Prevalence of IgG subclasses (IgG1/IgG3) in antenatal alloimmunized women

Experience in a tertiary hospital in South India

A dissertation submitted in partial fulfillment of M.D. Immuno Haematology and Blood Transfusion (Branch XXI) Examination of the Tamil Nadu Dr M.G.R. UNIVERSITY, CHENNAI to be held in 2015.

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Certificate

This is to certify that the dissertation “Study of prevalence of IgG subclasses (IgG1/IgG3) in antenatal alloimmunized women in South India” is a bonafide work of Dr Jui Choudhuri towards the M.D. (Immuno Haematology and Blood Transfusion) Examination of the Tamil Nadu Dr M.G.R. University, Chennai to be held in 2015.

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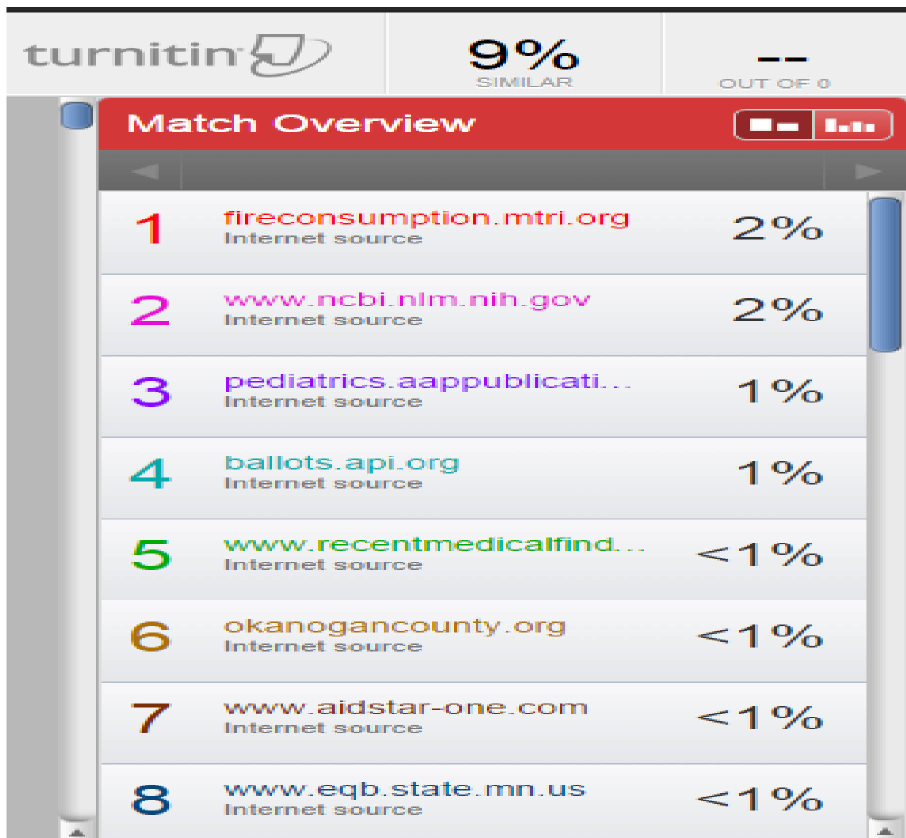

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ABSTRACT: To study the prevalence of IgG subclasses (IgG1 and IgG3) in antenatal alloimmunized women in South India

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Objective: To study the prevalence of IgG subclasses (IgG1 and IgG3) in antenatal alloimmunized women in South India and to correlate it with occurrence and severity of Hemolytic Disease of Fetus and Newborns (HDFN).

Methodology: 85 antenatal women with a positive antibody screen were included in this study, irrespective of parity and period of gestation. The antibodies were identified and IgG subclass (IgG1/ IgG3) was determined using the "DAT IgG1/IgG3 ID card from DiaMed Gmbh, BIO-RAD which utilizes the column agglutination technique. These pregnancies were followed up to see the outcome. The newborn was identified to "have or not have HDFN" using the DAT test and further categorized into Mild/ Moderate/Severe HDFN based on the modality of treatment required. The prevalence of IgG1/IgG3 subclasses was calculated in the alloimmunized antenatal. The categorical data including disease IgG subclass, severity of HDFN and correlation with DAT strength was calculated using Pearson's chi square test. The severity of HDFN was correlated with the IgG (IgG1 and IgG3) subclasses and antibody titre levels in maternal serum using the univariate and binary logistic regression analysis.

Results and Conclusion: The prevalence of IgG subclasses in our study was found to be, 20% for IgG1, 4% for IgG3, 25% for IgG1+ IgG3, 51% had neither IgG1 and/or IgG3. Among the 57 newborns at risk of HDFN, we found a highly significant difference between disease severity and absence / presence of IgG1/IgG3 – either singly or in combination ($p<0.001$). Assessing disease severity in low and intermediate antibody titre level groups showed a significant difference with more severe disease in those with IgG1 and / or IgG3 as compared to those who were negative for both these subclasses ($p<0.001$). The binary logistic regression analysis showed the odds of severe HDFN to increase an additional seven times stepwise , the lowest being in patients with neither IgG1/IgG3, to having either one of them, to having both these subclasses together ($p<0.05$, CI between 1.5-31.89). DAT was positive in 37 of 57 babies. Severity of HDFN was significantly associated with increasing DAT strength ($p<0.001$)

We conclude that the presence of IgG1/IgG3 subclass impacts significantly on the severity of HDFN. This effect continues to be significant even when titres are low. These mothers therefore require closer antenatal monitoring, and babies in this group require closer follow up to allow for appropriate and timely intervention. A stronger DAT reflects as HDFN with greater severity. This study identified the significance of IgG subclass as an independent prognostic marker for HDFN which can easily be incorporated into routine clinical practice.

AIM:

To study the prevalence of Ig G subclasses (IgG1 and IgG3) in antenatal alloimmunized women in South India

OBJECTIVES:

1. To study the prevalence of Ig G subclasses (IgG1 and IgG3) in antenatal alloimmunized women in South India

2. To correlate the IgG subclass with occurrence and severity of Hemolytic Disease of Fetus and Newborn (HDFN)

INTRODUCTION:

Haemolytic disease of newborn is a condition characterized by rapid destruction of an infant's red cells by antibodies transferred from the mother through the placenta, which develop as a result of sensitization to the paternal antigens. The symptoms may vary, ranging from mild anaemia and jaundice to a severe condition leading to cardiac failure, hydrops fetalis or still birth. It has been a major challenge for obstetricians and neonatologists for the longest time and since the process of destruction begins as early as during the intrauterine phase of pregnancy it has more accurately been termed as haemolytic disease of foetus and newborn (HDFN) .

In present day clinical practice even with the best of diagnostic and therapeutic measures available, HDFN should be considered as a possibility in the following situations,(1)

- A positive maternal antenatal antibody screening and/or diagnosed severely anemic/hydropic fetus;
- A positive DAT carried out on the fetus or newborn's sample
- Rapidly developing indirect hyperbilirubinaemia in a newborn, not explained by maternal antenatal antibody screening (as in the case of very rare antibody specificities)
- Haemolysis in the newborn detected on blood film examination, characterized by reticulocytosis or presence of increased nucleated red cells.

The outlook towards HDFN has shifted drastically over the last few decades. While earlier the focus was more towards cure and appropriate management of a newborn afflicted by the disease, presently many new methods are being implemented and studied for prevention of the same. Serological tests, radiological interventions and immunoglobulin prophylaxis are

all works in various fields which have contributed in decreasing the burden of HDFN globally. There have also been remarkable advances in the treatment modalities involved both prenatal and post-natal, in the form of Doppler monitoring and IV Immunoglobulin treatment.

HDFN is mainly identified by anaemia and indirect hyperbilirubinemia in a newborn with a Direct Antiglobulin Test (DAT) positive. DAT helps detect maternal antibodies that coat the foetal red cells; the presence of the corresponding alloantibody in the mother is identified by an Indirect Antiglobulin Test (IAT). As per BSCH guidelines 2008, "All infants born to women who have clinically significant antibodies should be closely observed for evidence of HDN. A DAT should be performed and if positive, hemoglobin and bilirubin levels should be measured".(2)

Antibody screening test is now routinely done for all antenatal women during the course of their pregnancy in our institution. However, a positive antibody screen does not necessarily correlate all the time with the occurrence or severity of HDFN. Numerous studies in literature have showed that the IgG subclass – particularly the presence of IgG1 and IgG3 either singly or in combination, impacts on the presence and severity of HDFN(3,4). However, there is very limited literature from India.

The primary objective of undertaking this study was to look at prevalence of IgG1 and IgG3 subclasses in all sensitized women and to observe if the presence of these IgG subclasses contributed to the severity of disease. This would have a great clinical impact if a correlate were to be found. If this test was found useful in predicting severity of HDFN, it would greatly impact clinical practice by allowing the clinician greater antenatal and neonatal monitoring / intervention.

Identifying IgG subclasses, as a predictor for severity of HDFN as both qualitative and quantitative assays have been described with most studies having been carried out in the West. Platforms that have been used include Enzyme linked immune sorbent assay (ELISA) and flow-cytometry. However these are not very viable options for routine use considering cost, ease of operation and the necessity to batch on the former. The Column agglutination technology (CAT) is an easily available and commonly used platform in the immuno-haematology arena. Reliable, robust and used for many tests including blood grouping, compatibility testing and antibody screening, when the IgG sub classing was introduced on this platform, it was found to gain wide acceptance. A study comparing the efficacy of detection of DAT positivity on CAT vs. flow flow-cytometry was done by Dittamar et al. in the year 2001, and they suggested that CAT was as sensitive a platform to detect red cell bound antibodies as the flow-cytometry. The CAT offers greater sensitivity than other platforms used in immunohaematology as demonstrated by Sudipta et al who have described their experience comparing CAT and tube technique for patients of AIHA. In addition to greater sensitivity, they also reported that the strength of reactions were stronger on this platform..(5) Not only is the CAT a more viable option for a developing country like India because of the cost benefit, they have also shown comparable results with flow cytometry. While the latter is mostly used as a research tool, it also requires expertise in carrying out the test. Keeping in mind the cost- benefit aspect of a new test and its role in serving our population, the column agglutination technology which is an easily available and a reliable platform used in many blood banks was considered an appropriate platform for the same.

The major advantages of using the CAT lies in its simple procedure, reproducibility of results, ease of standardization and familiarity of use in a blood bank set up. Moreover the test in comparison to other conventional methods requires less volume of sample.(6)

The catalytic factor for this study was the limited literature available about the prevalence of IgG subclasses in the Indian subcontinent. Considering the clinical impact this could have in terms of minimising morbidity and mortality secondary to HDFN, we aimed at studying the prevalence of IgG1 and IgG3 on the CAT platform and to correlate it with the severity of HDFN in our tertiary hospital in South India.

REVIEW OF LITERATURE:

The pathophysiological basis of HDFN began to be studied at the beginning of the last century to increase knowledge about the condition and achieve methods to prevent it. It has been an important cause of foetal loss and neonatal mortality, but the declining incidence in recent times is primarily attributed to the cooperation between pathologists, paediatricians, haematologists, obstetricians, immunologists, geneticists and transfusion medicine specialists. Despite all the advances in this field it remains a serious condition and is not managed successfully in all cases despite successful immunological prophylaxis or close monitoring.

HISTORY:

HDFN an important cause of foetal loss and death of newborn babies and is said to have been first described by a French midwife named Louise Bourgeois in 1906, who worked at the royal court of King Henry IV. While assisting in the delivery of twins she noted that—one baby was swollen and died soon after birth, while the other baby developed jaundice and died several days later.(7)(8) For the next 300 years, many similar cases were described in which newborns failed to survive due to unexplainable cause.

Hippocrates, mentioned a condition known as fetus carnosus, which is thought by medical historians to represent present day foetal hydrops.(9) The first descriptive work which reviewed the condition from the point of view of morphology and pathogenesis of the condition was that of Schridde in 1910. He suggested, that the anaemia was characteristic by extramedullary haematopoiesis in the spleen and liver and increased number of immature erythrocytes in the peripheral blood due to destruction of red cells(9) . The actual name for the phenomenon – “erythroblastosis”, was coined by Rautmann. Later in 1932 Diamond et al.

coined the term "erythroblastosis fetalis" and for the first time identified it as a syndrome linking three important features, that is, congenital anaemia, jaundice, and hydrops fetalis with evidence of extramedullary haematopoiesis and erythroblastemia(10).The fact that the anatomical and pathological part of HDFN could be contributed by an "antigen-antibody reaction" was a major path breaking work described by Ruth Darrow, and was accepted in the medical community only following much work and contemplation. (11) In 1900, Landsteiner and colleagues discovered the ABO blood group system, for which he also received the Noble prize and later in the 1930s and 1940s, Landsteiner and Weiner described the Rhesus (Rh) blood groups. Levine and associates described the link between the Rh groups and HDFN. Following which, Auden described a series of fatal cases of severe neonatal jaundice in families which formed a major contribution in understanding Rh antibody associated HDFN.(12)The work and studies of these scientists has been the stepping stone which has lead to the present day knowledge of HDFN.

EPIDEMIOLOGY:

Following the implementation of Rhesus D immunoprophylaxis, HDFN due to ABO incompatibility and other alloantibodies has emerged as a cause for concern other than the widely known Rh alloantibody (irregular/ unexpected antibody). ABO incompatibility between the mother and the baby occurs in about 15-20% of all pregnancies, which produces HDFN in 10% of these cases. Though ABO blood groups have predominantly IgM antibodies, 40-93% of the group O mothers also have IgG anti-A and anti-B antibodies which is able to cross the placenta and cause fetal cell destruction.(13)(14) In developing countries, anti-D still remains a common alloantibody encountered in pregnant women, and many Asian countries have identified alloantibodies other than anti-D as a cause of moderate-severe hemolytic disease.(15)

WORLD SCENARIO: Alloimmunization has been extensively studied in different countries and regions, with frequency in pregnant women being found to range from 0.4% to 2.7% worldwide.(16)(17) Published data suggests that the rate of alloimmunization may be greatly dependent on the geographical boundaries, and to this effect Lee et al, suggested that antenatal antibody screening for Chinese women may not be worthwhile due to prevalence of alloimmunization being as low as 0.79%, unless women were D antigen negative or had a significant history of HDFN. (18)

Table 1: Previous studies on alloimmunization in pregnant women

Authors	Place	Year	Overall Prevalence
Varghese et al	Vellore, India	2013	1.48%
Pahuja S et al.	Delhi, India	2008-09	1.25%
Koelewijin JM et al.	Netherlands	2008	1.232%
Al-Ibrahim et al.	Saudi Arabia	2008	1.92%
Gottvall et al.	Sweden	2008	0.4%
Lurie et al.	Israel	2003	RHD positive 0.2%, RHD negative women 0.9%
Lee et al.	China	2003	0.79%
Chandrasekar et al.	Ireland	2001	0.53%
De Vrijer et al	Netherlands	1999	2.71%
Howard et al	UK	1998	1%
Filbey et al.	Sweden	1995	0.57%

Courtesy: Pahuja S et al.(19)

INDIAN SCENARIO: There is limited data on the prevalence of alloimmunization in India. However one study conducted in Delhi, North India, found alloimmunisation in 1.2% with Anti-D antibodies contributing to 78% cases. This was followed by anti-C (11.76%), and Anti-M with a frequency of 3.92%.(19)

CMC, TAMIL NADU: According to a study done earlier in our tertiary hospital which looked at the profile in the South Indian population, the prevalence of alloimmunization was found to be 1.48% in pregnant women. In 37% of these women the allo-antibodies could not be identified; however in the remaining 63% of women, 54 antibodies were characterized. A total of 40 clinically significant antibody specificities were identified among 36 women, of whom four were Rh(D) positive. Allosensitization with clinically significant antibodies was found in 9.43 per cent Rh(D) negative and in 0.08 per cent Rh(D) positive women. Anti D was the most frequent antibody found in 8.85 per cent Rh(D) negative women. The remaining clinically significant antibodies identified included anti-C, c, E, Jk^a, Jk^b, M and S. In Rh(D) negative women, anti-D and antibodies of the Rh system contributed 83.3 and 94.4 per cent of clinically significant antibodies.(20)

DISEASE BURDEN IN INDIA: Rh alloimmunozation affects a large number of pregnancies every year, having both health and financial implications. It has been seen that without proper management and treatment 50% affected fetus of HDFN would succumb to death, and with timely measures and transfusion support, there is hope for 90% survival rate in affected pregnancies. In developing countries like India, without a proper antenatal prophylaxis programme in place, HDFN could lead to loss or damage of approximately 50,000 foetus per year.(21) This data highlights the need for evidence based universal guidelines for antibody screening in pregnant women, in developing countries such as India for proper management of child birth.

PATHOGENESIS OF HDFN:

The pathogenesis of HDFN includes the sensitization of a mother to a paternal antigen she is negative for, through the fetus which is positive for the same. This may occur with the slightest of fetomaternal haemorrhage, more commonly occurring during delivery or sometimes even during the third trimester. The event sensitizes the mother for life and she develops IgG antibodies. In subsequent pregnancies the mother develops these IgG antibodies in higher titres due to an amnaestic response, which cross the placenta and bind to the fetal red cells. These sensitized fetal red cells attach to the receptors of macrophages in the reticulendothelial system, particularly the bone marrow and are rapidly destroyed and removed from the circulation. This rapid destruction leads to haemolysis in the new born.

The pathogenesis of the disease may be broadly divided into three parts:

1. Alloimmunization of the mother

2. Transfer of these irregular antibodies to the fetus through the placenta

3. Destruction of fetal red cells.

BIOCHEMISTRY OF IgG ANTIBODIES:(22)

Immunoglobulin molecules are proteins made up of four polypeptide chains- two light chains with molecular weight of approximately 22,500 Daltons and two heavy chains of molecular weight 50,000-75,000 Daltons which are interconnected by covalent disulfide bonds. The heavy chains are also held together by disulfide bonds at their hinge region. There are a total five types of heavy chains (gamma, alpha, meu, delta, and epsilon) and two types of light chains, named kappa (κ) and lambda (λ) respectively. The light chains are the same in the five types of immunoglobulin molecules it is the heavy chain that varies for each type.

Immunoglobulin are protein molecules with an amino (-NH2) and a carboxyl (-COOH) end. When treated with papain enzyme an immunoglobulin is divided at certain specific points to form fragmented units which include: one crystalline Fc fragment and two antigen binding-Fab segments. The Fc region extends from the carboxyl terminal to the hinge region and is primarily responsible for monocyte binding and complement fixation, and in IgG subtype of immunoglobulin it is the Fc fragment which crosses the placenta and is responsible for the pathophysiology of HDFN. The Fab fragments extend from the amino terminal to the hinge region of the molecule.

The domains of an immunoglobulin molecule are made up of regions of both heavy and light chains folded into globular structures or loops, and these are made up of approximately 110-120 amino acids. The domains consist of variable (V) and constant (C) regions made up of heavy and light chains. The number of domains is dependent on the immunoglobulin isotype and IgG molecules have three constant heavy chain regions (CH1, CH2, CH3).The hinge region of the immunoglobulin structure exists between the CH1 and CH2 domains of the heavy chain. Minor differences in these hinge regions are used to categorize the four subclasses of IgG. In IgG molecules there is a specific constant heavy chain region (CH2 and CH3) which allow for attachment of Fc receptors of monocytes and macrophage cells.

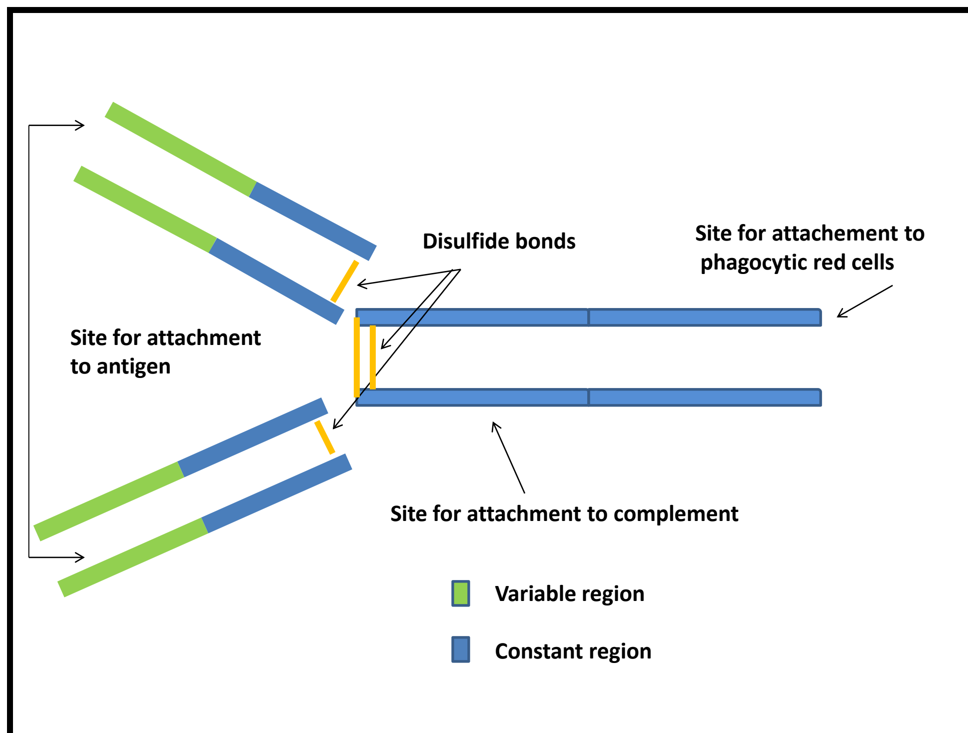


Fig 1: Depicting basic structure of an IgG molecule

IgG antibodies can be subdivided into four subclasses (IgG1, IgG2, IgG3 and IgG4), on the basis of minor structural differences in the hinge region of the IgG structure and these can be separated out by electrophoresis. The overall ratio of κ to λ in human IgG is 2:1, but the ratios is 1:1 and 8:1 for individual IgG2 and IgG4 subclasses respectively. The disulfide bonds linking the heavy chains also acts as factor for structural variation among the different subclasses. While IgG1 and IgG4 have two bonds each, IgG2 and IgG3 have four and five bonds respectively. The disulfide bond lends flexibility to the hinge regions of the subclasses of IgG molecule and the distance the Fab fragment can cover determines the antigen it can accommodate.(23)

Each of the subclasses exhibit differences in properties including placental transfer and complement fixation. While IgG1 and IgG3 bind to complement C1q molecule more strongly than IgG2. IgG4 doesn't bind at all and cannot activate the complement cascade. (24)

Among blood group antibodies it has been observed that one particular blood group system is not necessarily a particular subclass alone. The Rh blood group system associated HDFN has commonly been attributed to IgG1 and IgG3 subclass, while IgG1 and IgG3 alone have been found responsible in the Kell and Duffy blood group system respectively.

