the maternal serum and, in the event of a positive DAT, the IgG (anti-A and/or anti-B) are eluted from the neonate's red cells.	*2C*

Anti-D immunoprophylaxis

Background

A non-sensitised RhD negative woman who has not received IP has a 16% risk of becoming immunised in every pregnancy with a RhD positive neonate[65]. The most common immunising event is delivery, whether this is vaginal or by Caesarean section. However, potentially immunising events can also occur during pregnancy. Since IP has been introduced, HDFN secondary to anti-D immunisation has become a rare condition, with

the percentage of successful postnatal IP having reached 98-99%[66,67].

Nevertheless, the risk of maternal immunisation in non-sensitised RhD negative women who undergo IP in the post-partum period and during pregnancy after potentially immunising events has not been completely eliminated. This is mainly because of unrecognised bleeding, which can occur during the third trimester of pregnancy and leads to silent immunisation[68,69]. The frequency of silent FMH increases with the progression of pregnancy and has been reported to reach 73% in the third trimester[70,71]. A systematic review of randomised controlled trials showed that routine, systematic antenatal prophylaxis in RhD negative pregnant women, administered in the third trimester of pregnancy, led to a further reduction of the risk of immunisation from 1 to 0.2%[72]. Similar conclusions were drawn from a meta-analysis of non-randomised studies, in which it was estimated that there was an absolute reduction in risk from 0.9 to 0.3%[73].

General indications (Table VII)

- IP with anti-D Ig must be given to all non-sensitised RhD negative women immediately after the delivery of a RhD positive neonate[74,75], at 28 weeks of gestation and in the case of events that could cause immunisation[76]. The methods and doses to use are described in the following paragraphs.
- The TS that carries out the immunohaematological investigations aimed to prevent HDFN must indicate on the immunohaematological report form that IP should be performed, keep a register of RhD negative women to be given IP and, in close collaboration with the Obstetrics and Gynaecology units, monitor and record that the IP has been given[31,33].
- If IP has been given during a pregnancy, the possible presence of passively introduced anti-D Ig should be considered in subsequent serological tests; this Ig must be distinguished from active anti-D alloimmunisation. The TS must, therefore, always be informed of the administration of anti-D Ig and, in most cases, documentation of the administration of anti-D Ig will enable active and passive immunisation to be differentiated.
- Before administering anti-D Ig, the woman's informed consent must be obtained, recorded and archived, as required by law[34]. The woman must be informed that the procedure is not without risks and must give her consent or refusal to the procedure in writing. An informed consent form is presented in the appendix.
- Antenatal IP with anti-D Ig is not indicated in a RhD negative woman with a RhD negative partner, provided that the paternity has been ensured by private interview with the woman.
- If there is a serological discrepancy in the determination of RhD, in accordance with the SIMTI Standards[33], it is desirable that a partial D phenotype assigned by serological methods is confirmed by molecular biology techniques. In the event that it is not possible to identify the antigenic variant with molecular analysis of the RhD gene, the woman must be considered as RhD negative and given IP.
- In the light of current scientific knowledge, in cases in which molecular analysis can define the RhD antigen variant, IP with anti-D Ig is not indicated for weak D variants, type 1, 2, and 3. IP is necessary for all the other antigenic variants[77-79].
- Mistaken administration of anti-D Ig to RhD positive women does not cause appreciable adverse effects in any case.
- In the event of apparent anti-D+C specificity, the presence of anti-G must be carefully evaluated. In these cases it is important to distinguish anti-D+C antibodies from anti-G antibodies because pregnant women with anti-G but without anti-D can benefit from IP with anti-D Ig in the antenatal and postnatal periods, which would not be necessary if anti-D antibodies were actually present. In the above described cases, mistaken attribution of an anti-D specificity could lead to IP not being given to women at risk[80-82]. In this situation, samples should be sent to a reference laboratory to determine the real specificity of the antibodies present[83].
- The efficacy of the IP must be checked with a search for anti-D antibodies in the serum of the mother at least 6 months after delivery (to avoid interference by the anti-D introduced passively). A negative search for anti-D antibodies is evidence that the IP has been successful.

Table VII -Recommendations on anti-D immunoprophylaxis.

