Original Article

Monitoring fetal maternal incompatibility, a retrospective study – In a Spanish population attending the Parc de Salut, Mar in Barcelona

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Introduction: Hemolytic disease of the fetus and newborn (HDFN) continues to be a complication of early life in the newborn. Prophylaxis by administration of anti-D immunoglobulin IgG to Rh D negative pregnant women at 28 weeks and postpartum is a standard practice and the study of irregular antibodies (Ab) to all pregnant women has contributed to the detection of other Ab capable of inducing HDFN.

Aims: To determine the current frequency and incidence of maternal alloimmunization, severity of hemolytic disease and determine the frequency of Rh D negative pregnant resulting in newborn Rh D negative, where the administration of gamma globulin could have been avoided.

Methods: Retrospective study. We evaluated for two years, a total of 7087 tests in pregnant women and in 6224 newborns of the Blood Bank Parc Salut Mar of Barcelona. The positive results of indirect antiglobulin test and direct antiglobulin test were reviewed. The study of Ab was performed using plasma from pregnant women with 3-cell ID DiaCell I-II-III card. In positive tests it was used panel 11-cell ID with a DiaPanel autologous cell and 11-cell NaCl ID card, enzymatic assay and cold agglutinins and Media-Liss Coombs.

Results: The frequency of maternal active alloimmunization was 39 in 3.118 pregnant women (1.25 in 100). 2 cases of severe HDFN among 3.000 newborns were observed which yield a prevalence of severe hemolytic disease of 1 in 1500.

Conclusion: Most Ab detected corresponds to Ab against the Rh system. The anti-D Ab detected in our studies are mostly passive antibody administration remains IgG anti-D prophylaxis. The politics of fetal Rh-D genotyping in maternal plasma in all pregnant negative D should be implemented in order to prevent IgG anti-D administration of prophylaxis if the fetus is also D negative.

Key words: Newborn hemolytic disease, Rh-D, genotyping, maternal

Introduction

The hemolytic disease of the fetus and newborn (HDFN) arises when the mother lacks a red blood cell (RBC) antigen (Ag) and the fetus is a carrier of the respective Ag. During normal pregnancy, i.e., physiologically, vascular fissures communicating fetal blood in the mother's bloodstream occur. The mother immune competent lacking Ag recognizes the fetal Ag as foreign and a specific antibody (Ab) is produced.(1) With the progress of pregnancy, new contacts can be established and especially at the time of delivery a fetus-maternal bleeding can occur. HDFN remains a current complication of pregnancy. The anti-Rh (D) alloimmunization is still detected with a higher than expected frequency for a susceptible phenomenon to be avoided with the proper

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administration of anti-D, IgG anti-D gamma globulin to the standard doses and schedule. (1–3)

The most frequent and serious case is the Rh incompatibility group. The immune system of a Rh D negative mother becomes active against the Rh D positive fetus. During gestation, fetal cells can enter the bloodstream of the mother due to trauma, invasive procedures or spontaneously in the third trimester. The mother produces Ab against these foreign Ag, with capacity to cross the placenta actively and can be fixed on fetal RBC membrane. This sensibilization is followed by hepatosplenic uptake and destruction of the RBC by the macrophage system of the fetus, which may result in hemolysis and mild to severe anemia, hydrops fetalis, anasarca, hepatosplenomegaly, jaundice, heart failure or exitus. (4,5)

All pregnant women should be tested, and for those that turn out to be Rh D negative, prophylaxis should be offered. (6) Audits conducted in the United Kingdom have observed that such prophylaxis was not performed, although this intervention would protect the lives of their unborn children. The administration of anti-D IgG immunoglobulin may prevent HDFN due to Rh incompatibility. Furthermore, in a study conducted over 15 years (1996-2011) preventable errors in this regard have been detected. Health workers voluntarily reported 1.211 errors: regarding pregnant women that should have received the anti-D IgG immunoglobulin and did not. In half of the cases the prophylaxis wasn't administered or was administered too late. A quarter of the errors were due to laboratory errors. Nine newborns suffered hemolytic disease collateral effects, three needed urgent transfusions and one of them was exitus. That is why the authors of the University of Manchester expect progress in this field of the clinical laboratory. (7)

The role of the laboratory in the HDFN is therefore essential and must ensure that no mistakes occur with appropriate quality measures. Its role is multifactorial and very broad, covering all aspects of diagnosis, prevention and treatment of this immunologic complication during pregnancy. (8)