TRANSFER OF ANTIBODIES FROM MOTHER TO FETUS:

Of the five major classes of antibodies (IgG, IgM, IgA, IgD and IgE), IgG alone is capable of crossing the placenta. The transplacental movement of antibodies is an active process proved by the fact that that the immunoglobulin concentration in the fetus correlates positively with the gestational age, and the cord blood immunoglobulin level at term may exceed the respective maternal concentration. The movement of IgG is dependent on the interaction between the antibody molecule and the synctiotrophoblast Fc receptors and the process is most active in the third trimester.(25)(26). The structure of Fc receptors has been found to be unique and unlike those of other Fc receptors, and it is a distant member of MHC class I protein, dependent on β2 microglobulin for its functioning. Placental transfer of immunoglobulin G (IgG) has been demonstrated and studied by several investigators. It has been suggested that the α2 and β2 microglobulin domains of the Fc receptors interact with Cγ2 and Cγ3 domains of IgG molecule. (25) .

The physiologic process of IgG transfer to the fetus is responsible for providing the advantage of passive defence immunity to the newborn, but there are disadvantages in certain pathologic conditions. According to literature the Fc receptors transport IgG in a two-step process. IgG as a first step is engulfed non-specifically into acidic endosomes, which form

high affinity bonding between the respective receptors and IgG molecule at a pH of <6.5, following which, IgG-FcRn complexes are shuttled in transport vesicles to the basolateral surface where IgG is released at neutral pH of approximately 7.4. According to Martin et al. (2001) the pH-dependent interaction involves histidine residues on the Fc portion of IgG and residues on FcRn that are charged at acidic but not basic pH.(25)

IgG concentrations in the fetus rise slowly during the first half of gestation to reach levels between 10 and 20% of those found in the mother by 22 weeks gestation.(27) In the first twelve weeks of gestation the transfer of antibodies may be slow and quite minimal. However if the mother has relatively potent amount of anti-D in high titres, the DAT for the fetal cells may show positive results as early as 6 to 10 weeks into the pregnancy. From twenty fourth week of pregnancy the transfer of antibodies has been shown to rise exponentially.(28)

Lastly, Hughes-Jones et al. calculated that the placental transfer of anti-D may be the rate-limiting step in the reaction between anti-D in the maternal circulation and fetal red cells, and therefore diminished IgG transport on the corollary may result in unexpectedly mild HDFN.(29)

FETAL RED CELL DESTRUCTION:

Once the maternal irregular antibodies enter into the fetal circulation, they are free to bind to the corresponding antigens on the fetal red cells. However some of these antigens are not specific for erythroids for example the AB, Duffy and Kidd antigens which are also expressed on other tissues, thus binding of antibodies to the non-erythroid antigenic sites leads to decrease in effective antibody load which can cause red cell destruction. The Rh, MN and Ss antigens are expressed solely on erythroid cells, while Kell antigen is expressed both on erythroid and myeloid cells.

The placental barrier and variable expression of different antigens on the fetal red cells contribute to the degree of hemolysis which finally occurs. The red cell destruction can occur primarily by two methods, activation of the complement system or antibody mediated cytotoxicity. Complement activation is rare with IgG molecules, because it requires coming together of two molecules to bind to the C1q complement initiating protein.

The sensitized fetal red cells are primarily destroyed by binding to the Fc receptor of macrophages in the reticuloendothelial system of the newborn, primary site being the spleen. The destruction of red cells is primarily intracellular and thus characterized by anaemia and hyperbilirubinemia.(30)

QUANTITATIVE AND QULITATIVE ASPECT OF ALLOIMMUNE RESPONSE IN HDFN:(29)

A. **Antibody concentration/titer levels:** In present day clinical practice a sensitized antenatal woman undergoes close monitoring with antibody titer levels. The antibody titration is expected to indicate the concentration of antibodies and the capability of the same to cause HDFN. However the accuracy of titration is debatable according to literature. Gupte et al. in their work did not find a good correlation between Rh titer levels and disease severity. In recent advances other factors have been found to play a role in rise of Anti-D antibodies. The HLA phenotype of the mother is one such factor under consideration. HildCn et al. in a very advanced research have reported the HLA-DQB1 allele *0201 is associated with higher concentration of antibodies in maternal serum.(31) The significance of these and value as a prognostic marker are the challenges for the future.

B. Antibody Subclass: A great amount of emphasis is laid on the association between IgG subclass and the extent of haemolysis in utero in the last decade of scientific research. However studies have not brought up uniform results. The conflicting outcome seen through the various studies is still debatable and newer methods are being applied for not only qualitative but quantitative analysis of the IgG subtypes in alloimmunized women.

C. Antibody glycosylation: The IgG molecule contains a carbohydrate moiety within the Cy2 domains which plays a critical role in stabilizing the tertiary structure of the antibody. The biological activity of the carbohydrate molecule has gained importance lately following experiments which have shown that aglycosylated form of anti-D is less efficient in causing moncyte and K cell mediated hemolysis than glycosylated forms. Hence it may be expected that anti-D secreted under conditions which favor the incorporation of relatively high levels of galactose such as condition of pregnancy is relatively a high risk for hemolysis compared to serum from a male.

D. Placental transfer of antibodies: IgG antibodies cross over from the mother to the fetus by attaching to the Fc receptors. The IgG concentrations in the fetus rise rather slowly during the initial stages of pregnancy with a more steep incline in the third trimester or 22 weeks onwards when the levels in the fetus maybe almost 10-20% of that in the maternal serum. The rise is exponential after 22-24th week of pregnancy, till about term when the fetal levels of IgG may even surpass the maternal levels. The steep rise of IgG levels in the fetus is an indication of the on-going development of the immunoglobulin transport between mother and fetus. The efficiency with which maternal IgG alloantibodies are transported is also suggested to impact the severity of fetal hemolysis.

ALLOANTIBODIES CAUSING HDFN:

The most common IgG red cell antibodies are anti-A and anti-B, and ABO incompatibility leading to HDFN can occur when the mother is blood group O and it is seen in only 1% mothers with ante-partum high titres of IgG antibodies. ABO HDFN is less severe as compared to other antibodies such as Anti-D and all antenatal and postnatal tests are poor indicators of ABO incompatibility.(32) ABO incompatibility may often be DAT negative and the peripheral smear of these babies shows prominence of spherocytes in contrast to Rh incompatibility which is DAT positive and predominantly shows nucleated red cells as a peripheral smear finding.(32)

Presently more than fifty different red cell antibodies have been identified which can lead to HDFN and almost all IgG types can cause red cell lysis.(33) Many of the red blood cell alloantibodies of the Rh system have been associated with HDFN; however, the severity of the disease is usually the greatest with anti-D and anti-D continues to be the commonest cause especially in developing countries, despite wide prevalent use of prophylactic Anti-D immunoglobulin prophylaxis during antenatal and postnatal period. The other frequent alloantibodies, among: Rh antigens (anti-c, anti-C, anti-e, anti -E), Kell antigens, Duffy antigens, Kidd and MNS antigens. Anti-c and anti-E are the most commonly implicated non D (Rh) antibodies in the pathogenesis of HDFN. Anti E-induced HDFN may also cause severe disease and require prenatal management.(34) (35)(36) The failure of some antibodies of other blood group systems to cause HDFN may be attributed to lack of the corresponding antigens on fetal red cells.(28) or absorption of antibodies by fetal antigen in the placenta (eg. Cromer system antigens)(37)

The Kell blood group system which is the second most common blood group system associated with HDFN, was discovered in 1946 and was named after Mrs. Kelleher, a patient in who had borne a newborn with HDFN and had anti-Kell antibody identified. A total of 25

Kell antigens have been discovered over time and these are expressed in different frequencies in various populations. However the original K antigen is still considered the most significant in respect to incidence of HDFN. There are case reports due to other rarer Kell antigens. In contrast to the conventional Rh and ABO sensitization, HDFN due to Kell sensitization is caused by anti-K suppressing the fetal production of red cells. Kell antigens are unique in that they are expressed on the surface of red cell precursors, and anti-K promotes the immune destruction of K positive erythroid early progenitor cells by macrophages in the fetal liver. Since the red cell precursors do not contain hemoglobin, less bilirubin is released during the hemolysis, and jaundice in the newborn period is a less prominent feature, however, the underlying anemia may be severe due to overall suppression of the erythropoietic system.(38)

Alloanti-Kp^a and Kp^b, are examples of rare, but clinically significant antibody against high frequency red cell antigen Kp^a and Kp^b respectively from the Kell system. Anti-Kp^b is capable of causing severe late onset HDN. There is a case report by Elhence et al. from India in which the baby showed manifestations of disease after almost one month of age, suggesting that infants born to irregular antibody positive mothers may required a more intensive investigation and monitoring for a longer period of time to diagnose immune mediated HDFN even if the newborn is asymptomatic initially.(39) Kpa, another Kell blood group antigen, occurs in less than 2% of Caucasians.(40) There are case reports of HDFN due to maternal anti-Kpa alloimmunization. However the disease severity may range from mild form requiring only close monitoring to severe transfusion dependent disease.(41)(42) When a clinical picture of HDFN is encountered with DAT positive results, but a negative maternal antibody screen, the presence of antibodies to an uncommon red cell antigen must be considered.

In the MNS blood group antibodies, anti-S is more common than anti-s, and both are capable of causing severe hemolysis. Less common causes of HDN include anti-M, anti-N, anti-U, anti-Mi^a, anti-Mt^a, and anti-En^a. Anti-M antibody induced HDFN has been reported in literature. Though mostly they tend to be IgM type of antibodies, they can also be IgG and lead to severe HDFN leading to intrauterine death. In a case report Anti-M has been associated with red cell aplasia in the newborn and hence it is suggested that Anti-M induced hemolysis may affect early erythroid stages, similar to the action of Anti-Kell. (43)(44)(45)(46)(47)(48)(49). Other MNS antibodies implicated in HDN are anti-Vw, anti-Mur, anti-Hut, anti-Hil, anti-Mv, anti-Far, anti-s^D, anti-Or, and anti-MUT. Antibodies to low incidence MNS antigens once identified in an antenatal woman must be looked upon with caution and may require closer monitoring, since there are no set critical titre levels documented for these.(50)(51)(52)(53)

Maternal-fetal incompatibilities within the Duffy blood group system is an uncommon cause of HDN. The disease has usually been noted to be mild in nature. The Duffy antigens known to have caused maternal immunization and subsequent hemolytic disease are Fy^a, Fy^b, and Fy3, however the disease is milder.(54)(55)(56)

There are several case reports and studies about alloimmunization leading to HDFN occurring due to other rarer antibodies. Some of these include:

HDFN due Wra antigen from the Diego blood group system, which can occur even in the first pregnancy in newborn when a Wra negative mother is sensitized with a Wra positive neonate, inherited from the father. Severe HDFN with Wra antibodies have been described in literature and it is emphasized that these very rare antibodies should be suspected when the mother is IAT negative and the baby is positive for a DAT.(57) There is one more similar report highlighting a strong DAT positive in a newborn with mother being IAT negative

throughout pregnancy. However, at birth, using a collection of thawed red blood cells with rare phenotypes (private antigens), the presence of antibody anti-Wr(a) in the maternal serum was demonstrated. The antigen Wr(a) on the surface of the newborn and its biological father red blood cells was also confirmed. The concentration of IgG anti-Wr(a) on the baby's red cells was demonstrated by the presence of the antibody anti-Wr(a) in the eluate. This case illustrates the difficulties to detect antibodies against private antigens on baby erythrocytes, responsible of hemolytic diseases of newborn. The standard red blood cell panels used for irregular antibodies screening tests in blood banks do not express generally these private antigens and therefore these may get missed during routine screening.(58)

ALLOIMMUNIZATION DUE TO Rh ANTIBODIES:

Rh proteins are encoded by two separate genes encoded on short arm of chromosome 1, RHCE and RHD respectively. The RHCE gene encodes both the C/c and E/e membrane proteins associated with the Rh blood group system, while the RHD gene encodes for the D protein. The most significant of the Rh antigen is D antigen. The Rh negative phenotype results from deletion of the RHD gene on both chromosomes.

Alloimmunization due to Rh antibodies leading to HDFN occurs due to incompatibility between mother and the fetus, when an Rh negative mother carries a Rh positive fetus. Hughes et al have suggested that the D antigen is a highly immunogenic antigen and can is likely to provoke an immune response in 80% of D-negative people transfused to 200ml of D-positive blood.(59) Similarly a leakage of fetal red cells into the maternal circulation provokes the maternal immune system leading to formation of antibodies to the newly exposed foreign antigens. The transplacental movement of these antibodies followed by attachment to respective antigens on fetal cells leads to red cell destruction characterizing

HDFN. Often the sensitizing event is the first pregnancy which goes uneventful and hemolysis of newborn is worse in the subsequent pregnancies. Following sensitization, re-exposure to the antigen leads to an anamnestic response increasing the maternal anti-D titres.(60)

Although it is often implicated that the child of a primiparous does not suffer HDFN, there is literature to support that Rh haemolytic disease though rare can occur in 1% cases during first pregnancy, and according to a study by Dajak et al. (2011) they observed severe HDFN in three primiparous patients who had experienced no sensitizing event such as abortion or any transfusion in the past.(27) (61)

Factors influencing Rh(D) HDFN include:(62)

A. Size of feto-maternal haemorrhage: The fetal red cells that cross the placenta into maternal circulation during the third trimester is usually a small amount which is insufficient to stimulate antibody production in the mother. However during delivery, transplancental haemorrhage is commonly known to occur and fetal red cells varying from as less as 1ml to more than 10ml can cross into the maternal circulation leading to sensitization in upto 5-9% of pregnancies in Rh negative mothers. These mothers may form antibodies to the sensitizing event upto 6 months after the first delivery, and sometimes these antibodies may not be detectable initially but a fresh stimulus in the form of a subsequent pregnancy may increase it. When pregnancy with a second Rh (D) positive fetus occurs the fetal red cells may crossover through the placenta from as early as 24th week of pregnancy further increasing the sensitization and provoking increased antibody titre levels, which can lead to HDFN.(62) However there are studies and case reports which have found anti-D associated HDFN even in primiparous women.(61)

B. Parity: Parity has been found to correlate significantly with alloimmunization and risk of HDFN specially associated with second and third pregnancy.(63)

C. Effect of ABO incompatibility between mother and fetus: ABO incompatibility has been shown to provide some protection from Rh HDFN. It is suggested that an existing ABO incompatibility between mother and fetus may destroy the Rh (D) positive fetal red cells as soon as they enter the maternal circulation, before they can initiate any sensitization in the mother.

D. Zygosity of father: If the father is homozygous for a particular antigen to which the mother is sensitized then the fetus is at a definite risk of HDFN, however if the father is heterozygous the risk decreases by 50%.

E. History of blood transfusion: The risk of sensitization of an Rh negative mother who previously received Rh positive blood is high, due to greater antigen load in transfusion as compared to a small transplacental leak.

F. Medical Termination of pregnancy/abortion: Abortion or any interventional procedure during a pregnancy such as chorionic villous sampling can also be the sensitizing event leading to alloimmunzation in a woman, emphasizing the need for a detailed history.

LABORATORY AND CLINICAL PERSPECTIVE FOR MOTHER AND NEWBORN:

Antenatal testing for alloimmunized women:

The role of laboratory testing and fetal imaging studies is to guide the need for intrauterine transfusions, closer monitoring needs or early termination of pregnancy. However these tests have to be assessed in view of risk versus benefit for both mother and fetus. Antenatal assessment includes these basic steps:

1. Detailed obstetric history: Bad obstetric history (previous hydrop fetalis/ still births/ early neonatal deaths) or evidence of other sensitizing events.

2. History of blood transfusion
3. Grouping and typing
4. Antibody screening/ Indirect antiglobulin test (IAT):

   For an IAT, mother's serum is incubated with O pooled positive red cells at 37 degrees, allowing any antibodies present in the maternal serum to bind to the red cells. The cells are washed repeatedly to remove any free antibodies and Coomb's reagent is added. The anti-Ig antibodies agglutinate red cells if they have maternal IgG antibodies bound to them. The indirect Coombs test, as the name suggests finds "indirect" evidence of maternal antibodies.

5. Antibodies detected and identified, for its clinical implication.
6. Father's probable genotyping
7. Antibody dependent cell mediated cytotoxicity (ADCC) test: The result of ADCC correlates with the severity of HDFN.
8. Amniocentesis, when indicated.

The major role of an apt blood bank is to identify the alloantibodies present, to determine their clinical significance and to perform titrations to assist the clinician make a decision on the requirement of an invasive fetal monitoring procedure.

During the first prenatal visit, ABO grouping and Rh typing must be performed on the mother's sample. If the patient is found to be Rh negative with an initial antibody screen negative she needs to be given Rh prophylaxis. The antibody screen is a technique to detect IgG antibodies reactive at 37C and use separate screening cells representing all clinically important specificities. To avoid detection of IgM antibodies which do not cross the placenta, immediate spin and/or room temperature incubation should be omitted. A positive antibody screen must be followed up with appropriate antibody identification. Antibodies such as anti-I, -P1, -Le^a, -Le^b, can be avoided whether IgM or IgG since these are poorly expressed of

fetal red cells and do not contribute to HDFN. Treatment of mother's plasma with dithiothretiol (DTT) can be used to differentiate IgG and IgM since the latter is destroyed by it. If a clinically significant alloantibody is identified the next step would ideally involve establishing the father's zygosity (homozygous or heterozygous) for the corresponding antigen. If the father is homozygous for the antigen then the fetus is definitely at risk of HDFN, and if the father is heterozygous then the genotyping of the fetus maybe determined by polymerase chain reaction (PCR) of sample obtained by amniocentesis, chorionic villous sampling or cordocentesis. However these tests are expensive with added risk to the fetus.

The antibody titration is a critical step in decision making for alloimmunized pregnant women. Different methods are used for the same, including saline AHG with sixty minute incubation at 37C (recommended by AABB), standard saline method, and gel technique. According to some literature the gel technique may result in titres in higher dilution resulting in inappropriate invasive management. It is recommended that a previously frozen serum sample must be run parallel with a current sample to minimize errors due to difference in technique or reading of results. The critical titre for anti-D, the level below which HDFN is unlikely has been found to be 16 or 32 according to studies.(64)(65) If titres are lower than 8 except with respect to anti-K the pregnancy can be followed up with titres every 4 to 6 weeks until delivery. A difference of two dilutions is considered significant. If the titres approach critical levels and pregnancy is 18 weeks complete and invasive procedure such as ultrasound, Doppler imaging, amniocentesis or cordocentesis maybe used as per the clinical scenario. The critical titres levels are applied for all clinically significant antibodies, however for anti-K a titre as low as 8 can cause HDFN since these antigens are expressed even on early precursor red cells and can lead to fetal anaemia.

Serological testing for newborn:

Investigation on a newborn suspected of HDFN broadly includes the following:

1. ABO grouping
2. Rh typing, including weak /partial D
3. DAT: The principle steps of the test involves repeated washing of fetal red cells to remove unbound antibodies, followed by addition of the Coomb's reagent (anti-Ig), which leads to agglutination of fetal red cells to by attaching to maternal antibodies which are already bound. Direct Coombs test is a synonym for DAT, the "direct" implies for the anti-IgG binding to the maternal antibodies coating fetal cells.

   In a study by Dinesh et al. they found DAT to have a sensitivity of 86% for the diagnosis of HDFN and it provided the first alert, even before jaundice for patients eventually requiring phototherapy.(66)

4. Screening and identification of antibodies in cord serum
5. Haemoglobin estimation on cord blood
6. Cord blood bilirubin
7. Hematological parameters

After birth, neonatal grouping and typing can be performed on cord blood sample, which needs adequate washing to avoid contamination for Wharton jelly. The D typing on cord cells maybe falsely negative if the fetal cells are coated heavily with anti-D due to prophylaxis rendered to the mother or due to repeated intrauterine transfusions. The DAT is one of the hallmarks for diagnosis of HDFN. It detects maternal IgG antibodies coating the surface of fetal red cells and the number of antibody molecules bound to the red cells determines the strength of the reaction. However the strength of the DAT has not found to correlate well with the severity of the disease. Infants may have a positive DAT with no signs of HDFN. A

positive DAT with negative maternal antibody screen may occur due to ABO antibodies or alloimmunization to a low prevalence antigen.

CLINICAL MANIFESTATIONS OF HDFN:

The clinical manifestations of HDFN may vary greatly depending on the various factors discussed above and finally the strength of the immune response. The disease may only lead to mild anemia diagnosed on the basis of laboratory parameters, to severe anemia leading to hepato-splenomegaly as a result of compensatory erythropoietic tissue hyperplasia. In case the anemia surpasses the compensatory mechanisms it leads to pallor, signs of cardiac failure (cardiomagaly and respiratory distress), anasarca and shock. “Hydrops fetalis” is characterized by accumulation of excessive fluid in two or more fetal compartments, such as skin, pleura, pericardium, peritoneum. The severity of hydrops is dependent on the hypoalbuminemia, which occurs due to the liver dysfunction and also due to right heart failure leading to edema and ascites. In the case that there is a failure to initiate effective ventilation at birth due to pleural effusion or pulmonary edema it may lead to birth asphyxia.

Thrombocytopenia may also be a manifestation of HDFN or occur as a result of disseminated intravascular coagulation and may lead to bleeding manifestations such as petechiae and purpuras. Jaundice which is the other significant and common finding, is usually evident on the first day of birth itself and the amniotic fluid may also be stained by bilirubin if the hemolysis is severe enough to exhaust the placenta’s capacity to clear it. The levels of unconjugated bilirubin may rise rapidly and put the baby at risk of developing encepahalopathy. These newborns may also suffer from hypoglycaemia and acidosis due to related hyperinsulinemia and hypertrophy of the islet cells of pancreatic tissues.