Rec. n.	Recommendation	GoR
16	It is **suggested** that IP is performed with anti-D Ig in women with a D variant phenotype poorly defined from a molecular point of view.	***2C***
17	Before administering anti-D Ig it is **recommended** that informed consent is obtained from the woman	***1B***
18	It is **suggested** that IP is not performed (since it is not indicated) with anti-D Ig in a RhD negative women with a RhD negative partner, provided that paternity has been ensured by a private interview with the woman.	***2C***
19	It is **suggested** that the efficacy of the IP is checked by a search for anti-D antibodies in the maternal serum at least 6 months after the delivery. The absence of anti-D antibodies at this point is evidence of the success of the IP.	***2C***

Immunoprophylaxis following potentially immunising events during pregnancy (Table VIII)

- Prophylaxis with anti-D Ig is recommended in non-immunised RhD negative women in the following circumstances that can promote the passage of foetal red blood cells into the maternal circulation[21,75,84-89]:
  - invasive antenatal diagnosis: amniocentesis, cordocentesis, chorionic villus biopsy;
  - dilation and curettage, elimination of one or more embryos, treatment to the foetus (introduction of shunts, intrauterine transfusion);
  - direct, indirect, open or closed abdominal trauma;
  - external cephalic version;
  - antenatal haemorrhage;
  - intrauterine foetal death;
  - therapeutic termination of pregnancy, with surgical and/or medical techniques;
  - complete or incomplete spontaneous abortion followed by dilation and curettage, regardless of the gestational age of the foetus;
  - ectopic pregnancy[90].
- In the first trimester (<12 weeks of gestation), even in the absence of traumatic manoeuvres to the uterus, the reported risk of alloimmunisation following abortion or blood loss is 1.5-2%. On this basis, some recommendations advise giving IP also following spontaneous abortion without dilation and curettage and for pharmacologically induced abortions[85,89,91-98]. In these cases, a minimum dose of anti-D IgG is sufficient, i.e. from 250 to 600 IU (50-120 μg)[21,83-84,88].
- In the event of copious blood loss, if the bleeding recurs in subsequent weeks, the anti-D Ig should be administered at intervals of 6 weeks[99]. After the 20th week the volume of FMH must be determined.

Table VIII - Recommendations on immunoprophylaxis following potentially immunising events during pregnancy.

Rec. n.	Recommendation	GoR
20	In circumstances that can promote the passage of foetal red blood cells into the maternal circulation, it is **suggested** that prophylaxis with anti-D Ig is offered to all non-immunised RhD negative women.	*2C*

Systematic antenatal immunoprophylaxis (Table IX)

The aim of systematic antenatal IP is to protect against unpredictable, silent sensitisation which can occur in the third trimester of pregnancy, in order to prevent immunisation in subsequent pregnancies. In the absence of this type of IP, about 1% of RhD negative women who give birth to a RhD positive neonate could be sensitised[87,95,96,100,101].

Various recommendations suggest systematic administration of antenatal IP to non-immunised RhD negative women[1,21,70,75,83,86,88,91,102-105]. Given that IP is effective for about 12 weeks, as indicated by pharmacokinetic studies on anti-D Ig[97], and that the risk of significant FMH before 28 weeks is low, antenatal prophylaxis is advised from 28 weeks of gestation onwards[106].

- It is recommended that all non-immunised RhD negative women are offered systematic antenatal IP with a dose of anti-D Ig of 1,500 IU (300 μg)[98-106]. In cases in which it is not possible to administer IP at 28 weeks for organisational/logistic reasons, it can be give a few days earlier or later (27-29 weeks of gestation).
- Systematic antenatal IP should be performed even if IP has been given in the preceding weeks because of events that could cause immunisation.
- In the same way, sensitising events occurring after the administration of systematic antenatal IP should be managed with an additional dose of anti-D IgG and an evaluation of the FMH.

Table IX - Recommendations on systematic antenal immunoprophylaxis.

Rec. n.	Recommendation	GoR
21	It is **recommended** that all non-immunised RhD negative women are offered IP at 28 weeks of gestation with a dose of anti-D Ig of 1,500 IU (300 μg). IP at 28 weeks of gestation should be proposed even if IP was given in the preceding weeks because of a potentially immunising event.	*1B*

Post-partum immunoprophylaxis (Table X)

- All non-immunised RhD negative women who have delivered a RhD positive (or weak D) neonate (or stillborn baby) must be given a dose of anti-D Ig within 72 hours of the delivery[8,10,11,15,21,27,36,42,70-76,83,86-90,94,104-107].
- Anti-D Ig should also be given in cases in which the neonate's RhD group is not available.

Table X - Recommendations on post-partum immunoprophylaxis.