ABO incompatibility HDN (ABO-HDN) between mother and newborn is the most frequent of the HDN. This occurs most often in pregnant women with group O, A, B or AB. This is because pregnant group O has naturally occurring immunoglobulin (Ig) M against ABO, but they have also certain quantity of IgG anti-ABO. Thus, the IgG anti-A or anti-B present in the serum of the pregnant group O can cross the placenta and bind to fetal or newborn RBC.(9)

Although D alloimmunization remains a current complication, at our hospital there is no reliable data about the frequency of maternal alloimmunization or the severity and their consequences in the newborn. Regarding preventive treatment being applied there are no data. (10)

Only a review published in the Spanish Pediatric Journal was found. Nevertheless, we can't find the answers to some questions, such as: how can we know which Ab are the most frequent in pregnant women? And which ones are the most important for the fetus? [10]

No wonder that a local initiative is developing in this regard, as the MAMI project (initiative for managing the HDFN in Valencia, Spain), which was exhibited at the recent National congress of Blood Transfusion. (11)

Besides, knowing the frequency of alloimmunized mothers, severity and frequency of HDFN, we should know if the only existing preventive treatment for more severe hemolytic disease is being correctly implemented and as expected no data were available. (10,12–16)

In our center we follow "the protocol for pregnant women" and our aim was to is identify all the non-sensitized Rh D negative women should benefit with prophylactic administration of D IgG anti-D gamma globulin as well as the early detection of maternal RBC alloimmunization to identify women at risk of inducing a HDFN. Since we haven't found any recent data about it, with this study we aim to analyze and clarify the prevalence of maternal alloimmunization and HDFN in Catalonia and Spain.

MATERIAL AND METHODS

STUDY POPULATION

Retrospective study in pregnant women and neonates, our sample was constituted by all pregnant women at the Obstetrics and Gynecology service of the influence area of the Hospital del Mar, which also includes the primary care centers in the area. (17) We received 7087 samples from pregnant women and 6808 from newborns from January 1st, 2011 to December 31st, 2012.

According to recommendations of the scientific societies, a blood group test group and indirect antiglobulin test (IAT) should be performed to all pregnant women at the first trimester. (18) We selected all cases of pregnant women with IAT in this population. Once our laboratory is certified by the ISO Quality Standard 9008: 2000 all procedures are normalized and we enter the cases in a specific recording system (Register Gestates, R -116). We collect data from electronic medical records in computer

application Imasis first from the mother: NHC clinical history number, patient age, identified Ab, gestation week and Ab first titer and immediately after delivery, from the newborn: date of birth and direct antiglobulin test (DAT). (8,19)

Newborn blood test group and DAT were performed in the cord blood samples. When the result was positive and there was ABO incompatibility with the mother group, a Lui elution was performed. (20) Whenever DAT was positive and the mother had a positive IAT, the acid elution technique (Bio-Rad, Cressier, Switzerland) in cord blood was performed. The results of these tests are recorded in the Eyra program from the Analysis Laboratory Hospital (Reference Laboratory Catalonia, LRC).

Concerning the second objective, we retrospectively collected cases from Rh negative mothers who received anti-D immunoglobulin and had a Rh negative newborn from historical analytical data and Eyra and Imasis software. (3)

For descriptive statistics, statistical functions of Microsoft Excel program were used.

SAMPLES

COLLECTION AND PROCESSING

Blood sample from pregnant women were collected to 6 ml tubes containing ethylenediamine tetra-acetic acid tripotassium (K_3EDTA) appropriate for Ag typing of ABO and Rh. Blood group was performed by two different methods: reactive Novaclone tube (Murine Monoclonal IgM, Dartmouth, Canada) and gel card ABD-Confirmation for Patients monoclonal (Bio-Rad, Cressier, Switzerland). (21,22)

The IAT, which was performed as screening to detect irregular anti-RBC Ab, is an agglutination technique. For this test maternal plasma was obtained by centrifugation of the 6 ml K_3EDTA tube (3500g for 3 minutes). (15) Plasma was dispensed into the Liss-Coombs of (Bio-Rad, Cressier, Switzerland) card and RBCs were added (25 µl of plasma and 50 µl of each of the cells of the three types of RBCs screening (Bio-Rad, Cressier, Switzerland). To avoid any types of interference they were pre- heated at 37°C. After dispensing plasma and RBCs, cards were incubated for 15 minutes at 37°C and centrifuged for 10 minutes at 910g.