DIAGNOSIS OF ANEMIA, HEMOLYSIS AND HYDROP FETALIS:

Fetal monitoring is used to detect the early features of HDFN or clinical features suspicious of hydrops fetalis, which can be associated with high risk of mortality and to determine the needed for transfusion requirements. This is accomplished by three methods:

-Doppler ultrasonography

-Amniocentesis

-Fetal blood sampling eg.Cordocentesis

DOPPLER ULTRASONOGRAPHY: It is a much preferred non-invasive method which has largely replaced the invasive aminocentesis test. It was first proposed by Mari et al. and measures blood flow velocity. Fetal anemia results in increased cardiac output and decreased viscosity which increases the blood flow. Fetal middle cerebral artery is commonly chosen for study purpose since can be used with minimal angle of insonation. It was established by the pioneers of this method through studies on 265 normal fetus, that values of 0.84 to 0.65Mom corresponded to mild anemia, 0.65 to 0.55Mom to moderate anemia for the gestational age and severe anemia was less than 0.55 Mom. Following this, more than thousand fetus at risk of HDFN with alloimmunized mothers were evaluated prospectively and a value of greater than 1.5Mom was found to correlate with severe anemia.(67) It is therefore considered critical for establishing need for a further fetal blood sampling. The measurement of middle cerebral artery peak systolic velocity (MCA-PSV) has been shown to correlate with the severity of anemia and predict the need for invasive procedures, such as intra uterine red cell transfusion. It has specially been found to correlate well with diagnosing moderate to severe anemia in the fetus.(65)

AMNIOCENTESIS: Before the development of colour Doppler ultrasonography, the severity of HDFN was monitored by serial ultrasound guided amniocentesis, to follow the bilirubin levels in amniotic fluid by spectral analysis. The aspirated fluid is tested by a scanning spectrophotometer at 450nm optical density (▲OD 450), to measure bilirubin levels as an indicator for degree of hemolysis. The result is plotted on a graph according to gestational age, however it is useful only after 27 weeks of gestation. This method was first clinically introduced by Liley in 1961, who proposed a graph divided into three zones: top (zone 3) with the highest value, indicating most severe disease, needing intrauterine transfusion if less than 32 weeks into the pregnancy or termination of pregnancy depending on the maturity of the fetus. Middle area (zone 2) indicated a moderately affected fetus, and may need exchange transfusion following delivery and the lowest (zone 3) was for mild form of disease in which the newborn may or may not be affected.

Amniocentesis is usually carried out between 28 and 32 weeks of pregnancy, and can be repeated after two weeks. (68)The optical density of amniotic fluid (at 450-460nm) is about 0.05 at the 30th week of pregnancy, and falls to about 0.02 near term. The OD readings can help estimate the risk of HDFN and take decisions on the need for intrauterine transfusion or early termination of pregnancy.(62)(69) The risk of fetal loss per procedure is 0.25-1% and it also adds to the risk of increased fetomaternal haemorrhage.(70)

A graph for lower gestational age was proposed by Queenan et al, and involves four zones instead of the earlier three.

Fetal blood sampling includes various methods of testing such as cordocentesis, funipuncture and these involve direct access to the fetal circulation. These are used to obtain fetal blood samples for crucial tests such as DAT, hematocrit levels and for estimation of fetal acidosis. Though still considered the gold standard test for assessing fetal anemia

however, it is associated with 1-2% risk of fetal loss during procedure. (68) A hematocrit of less than 30% is a strong indicator for the need of intrauterine transfusion.(65)

O blood group, RhD-negative, leukodepleted irradiated blood to prevent risk of transfusion associated graft versus host disease is the standard requirement for an intrauterine transfusion. The purpose of intrauterine transfusion is to maintain the fetal hemoglobin value at more than 9 g/dL.(65)

HEMOLYSIS AND IgG SUBTYPES:

Rate of hemolysis and severity of HDFN is determined by various factors such as, IgG subclass, amount of antibody and the number of antigenic sites on red cells .One factor that contributes to the degree of hemolysis and has not been evaluated enough in the Indian context is the IgG subclass. IgG is the major immunoglobulin in normal human serum. Four human subclasses of the IgG molecule can be differentiated, IgG1, IgG2, IgG3 and IgG4, of which IgG1 is predominant. Although all 4 subclasses are able to cross the placenta, types 1 and 3 bind to the Fc receptor of phagocytic cells with greater affinity than do types 2 and 4. Hemolysis is therefore expected to occur to a greater extent in association with the former.(28),(32)

According to studies by Garty et al. there is a close linear relationship between placental transfer of each of the IgG subclass and gestational age, and although all the IgG subclasses can cross the human placenta, their transport is not uniform: the transport of IgGl and IgG4 is more efficient than that of IgG3 and IgG2, it is noted to be least for IgG2 antibodies. Reduced passage of IgG2 from mother to fetus was reported earlier by Wang et al. and Hay et al., whereas Pitcher-Wilmott et al. and Morell et al. reported similar passages for all four subclasses.(71) There is no satisfactory explanation for these discrepant results in studies.

Despite these findings it has been clearly demonstrated that the subclasses IgG1 and IgG3 are more efficient at causing hemolysis than IgG2 and IgG4.It has been observed that IgG1 shows greater affinity to the receptors on placental tissue than IgG2. Morell at al. and Schur et al. observed as early as in 1971, documented that among the IgG1 and IgG3 subclasses it was the former that rose at an earlier stage of gestation. (28)

There is varied literature on the opinion of severity of HDFN in relation to IgG1 and IgG3. While some authors have shown that it is mainly IgG1 antibody that causes severe disease, others have suggested that severe HDFN occurs when both IgG1 and IgG3 are present in the maternal serum. However the respective contribution of IgG1 and IgG3 in the pathogenesis is still unclear.(72)(4)(73)

According to a study by Iyer et al. conducted in KEM hospital, Mumbai, they found that in 107 Rh (D) immunized antenatal women screened between 34th and 36th week of pregnancy they found all the four IgG subtypes detected in the sera using polyclonal IgG subtype specific reagent, however IgG1 and IgG3 were the predominant classes. They observed that IgG3 anti-D was usually found in low titre patients, and at the same titre levels IgG1 alone caused more severe HDFN than IgG3 alone. HDFN was found to be most severe when IgG1 and IgG3 co-existed and there was highest risk of having a still born (IgG1 +IgG3 34.8%, IgG1 19.2% and IgG3 15.4%).(74)

With similar findings is another study conducted in Canada, by Pollock et al. published in Vox Sanuinis in 1990, which examined the influence of IgG subclasses and severity of HDFN. They found that disease was severe in 19 of 32 cases with only IgG1, 40 of 63 cases with both IgG1 and IgG3 and 0 of 3 with only IgG3 detected. Therefore when both IgG1 and IgG3 existed disease was most severe than IgG1 alone.(4)

According to a major study by Lamin et al published in 2002, correlating IgG1 and IgG3 anti-D in maternal serum with severity of disease, they found HDFN correlating with IgG1 anti-D for several reasons. Primarily they found, a negative correlation between infant's hematocrit (Hct) and IgG1 anti-D concentrations in maternal serum, in contrast, there was a positive correlation between infant's Hct and the amount of IgG3 anti-D bound to infant's red cells. Secondly, a higher concentration of IgG1 anti-D in maternal sera was observed in the 26 cases of HDN necessitating intrauterine transfusion (IUT) than in the 14 cases of HDN requiring post natal transfusion (PNT) alone. In contrast, on infants' RBCs, the amount of IgG3 anti-D was significantly higher in the 14 cases of HDN necessitating only PNT than in the 26 cases necessitating IUT. Lastly, in the 12 cases of HDN that required IUT and in which the maternal serum had a proportion of IgG3 anti-D above 5 percent, it was found that the highest proportions of IgG3 anti-D were associated with the highest Hcts and with later times of gestation for the first IUT. Therefore, the quantitative analysis of IgG and IgG subclasses of anti-D in 40 cases of HDN helped to conclude that the amount of anti-D fixed on infants' RBCs increases with the concentration of maternal anti-D but that the ratio of the concentration of anti-D on infants' RBCs to that in maternal serum diminishes as the maternal concentration increases. In addition, the proportion of IgG3 anti-D on infants' RBCs is about three times lower than in maternal sera. Finally, correlations between anti-D IgG subclass concentrations (and percentages) in maternal serum (and on infants' RBCs) with the intensity of fetal anemia suggested that IgG1 rather than IgG3 played a more important role in the pathogenesis of this condition.(75)

Most of the studies have focused on Rh hemolytic disease, and found that infants who are sensitized primarily with IgG1 are more likely to be severely affected than their IgG3 predominant counterparts. IgG3, however, may also be associated with rapid postnatal rise of serum bilirubin concentrations. Combination of IgG1 and IgG3 seemed to result in the most

severe form of hemolytic disease. It has been suggested that identification of IgG subclasses may yield prognostic information regarding the development of hemolysis and hyperbilirubinemia.

However there is limited literature looking at the significance of IgG subclasses in correlation with other antibodies which are known to cause HDFN. According to Thomas et al., (1995), they studied some of the other clinically significant antibodies encountered in a series of antenatal women, which included E, Fya, Jka and S. According to this study IgG1 and IgG3 accounted for greater than 50% of the antibodies subclass distribution in 5 cases that resulted in HDFN. Although no conclusive result could be documented due to a small sample size.(73) Though standardization of these tests is difficult, there are studies which have explored ELISA, Flow cytometry and gel centrifugation technique to categorize IgG subclasses.(76)(77)

There is a study by Ahaded et al.(2002) which looked at the IgG sublclass distribution in the second most important alloantibody group cauing HDFN, that is the Kell system. They used ELISA to quantitate anti-K (KEL1) IgG subclasses in serum of severely alloimmunized women and found that in comparison to the anti-D system, 95% of anti-K antibody was IgG1 subclass. There were only small amounts of IgG2, IgG3 and IgG4. This is in contrast to anti-D group where both IgG1 and IgG3 have been found to be significant. (78)

Column Agglutination technique: Lapierre et al, was the first to describe this technology which aimed to standardise red blood cell agglutination reactions and help in uniform interpretation of results. It involves trapping the agglutinates, which can be simply read and graded by naked eye.

One column usually consists of six special microtubes containing a dextran gel matrix. Red and serum or red cells alone depending on the test being carried out is added into the microtubes in a specific concentration as per manufacturer and test protocol, followed by incubation when necessary, and then centrifuged under stringently controlled parameters. The gel contained in each of the microtubes acts as a sieve, to trap the antigen antibody complexes formed, while the unagglutinated red blood cells form a pellet at the bottom of the tube. The gel in the microtubes may be neutral or contain specific reagents as per the test being carried out.(6)

GUIDELINES FOR ANTIBODY SCREENING AND IMMUNOPROHPHYLACTIC MEASURES:

Recommendations from various bodies state that all women during their first antenatal visit must have a blood grouping and antibody screening performed. It has been reported that 1.5%–2% of pregnant women show atypical blood group sensitization.(79) There is however a mixed opinion on the clinical significance of a repeat anti-D antibody screen at 28 week of gestation. Clinicians who are of the view that a repeat testing should be carried out at 28 week suggest that there is the potential advantage to identify about 0.18% or fewer women particularly Rh-negative who become alloimmunized after their first antenatal screen possibly as a result of potential sensitizing event occurring after the first antenatal visit and may otherwise go undiagnosed until too late.(80) Presently antibody screening is recommended for all antenatal women at 12 weeks of gestation or the first antenatal visit. Those found to be Rh positive and with a negative antibody screen, undergo a repeat screening at 28 weeks and no further action needed if same results continue. Those found to be Rh negative with a negative antibody screen at 12 weeks, are retested at 28 weeks and if remain negative are provided routine Anti-D prophylaxis. If the baby is found to be Rh positive, Rh prophylaxis post delivery is given and feto-maternal haemorrhage quantified.

For mothers found to have a positive antibody screen at initial check up, antibody identification and titration is recommended. Titres are done every four weeks until 28 week of gestation, followed by once every two weeks till delivery. A rising trend in titres (>16) or a jump of more than two dilutions requires close follow up which may include Doppler test, intrauterine transfusion or any early termination of pregnancy.(81)

Rh immunophrophylaxis rarely leads to Anti-D titres levels of more than 4, which is not considered to be clinically sufficient to cause fetal red cell hemolysis.(69)(65) The half life of Rh immunoglobulin is approximately 24 days, therefore if received at 28 weeks, 15-20% women receiving it will have very low titres of anti-D (Titres of about 2 to 4), detectable at time of labour.

RH PRPHYLAXIS RECOMMENDATIONS: according to the American Society of Clinical Pathology the testing for unexpected antibody should be carried out before a prophylactic antenatal anti-D is given to Rh-negative pregnant women. Repeat testing is not needed if the Rh negative status is documented twice.(82) Since 1990, with the advent of Rh immuno-prophylaxis in the clinical management there has been a noticeable reduction in mortality due to HDFN. A further reduction was brought about by introducing prophylaxis during the third trimester of pregnancy.(83) These findings contributed to the National Institute for Clinical Excellence (NICE) recommendation that all D-negative pregnant women who do not have immune anti-D should be offered anti-D immunoglobulin routinely during the third trimester of pregnancy.(84) Initially anti-D immunoglobulin was administered antenatal only when a sensitizing event occurred that could be associated with a feto-maternal hemorrhage.

According to BCSH guidelines a sensitizing event could include following events:

- Amniocentesis

- Cordocentesis
- Any in-utero therapeutic interventions or measures (e.g. intrauterine transfusion)
- Chorionic villus sampling
- Ectopic pregnancy
- Ante partum haemorrhage (APH)
- External cephalic version
- Termination of pregnancy
- Intrauterine death
- Miscarriage
- Fall / abdominal trauma

NICE recommends that Rh immune-prophylaxis should be offered as two doses of anti-D immunoglobulin of 500 IU at 28 and 34 weeks respectively or as a single dose of 1500 IU dose between 28 and 30 weeks. There is no clear evidence based guidelines comparing the advantage or efficacy of single dose prophylaxis to divided dose.(85)

It is part of modern day antenatal care to offer all RhD-negative pregnant women an anti-RhD immunoglobulin IgG injection at about 28 weeks' gestation with or without a booster at 34 weeks' gestation. This reduces the effect of the vast majority of sensitizing events which mostly occur after 28 weeks' gestation. Anti-RhD immunoglobulin is also given to all Rh-negative women immediately after or within a span of 72 hours after pregnancy, since the delivery could be the major sensitizing event. The prophylactic immunoglobulin rarely leads to titres of anti-D usually greater than four, which should not cause HDFN. (69) A titre of 16 or above is considered critical for all alloantibodies, except Kell, in which a titre of more than eight can lead to severe suppression of fetal hematopoiesis.(86)

The mechanism of action of anti-D immunoglobulin (Ig) is not very clearly understood. It is known to suppress the immune response activity of an RhD-negative mother, when exposed to RhD-positive fetal cells. The suggested mechanisms of action include, primarily, Anti-D Ig induces rapid clearance of the RhD-positive fetal cells, interfering with the presentation of Rh antigens by dendritic cells and macrophages; secondly, it also suppresses the RhD antibody production by the reactive B cells.

According to a major work presented by Koelewijin et al.which looked at risk factors for Rh sensitization and the pit falls of prophylaxis measures. They interestingly found twin pregnancy as a risk factor following univariate analysis. Non-spontaneous deliveries which included both caesarean section and assisted vaginal deliveries were also seen as a risk factor for increased FMH, exceeding the amount of fetal cells that could be neutralised by a single dose of anti-D Ig of 1000 iu (200 Ig) as recommended in some guidelines. Most national guidelines also support the use of a Kleihauer–Betke test as a routine modality for quantifying the FMH after caesarean section to guide the accurate dosage of postnatal anti-D Ig.(87) However it has not been universally supported or applied since various studies have shown different results for the sensitivity and specificity of the test, probably due to manual performance and factors such as small amount of FMH which may not be picked up by this method.

The Kleihauer–Betke test is performed in most hospitals specially in a developing country by the manual method with stained blood smears, however more sensitive and accurate results may be obtained using a flow cytometry- based method and the technical merit and validation of the used method may be of influence on test performance. In the conventional method the fetal blood loss into the maternal intraperitoneal cavity, may not be detectable by the Kleihauer–Betke test, which may lead to further RhD immunisation, not covered by

routine prophylaxis, since 300ug is suppresses alloimmunization by 30ml of fetal whole blood(69).

Kleihauer Betke test works on the principle of resistance of fetal red cell haemoglobin to acid treatment. A thin smear of maternal blood is made, which is treated with acid, rinsed and counterstained, and finally read under the microscope. The maternal red cells appear as "ghosts" and the fetal red cells appear darker pink, as depicted in Fig 2.

The FMH is estimated using a simple formula proposed first by Mollison in 1962, which was based on three assumptions: (88)

- The red cell volume in the other is estimated to be approximately 1800mL
- Fetal cells are estimated to be 22% larger than maternal red cells
- Only 92% of fetal cells are found to stain darkly

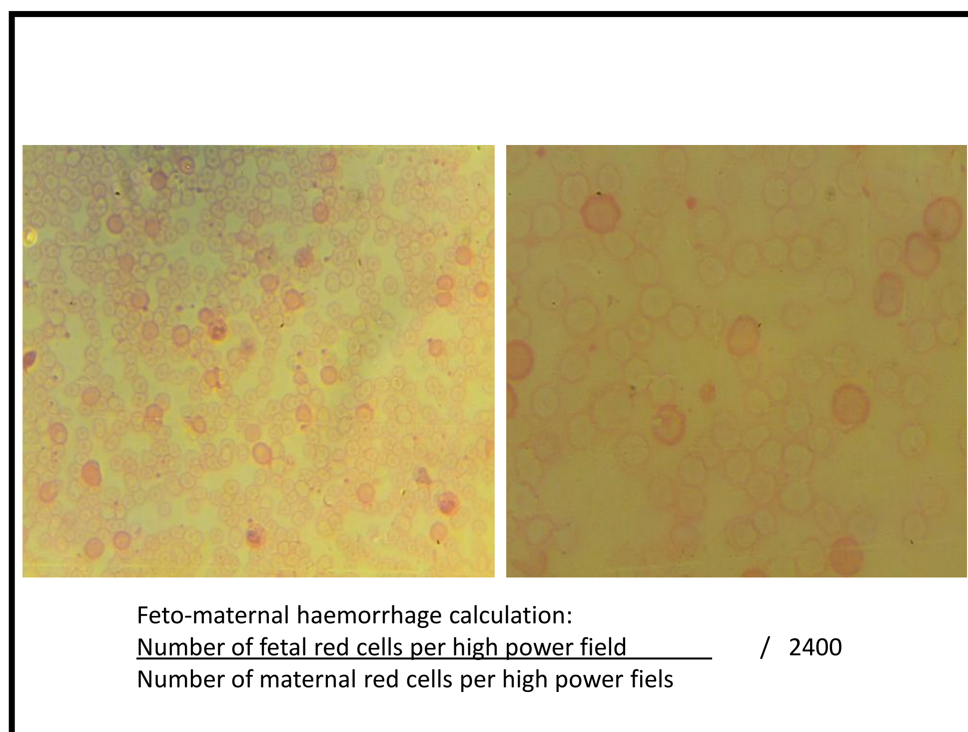


Fig 2: Acid elution technique for Kleihauer Betke test and the formula to calculate feto-maternal haemorrhage.

The post maturity of fetus also contributes as a risk factor in HDFN susceptible pregnancies since, in addition to the prolonged affect of the to the immunogenic effect of fetal red cells in the maternal circulation and as stated by some studies the drop in levels of anti-D Ig with time. There are studies which have shown undetectable levels of anti-D Ig more than 12 weeks after administration, even when a 300 lg of anti-D Ig was administered in a single dose. This emphasizes the fact that, anti-D Ig levels may drop too low if a pregnancy exceeds 42 weeks increasing the risk of sensitization.(87)

Despite all these stringent measures to reduce risk of sensitization and, the threat of HDFN continues to persist. It has been found that in present scenario one of the most important causes of anti-D antibodies sensitization is due to a mild event during the pregnancy itself, best explained as a no noticeable overt sensitising event. A late sensitizing event, during the third trimester of a first pregnancy, has been found to be responsible for 18–27% of cases. Sensitization events during second or subsequent pregnancies may account for a similar proportion of cases. However clinically it is not feasible to distinguish a late sensitising event from failure of prophylaxis at the end of the preceding pregnancy as the precipitating event.(89)

INDIAN RECOMMENDATIONS FOR PROPHYLAXIS: The Federation of Obstetrical and Gynaecological Societies of India (FOGSI), recommendations state that Rh immunoprophylaxis should include: a dose of 50-100 µg of anti-D Ig in case of any sensitizing event in the first trimester. A further routine antenatal prophylaxis in the second trimester is advocated, that includes all women tested at registration and 28 weeks. They should either be administered 100µg at 28 and 32 weeks each respectively or a single dose of 300µg at 28weeks and followed by a post partum dose of 300 µg for all pregnancies within 72 hours. Women with a weak D identified need not receive Anti-D.

THERAPEUTIC MEASURES FOR NEWBORN:

Management of HDFN is broadly categorized into two- the antenatal and post-natal aspect. The management of the newborn is basically described to be supportive and symptomatic.(90) Most previous studies have categorized the disease on the basis of combination of DAT result, hemolysis seen and the treatment modality needed.(4,74)(90) While intrauterine transfusion is the corner stone of antenatal management, post-natal initially consisted primarily of phototherapy and exchange transfusion, but in present day IV immunoglobulin also plays a major role. Phototherapy was initially started in 1956, and since then has come a long way in managing jaundice in newborns. It had also lead to a decline in frequency of exchange transfusion and mortality related to it.(91, 92)

Phototherapy: Despite phototherapy becoming such an integral part of management of jaundiced newborns, there is limited evidence based guidelines for its dosing. The conventional phototherapy to start with utilized a source of white light, exposing the newborns to 7-10$\mu W/cm^2/nm$. It helped in a decline of 6-20% of serum bilirubin in the initial twenty four hours. However presently there are much more intensive phototherapy methods available. These basically include using blue lamps and placing the baby at a distance of not more than 10cm and generating a spectral irradiance of greater than 50$\mu W/cm^2/nm$. Double surface phototherapy is also used to increase the surface area exposed to the irradiance with the application of fibre-optic pads these are routinely used in specialized neonatal set ups these days. (93)

Exchange transfusion: It comes into play when phototherapy has not been effective and there are recommendations for its appropriate use elaborated by the American Academy of Paediatrics(AAP).(94) In addition to removing excess bilirubin it also contributes in removing maternal antibodies in the circulation, and reducing chances of further on-going

hemolysis. It is done with double volume (160ml/kg) irradiated, leukocyte depleted compatible blood, ideally negative for cytomegalovirus (CMV) via an intravenous catheter inserted in the umbilical vein. Exchange transfusion has its independent hazards and is associated with a high risk of procedure related morbidity. The requirement of exchange transfusion in HDFN may vary from 20 to 70%, and while in a study by Birchenall et al. they found the rate of transfusion as high as 37%, another study by Loporiore et al. found it 15-17% and managed more cases with intensive phototherapy and top up transfusion.(95)(96)

There are no international guidelines for management of HDFN using uniform triggers or thresholds for transfusion requirements. This is mostly because of the comparatively low prevalence of the disease, because of which large evidence based studies have been limited. This has lead to lack of literature to compare transfusion requirements and to assess variability in regard to transfusion thresholds, volumes, products and different outcome measures.