Rec. n.	Recommendation	GoR
22	It is **recommended** that all non-immunised women who have given birth to a RhD positive (or weak D) neonate are given immunoprophylaxis with anti-D Ig.	*1A*

Times and methods of administering anti-D immunoglobulin (Table XI)

- Anti-D Ig must be given as soon as possible after the

delivery or potentially sensitising event, and at the latest within 72 hours. If IP is not performed within 72 hours, an attempt to avoid immunisation must be made in any case with the administration of anti-D Ig up to 10 days after the event[108]. According to some authors, IP can still be attempted even with a delay of as much as 28 days after the sensitising event[36].

- The route of administration of choice for anti-D Ig is intramuscular. The deltoid muscle is an appropriate and safe site to maximise absorption[70], but the gluteal region can also be used, taking particular care to ensure that the injection is made into muscle, since absorption can be delayed if the injection only reaches subcutaneous tissue.
- In the event of disorders of haemostasis (for example, severe thrombocytopenia or other bleeding diatheses), when intramuscular injections are contraindicated, it is recommended that the anti-D Ig are administered intravenously. It seems that both routes of administration are equally effective and that there are not significant differences between the two[109].
- It is suggested that anti-D Ig preparations for intramuscular administration are kept available for the cases set out in the previous section and the cases in which it is expected that high doses of anti-D Ig might have to be given because of massive FMH (see the last three points in the next paragraph).

Table XI - Recommendations on times and methods of administering anti-D immunoglobulin.

Rec. n.	Recommendation	GoR
23	It is **suggested** that anti-D Ig is given as soon as possible after the delivery or potentially immunising event, and at latest within 72 hours. If the anti-D Ig is not given within 72 hours, it must be administered as soon as possible (up to 10 days afterwards).	***2B***
24	It is **suggested** that the anti-D Ig is given by intramuscular injection into the deltoid muscle. In the event of altered haemostasis, when intramuscular injections are contraindicated, or for doses greater than 3,000 IU (600 μg) it is suggested that the anti-D Ig is administered intravenously.	***2C***

Dose of anti-D immunoglobulin (Table XII)

The doses of anti-D reported in these recommendations are the minimum indicated for the specific situations mentioned. The dose of anti-D Ig should be commensurate with the amount of FMH. In the large majority of cases, a dose of 625 IU (125 μg) is considered sufficient to prevent active immunisation when the FMH does not exceed 4 mL of foetal red blood cells (99% of FMH).

- For prophylaxis following potentially immunising events occurring up to 19^{+6} weeks of gestation, a dose of 625 IU (125 μg) of Ig* is considered sufficient[21,86-87,101].
- For all potentially immunising events occurring after 20^{+0} weeks of gestation and after the delivery of a RhD positive neonate, a minimum dose of 625 IU (125 μg) of anti-D Ig should be administered, followed by evaluation of the FMH.
- If quantification of FMH shows that the volume of bleeding exceeded that covered by the dose administered, a further dose of anti-D Ig (125 IU every millilitre of RhD positive foetal red blood cells) must be injected[110]. The supplemental doses of anti-D Ig (rounded to the nearest dose of available packages) must take into account the dose of anti-D already administered.
- If the volume of FMH is not evaluated, for events occurring after week 20^{+0} of pregnancy and in the post-partum period, a dose of 1,500 IU (300 μg) of anti-D Ig should be adminstered.
- In the event of a Caesarean section, twin birth or dystocia, the dose of anti-D Ig should always be 1,500 IU (300 μg).
- If the total dose of anti-D Ig to administer is greater than the contents of two intramuscular injections of 1,500 IU (bleeding >24 mL of foetal red blood cells) it is advisable to give the Ig intravenously.
- The total dose administered within a 24-hour period must not exceed 10,000 IU (2,000 μg).
- The maximum individual dose to be given intravenously must not exceed 4,500 IU (900 μg); any additional doses must be administered at intervals of 12 hours. This indication is based on the known risk of haemolysis associated with the dose of anti-D Ig given intravenously in regimens used in RhD positive idiopathic thrombocytopenic purpura[94].

Table XII - Recommendations on dose of anti-D immunoglobulin.