For the reading we compare the agglutination in the working pattern Negative O/++ ++ to positive ++++/++++.

Screening cells displayed the following Ag at their surface: C, c, D, E, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b M, N, S, s, Le^a. (23) As recommended, a cell was R_1R_1 and another was R_2R_2 and the Fy^a, Fy^b, Jk^a, Jk^b, S Ag were present in homozygous in one of the cells I, II, III (Bio-Rad, Cressier, Switzerland).

This technique allows the detection of anti-K and anti-c Ab which are the most frequent Ab after anti-Rh D specificity. When plasma reactivity was detected we proceeded to the identification of irregular Ab performing further investigations. A panel of 11 RBC card is used in Liss-Coombs and included autologous RBCs to identify an eventual autoantibody (dilution of 10 µl of maternal blood suspended in 0.5 ml of diluents LISS ID-2), (Bio-Rad, Cressier, Switzerland). A second 11-cell panel with RBCs treated with papain was used (NaCl card, Enzyme Test and cold agglutinins, (Bio-Rad, Cressier, Switzerland).

In order to confirm the presence of an alloantibody, we performed maternal RBC phenotyping with 50 µl of maternal RBCs diluted in 0.5 ml of diluent solution enhancer ID-1 (Bio-Rad, Cressier, Switzerland) to work on gel cards (Rh - card Subgroups C_w + K + Bio-Rad, Cressier, Switzerland). (14) Thus, every time a patient is a carrier of an Ab, we verify if she lacks the corresponding Ag. Once the Ab was identified we proceed for a titration using appropriate RBC and serial dilutions of plasma. For Ab anti-D titration the R_2R_2 phenotype RBCs (cDE/cDE) in homozigoty should be selected. We observed, interpreted and validated the results always with the same biomedical laboratory scientist and we examined in parallel the current sample with the previous one which is always frozen within 48h after extraction so the Ab is preserved.

The cord blood samples are obtained in the Obstetrics and Gynecology service. In the delivery room, 4.5 ml of blood is collected in K_3EDTA tube. The infants and pregnant women ABO groups and the IAT were processed with Techno 445 autoanalyser from Diamed Iberian along with two controls, sample 1 (group A, D negative with anti-D) and sample 2 (group B, D positive with anti-Fy^a) in parallel (Bio-Rad, Cressier, Switzerland).

All maternal blood groups and IAT data, cord blood groups and cord blood DAT were recorded at the hospital data base (Eyra program) and the result was validated.

The ABO group and the DAT were performed on gel card DiaClon ABO/Rh for Newborns DVI^+ monoclonal Ab (Bio-Rad, Cressier, Switzerland). (21) The blood type was verified using monoclonal reagents (Novaclone Murine Monoclonal IgM, Dartmouth, Canada).

The acid elution technique was performed with a cord blood sample collected to K_3EDTA with at least 1 ml of packed cells. Cells were washed with isotonic saline and 0.9% reagent and *"DiaCidel for acid elution of serological antibodies*" (Bio-Rad, Cressier, Switzerland) was used. This is a simple procedure for the elution of the most common Ab.

To determine fetus-maternal ABO incompatibility we use the Lui elution with 1.5 ml cord blood drawn from a K_3EDTA tube. It is a manual procedure which takes off the red cell Ab washing them with 0.9% isotonic saline and through a process of freeze-thaw. Then we pit them against A_1, B and O red cells as recommended.

Fetus genotype was performed on maternal plasma in an external reference laboratory using the "*Free DNA Fetal Kit® RhD*" (Diamed AG, Florida, United States).

All procedures were performed according to the manufacturers.

RESULTS

A total of 7807 immunological tests were performed between 2011 and 2012 from these 3118 correspond to pregnant women. Being 50.75% ABO blood group Rh (D) test and 49.25% to the IAT. Among the IAT 91 were positive (2.36%) and 3753 tests were negative (97.63%).

SAMPLE FROM MOTHERS

The frequency of maternal active alloimmunization was of 39 in 3,118 pregnant women (1.25 in 100). Among the 91 positive results, 16 different Ab specificities were identified 3 classified as unspecific. The most frequent Ab was the anti-D cases followed by anti-E 9 cases Anti -Kell 7 cases and anti-M 7 cases. The other Ab in combination are: anti-D+C^W (1), anti-D+ Kell (1), anti-E+C^W (1), anti-C+e (1) and anti-D+C+G+ (1). Anti-D+E 4 cases and anti-Le^a (1), anti-Kp^a (1), anti-Lu^a (1). Nonspecific 3 cases.