There have been significant steps made in antenatal and postnatal management strategies since the journey began to overcome HDFN. New diagnostic techniques, monitoring tools and management options are constantly under investigation. While intravenous immunoglobulin has also been recommended by AAP in infants in whom bilirubin is on the rise despite intensive phototherapy the evidence is still inconclusive according to some studies. There is also research work going on looking at erythropoietin to stimulate fetal hematopoiesis in HDFN.(97)(94)

Against the complex scenario of HDFN, it’s variable clinical presentation and differing levels of management, it would add great clinical value to know if an antibody when identified is clinically more likely to cause disease or not. Literature from the west suggests that the IgG1 IgG3 subtype testing offers this value addition. This study was performed to

assess prevalence of IgG1 and IgG3 subtypes in alloimmunized antenatal women in a tertiary care centre in South India using the DAT IgG1/IgG3 card test on the column agglutination platform. In addition we aimed to correlate severity of disease with the test results to see if this could be used to prognosticate severity of HDFN, This would allow for deciding frequency of antenatal follow up, antenatal intervention and proactive management of the newborn. Being a simple test on a platform familiar to most transfusion personnel, if found to be clinically relevant, this could easily be implemented if required.

METHODS AND MATERIAL

SETTING:

1. The study was conducted in Christian Medical College, Vellore, Tamil Nadu. It is a 3000 bed teaching hospital providing tertiary medical care to the residents of Vellore and surrounding districts of Tamil Nadu, Andhra Pradesh and Kerala. It also serves as a referral centre for patients from rest of India and South East Asia. This observational cohort study was carried out in the Department of Transfusion Medicine and Immunohematology (Blood bank), with the department of Obstetrics and Neonatology.
2. The primary objective of this study was to find out the prevalence of IgG subtypes (IgG1 and IgG3) in antenatal women in South India. It required inclusion of all antenatal women identified to be alloimmunized at the blood bank during a routine antenatal check up irrespective of period of gestation, over a period of one and a half year, spanning between, January 2013 – July 2014.
3. The second objective of the study was to correlate IgG subtypes and severity of HDFN. Women included in the first part of the study were followed up through their pregnancy to observe the outcome. The blood group and DAT done on the newborn's sample was used to label "HDFN or NO HDFN" and severity was graded into mild/ moderate/ severe based on treatment required.
4. This study involved a novel test for the blood bank, IgG subtyping by column agglutination technique to identify the IgG subclass (IgG1 or IgG3) in the maternal serum.

PARTICIPANTS:

All antenatal alloimmunized women identified during a routine antenatal check up to have a positive antibody screening test were enrolled as study participants. This was irrespective of the antibody identified, titre levels or parity

Inclusion criteria for the study:

1. All alloimmunized Rh negative antenatal women, irrespective of parity.
2. All antenatal women with antibody screen positivity

Lost to follow up: Antenatal women enrolled in the study during an antenatal check up, but who did not deliver in Christian Medical College and Hospital were categorised as lost to follow up. They were included only for the calculation for IgG subtype, but not analysed further in correlation with HDFN.

METHODOLOGY:

All antenatal women during their antenatal visit have a blood grouping and typing and antibody screen performed as per routine protocol. Those found to be alloimmunized by virtue of having a positive antibody screen were recruited for this study, irrespective of period of gestation. (Fig 3) These samples were further evaluated as per usual protocol to identify the antibody specificity. The serum sample was then stored for processing the same for IgG subclass testing.

Mothers identified to have a positive antibody screen were then followed up with help of support departments which included Medical records and Neonatology. Clinical details and relevant laboratory results were accessed from the clinical work station of the hospital, both

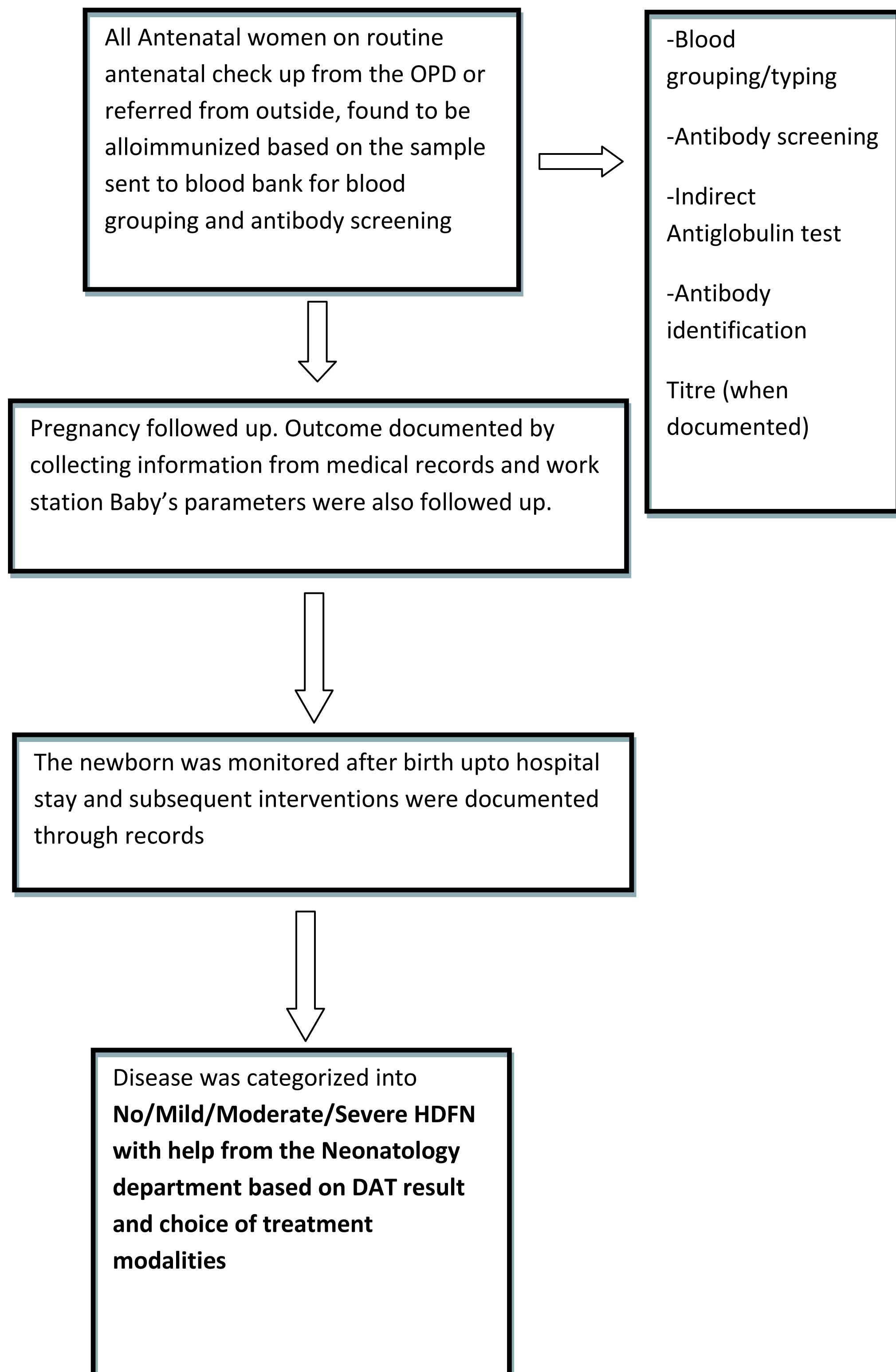
for the mother and subsequently for the baby. The course and outcome of the pregnancy was followed up in consultation with neonatology and details from the clinical workstation

The newborn was evaluated for presence or absence of HDFN. If present, severity was assessed. Classification of hemolysis and severity of disease was done in consultation with the department of Neonatology into one of three grades - namely Mild/ Moderate/ Severe HDFN. This was based on therapeutic interventions as per guidelines laid down by the Department of Neonatology for this study, since modalities of therapy are decided based on severity of disease. There were no clear guidelines available to categorize the disease. In a previous study from India, the severity of disease was categorized on the basis of still birth, live but requiring exchange transfusion and live and not requiring exchange transfusion, however the criteria for exchange transfusion was based on haemoglobin and bilirubin and varied between clinicians.(74) Another study from the west has used haemoglobin, bilirubin, treatment modality and outcome of the baby as the criteria for classifying severity.(4)

SAMPLE PROCESSING:

Following blood grouping, antibody screening, antibody identification and titration done as per routine protocol or requirement, all samples which had been collected in EDTA vacutainers were centrifuged at 3000 rpm for 3minutes and plasma separated and stored in eppendorf tubes at -70 degreesC, until a later date to perform the IgG subtyping, by the column agglutination technique using ID card “DAT IgG1/IgG3” DiaMed GmbH 1785 Cressier FR Switzerland (BIO-RAD).

Fig 3: Algorithm to demonstrate the methodology of this study:



EXPECTED OUTCOMES:

Primary:

1. Classify IgG subtypes (IgG1 and IgG3)
2. Outcome variable, namely HDFN present or absent
3. Severity of HDFN into Mild/Moderate/Severe

DATA COLLECTION and SOURCE OF DATA:

A. Data of the mother:

Demographic

Personal:

1. Blood group
2. Period of gestation
3. Obstetric history
4. History of transfusion
5. Indirect Antiglobulin test result
6. Antibody identified
7. Titre levels
8. Outcome of pregnancy

B. Baby's data:

1. Live/ Still birth/ Intrauterine death
2. Preterm/ Term/ Post-term
3. Gender
4. Weight at birth
5. Blood group
6. Direct antiglobulin test

7. Cord blood haemoglobin (if done)
8. Cord blood bilirubin (if done)
9. Features of HDFN
    a. Jaundice
    b. Pleural effusion
    c. Ascites
    d. Hypoalbuminemia
    e. Acidosis
    f. Hydrops
    g. Kernicterus
    h. Coagulopathies
    i. Sepsis
10. Haemoglobin (monitored over hospital stay; lowest level documented)
11. Bilirubin (monitored over hospital stay; highest level documented)
12. Reticulocyte count (monitored over hospital stay; highest level documented)
13. Treatment required- including admission to the Neonatal intensive care unit
14. Treatment details:
    a. Only observation and serial monitoring of parameters
    b. Phototherapy
    c. Exchange transfusion
    d. Intrauterine transfusion
    e. Any top-up blood/component transfusion
    f. None

We analyzed the above mentioned data from medical records and work station. The HDFN was graded according to guidelines set with help from Department of Neonatology.

HDFN was characterized into Not present /Mild/ Moderate and Severe

1. No HDFN- DAT negative
2. Mild- Requiring observation only (DAT positive)
3. Moderate- DAT positive, requiring phototherapy (Single/Double surface)
4. Severe-DAT positive, requiring Intrauterine transfusion/ exchange transfusion/ blood transfusion/perinatal death

Tests done in the blood bank:

A. On mother's plasma sample:

1. Blood grouping and Rh typing
2. Antibody screen/ Indirect Coomb's test
3. Antibody identification using the three cell panel and eleven cell panel, using column agglutination technique
4. IgG subtyping (IgG1 and IgG3) using Column agglutination technique, finding high (1:100) and low titres (1:1) for each respectively.
5. Antibody titration was done when requested by the clinician

B. On newborns' sample:

1. Blood grouping and Rh typing
2. Direct Antiglobulin test

COLUMN AGGUTINATION TECHNOLOGY: It is a commonly used platform in blood banks presently, and it uses gel or glass beads to trap agglutinated red cells following an antigen antibody reaction. Test can be performed on this platform manually or by automated means, which allow for processing multiple samples at the same time. CAT as it is commonly known as, can be used for tests like blood grouping, antibody screening, DAT, IAT and cross-matching. Antigens expressed on the red cells interact with antibodies in the chambers at the top of a column, and after proper incubation and centrifugation as recommended by the manufacturer, the test can be interpreted. The agglutinated red cells remain at the top, and are read as a positive reaction, while the free red cells are forced through the column to the bottom and read as negative. This forms the principle of many tests carried out via this platform in our blood bank.

STANDARD OPERATING PROCEDURE:

A. IgG1 and IgG3 SUBTYPING:

Principle: The risk of hemolysis in HDFN depends on the amount of IgG coated on the red cells as well as on the IgG subclasses involved. To induce phagocytosis it is suggested that approximately 1000-4000 IgG1 molecules or 135-500 IgG3 molecules must coat the red cells. Therefore it is not only important to identify the presence of these subclasses but may require an estimation of the titre levels to comprehend its clinical significance.

Principle: The ID-Card "DAT IgG1/IgG3" has two dilutions (1:1 and 1:100) of the both subclasses and works on the principle of CAT.

Materials: ID-card with 6 columns as in Fig 4 (having four microtubes for IgG 1 and IgG3 in two dilutions each, a negative control well, and a positive control well), ID-Dia cell (0.8%) suspension, Pipettes, Tips, Incubator and Centrifuge.

Procedure:

1. Serum or plasma sample stored previously at -70degress C is thawed to room temperature and centrifuged at 1500g for ten minutes to avoid fibrin residues which can interfere with reaction pattern.
2. Label the ID-card with appropriate patient hospital/identity number.
3. Remove aluminium foil, holding the card in upright position.
4. Pipette 50µl of ID-DiaCell into each microtube.
5. Add 25µl of patient plasma/serum to each microtube.
6. Incubate the card for 15mintues at 37degreesC in the incubator.
7. Centrifuge the card for ten minutes.
8. Interpret and record the agglutination reaction.

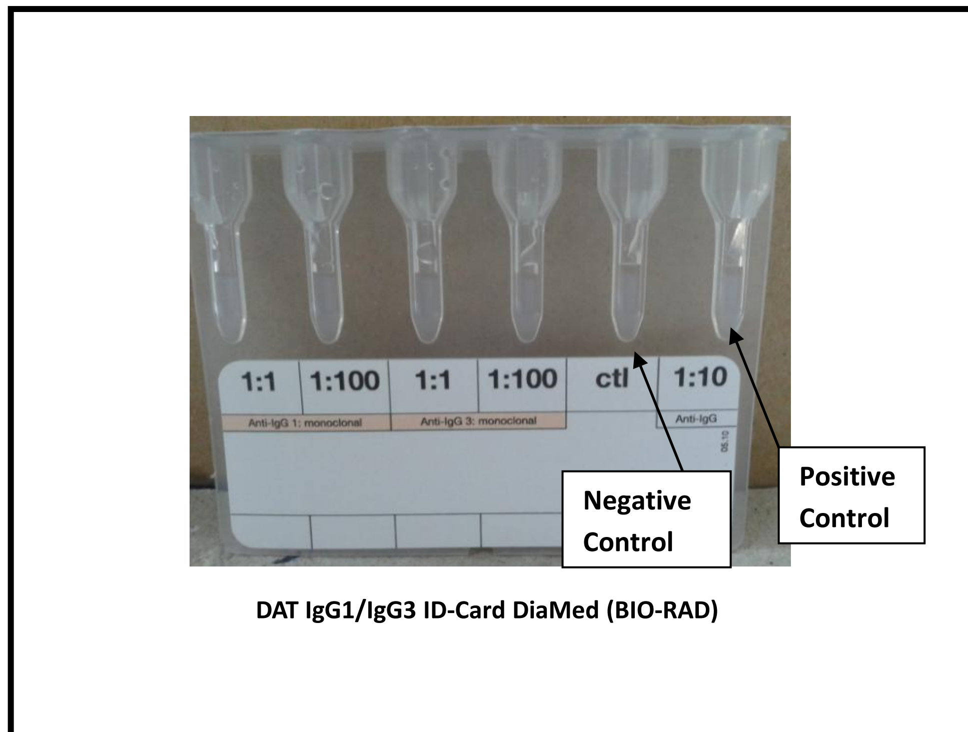
If the positive or negative controls failed the test was considered un-interpretable. The test was repeated for these patients with DTT (0.01N in 1:1 concentration, following incubation for 30min at 37 degrees) treated plasma and read when the control came negative.

Interpretation:

A. Positive: Agglutinated cells form a red line on the surface of the gel (strong reaction) or the agglutinates tend to disperse in the gel in a weaker reaction.

Negative: Compact button of cells at the bottom of the microtube.

A positive reaction with the 1:1 dilution has a sensitivity of approximately 1000 Ig1 molecules and 125 anti-IgG3 molecules respectively. A positive reaction in 1:100 dilution is indicative of a higher strength of antibodies in the plasma.



DAT IgG1/IgG3 ID-Card DiaMed (BIO-RAD)

Fig 4: The DAT IgG1/IgG3 card

DAT IgG1/IgG3 card consists of six microtubes as depicted in the figure above. The first two tubes are for detection of IgG1 in titres of 1:1 and 1:100 respectively. The next two wells are coated for IgG3 in 1:1 and 1:100 titre. The fifth tube acts as the negative control for the test, and should be negative for the test to be interpretable. The last well is for overall IgG 1:10 (IAT) which acts as the positive control for the test.

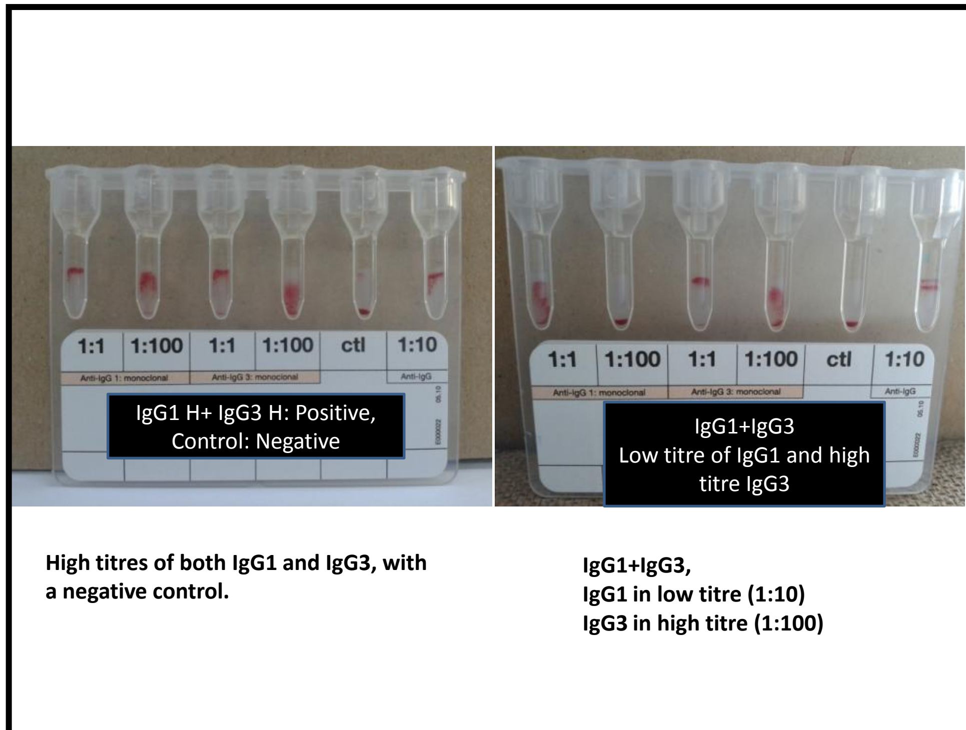


Fig 5: Test results on the card

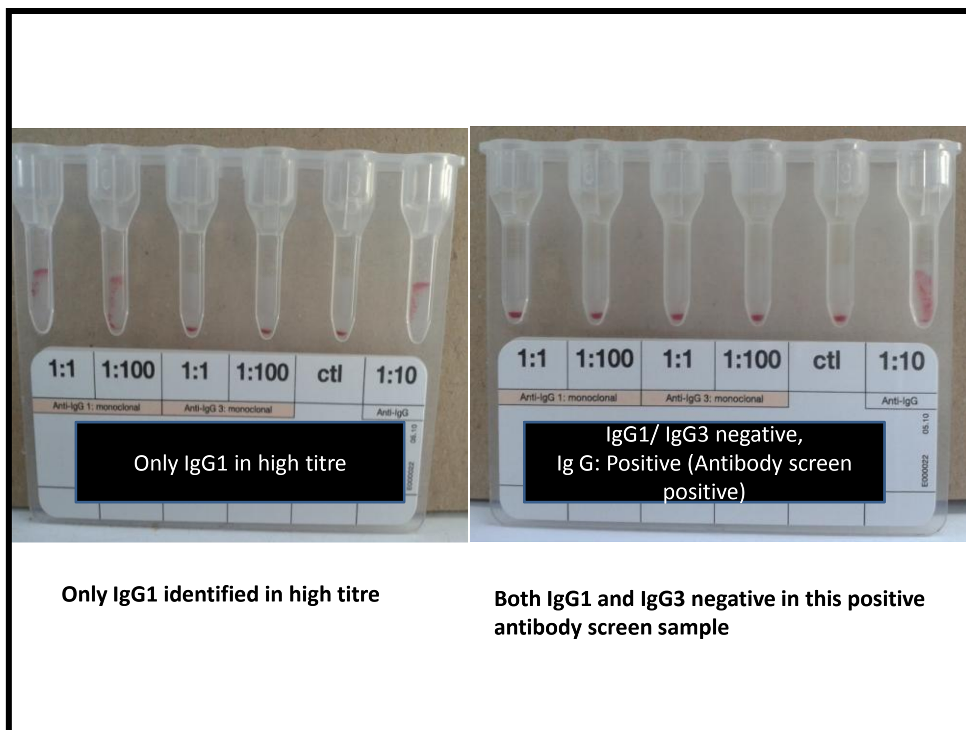


Fig 6: Test results on card

Table 2: Result interpretation on the DAT IgG1/IgG3 card

B. Reactions for Anti-IgG1/Anti-IgG3 subclasses

	IgG1		IgG3		Control	IgG
	1:1	1:100	1:1	1:100		1:10
Reaction	2+	-	-	-	-	3+
Interpretation	Moderate risk of hemolysis (Subclass IgG1; low concentration)					
Reaction	3+	2+	-	-	-	3+
Interpretation	High risk of hemolysis (Subclass IgG1; high concentration)					
Reaction	3+	2+	2+	-	-	3+
Interpretation	High risk of hemolysis (IgG1 high concentration; low concentration IgG3)					
Reaction	-	-	3+	2+	-	3+
Interpretation	High risk of hemolysis (Subclass IgG3; high concentration)					
Reaction	-	-	2+	-	-	3+
Interpretation	Moderate risk of hemolysis (Subclass IgG3; low concentration)					
Reaction	2+	-	3+	2+	-	3+
Interpretation	High risk of hemolysis (IgG1, low concentration, IgG3 in high concentration)					
Reaction	2+	-	2+	-	-	3+
Interpretation	Moderate risk of hemolysis (Subclasses IgG1 and IgG3, low concentration)					
Reaction	3+	2+	3+	2+	-	3+
Interpretation	High risk of hemolysis (Subclasses IgG1 and IgG3, high concentration)					

Note: The first two mircotubes contain Anti-IgG1 (cell line M345/795) in two dilutions and third and fourth microtubes contains Anti-IgG3 (cell line M346/805) in two dilutions respectively.