Rec. n.	Recommendation	GoR
25	It is **suggested** that prophylaxis following sensitising events occurring up to 19 weeks of gestation consists of the administration of a dose of 625 IU (125 μg) anti-D Ig.	***2B***
26	For events occurring after 20^{+0} weeks of gestation and in the post-partum period, if the FMH is not quantified, it is suggested that the dose of anti-D Ig used is 1,500 IU (300 μg).	***2B***
27	If the amount of FMH is evaluated routinely, for events occurring after 20^{+0} weeks of gestation and in the post-partum period, it is suggested that the dose of anti-D Ig used is 625 IU (125 μg).	***2B***
28	If the FMH is greater than that covered by the dose of anti-D Ig given, it is **suggested** that the woman is administered supplementary doses of anti-D Ig commensurate with the volume of the FMH.	***2B***

* In Italy the only Ig available at a dose of 625 IU (125 μg) (RHESONATIV®, Octapharma Italy S.p.A., Pisa, Italy) carries the indication to use this dose up to the first trimester of pregnancy, whereas most Guidelines and Recommendations[21,75,86-87] propose its use until the 20th week of gestation.

Anti-D immunoglobulin preparations available in Italy

Anti-D Ig are blood derivatives obtained from the plasma of RhD negative donors with high levels of circulating anti-D, after voluntary immunisation.

There are various anti-D Ig preparations commercially available. Some are used exclusively by the intramuscular or subcutaneous route, while others can also be administered intravenously. The following preparations are currently available in Italy[111]:

- **IGAMAD**® (Grifols Italia S.p.A., Ghezzano-Pisa, Italy): available at the dose of 300 μg (1,500 IU) in type 1 glass syringes for intramuscular or subcutaneous use (MA number 033867021).
- **IMMUNORHO**® (Kedrion S.p.A., Lucca. Italy): available at the dose of 200 μg (1,000 IU), in a vial, and at the dose of 300 μg (1,500 IU), in a vial or a pre-filled syringe for intramuscular or subcutaneous use (MA number 022547018).
- **PARTOBULIN**® (Baxter Ag, Vienna, Austria): available at the dose of 250 μg (1,250 IU) in a pre-filled syringe, exclusively for intramuscular use (MA number 021974035).
- **RHESONATIV**® (Octapharma Italy S.p.A., Pisa, Italy): available at the dose of 125 μg (625 IU), in a vial for intramuscular or subcutaneous use (MA number 039596010) and at the dose of 250 μg (1,250 IU), in an ampoule for intramuscular or subcutaneous use (MA number 039596022).
- **RHOPHYLAC**® (CSL Behring GmbH, Marburg, Germany): available at the dose of 200 μg (1,000 IU) or 300 μg (1,500 IU) in a pre-filled syringe, for intramuscular or intravenous use (MA number 036161026).

Evaluation of foetal-maternal haemorrhage

In 99% of pregnancies the amount of bleeding at birth is less than 4 mL of red blood cells. Various studies have, however, shown that about 0.3% of pregnancies are associated with a FMH greater than 15 mL at birth, an amount not covered by the maximum dose of 1,500 IU (300 μg) of anti-D Ig[112-114]; furthermore, large volumes of FMH (>15 mL foetal red blood cells) can also occur during normal childbirth[115]. This means that as many as three out of every 1,000 RhD negative women could be at risk of alloimmunisation.

Clinical practice and the recommendations on IP differ between North America, Australia, the United Kingdom and continental Europe[116]. In countries such as the United Kingdom and Australia, in which the recommended, standard post-natal dose of anti-D is, respectively, 500 IU (100 μg) and 625 IU (125 μg), the test for evaluating FMH is recommended routinely, whereas in Italy and many other European countries, in which the dose of anti-D Ig used for postnatal prophylaxis is 1,250-1,500 IU (250-300 μg), tests to evaluate FMH are not always used[2,117]. The AABB Standards require that a test is adopted that is able to determine the volume of an FMH so that further doses of Ig can be given if the FMH was not covered by the dose of anti-D Ig already administered[118].

Main tests used to evaluate the volume of foetal-maternal haemorrhage

Various different laboratory techniques have been proposed for the determination of whether a FMH has occurred and, if so, its volume. The most widely used screening test is the acid elution test described by Kleihauer and colleagues[119], but there are others, including the consumption of anti-D monoclonal antibodies using a gel agglutination technique[120] or a rosette test[121]. Flow cytometry techniques[122], unlike the screening tests, can give a measure of the volume of the bleeding.

Kleihauer's acid elution test consists in identifying the cells, by microscopy, which contain foetal haemoglobin (HbF) present in a sample of the pregnant woman's blood. In some circumstances, such as congenital conditions characterised by high levels of HbF in the mother's blood, the results may be falsely positive. The test is based on a subjective evaluation by the person carrying out the test and its precision is limited to small volumes of transplacental bleeding.