Titrations for maternal anti-D Ab showed titers <8 (86.79%), 16 (3.7%), 64 (5.6%), 256 (1.8%) and 512 (1.8%), as can be observed in Figure 1. Two cases (antibodies Anti-Kp^a and anti-Lu^a) were lost after follow up. Table 1 describes the distribution of specificities of alloimmunized mothers with multiples Ab.

The study of fetal Rh genotype in maternal plasma was performed in the case where the titer was higher than 512 and an RH D positive fetus was typed. Table 2 describes the other Ab detected and respective titer course of pregnancy clinical and analytic impact on the newborn.

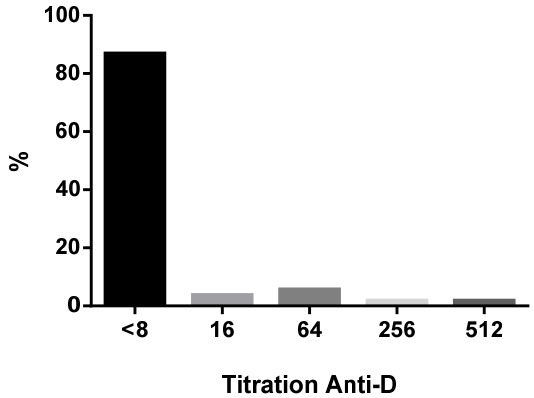

Fig.1 *Titration of anti-D antibody, to all pregnant women was administrated anti-D IgG gamma globulin.*

SAMPLES FROM CORD BLOOD

From the 3099 tests for blood group the following results were obtained: group A (36.01%), group B (16.98%), group AB (5.00%) and for the O group (42.01%) (Figure 2).

The results for D in newborns were: D positive 89.84%, D negative 10.16% (Figure 3).

As for cord blood DAT 3.19% were positive and 96.57% negative. Among this 3.19% of cases with a positive DAT we had to perform supplementary studies, Lui elution test for ABO incompatibility (105 test) and the Test of acid elution (7 test) for the other.

Lui elution test was performed when requested for fetus-maternal ABO incompatibility and positive cord blood DAT and when a negative DAT was obtained but there was a clinical indication.

Table 1 Distribution of specificities of alloimmunized mothers with multiples antibodies.

Antibody	D	E	Kell	M	Lu^a	Kp^a	Le^a	D + E	D + Kell	D + C^w	D + c + G	E + C^w	C + e	Unspecific
Number of cases	53	9	7	7	1	1	1	4	1	1	1	1	1	3

N=100

Table 2 Mothers antibodies and respective newborns titration.

	Mothers Titration		New-borns Titration			
Antibodies	1 - 128	>128	DAT -	DAT +	Acid Elution	HDFN
anti-D	51	2	33	3	3	1
anti-E	9	0	3	0	0	0
other Rh	9	0	3	1	1	0
anti- Kell	5	2	5	0	1	0
anti-M	7	0	3	0	0	0
anti-Kp[a]	1	0	1	0	0	0
anti-Lu[a]	1	0	1	0	0	0
anti-Le[a]	0	0	0	0	0	n.a.
Nonspecific	0	0	1	0	0	n.a.

DAT – Direct antiglobulina test, HDFN – Hemolytic disease of the fetus and newborn, n.a. – not applicable

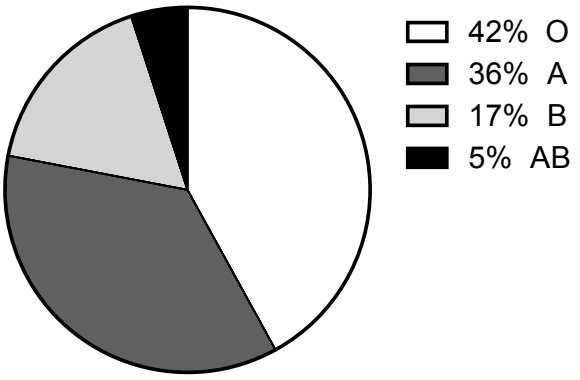

Fig.2 *Distribution of specificities of alloimmunized mothers with multiples antibodies.*