Reference: DAT IgG1/IgG3 ID-card, (Diamed GmbH 1785 Cressier FR Switzerland) BIO-RAD manual

STANDARD OPERATING PROCEDURES FOR ALL THE OTHER TESTS:

B. ANTIBODY SCREENING:

It is a test done routinely in our blood bank for all donor and patient samples to detect irregular antibodies. It is based on the IAT. This test uses patient's plasma/serum against reagent red cells to detect unexpected antibodies.

Screen cells used for this test are commercially prepared 2-5% suspension of group O cells obtained from individual donors and phenotyped for the most commonly encountered and clinically important red blood cell antigens, which is in contrast to the IAT which utilizes in-house preparation of O-pooled cells from three phenotyped donors. Screen cells require the expression of the following antigens as per the BCSH guidelines for antibody screening in antenatal women: D, C, E, c, e, M, N, S, s, Le^a, K, k, Jk^a, Jk^b, Fy^a, Fy^b.(2) Group O red cells are preferred since it does not contain A and B antigens on the cell membrane, minimizing the chances of reacting anti-A and anti-B antibodies, and making it insensitive to the "regular or expected antibodies".

Both Rh Positive and Rh Negative cells are used in the screen cells. In the three cell vial which is commonly used in our blood bank, the Rh genotypes expressed are: (homozygous) R1R1, R2R2, R1R1. The homozygous expression of antigens for the major and clinically significant blood groups is crucial in choosing the appropriate screen cells to detect antibodies to antigens which even show dosage effect (eg. Kidd, MNS and Duffy). red cell.

Material Required:

1. Coombs cassette
2. Biorad system centrifuge.
3. Micropippette and tips

4. Low ionic strength solution (LISS)

5. DiaCel – I-II-III Asia cells (Three cell panel reagent cells)

6. Heat block (Incubator)

Procedure:

Antibody screening is done using commercial 3 cell panel DiaCel-I-II-III Asia

Antibody screening is done using a Coombs cassette

1. Label the coombs cassette with patient/donor ID
2. And label three columns S1, S2, S3 respectively
3. Pipette 50 microlitres of reagent cells into columns labelled as S1,S2,S3 respectively
4. And pipette 25 microlitres of patients / donor's serum or plasma into all three wells.
5. Incubate for 15minutes at 37 degrees.
6. Centrifuge the card in Biorad centrifuge for 10 minutes at 900 rpm.
7. Read the cassette from both sides
8. Read and record the results.

<u>Gel card technique and range of reactions</u>

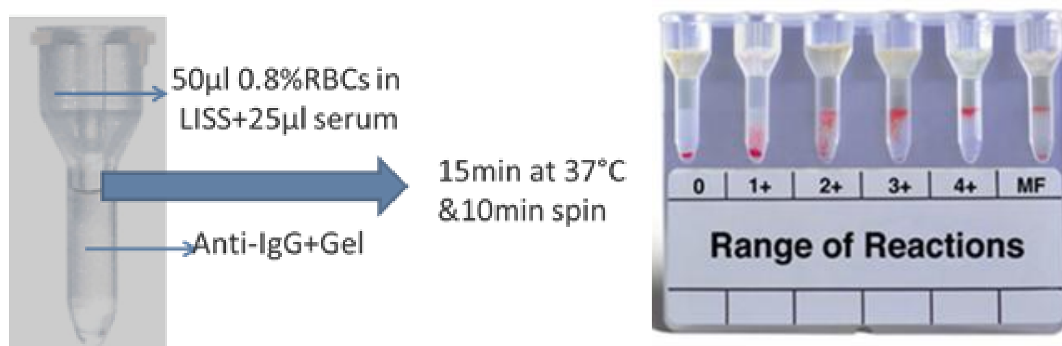


Fig 7: Interpretation of results using CAT

Interpretation of column agglutination testing:

- **Negative** – All cells settle at the bottom.

- **1+ Reaction** - A button of cells at the bottom with most aggregates is remaining at the lower half of the beaded column
- **2+ Reaction** - A small button of cells may also be visible at the bottom while aggregates observed throughout the length of the beaded column
- **3+ Reaction** - Most aggregates remains in the upper half of the beaded column.
- **4+ Reaction** - Agglutinated cells form a band at the top of the beaded column.
- **+/- Reaction** - Indeterminate.
- **Weak Reaction** - Granular suspension
- **MF Reaction** **-** Mixed field agglutination / double population.

Reporting of results:

Antibody screen – positive or negative –mentioning which screen cells are positive and strength of positivity. Entry of results to be made on the patient card filed in blood bank.

Quality Control:

To check the proficiency of screen cells and the coomb's card, antibody screening is done with diluted anti-D IgG (1 in 10 dilution) as positive control and BLISS as negative control.

C. ANTIBODY IDENTIFICATION

Scope: This test enables identification of clinically significant antibodies and thus enables physicians to make clinical decisions regarding transfusions and antenatal interventions as appropriate

Sample required: Properly labelled 4ml EDTA samples /6ml clotted serum sample is required.

Material Required:

1. Coombs cassette

2. Ortho Biovue system centrifuge
3. Micropipette and tips
4. Work rack/card holder
5. LISS
6. Heat block (Incubator)/ID Incubator
7. 11 cell panel cells
8. Patient cells – for auto control

Procedure – Using 11 Cell panel cells from Ortho Clinical Diagnostics:

1. Antibody identification is done on the Ortho Coombs cards in our blood bank
2. Label the Coombs cards with patient hospital number and also mark the wells.
3. Pipette 10 microlitres of panel cells (from cell vials 1-11) into columns labeled 1-11 and add patient cells (3% suspension) into well 12.
4. Pipette 40 microlitres of patients / donor's serum or plasma into all 12 wells followed by 40 microlitres of BLISS
5. Incubate at 37ºC for 10 minutes in the heat block
6. Centrifuge the card for 5 minutes which will spin at 800 rpm for the first 2 minutes followed by 1500 rpm for the next 3 minutes (biphasic centrifugation)
7. Read the cassette from both sides
8. Grade and record the reactions
9. Enter the reaction pattern onto the 11 cell panel worksheet

Interpretation:

1. The first well to be looked at prior to interpretation is well 12 – (the auto control)

2. If the auto control is positive – the test is usually un-interpretable unless it is extremely weak compared to the positive reactions in other wells
3. If the auto control is negative – then look at all the negative wells and cross out the antigens corresponding to the cells in that well
4. Following this step look at the remaining antigens which are uncrossed out at the top row of the worksheet and see if the pattern of positivity fits into an antibody present against anyone / mixture of antigens
5. If the auto control is positive, only wells with a higher grade of positivity should be identified as positive. Attempt to follow the process of interpretation as described above
6. A standard approach has been to require that 3 antigen positive red cells react and that 3 antigen negative cells fail to react for each specificity identified
7. Correlate result by also doing a phenotype of the patient's red cells – they should be negative for the antigen to which a corresponding alloantibody has been identified.

Reporting of results: Report should state that the antibody that was finally identified.

D. COOMB'S TEST (DIRECT ANTIGLOBULIN TEST)

Scope: To detect the presence of incomplete antibodies and compliment binding antibodies in serum/plasma or those coated on patients red cells

Principle: Washed red cells which are coated with immune antibodies (commonly IgG) and complement components (generally C3) will show agglutination with broad spectrum AHG

reagent. The coating (sensitization) of red cells can occur in vivo or in vitro following incubation at 37deg C with serum containing antibody.

Tests performed: Direct Antiglobulin test (DAT)

Methodology:

Coombs test can be done by two different techniques

A) Tube technique

B) CAT

Sample: Properly labeled 4ml EDTA samples /6ml clotted serum sample is required.

Haemolysed samples are not acceptable.

Material Required for Tube Method for DAT:

Test tube 10X75 mm

Test tube rack

Marker

Centrifuge / Serofuge

Pasteur pipette

Slide

Normal saline

Microscope

Water bath at 37ºC

Reagents for DAT:

1. Coombs serum
2. Negative control for DAT – 3-5% suspension of saline washed pooled O group cells

3. Positive control for DAT - Sensitized O pooled cells – (made by adding 0.5ml of IAT positive control to 1 ml of washed and packed O pooled cells and incubating at 37 deg for ½ an hour.

Procedure for DAT:

Tube technique:

1. Take 3 tubes and label as patient ID, Positive control and Negative control respectively
2. Add one drop of patient cells, positive control, negative control to the respective tubes
3. Wash three times with normal saline
4. After a final wash, decant the supernatant completely
5. Add one drop of coombs serum to all the three tubes, mix and centrifuge at 3000rpm for 15 seconds
6. Then dislodge the button gently and read both macroscopically and microscopically and grade the reactions as above.

GRADING OF THE REACTION FOR ALL TESTS DONE BY TUBE TECHNIQUE: (Reference AABB Manual)

No agglutination	Negative
Small aggregation	1+ Positive
Medium size aggregates	2+ Positive
Several large aggregates	3+ Positive
One solid aggregate	4+ Positive

The presence of haemolysis in the supernatant also indicates a positive reaction.

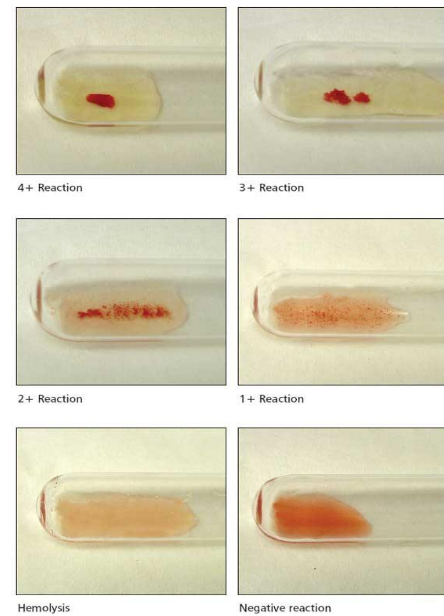


Fig 8: Grading tube test reaction

Column Agglutination Technique:

Materials Required: Coombs card

ID Card Centrifuge

Normal saline

Micropipette and tips

Biovue system work rack/ID card holder

LISS

Heat block (Incubator)/ID card Incubator

Procedure for DAT using Bio-Rad card

1. Ensure that the cassette is at room temperature before use
2. Label the cassette appropriately with patient ID, and control ID
3. Add 50µl of 0.8% suspension of patient, positive and negative control cells respectively to all the 3 wells.
4. Centrifuge the card in ID Card centrifuge for 10 minutes at 910rpm
5. Read and record the reactions (read the card from both sides)

Grading of results by CAT as mentioned previously.

Precautions:

1. Adequate washing of sensitized cells is the critical part of the test. A trace amount of residual globulin, due to improper washing causes neutralization of the AHG Serum.

2. A false negative reaction can occur if testing is interrupted or delayed. Washing of sensitized cells, addition of AHG serum and centrifugation must be quick and uninterrupted to avoid elution of antibodies attached to red cells and neutralization of AHG Serum.

3. Inappropriate serum to cell ratio.

4. Failure to add AHG reagent may give false negative results.

5. Under centrifugation may not provide optimal conditions for agglutination.

6. Failure to check the negative reaction microscopically.

7. Over centrifugation packs red cells so tightly that they cannot be dispersed completely.

8 Red cells may be agglutinated before washing and this may persist through the washing phase i.e. adsorption of complement during storage. In such cases a positive antiglobulin test may be incorrectly interpreted as a result of the IgG or complement coating.

Documentation and Result entry: All reactions to be entered in the grouping worksheet All results to be entered with strength of reactivity on the patient grouping card.

E. ANTIBODY TITRATION:

Principle: Titration is a semi-quantitative technique of measuring the concentration of an antibody in a serum. The titre of an antibody is usually determined by testing the serial two fold dilution of the serum against selected red cells

Specimen required: Properly labeled 4ml EDTA samples /6ml clotted serum sample is required. Haemolysed samples are not accepted

Materials:

1. 100µl automatic pipette and pipette tips
2. Test Red blood cells: 3-5%
3. Sterile 12X75ml test tubes
4. Sterile saline 0.9%
5. Serofuge
6. Glass slides
7. Water bath
8. Microscope

Procedure:

1. Label the tubes according to the serial dilution usually 1:2 through1:1024
2. Deliver one volume (100ul) of saline into all tubes
3. Add one volume (100 µl) of the DTT treated serum/ plasma to tube 1:2
4. Mix the contents of the tube several times and transfer one volume (100 µl) of the mixture from 1:2 to 1:4 and continue through the remaining tubes.
5. Discard 100 µl from the tenth tube i.e. 1:1024
6. Add one volume (100 µl) of 3-5% saline cell suspension of appropriate red cells to each tube
7. Mix well and Incubate the serum cells mixture at 37deg C for one hour
8. After one hour wash three times with normal saline
9. Soon after the third wash decant the supernatant and add AHG serum
10. Spin at 3000rpm for 15 seconds.
11. Gently dislodge the button. Examine the test results macroscopically and grade and record the reaction.

12. If there is agglutination in the tube containing the most diluted serum, an end point has not been reached. Prepare and test additional dilutions.

Grading of the Reaction: As for all test done by tube technique

Interpretation: The titre is the reciprocal of the highest serum dilution at which macroscopic agglutination is observed.

Reactivity in the dilution control serum and no reactivity in the DTT treated indicate an IgM antibody

Reactivity in the dilution control serum and in the DTT treated serum indicates an IgG and IgM mixture

No reactivity in the dilution control serum indicates dilution of weak antibody reactivity and an invalid test

Table 3: Antibody titration using DTT treated and not treated serum and interpreting results.

Test Sample	1:2	1:4	1:8	1:16	1:32	Interpretation
Serum+DTT	3+	2+	2+	1+	0	IgG
Serum+PBS	3+	2+	2+	1+	0	
Serum+DTT	0	0	0	0	0	IgM
Serum+PBS	3+	2+	2+	1+	0	
Serum+DTT	2+	1+	0	0	0	IgG+IgM
Serum+PBS	3+	2+	2+	1+	0	

Prozone Reaction: In a serum or plasma with a very high antibody concentration the prozone phenomenon may cause a weaker reaction in the lower dilutions than in the higher dilutions.

Results: The titre of the antibody should be reported as follows:

Coombs titre performed with DTT treated serum –

Serum with PBS - 512   Serum with DTT -128

Results suggest a mixture of IgM and IgG antibodies.

BIAS:

All efforts were be made to eliminate any bias in selection of patients. Despite this the prevalence of alloimmunization in antenatal women may show skewed results since CMC is a tertiary hospital and cases at risk are referred here. Instrument bias was likely to be negligible as variables were measured using calibrated and automated analyzers in the Clinical Pathology department and blood bank and all the serological tests done in the blood bank were run in duplicates. The column agglutination gel card, which was the novel test done in our study was interpreted by two individuals (principal investigator and an experienced technologist) independently. Investigator bias in interpretation of severity of HDFN was avoided as the investigator was not involved in the treatment directly.

SAMPLE SIZE:

Sample size was estimated to be approximately 84.

Since severe HDFN due to IgG (IgG1 +IgG3) antibodies occurs in 30% pregnancies with Rh alloimmunization, according to a study conducted in KEM hospital, Mumbai.[1]

Sample size = $4pq/d^2$ (p=30%, q=70% and d= 10%)

= 4x30x70/ 10x10 = **84**

Therefore if IgG1+G3= 28 and rest= 56, we expect 50% **(P_1)** of the first group to be severe HDFN and 10% **(P_2)** of the 56 may have severe disease. To suggest IgG1+1gG3 is more significant than

IgG1 alone: $Z\alpha=1.96$, $Z\beta= 0.84$

Sample size= $2pq\,(Z\alpha-Z\beta)^2/(P_1-P_2)^2$

$= 2x30X70X8/ 40X40$

$= 21$

However in our study sample size achieved 68, since of the 85 women subtyped for IgG, 17 were lost to follow up.

Reference for calculation of sample size: Iyer YS, Kulkarni S.V.,Gupte S.C. KEM hospital, Mumbai; Distribution of IgG subtypes in maternal anti-D sera and their prognostic value in Rh haemolytic disease of newborn: Acta Hematologica 1992;88(2-3):78-81

STATISTICAL ANALYSIS:

Mean, Standard deviation and 95% confidence interval (CI) was calculated for all continuous variables and Pearson and Fischer's exact chi square was used for comparison of categorical data.

Univariate and binary logistic regression analysis was used for comparing the categorical data and to calculate the odds ratio. P value <0.05 was considered significant.

RESULTS:

A total of 3900 antenatal women had blood groups and antibody screens performed at CMCH between January 2013 and July 2014 and 85 of them who were antibody screen positive (2.17%) were included in the study. This was irrespective of their period of gestation.

Demographics:

Our cohort of patients included women mostly from Tamil Nadu (84%), and some from Andhra Pradesh (12%) and a few other states including Jharkhand, West Bengal and Karnataka

Age:

The mean age of the study population was 27 years (Max. 38, Min. 20years).

Parity / Obstetric history:

With regards to parity, 57 (74%) of these patients were multiparous, and 20 (26%primiparous (Fig 9). History on parity was not available for 8 women.

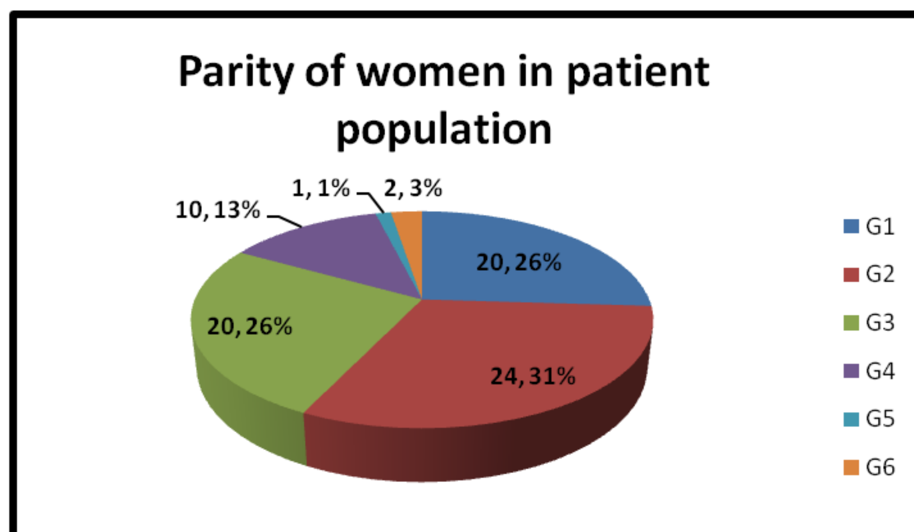


Fig 9: Pie chart depicting the parity of the women found to be antibody screen positive

Previous bad obstetric history/sensitizing event:

A bad obstetric history such as previous 1st or 2nd trimester loss, still births or intrauterine deaths or other fetal anomalies was recorded for 36 (63%) women among the multiparous group as shown in Fig 10.

It was noted that among the multiparous group of women, those with gravid of 4 and more, all had a BOH (100%). However it is important to note that these numbers are small. In women who are G2 / G3 bad obstetric history was noted in 36% and 56% of women respectively.

A transfusion history was documented for only two patients of our entire study cohort.

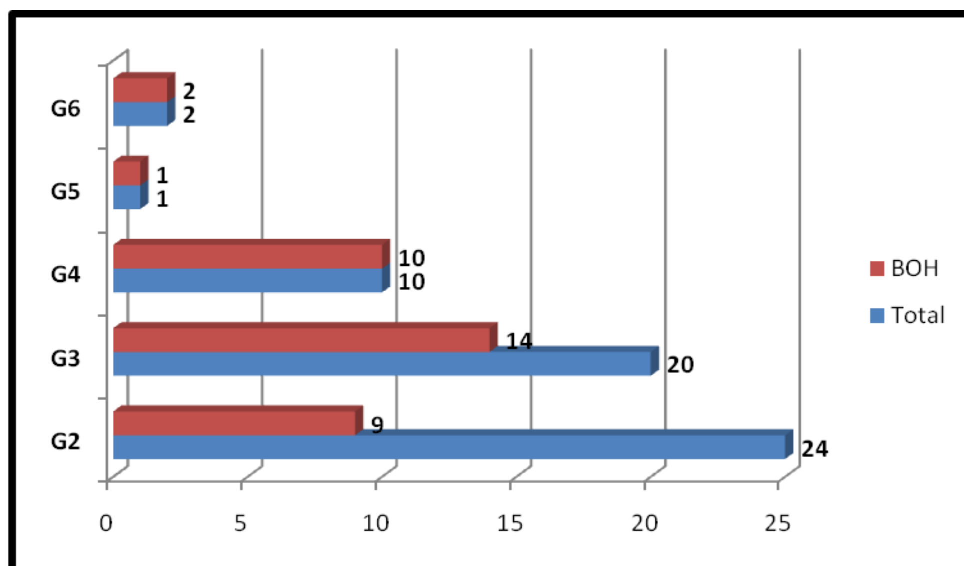


Fig 10: Depicting the BOH among women of different parity groups

Immuno-haematology parameters:

All blood groups were performed on two different platforms which included the microtitre plate and the column agglutination technique. For definition of D positivity D VI negative cards and D VI positive cards were both used on all samples in order to define any DVI positive individuals. However, in our study population no D VI positive individuals were identified.