For all these reasons, the Kleihauer test is only indicated as a screening test and in the event of a FMH greater than 2.5 mL, the amount of bleeding must be re-evaluated with a more precise method, such as flow cytometry[123]. Flow cytometry exploits monoclonal antibodies against RhD positive cells or, alternatively, monoclonal antibodies against HbF and the isoenzyme carbonic anhydrase, which is expressed in the first months of life. In this way it is possible to distinguish foetal blood cells from adults ones containing HbF (because woman who have haemoglobinopathies, even if they are heterozygous carriers, may have HbF in their blood cells, giving rise to false positive results). This type of technology is not available in all hospitals and in these cases, the samples must be sent to external laboratories.

When to evaluate foetal-maternal haemorrhage (Table XIII)

- FMH must be evaluated in non-immunised RhD negative women after the birth of a RhD positive

neonate or after potentially immunising events that occur after 20 weeks of gestation, particularly if a dose of 625 IU (125 μg) anti-D Ig is used.

- The evaluation of FMH is not advised in the event of potentially immunising events occurring before 20 weeks of gestation[124].
- A FMH must always be evaluated, independently of the dose of anti-D Ig administered, in cases in which there are factors associated with large volumes of bleeding:
  - abdominal trauma during the third trimester;
  - unexplained hydrops foetalis;
  - placental abruption;
  - external cephalic version;
  - multiple pregnancies;
  - stillbirths and intrauterine deaths;
  - instrumental delivery;
  - Caesarean delivery;
  - manual removal of the placenta.
- The sample of blood on which to evaluate the FMH should be taken at least 30 minutes after but no later than 2 hours after the birth or potentially immunising event, in order to allow the neonatal or foetal blood to spread through the maternal circulation[125,126]. The sample must, in all cases, be taken before the administration of the anti-D Ig.
- The sample must be stored at a temperature between 2 °C and 10 °C for no more than 2 days, but in any case, the test must be performed as early as possible, to enable administration of further doses of anti-D Ig within 72 hours of delivery or the sensitising event.
- The laboratories that quantify FMH must demonstrate acceptable performance in internal and external quality audits, use validated methods and have staff training programmes to guarantee the precision and reproducibility of the results.
- The results of the evaluation of FMH should be reported in a form that enables easy calculation of any supplementary dose of anti-D Ig that it is necessary to give or indicate the total dose of anti-D Ig to administer.
- Following administration of a supplementary dose of anti-D Ig, the test evaluating FMH must be repeated to determine the actual clearance of RhD foetal red cells from the maternal circulation. This re-evaluation must be conducted 48-72 hours after the administration of the anti-D Ig.

Table XIII -Recommendations on when to evaluate foetal-maternal haemorrhage.

Rec. n.	Recommendation	GoR
29	With the aim of calculating the appropriate dose of anti-D Ig to administer, it is **suggested** that FMH is evaluated in RhD negative women after giving birth to a RhD positive neonate or after potentially immunising events that occur after 20 weeks of gestation.	***2B***
30	Independently of the dose of anti-D Ig administered, it is **suggested** that FMH is evaluated in cases in which there are risk factors associated with large volumes of FMH.	***2B***
31	It is **suggested** that the blood sample in which to evaluate FMH is taken at least 30 minutes afer but no later than 2 hours after the delivery or potentially immunising event in order to allow the foetal or neonatal blood to spread throughout the maternal circulation. In all cases, the sample must be taken before the anti-D Ig is given.	***2B***

8. Monitoring and evaluation indicators for the clinical audit

With the aim of controlling some aspects of the management of the prevention of HDFN due to anti-RhD it is suggested that the following monitoring indicators are adopted:

- percentage of non-immunised RhD negative women who receive anti-D Ig after a sensitising event;
- percentage of non-immunised RhD negative women who receive antenatal prophylaxis at 28 weeks of gestation;
- percentage of non-immunised RhD negative women who receive anti-D Ig within 72 hours after the delivery of a RhD positive neonate;
- percentage of non-immunised RhD negative women who receive anti-D Ig more than 72 hours after the delivery of a RhD positive neonate;
- percentage of tests to evaluate FMH performed because of sensitising events after 20 weeks of gestation;
- percentage of tests to evaluate FMH performed after the delivery of a RhD positive neonate;
- percentage of immunised RhD negative women despite a complete programme of antenatal and postnatal prophylaxis;
- percentage of neonates who undergo ET for HDFN due to anti-D out of all neonates.

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