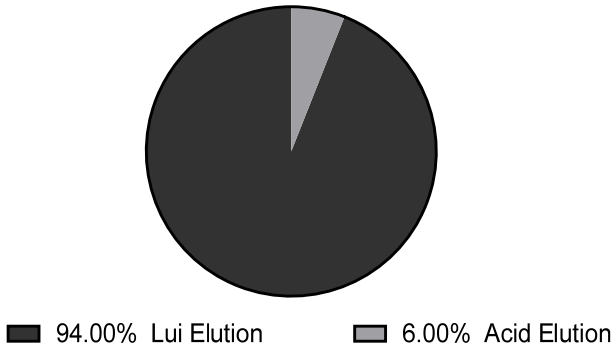

Fig.4 *Antibody titration in mothers, antibody elution techniques in newborns and respective titre.*

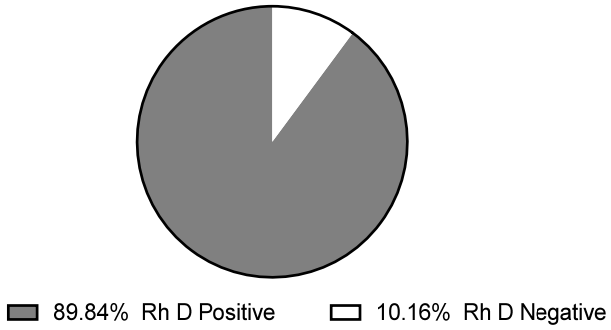

Fig.3 *ABO blood groups in cord blood*

There are cases the fetus-maternal incompatibility in which the DAT was negative, only one was detected (15.23%) tested positive for the eluted (Figure 4). The most common Ab contained in the determinations of Acid Elution Test was: Anti-D and anti-D+C.

HAEMOLYTIC DISEASE OF FETUS AND NEWBORN

We detected only 2 cases of severe HDFN in 2 years. Among 3099 live births, an incidence of 1 in 1550. These cases required transfusions and/or exchange transfusions. In spite of a weakly positive DAT, the newborn at 12 hours of life showed a serum bilirubin of 10.76 mg/dl (+2=12.76 mg/dl) as phototherapy, phenobarbital and fluid began. At 30 hours of life, there was an increase in bilirubin 16.73 mg/dl (+2=18.73 mg/dl) as treatment began with gamma globulin 500 mg/kg (4 doses). At 76 hours of life, increased bilirubin 23.66 mg/dl, it was decided to administer the fourth dose of gamma globulin and proceed with the exchange transfusion.

DISCUSSION

The present study confirms that the HDFN is a disease with a low incidence in Spain (1 in 1500) similar to what was previously reported in the Spanish literature of 1 per 1000 live births. One of HDFN cases detected by anti-D was from an immigrant pregnant women. This case debuted with high titers of 512 before the 28th week of gestation and corresponds to isoimmunization by the Rh D. In this case, it is unnecessary to administer preventive doses of IgG anti-D, once it would be useless. This was a serious case of HDFN with fetal distress, despite a scheduled cesarean section at 37.3 weeks of gestation. The neonate made three exchange transfusions due to anemia and hyperbilirubinemia severity.

The introduction of anti-D gamma globulin prophylaxis to prevent fetus-maternal isoimmunization by anti-Rh (D) represents one of the largest most important medical advances in modern times. (24)

In this study, the most frequently detected alloantibody laboratory was anti-D (58.24%). These Ab have greatest clinical impact, with the peculiarity that 98% of cases were low titers of anti-D, associated with the history of administration of IgG anti-D prophylaxis to all pregnant D negative at 28 weeks of gestation. (25)

Between 2011-2012 studies we also detected positive studies regarding specificity other than D Ag. Rh antigens are highly immune system proteins capable of eliciting an immune response in those individuals who do not have. (26) Although the D antigen is the most common cause of alloimmunization, 43 antigens are described capable of producing hemolytic disease. The most important are, apart from D, the antigens C, c, E, e, Kell, Duffy and Kidd Ag also have clinical significance. (19) The second Ab infrequency was anti-E (9.9%) with low titers (<2) was pregnant D positive and were followed monthly as the title remained stable. The third most frequently detected Ab was anti-Kell (7.7%); pregnant women were D positive with initial titles 64 to 1024. Literature suggests that from the title 1/32 should be considered a "critical degree", but there is certainly a "critical degree" to anti-K, specificity because very low titers have been associated to the inhibitory effect of the fetal erythropoiesis. (27)