Antibody screen: 85 women were included in the study having a positive antibody screen on the commercial three cell panel from ID- DiaCell I-II-III Asia, DiaMed.

The three cell panel included the following phenotypes - and a Mia phenotype as well:

- R1R1 (CCD.ee)
- R2R2 (ccD.EE)
- R1R1 (CCD.ee)

Antibody identification: Antibodies were further identified using the eleven cell panel from Ortho Clinical Diagnostics. 76 of these women were Rh negative. 73 of these 76 (85.8%) women had an anti D identified. 3 of 76 (3.53%) had more than one antibody identified namely anti-D and C.

The remaining 9 antibodies were identified in Rh positive women. The antibodies identified included Anti-M in 3 (3.53%), Anti-E in 1 (1.17%), Anti-S in 1(1.17%), Anti-c in 1 (1.17%) and Anti-Lea in 2 (2.35%)

The antibody specificity could not be identified with the panel on hand in one patient. The diagrammatic representation of antibodies identified is shown in the figure below (Fig 11).

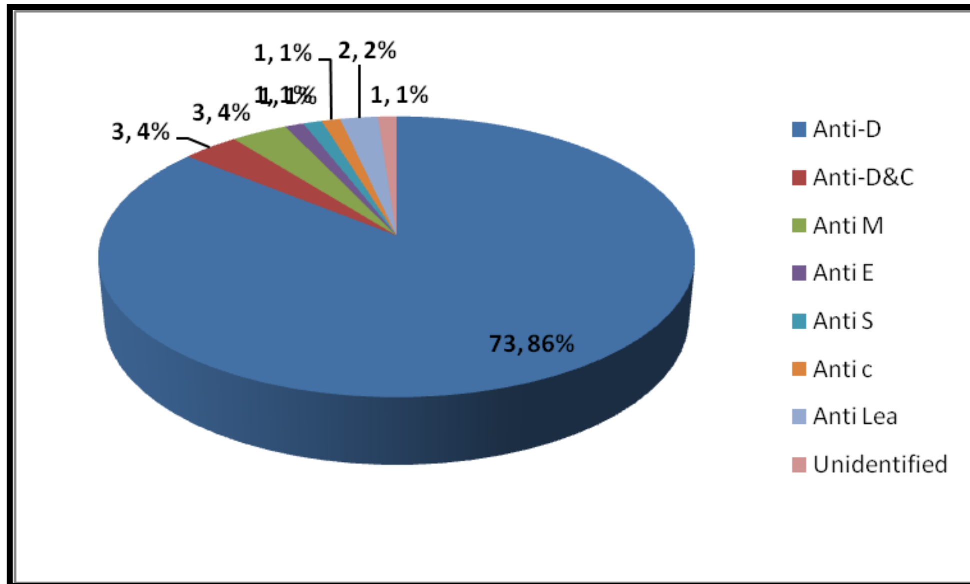


Fig 11: Pie chart showing the profile of antibody specificities in our study population

Antibody titration:

This test was performed when requested by the clinician and was available for 58 alloimmunized women who were part of our study. 31 women (53%) had titres of less than or equal to 64, followed by 15 (26%) with titres between 128 and 256, while the rest 12 (21%) had titres of greater or equal to 512.

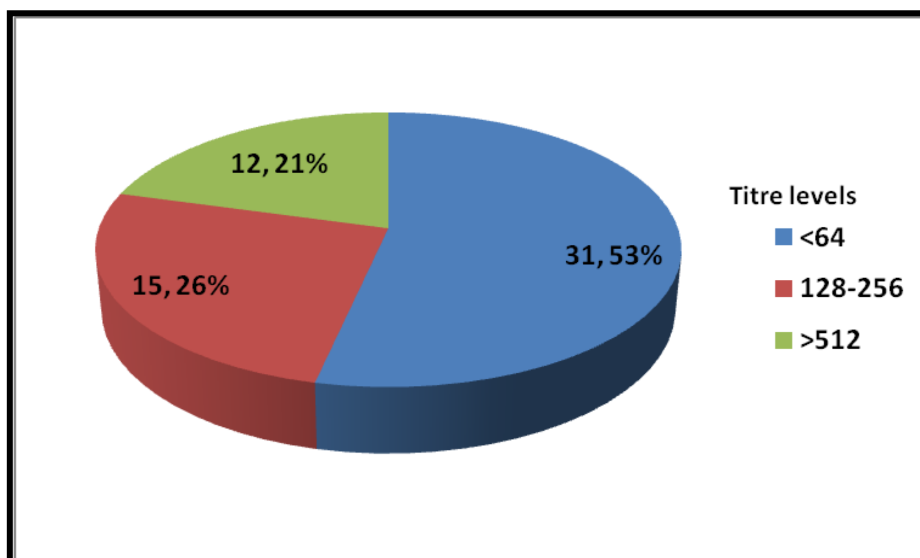


Fig 12: Representing spread of antibody titre levels in the antenatal women

IgG subclasses identified (IgG1 and IgG3):

This was performed using a DAT IgG1/IgG3 ID-Card from DiaMed GmbH, using the CAT. This test contains dilutions of IgG1 and IgG3 in 1:1 and 1:100 strengths alongside a positive and negative control. The strength of each of the subclass IgG1 and IgG3 could therefore be interpreted according to positivity recorded on the relevant microtubes representing strengths of either 1:1 (low titre) or 1:100 (high titre).

Our study showed the overall prevalence for IgG1 and /or IgG3 subclasses in our population of alloimmunized antenatal women to be 48.23%.

Table 4: IgG1 and IgG3 results in women with a positive antibody screen

IgG1/IgG3 result of patients with antibody screen positivity (n= 85)	
IgG1/IgG3 Positive	**IgG1/IgG3 Negative**
41 (48.23%)	44 (51.76%)

Analysing it within subclasses showed the prevalence of IgG1/IgG3 as follows:

- IgG1 only: 20%
- IgG3 only: 4%
- IgG1 + IgG3: 25%
- Negative for both IgG1 and IgG3 - 51%

** 32 women had high titres (in 1:100 dilution) for either one or both the IgG subclasses.

The positivity was contributed predominantly by a mixture of IgG1 and IgG3 (51%followed by IgG1 alone (42%), as shown in the diagram below.

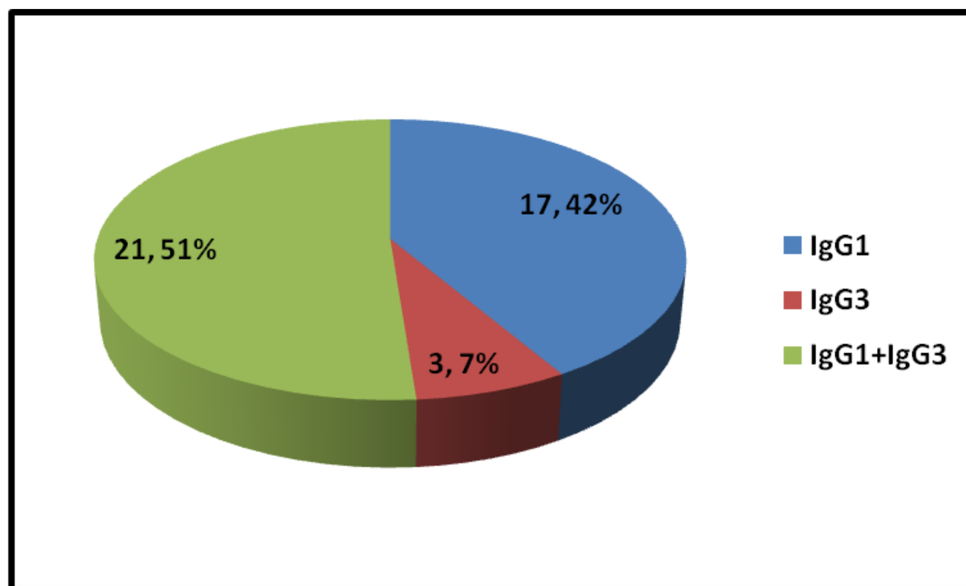


Fig 13: Representation of IgG1/IgG3 subclasses in our population

IgG1 and IgG3 titre levels:

Our results show, that 44% of our study group did not have either IgG1 or IgG3, while for 12% both these subclasses co-existed in high titres. It was interesting to note, that all three patients with IgG3, had the antibodies in low titres. (Table 5)

Table 5: Titre of IgG1 and IgG3 antibodies in patients (n=85)

IgG1	IgG3		
	Absent	**Low titre (1:1)**	**High titre (1:100)**
Absent	44 (51.8%)	3 (3.5%)	0
Low titre (1:1)	5 (5.9%)	1(1.2%)	5 (5.9%)
High titre (1:100)	12 (14.1%)	4 (4.7%)	11 (12.9%)

Antibody specificity and IgG subtypes:

We performed IgG1 and IgG3 subclass testing on all antibody screen positive women and tabulated it against the antibody specificity identified.(Table 6)

We noted that for women with only anti-D, 16% had IgG1 and 29% had both IgG1+IgG3. 51% had neither. However in the three women with anti-D and C, all had only IgG1 identified. In the patients with anti-M, 2 (67%) had IgG1. The remaining numbers are too small to derive any conclusions from. .

Table 6: IgG subclass for all antibody specificities

Antibody	IgG1	IgG3	IgG1+IgG3	None	Total
D	12 (16.4%)	3 (4.10%)	21 (28.77%)	37 (50.68%)	73
D&C	3 (100%)	0	0	0	3
M	2 (66.67%)	0	0	1 (33.33%)	3
E	0	0	0	1 (100%)	1
S	0	0	0	1 (100%)	1
C	0	0	0	1 (100%)	1
Lea	0	0	0	2 (100%)	2
Unidentified	0	0	0	1 (100%)	1
Total women with antibody screen positive=					**85**

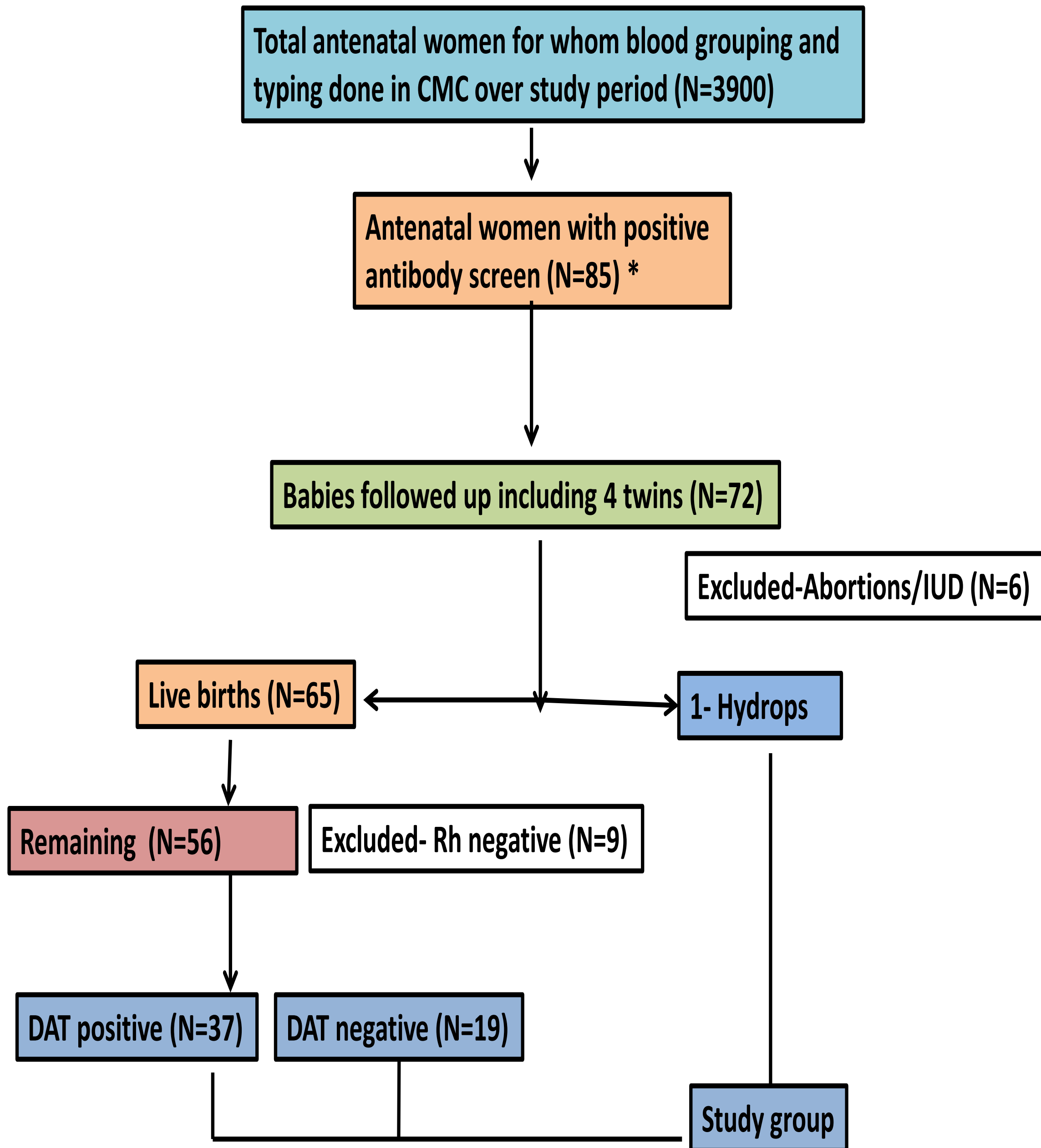
Follow up of Pregnancy and Diagnosis of HDFN:

65 babies were live births in our study group, while loss of pregnancy was seen in 6 pregnancies. There was an additional baby with clinically diagnosed hydrops with severe fetal anemia prescribed for intrauterine transfusion (which was not done) at 25 weeks of gestation, and further lost to follow up. This case has been included in further analysis as "severe" HDFN, even though the outcome could not be documented. In this case the mother had anti D, and had both IgG1 and IgG3 in high titres. The antibody titre level for this mother was 512.

Among the loss of pregnancies 2 were intrauterine deaths (in the first case, the fetus was Rh negative and the second was a primiparous woman with fetal movement not perceived at 22 weeks of gestation), 2 were missed abortions, and 1 was a termination of pregnancy due to severe –pre-eclampsia. Another baby was a still birth among the twin pregnancies, but there was no clinical evidence of hydrops. These six cases were excluded due to causality being excluded or unestablished.

Among the four twin pregnancies, two women delivered babies with a negative DAT. For one woman, both babies had a positive DAT. In the last case, one baby was DAT positive and the second was a still birth (as mentioned above). Each baby has been dealt with separately in the analysis.

Additionally, nine Rh negative women with only Anti-D antibody identified, delivered Rh negative babies. They were excluded from correlation with severity of HDFN.



*17 pregnancies were lost to follow up.

Fig 14: Flowchart to illustrate patient follow up and results

Data about the newborns:

Among the 65 babies followed up in the study, we observed that approximately 40% required a caesarean section while the rest had a normal or assisted delivery. 46% of the births were prior to 37 weeks and the rest were term or post term deliveries. In our newborn population 58% newborns were of normal weight (>2.5kg), 37% were less than 2.5kg (Low birth weight), 2% with very low birth weight (between 1.5 and 1kg) and 3% were extremely low birth weight (less than 1kg).

Of the 37 DAT positive live births, 10 required no treatment and were only followed up with haemoglobin and bilirubin levels, while the remaining 27 required either an IUT, phototherapy, exchange transfusion or multiple treatment modalities. It was noted that 3% (1/37) required an intrauterine transfusion and 14% (5/37) needed an exchange transfusion, and one required a top up transfusion

.Table 7: The baseline features and treatment requirement of the live births in the study population

Variables	Groups	n(%)
Delivery (Live births)	Normal	38(58.46)
	LSCS	27(41.53)
Term	Pre term	30(46.15)
	Term	35(53.84)
Weight	ELBW	2(3.07)
	VLBW	1(1.53)
	LBW	24(36.92)
	Normal	38(58.46)
Sex (Live births)	Male	36(55.38)
	Female	29(44.61)

HDFN in Newborn:

The newborn was evaluated for presence or absence of HDFN. If present, severity was assessed. Classification of hemolysis and severity of disease was done in consultation with the department of Neonatology into one of three grades - namely Mild/ Moderate/ Severe HDFN. This was based on therapeutic interventions as per guidelines laid down by the Department of Neonatology for this study, since modalities of therapy are decided based on severity of disease

Table 8: Categorization of disease severity on the basis of treatment modality required in DAT positive live newborns

Categorization of disease severity on the basis of treatment requirement (DAT positive; n= 37)		
Mild disease	**Moderate**	**Severe***
10 (27.00%)	20 (54.05%)	7 (18.91%)

*Total patients with severe disease were 8, seven live births and one hydrops with outcome not known.

Correlating IgG1 IgG3 subclass typing results with presence / severity of HDFN

Women with IgG1 only:

There were 17 women who had IgG1 only, 5 with low titre and 12 with high titre. Of these, 5 with high titre were lost to follow up (all with high titre of IgG1).

Follow up was therefore available for 12 women (2 who delivered twins, one of which was a still birth).Of these 5 were low titre and 7 were high titre IgG1.

Among the 5 patients with low titre IgG1, one baby was Rh negative born to an Rh negative mother with anti D and was thus not included for follow up. The remaining 4 babies born to mothers with low titre IgG1 developed moderate disease, requiring phototherapy. Among the 7 mothers (8 babies, including the twin) with high titre of IgG1, one baby had mild disease, five babies had moderate HDFN, while two had severe HDFN category.

The two newborns with severe disease were born to mothers with Anti-D, with antibody titres of 128 and 512 respectively.

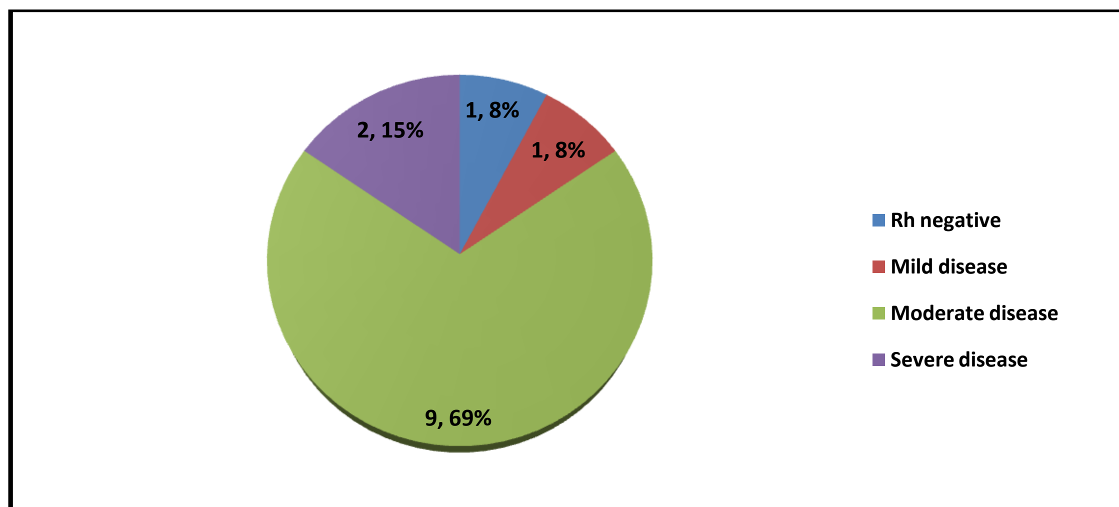


Fig 15: To illustrate the outcome of pregnancy in women with only IgG1 antibodies (5 with high titre IgG1 were lost to follow up). Both severe HDFN cases had high titres of IgG1.

Women with only IgG3:

Three patients were identified to have only an IgG3 antibody in our study population and interestingly all three of these patients had the antibody in low titres (1:1). One of them was lost to follow up. The two newborns in the category of IgG3 alone in maternal serum, suffered from mild and moderate form of disease respectively.

Women with both IgG1+IgG3:

Among the 21 women with IgG1+ IgG3, 12 of the 21 (57%) had high titres of both IgG1 and IgG3, and hence showed positive results in the 1:100 dilution microtubes of both IgG1 and IgG3. There were four patients each in the category with high IgG1 and low strength IgG3 and vice-versa. There was only one patient who had low levels of both the subclasses.

Table 9: Distribution of IgG1 and IgG3 titres in patients with a combination of these subclasses

In patients with IgG1 + IgG3 (n=21)			
IgG1 H + IgG3 H IgG1 1:100 IgG3 1:100	**IgG1 H + IgG3 L** IgG1 1:100 IgG3 1:1	**IgG1 L + IgG3 H** IgG1 1:1 IgG3 1:100	**IgG1 L + 1gG3 L** IgG1 1:1 IgG3 1:1
11 (52.38%)	4 (19.04%)	5 (23.81%)	1 (4.76%)

H-High titre (1:100), L- Low titre (1:1)

Of the 21 patients with both IgG1 and IgG3 identified, 4 were lost to follow up (1- IgG1H +IgG3H, 1- Ig1 H+IgG3L, 2- IgG1 L+ IgG3 H).

Of the ten patients with high titres of both IgG1 and IgG3 who delivered in CMCH, five had newborns with moderate HDFN, and five had the disease in the severe form.

Of the three patients with high titre of IgG1 and low titre of IgG3, one newborn was DAT negative; the other two had had mild and moderate disease respectively.

Of the three with IgG3 in high titre and IgG1 in low titre, two had babies with moderate HDFN and one was severe.

The one mother with both the antibodies in low titres, gave birth to a baby with moderate HDFN

Overall more than 90% of babies born to mothers with a combination of IgG1 and IgG3 developed moderate to severe disease irrespective of the titre of antibody.

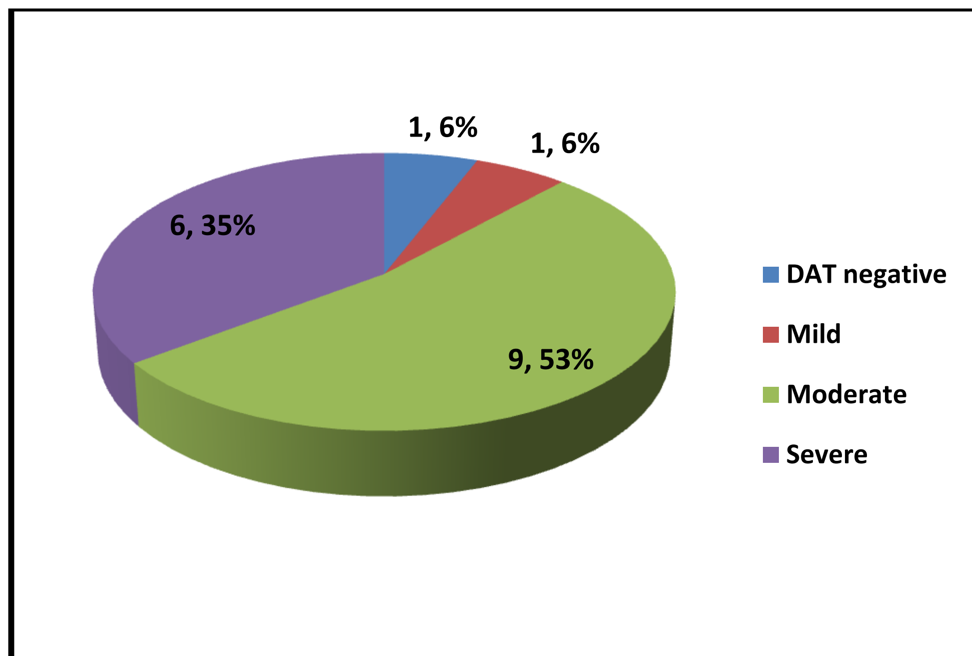


Fig 16: Pie chart to illustrate outcome of pregnancies in women with IgG1+IgG3 (of 17- five were lost to follow up).

Negative for both IgG1/IgG3:

There were 44 mothers negative for IgG1 or IgG3. Among them 7 were lost to follow up. Of the remaining 37, two mothers had twin deliveries. There were also 5 IUD/abortions in this group which were excluded for other causes. Of the 34 babies present, 8 were Rh negative babies born to mothers with Anti-D. Of the remaining 26, 18 were DAT negative and 8 were positive. Of these 8 were babies, 7 were categorized as mild disease (88%), except one who was labelled as moderate HDFN (12%).

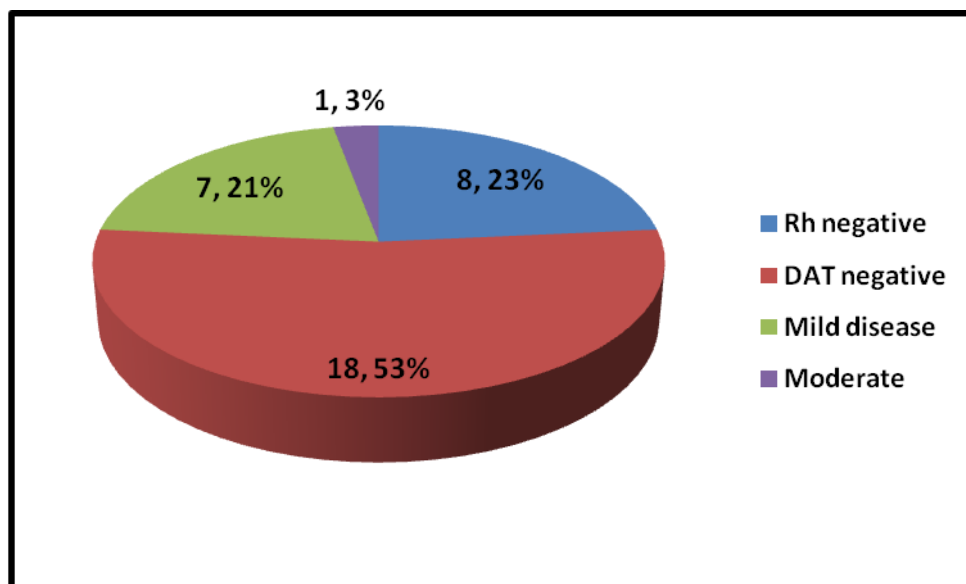


Fig 17: Pie chart to illustrate the severity of disease in babies born to mothers, negative for IgG1 or IgG3

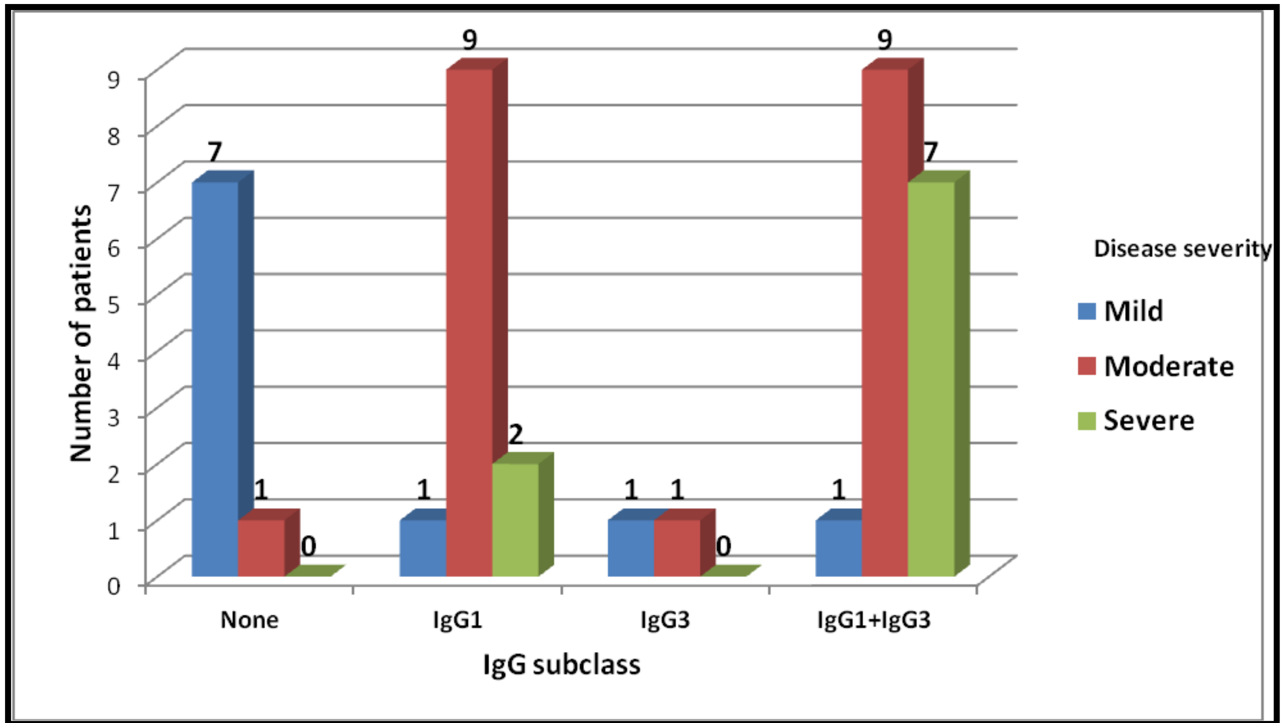


Fig 18: Severity of HDFN in comparison to IgG1/G3 subclass in mother (baby with clinical hydrops included)

Of the total eight severe cases of HDFN seen in our study (including the baby with clinically diagnosed hydrops), we noted that two were born to mothers with high titres of IgG1 alone while the rest were born to mothers having both IgG1+IgG3.

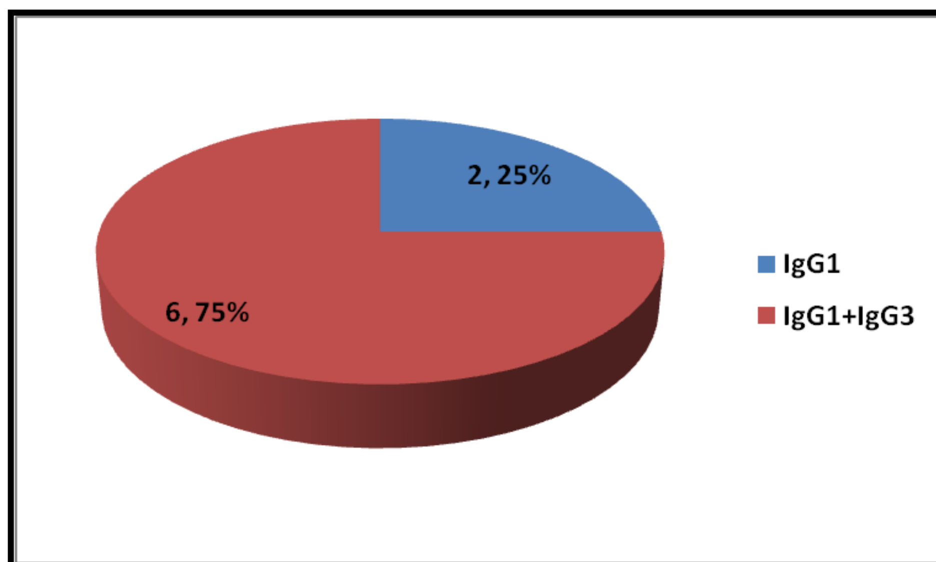


Fig 19: Pie chart to show the patients of Severe HDFN and the IgG subclass in the maternal serum

Analysis:

Correlating disease severity with the presence or absence of IgG1/ G3 subtypes

The Pearson's Chi square test was applied to assess significance of the impact of IgG1 subtype positivity on HDFN severity.

The table below compares severity of HDFN in babies born to mothers with

1. No IgG1 or IgG3
2. IgG1 or IgG3
3. Both IgG1 and IgG3

We observed a highly significant difference ($p<0.001$) in severity of disease when comparing mothers negative for IgG1 and G3 as against mothers with one subtype IgG1/ IgG3 and a combination of IgG1+IgG3 which was progressive with P values which were significant between each subgroup ($p<0.001$).

Table 10: Comparing the IgG subclass and disease severity in all newborns

IgG type	**Disease outcome**		**Total**
	No/Mild	**Moderate/Severe**	
None	25	1	26
	86.20%	3.57%	45.61%
IgG1/IgG3	2	12	14
	6.89%	42.86%	24.56%
IgG1+IgG3	2	15*	17
	6.89%	53.57%	29.82%
Total	29	28	57
	100.00%	100.00%	100.00%
p value <0.001			

*Baby with hydrops with documented clinically diagnosed hydrops was included in the analysis under severe disease category, with mother having IgG1+IgG3

This difference was still significant when comparing only DAT positive cases ($p<0.001$).

Table 11: Comparing severity of HDFN with respect to IgG subclass in live newborns with DAT positive result.

IgG type	Disease outcome		Total
	Mild	**Moderate/Severe**	
None	7	1	8
	70.00%	3.70%	21.62%
IgG1/IgG3	2	12	13
	20.00%	44.44%	35.13%
IgG1+IgG3	1	14	15
	10.00%	51.85%	40.54%
Total	10	27	37
	100.00%	100.00%	100.00%
P< 0.001			

*"None" - patients with IAT positive but not having IgG1 or IgG3,

For eight of our maternal samples, the negative control showed a positive reaction while performing the test. These samples were further treated with DTT (0.01N) in equal volume for 30 minutes at 37 degrees, and the test repeated. The negative control was negative after DTT treatment and the test interpretable. This highlighted an underlying IgM component of Anti-D along with the IgG. Subsequently to confirm the same, titre levels were done on both DTT treated and untreated serum, in a normal saline and Coomb's card. There was a difference between the saline titre and coombs titre – albeit small – being a one titre level difference , indicating a low titre of the IgM, Anti-D alongside the IgG component.

We compared the severity of disease with the IgG subclass in the mother, and we noted that 75% (9/12) of mothers with IgG1 had babies with moderate disease and an additional 17%

(2/12) had severe disease. In the case of IgG1+IgG3, 50% babies had moderate disease, and 44% had the disease in severe form. Overall the Pearson's chi square did not show a significant difference between the impact of IgG1 alone as compared to a combination of IgG1 and IgG3. However, logistic regression analysis shows the odds of developing severe HDFN to increase 18 fold when IgG1 and IgG3 are present in combination.

Antibody titre levels in different groups of IgG subclasses:

Below is the diagrammatic representation of the antibody titre levels in comparison to the IgG subclass which was identified.

Of the 23 women with no IgG1 or IgG3, 21 (91%) had the titre levels of less than or equal to 64, while the remaining 2 (9%) had levels between 128 and 256.

In the group of patients with both IgG1 and IgG3, 8 (38%) had titre levels between 128 and 256, followed by titres of greater than 512 in 7 (33.33%) and less than or equal to 64 in 6 (28.6%) patients.

Table 12: Distribution of the antibody titre levels in the antibody screen positive women

Titre	**Not IgG1/G3**	**IgG1 alone**	**IgG3 alone**	**IgG1+IgG3**	**Total**
≤64	21	3	1	6	31
128-256	2	5	0	8	15
≥512	0	4	1	7	12
Total	23	12	2	21	58

To understand if the IgG1 and IgG3 class contributed to HDFN independent of the overall titre levels, we compared disease severity among those with no IgG1 or IgG3 to those with one or both of these, in the group of patients with overall low to intermediate titre levels(<64 and 128-256). An extremely significant difference was noted ($p<0.001$), which helped infer that even in low titres IgG1/ IgG3 identified could cause more severe disease than in those in which it was absent. In low/intermediate titre levels, we noticed that only one patient with neither IgG1/IgG3 had a baby with moderate disease, however in those with either IgG1 and/or IgG3 despite having low to intermediate titre levels, 16 babies developed moderate to severe HDFN.

Table 13: Comparing disease severity in patients with neither IgG1/IgG3 to those with one or both of these subclasses, in patients with a low or intermediate antibody titre level

IgG subclass	**Disease severity (low/intermediate titre)**		**p value**
	Moderate/Severe	**No/Mild disease**	
Negative for IgG1 and /or IgG3	**1**	**12**	**$p<0.001$**
IgG1 and/ or IgG3 present	**16**	**2**	

*Rh negative babies not at risk were excluded from this analysis

We noted that antibody titre levels also correlated significantly with severity of disease ($p<0.05$). This analysis included all newborns including those with a final DAT negative result. The difference was more significant between <64 titre and the rest because of greater percentage of patients in this group.

Table 14: Comparing the antibody titre levels in maternal serum to the severity of disease in newborns in all patients with titres documented during antenatal visit.

Titre	Disease outcome		Total
	No/Mild	Moderate/Severe	
<64	12	09	21
	75%	37.50%	51.21%
128-256	3	7	10
	18.75%	29.17%	24.39%
>512	1	8	09
	6.25%	33.33%	21.95%
Total	16	24	41
	100.00%	100.00%	100.00%
	p = 0.006		

*Rh negative babies born to mothers with Anti-D were excluded

In view of the significant difference in disease severity among babies born to mothers with no IgG1/ IgG3 identified compared to those with IgG1/IgG3, and also a noticeable difference in titre levels of these two groups, we further compared the titre levels of patients with either IgG1 or IgG3 and those with a combination of the two antibody subclasses.

Due to the small numbers available the Fischer's exact test was used for comparison between titre levels and IgG subclass. There was no significant difference noted in the antibody titre levels of women with only antibody screen positivity and no identifiable IgG1/IgG3, and group of women who had one or both the IgG antibodies ($p>0.05$). Only patients who gave birth to DAT positive newborns were included for this analysis.

Table 15: Comparison of titres in mothers who were IgG1 / IgG3 positive vs those who were negative who both delivered DAT positive babies

Titre	IgG subclass		Total
	None	IgG1/IgG3/both	
<64	5	10	15
	83.33%	40.00%	48.39%
128-256	1	7	8
	16.67%	28.00%	25.81%
>512	0	8	8
	0.00%	32.00%	25.81%
Total	6	25	31
	100.00%	100.00%	100.00%
	p= 0.192		

When we compared the available antibody titre levels between IgG1 or IgG3 (12) patients and those with IgG1+IgG3 (21) we did not find it to be significant (p>0.05).

Table 16: Comparing the antibody titre levels of patients with IgG1 or IgG3 and IgG1+IgG3

IAT titres	IgG subclass		Total
	IgG1/IgG3	IgG 1+ IgG3	
<64	4	6	10
	33.33%	28.57%	30.30%
128-256	4	8	12
	33.33%	38.09%	36.36%
>512	4	7	11
	33.33%	33.33%	36.67%
Total	12	21	33
	100.00%	100.00%	100.00%
	p = 0.934		

Further univariate logistic regression analysis was done to compare the disease severity and the IgG sublclass. Our results show that the risk of newborn developing severe HDFN increases 18 times when IgG1 or IgG3 is present as compared to none of these (only antibody screen positive). When IgG1+IgG3 is present there is an additional 18 times more risk of severe disease as compared to either of them alone. Thus IgG1+IgG3 served as indicators for more severe form of HDFN as compared to each of them alone.

Our results highlighted the significance of identifying IgG subclass as an independent prognostic marker for HDFN.

Table 17: Univariate logistic regression analysis, showing the odds- of HDFN when IgG1/IgG3 or IgG1+ IgG3 is present in the mother. Compares the HDFN in all live newborns

Logistic regression analysis (Univariate)			
Outcome	**Odds ratio**	**P**	**95% Confidence interval**
IgG1/IgG3/IgG1+IgG3	18.18	<0.001	4.70 - 70.29

A univariate logistic regression analysis was also used to calculate the odd of HDFN when compared to the antibody titre levels to look upon it as an independent prognostic marker of HDFN. Our results showed that the risk of HDFN increased 7 times (odds ratio, with a CI of 4.7-70.3) when titre was 128-256 when compared to that of less than 64, and a further 7 times when the titres were greater than 512 as compared to the range of 128-256.

Table 18: Univariate logistic regression analysis for titre of antibodies detected and odds of developing HDFN

Logistic regression analysis (Univariate)			
Outcome	**Odds ratio**	P	**95% Confidence interval**
Titre	7.43	0.008	1.69 – 32.64

Since IgG subtypes independently showed significant difference with severity of disease and so did the antibody titre levels with an odds ratio of approximately 7 and it tended towards significance. It helped realize that both played a vital role in predicting severity of HDFN and could not be looked upon in isolation. This lead to us to do a binary logistic regression analysis, to see the odds of developing severe HDFN, using IgG subclass, antibody titre levels and the IAT strength of reaction.

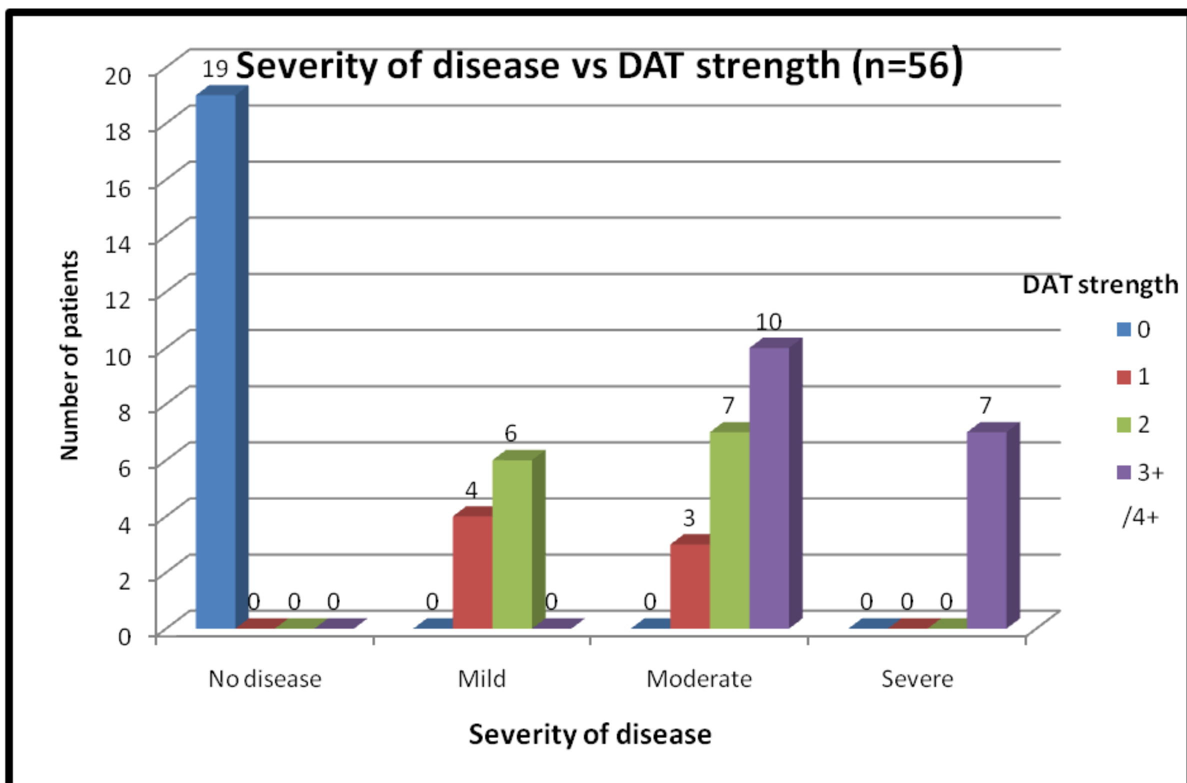
Table 19: Binary logistic regression to compare severity of HDFN in relation to IgG subclass, antibody titre levels and IAT strength

Binary logistic regression analysis			
Outcome	**Odds ratio**	**P**	**95% Confidence interval**
IgG1/IgG3/IgG1+IgG3	6.91	0.013	1.50 - 31.89
Antibody Titre	2.30	0.437	0.281 – 18.83
IAT	7.19	0.106	0.656 - 78.92

The overall odds of severe HDFN increased 7 times when IgG subclass, antibody titre levels and IAT strength were compared together, through binary logistic regression analysis. The odds of having severe disease was seven times more when IgG1/IgG3 was present in comparison to none of the antibodies, and a further seven times when IgG1+IgG3 was present in comparison to IgG1/IgG3 alone.