We also studied either the father's Rh phenotype in peripheral blood, or the fetal genotype in amniotic fluid. Nevertheless, controls were performed biweekly. In all cases, the sonographic follow-up was correct and fetus was Kell negative as expected. In cases of maternal anti-M (7.7%) titles were lower than 8. We determined that Ab were IgG type. HFNB never occurred. Usually anti-M Ab are of IgM nature and do not require a special serological monitoring, so our protocol for pregnant D negative non-sensitized applies. (28) We didn't observe at the present study anti-D in combination with other red cell Ab associated with HDFN. (25)

The Ab in combination such as anti-C^W D+, D+ anti-Kell and anti-D+E remained at low titles. Samples that appeared to contain anti-C+D, in which the anti-C is more potent than the anti-D, one must suspect the presence of anti-G or anti-G+C. (28) In our case, the new born had a positive DAT and no signs of mild anemia jaundice or neonatal problems.

The acid elution test clarified the cause of hyperbilirubinemia, eluting with positive anti-D+C. with anti-D (1/64) and anti-C (1/1) titles, excluding anti-G. For other mixtures of Ab anti-E and anti-C^W+C+ e+ newborns had DAT with negative results, and Ab wasn't eluted. While all Ab-sensitized pregnant women require a special assessment and monitoring as obstetric both immune, one can see that our laboratory has actively contributed to the identification and quantification of Ab detected in 91 determinations. (26)

The ABO blood group of newborns was 42% 0, 36% group A, group B 17% and 5% group AB. This frequency is similar to the frequency of the Spanish population. (29)

Regarding the Rh group of infants, the proportion of Rh positive fetuses born to D negative mothers negative is greater than what is described: in the Caucasian population it has been reported that the frequency of fetal Rh (D) negative is 40 % of cases, while in the study we found only 25% of Rh (D) negative fetuses. This difference can be attributed to the variable gene frequency of Rh (D) in our study population - Gynecology Service data show a high amount of mothers from Eastern, African and Central American origin. Regarding ABO incompatibility, a small proportion of newborns was detected with positive DAT (3.19%) among 3.128 births.

In all cases of group O mothers an elution test was performed, with 7 particular cases in which anti-AB was eluted and one strange case in which the mother was group O and the child was group AB. We investigated this special case and learnt that it corresponded to an IV in vitro fertilization case with an oocyte donation.

In the study we found an outstanding case of anti-B HDFN. It is rare to find this aggressiveness in fetus-maternal ABO incompatibility. The other case, also an aggressive case, was caused by anti-D originated in a mother immunized in previous abortions.

With regard to titrations performed into gel cards, we agree this is not the reference test. We observed that they are more sensitive than the standard tests made in tube.

(28) This means that a higher titer is obtained in comparison with the reference tube. In this regard, a recent article has shown that the application of gel cards is the technique of improved reliability and reproducibility for the comparison of results between laboratories, in the specific field of blood group IgG Ab in ABO incompatible kidney transplantation. (30)

Furthermore, in a comparative study at the Johns Hopkins Hospital, it was recently shown that the gel-card technique reduces the variability of the Ab titration results compared to those obtained with the test-tube assays, besides having the advantage of being a faster technique. (31,32) This simple and economic method of indirect haemagglutination in gel, used at the transplant center in Stockholm, seems to offer at least the essential guarantee of reproducibility. (31)

CONCLUSION

We can conclude with our study that HDFN in Spain has stabilized in frequency, with 1 in 1500, although we detected cases from immigrant pregnant women.

While we found a single case of alloimmune anti-D, other alloimmune Ab detected in maternal plasma corresponded to antigens belonging to the Rh system. The rest of anti-D Ab detected in our study are passive Ab, IgG, meaning that they were remainders of the administration of anti-D prophylaxis.

Most infants with a positive DAT were ABO incompatibility. DAT positive leads us that there is a hemolytic and negative DAT process is strongly suggestive that the newborn is not affected, but not absolutely rule that is immune cause hemolysis. If there is a suspected diagnosis, additional tests should be performed to extend the information such as elution techniques that can confirm or rule out the presence of Ab in cases of DAT to be negative. Regarding the secondary objective, the proportion of D negative mothers who had children with D negative is slightly lower compared to that reported in the literature, which is about 40%.

We conclude that the initiative of screening for *fetal RHD genotype* in maternal plasma in all pregnant D negative women, to prevent the administration of IgG anti-D prophylaxis at 28 weeks if the fetus is also D negative, could be more cost-effective in populations with higher frequency of D negative fetal groups. Otherwise, routine scheduled antiglobulin injection for non-sensitized Rh negative mother with fetus which had Rh positive or unknown father is more reasonable.

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