DAT result compared to severity of disease:

We compared the strength of the DAT result with the severity of disease in the newborns, In patients mild disease the DAT strength ranged from 1 to 2 only, while those with moderate disease had DAT ranging from 1 to 3+ and finally all patients with severe disease also had a corresponding strong DAT results (3+/4+).



DAT was not available for baby with hydrops and outcome not known

Fig 20: Severity of disease versus the DAT strength observed

The DAT strength in the Rh positive newborns was correlated with the disease severity. It was found to be highly significant ($p<0.001$) through Pearson chi square test, suggesting that a positive DAT in a high risk or clinically suspected HDFN is a strong indicator.

Table 20: Association between DAT and outcome of disease in all newborns

DAT outcome	Disease outcome		Total
Strength of reaction	Mild	Mod/Severe	
0	19	0	19
	65.51%	0.00%	33.92%
1	4	3	7
	13.79%	11.11%	12.5%
2	6	7	13
	20.68%	25.92%	23.21%
>3	0	17	17
	0.00%	62.96%	30.36%
Total	29	27	56
	100.00%	100.00%	100.00%
p < 0.001			

Of the 26 births, in mothers found to be antibody screen positive but negative for IgG1/IgG3, 8(31%) had newborns who were DAT positive In comparison, among the 30 pregnancies with antibody screen positivity and positive results for IgG1/IgG3 subclass or both , 29(96.6%) newborns were DAT positive.

Table 21: IgG subclass and DAT results of newborns

IgG subclass in maternal serum	DAT positive	DAT negative	Total (n=56)
IgG1	12 (1-Mild, 9-Moderate, 2-Severe)	0	12
IgG3	2 (1-Mild,1-Moderate)	0	2
IgG1 + IgG3	15 (1-Mild,8-Moderate, 6-Severe)	1	16
Negative (Antibody screen positive)	8 (7-Mild, 1-Moderate)	18	26

The above table is self explanatory, in that the greatest impact comes from a combination of IgG1 and IgG3. However, in the group of mothers who were antibody screen positive, but IgG subclass negative on this card, we still identified 8 babies with DAT positivity. However, 7of the8 babies (87.5 %) manifested as only mild HDFN, with one baby showed moderate disease. This is in contrast to the other 3 groups described in detail above.

We compared the IgG subclass, titre levels, other haematological and biochemical parameters and treatment modality in the severe cases. Table below describes each of these seven cases.

Table 22: Profile of babies with severe HDFN in our study

Sno.	IgG subclass (Titre)	Gestation (weeks)	Wt (gm)	Hb (gm%)	Retic (%)	Bil (mg /dl)	Max Bil.	ET/IUT/ BT	Outcome/Remarks
1	IgG1+IgG3 (1024)	35	2000	7.6	18.8	10	17.5	ET	Live
2	IgG1+IgG3 (512)	38	2500	6.5	22.5	9.2		ET	Live
3	IgG1 (512)	33	2020	11.8- 1st unit 5.9- 2nd unit	16.8	6.8	17.8	2 top up transfusion	Live Cord Hb- 12.1, Cord bil-3.6, ET avoided since rate of rise <0.5gm/dl/hr
4	IgG1+IgG3 (1024)	38	3400	13.3	-	53.6	53.6	ET	*Outside delivered, Bil. encephalopathy,
5	IgG1+IgG3 (128)	32	1960	-	-	-	8.1	IUT thrice	Ascites 1st IUT- 29 weeks , 2nd IUT (Hb 10.3), 3rd IUT (Hb 6) Live
6	IgG1+IgG3 (1024)	38	2760	10.3	15.9	16.0	-	ET	Live, Pericardial effusion and ascites
7	IgG1 (128)	39	3100	11.7	6.74	5.4 to 10.4 in 6 hrs	-	ET	Live

The table above depicts the profile of all severe live birth HDFN cases encountered in this study. The IgG subclass identified in the maternal serum, with the titre levels of antibodies in (). All these babies survived except 4th newborn who developed bilirubin encephalopathy. ET- Exchange transfusion, IUT- Intrauterine transfusion, BT- blood transfusion

DISCUSSION:

Red cell alloimmunization in the antenatal population has been a challenge for many decades. However with better anti D prophylaxis programmes, greater awareness of the problem, and better tools to diagnose the same, this has been declining. However, there has been constant work to develop tests which would predict the onset of HDFN, and also assess severity, in order to put therapeutic interventions in place on time.

It is against this background that we initiated this study. The IgG subtypes have been studied by many groups worldwide as they tend to impact on the presence/ severity of HDFN. Many groups have used various methods to qualitatively and quantitatively determine the subclasses. There is western literature which has looked at the significance of subclasses and the clinical outcome of pregnancy, but only one major study from India by Iyer et al. (1992) which explored the prognostic value of the IgG subclasses.(3,4) (74).

Our study was done in a tertiary hospital in South India. The prevalence of alloimmunization was found to be 2.17%, this is slightly higher than the prevalence of 1.48% reported in our previous study and also as compared to 1.25% prevalence reported by Pahuja et al. from Delhi, North India.(19,20)The reasons for this could be multi-factorial. It could be attributed to greater awareness as antibody screening has become routine protocol in many centres, and hence greater pickup of antibodies, and referrals to our institution as a tertiary care centre, being a reason. It could also be due to the fact that antibody screening with the standard three cell panel has become more extensively used. All previous literature have, reported a varying range of prevalence of alloimmunization, which differs in different geographical areas and populations. It has been found to be as high as 2.71% reported by De Vrijer et al.

(Netherlands) to as low as 0.4% as reported by Gottvall et al. from Sweden. Among Asian countries Lee et al. (2003) from China have reported a prevalence of 0.79%.(19) (98)

The most common alloantibody in our antenatal population was identified to be Anti-D. Of the 85 women found to be antibody screen positive during an antenatal visit, 85% had Anti-D, this is similar to the study by Pahuja et al. who found anti-D alone to contribute 78.43% of all alloimmunization(19). However Al-Ibrahim et al from Saudi Arabia found Anti-D to contribute only 52.38% to the alloimmunization of antenatal women and similarly Dajak et al. from Croatia reported Anti-D in 55% alloimmunized women.(27)(99) This was a contrast from results from the Indian subcontinent. In a report from America in 2000, it was reported that almost 6.8 per 1000 live births in America were affected by Rh sensitization.(68)

It was interesting to note the presence of other clinically significant antibodies, such as Anti-C, Anti-M, Anti-E, Anti-S and Anti-c in our antenatal alloimmunized population. Anti C and Anti-M made up 3.5% of the cases each respectively. There were only three patients in our study group with multiple antibodies identified, and all of them had a combination of Anti-D and C. Our profile of alloantibodies was very similar to that of Pahuja et al. , Anti-D in their study was followed by anti-C, and among the other significant blood group systems was the MNS with Anti-M having a frequency of 3.5%.(19) According to our previous study from CMC by Varghese et al. Anti-D contributed 34.2% of the alloimmmunized women, followed by Anti-Lea and Anti-Leb, which made up 10% and 8% respectively. Among the clinically significant alloantibodies, were those with a combination of anti-D and C (4%) or Anti-E and Anti-K (1.3%), the other antibodies reported were, Anti-c, Anti-C, Anti-M and Anti-Jka and Jkb, there were almost 37% cases in which antibodies could not be identified.(20) However now with antibody screening having become a routine, standardized practice followed by identification, we had only one case in which the antibody could not be identified using the 11 cell panel available.

We found that among our primiparous population of antenatal women, five had babies with HDFN. The severity of disease ranged from mild (2/5) to moderate (3/5). In contrast to long standing understanding of Rh related HDFN, which states that the first pregnancy is not affected, it has been reported in literature to occur in primiparous women with no history of transfusion or any known sensitizing event.(27)(100)

There was a bad obstetric history for almost 63% of our study group, which seemed more likely to have caused the sensitizing event, for the subsequent pregnancies, this was similar to the observation of Koelewijin et al. (Netherlands) who reported it to be approximately 54.28% in the alloimmunized antenatal women.(101). Pahuja et al. from Delhi have also reported a bad obstetric history in 62% of their alloimmnuzed antenatal women, which is very similar to ours.(19) In a study by Koelewijin et al. looking at profile of antenatal women at risk of HDFN, they observed that the Rh sensitization in antenatal women was most likely to be due to inadequate dose of Anti-D immunoglobulin, since most guidelines suggest a Kleihauer Betke test for calculating the dosage, which itself does not have sufficient sensitivity and specificity. They have attributed the inadequacy of the test in various studies to factors such as, different cut-offs in different laboratories, that the small amount of FMH capable of causing sensitization could be missed by the manual form of the test and lastly- that for evidence based guidelines larger number of cases need to be studied.(87)

IgG subclasses: We found the overall prevalence of IgG1 to be 20%, IgG3 4% and 25% for IgG1+IgG3 together in our population of antenatal women. The remaining almost 50% were antibody screen positive but did not have either IgG1 or IgG3. There are studies done in the Caucasian population, which have reported that the predominant category of antibodies in patients alloimmnunized with anti-D are IgG1 and IgG3, and rarely IgG2 and IgG4.(3,4,75) A study by Lubenko et al. has further gone on to report that IgG2 and IgG4 tend to decrease

in pregnancies at risk of HDFN as compared to those not at risk. (102) It was interesting to compare our results to those published by Pollock et al. in their study from Canada. They found that of 98 women identified to have Anti-D, 32 (33%) had IgG1, 3 (3%) had IgG3 and 63 (64%) had both IgG1+IgG3.

According to their results, in patients with combination of both IgG1+IgG3, IgG1 was the predominant subclass. They did not report on any women with IgG2 and IgG4. In our study it was IgG1 and IgG3 which were looked at to prognosticate HDFN in view of all previous literature, which state that these are the predominant subclasses involved in causation of HDFN. In contrast, IgG2 the least efficient among all the IgG subclasses in crossing the placenta, and also the contribution of IgG2 and IgG4 in HDFN is limited due to absence of receptors for these on the monocytes.(74)

We identified IgG1 alone in 17 of our 85 (20%) antenatal women including all antibody specificities. It was in 12 of 73 (16%) in those with Anti-D alone. This is however lower than that reported by Iyer et al. from Mumbai who studied only Anti-D alloimmunized women, and found IgG1 to be the major subclass identified in their study population which was in 52 of 107 women (48.6%).(74) Among western studies, Pollock et al. have reported isolated IgG1 in 32.6% cases.(4) Overall the prevalence of IgG1 alone appears much less compared to other published literature.

IgG3 alone was found in only three of our 85 study patients (3.5%). Among two women who were followed up, they had babies with mild and moderate disease respectively. Previously Iyer et al. and Pollock et al have reported the prevalence of IgG3 in their studies to be 12% and 3% respectively. In both studies they found that when IgG3 was present alone these women also had low antibody titres. In the study by Pollock et al. all of them had titres not

more than 4.(4,74) In our patients two had documented titre levels of 16 and 512 respectively. The patient with titre of 512 was eventually lost to follow up.

Our study population had majority with IgG1+IgG3 (25%) in all antibody specificities together and it was 29% (21 of 73) in those with Anti-D alone. This was higher as compared to Iyer et al. who reported IgG1 and IgG3 together to be in 23 of 107 cases (21.4%).(74) Pollock et al. found IgG1 and IgG3 in combination to be present in the highest proportion of patients, 64.2%. In our study also IgG1 and IgG3 together contributed the majority of patients who had either IgG1 or IgG3 present.

The majority of our antenatal alloimmunized women, 44 of 85 patients (52%) were antibody screen positive, but negative for both IgG1 and/or IgG3. Iyer et al reported this group as 19 of 107 patients (18%), but interestingly Pollock et al have no patients from their study group of 98 who had neither IgG1 and/or IgG3.(4,74)

Our study looked at IgG1 and IgG3 subclassses in all antibody specificities that were encountered. However due to very small numbers in patients alloimmunized with antibodies other than Anti-D it was difficult to draw any inference. Most studies have looked at IgG sublclasses in Rh (Anti-D) sensitization alone. We found that in patients with both Anti-D and C, all 3 cases (100%) had IgG1 alone, while 2 of 3 (67%) cases with Anti-M alone had only IgG1 and one case had neither IgG1 and/or IgG3. All other antibody specificities were negative for both these subclasses of IgG. There is one study by Thomas et al (1995), which carried out a quantitative assay of IgG subclasses in alloantibodies other than Anti-D. In their profile of antibodies studied which included Anti-S, -E, -Jka, and –Fya, IgG1 or IgG3 accounted for greater than 50% of the antibody subclass in babies which finally developed HDFN.(77)

We followed up 68 pregnancies to correlate the IgG subclass identified with the outcome of pregnancies. 56 newborns were Rh positive (and of these 37 newborns were DAT positive and 19 DAT negative). Of the live births, there were overall, 10 babies with mild disease, 20 with moderate and 7 with severe HDFN. There was also an additional case of severe HDFN who was planned for an IUT, but was subsequently lost to follow up and therefore whose outcome was not known.

It was observed that in patients with severe HDFN babies were born to mothers mostly with high titres of both IgG1 and IgG3, 75% (6/8), while two were born to mothers with high titres of IgG1 alone (25%). Iyer et al reported severe disease in 68% of the newborns followed up and the majority were born to mothers with IgG1 alone (53%), as compared to those with IgG1 and IgG3 together (25%). This was found to be in contrast to our findings.(74) However Pollock et al. have reported severe HDFN to be more frequently encountered in mothers with IgG1 and IgG3 together (67%) as compared to those with IgG1 alone (32%).(4) Of the five primiparous women who had babies with HDFN, it was noted that three had IgG1 alone, while two were negative for IgG1 and/or IgG3.

We used logistic regression analysis to calculate the odds of severe HDFN in respect to the IgG subclass in maternal serum. We observed that the odds of developing severe HDFN were eighteen times higher ($p<0.005$, CI 4.7-70.3) in patients with no IgG1/IgG3, to those with one of the latter and the odds further increased 18 times when the two were present in a combination. Our results were similar to that reported by Pollock et al and Zupanska et al. who reported that when IgG3 was present along with IgG1, hydrops developed earlier as compared to IgG1 alone and they reported that presence of IgG1 and IgG3 in combination resulted in greater rate of hemolysis in the fetus.(4)(103) There are studies with conflicting results and while some have found IgG1 and IgG3 together to be more significant, others have reported IgG1 alone as a major cause of severe HDFN.(3,74)(104)

The odds of developing severe disease also correlated with the antibody titre levels, and was found to be seven times higher when titre levels increased from <64 to 128-256 and finally greater than 512 (p=0.008, CI 1.69-33). This helped understand that each of these factors plays a role in prognosticating the severity of HDFN and either cannot be looked upon in isolation. The reports on HDFN severity and IgG subclass have been controversial, and against this background Shanfield et al.in 1980, reported that the severity of HDFN in mothers with IgG1 alone was dependent on the titre levels of the antibodies. However Iyer et al found no such correlation and observed that even mothers with low titre levels of IgG1 had babies with severe HDFN.(74) We did not find a significant difference in titre levels of antibodies between patients negative for IgG1/IgG3 and those having one or both of them. A similar result was observed between women with one of the IgG subclasses (IgG1 or IgG3) as compared to those with both. However Pearson Chi square and logistic regression analysis helper to infer that the titre level independently continued to impact the severity of HDFN, which was not associated with the subclass present.

In present clinical practise high risk pregnancies with antibody screen positivity are followed up with antibody titre levels. We found the antibody titre levels to be significantly different between newborns with “no/mild” disease and those with “moderate/severe” disease. Since we observed the odds of disease to increase when IgG1 was present in a combination with IgG3, we analysed the difference in titre levels between patients with one of these subclasses (IgG1 or IgG3) to those with both these subclasses, there was no significant difference noticed in these two groups.

We further compared the disease severity among low and intermediate titre groups of patients with neither, IgG1 or IgG3 and those with one or both of these. This was found to be extremely significant- helping us infer that even in low titres IgG1 and IgG3 might required closer follow up and are more likely to cause moderate to severe disease.

A binary logistic regression analysis was done to compare the odds of developing severe disease when IgG subclass, antibody titre levels and the IAT strength were taken together, and this came to be 6.9 ($p < 0.05$ and CI of 1.9 to 32). The odds of severe HDFN increased seven times through binary logistic regression when comparing No IgG1/IgG3 to IgG1/IgG3 to further a combination of the two.

In view of no clear cut guidelines in grading HDFN and since the treatment modality is guided by disease severity and subjective clinical decision, we also compared our DAT results to the HDFN in newborns. We found a strong correlation between the DAT result of the newborn and the severity of HDFN ($p<0.001$). DAT results itself have been controversial in their predictive ability of HDFN and there are different studies with contrary results. According to Song et al. they found that 96% of cases with a non-ABO incompatibility leading to HDFN the DAT was positive.(105) However Dinesh et al. who looked at the positive predictive value of DAT for HDFN found it to be only 23%, with the sensitivity of the test itself being, 86%.(66) But for our patient population of the 36 newborns with a DAT positive result, 27 required further treatment and close monitoring.

Our results helped us identify, that almost 50% of our antenatal alloimmunized population have either IgG1 or IgG3 or a combination of these two subclasses. We also inferred that that the odds of developing HDFN progressively increased when either one or both these subclasses were present in the maternal serum. Since even in overall low titres IgG1and/or IgG3 was found to cause significant disease as compared to those who had neither, the relevance of this test was highlighted. We realized that CAT being a simple and easily available platform can be used in blood banks to offer clinicians another mean to monitor pregnancies at a higher risk of HDFN.

LIMITATIONS OF THE STUDY:

The limitations of this study included,

1. Most of our patients (85%) are from Tamil Nadu, and hence it may not be able to extrapolate our findings regarding IgG sub class as representative of the South Indian population, as we had hoped it to be.
2. Secondly, 17 of our patients were lost to follow up. Since ours is a tertiary referral centre, many patients do not come for the final delivery if the antenatal period was uneventful or after an initial assessment here they carried on their antenatal check up at a place closer to home.
3. Assessment of IgG subclass effect for antibodies other than anti D was not possible as they formed a very small part of the study group and could not be studied separately.
4. The severity of disease was graded based on the treatment modality required. However varied studies have used different methods of grading and there is no evidence based guideline for the same.

CONCLUSION:

- The IgG1/ IgG3 subclass prevalence in our antenatal alloimmunized population was overall 49%, with 20% being contributed by IgG1 only, 4% by IgG3 alone and 25% by a combination of IgG1 and IgG3. The remaining 51% were negative for both, IgG1 or IgG3 subclass.
- A strong association is seen between the severity of HDFN and IgG1/IgG3 subclass positivity ($P < 0.001$) when compared to women negative for IgG1 /IgG3)
- The odds of developing severe HDFN increased progressively when comparing women who were negative for IgG1 / IgG3 to those with IgG1 or IgG3 and further increased when comparing those who had both IgG1 and IgG3(Odds ratio 6.9, $p < 0.05$ and CI between 1.9-32).
- Comparing outcome in women with titres of $\leq$256, severity of HDFN was significantly greater in babies born to mothers with IgG1 / IgG3 subclass positivity ($p < 0.001$).
- Strength of DAT (3-4+ compared to $\leq$2+) correlated significantly with severity of HDFN($P < 0.001$).
- The test, performed using the CAT is a feasible test, which if implemented can contribute significantly to clinical decision making algorithms and appropriate timely interventions in the context of antenatal alloimmunization and HDFN.

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ANNEXURE

INSTITUTIONAL RVIEW BOARD APPROVAL LETTER

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

December 17, 2012

Dr. Jui Choudhuri
PG Registrar
Department of Transfusion Medicine and Immunohaematology
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
Prevalence of immunoglobulin G subtypes (IgG1 and IgG3) in alloimmunized antenatal women in South India.
Dr. Jui Choudhuri, PG Registrar, Transfusion Medicine and Immunohaematology, Dr. Dolly Daniel, Dr. Mary Purna Chacko, Transfusion Medicine and Immunohaematology, Dr. Anil Kuruvilla, Neonatology, Dr. Annie Regi, Dr. Ruby Jose, Obstetrics.

Ref: IRB Min. No. 8119 dated 05.12.2012

Dear Dr. Jui Choudhuri,

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Dolly Daniel, Department of Transfusion Medicine and Immunohaematology

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

A sum of Rs. 60.000 (Rupees Sixty Thousand only) sanctioned for one and half year.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Dolly Daniel, Department of Transfusion Medicine and Immunohaematology

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

Thesis Data Form

Study of IgG subclass (IgG1/IgG3) in antenatal alloimmunized women and severity of HDFN

Department of Transfusion Medicine and Immunohematology
Christian Medical College, Vellore

Name of patient		Unique study no.	
Age/DOB		Telephone no.	
Husband's name		Address	
Hospital no.			
EDD			

Mother's information			
Blood group		ICT	
Period of gestation		Antibody-identified	
Obstetric formula/ obstetric history		**IgG subytpe**	
		Outcome of pregnancy	
H/O transfusion		Husband's group	

Newborn's information					
Hospital no.		**Hospital stay**	**Day1**	**Day2**	**Day3**
Live/Still born/ IUD		Haemoglobin			
Pre/Term/Post		Bilirubin (D/I)			
Normal/LSCS		RBC count			
Sex		Retic count			
Weight at birth		Peripheral blood smear			

<table>
<tr><td>(in gms)</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Blood group</td><td></td><td rowspan="2">Treatment required</td><td colspan="3" rowspan="2">YES / NO</td></tr>
<tr><td>DCT</td><td></td></tr>
<tr><td>Cord blood Hb</td><td></td><td rowspan="3">Treatment details
IUT
Phototherapy
Exchange transf.
None</td><td colspan="3" rowspan="3">(Including no. of times and other details)</td></tr>
<tr><td>Cord blood Bilirubin</td><td></td></tr>
<tr><td rowspan="2">Jaundice
Pleural effusion
Ascites
Hypoalbuminemia
Acidosis
Hydrops
Kernicterus
Coagulopathies
Sepsis</td><td rowspan="2">Y / N
Y / N
Y / N
Y / N
Y / N
Y / N
Y / N
Y / N
Y / N</td></tr>
<tr><td>Remarks</td><td colspan="3"></td></tr>
</table>