



# The combined effect of anti-D and non-D Rh antibodies in maternal alloimmunization

## Maternal alloimmünizasyonda anti-D ve D dışı Rh antikorlarının kombine etkisi

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### Abstract

**Objective:** This study aims to investigate the distribution of antibodies that cause hemolytic disease of the fetus and newborn (HDFN) and compare the clinical outcomes of pregnancies affected by anti-D and anti-D combined with non-D Rh alloimmunization.

**Materials and Methods:** We retrospectively searched and obtained the perinatal and neonatal data of patients with anti-D antibodies and anti-D combined with non-D Rh antibodies (anti-c, -C, -e, -E, and -Kell) from October 2015 to December 2018 at the University of Health Sciences Turkey, Kanuni Sultan Süleyman Training and Research Hospital. Univariate and multiple logistic regression analyses and adjusted odds ratios with their confidence intervals were used to define independent risk factors for non-D antibody positive.

**Results:** The severe fetal hydrops rate was significantly higher in the anti-D combined non-D group (3/25, 12%) than in the anti-D group (1/128, 0.08%,  $p<0.001$ ). The intrauterine transfusion (IUT) requirement in the anti-D combined non-D group (16/25, 64%) tended to be significantly higher than that in the anti-D group (5/128, 7.46%,  $p<0.001$ ). The incidence of neonatal exchange transfusion, top-up transfusion, and postnatal phototherapy frequency in the anti-D combined non-D group was significantly higher than in the anti-D group.

**Conclusion:** Anti-D combined with another non-D Rh alloantibody resulted in significantly higher HDFN rates than the anti-D alloimmunized pregnancies. Also, anti-D in association with non-D Rh antibodies resulted in more severe HDFN requiring more invasive treatment procedures, including IUT, neonatal exchange transfusion, or top-up transfusion.

**Keywords:** Fetal anemia, hemolytic disease of the fetus and newborn, non-D antibodies, Rh alloimmunization

### Öz

**Amaç:** Bu çalışma, fetüs ve yenidoğanın hemolitik hastalığına (FYHH) neden olan antikorların dağılımını araştırmayı ve anti-D ile birlikte D dışı Rh antikorlardan etkilenen gebeliklerin klinik sonuçlarını anti-D tarafından etkilenen gebeliklerle karşılaştırmayı amaçlamaktadır.

**Gereç ve Yöntemler:** Sağlık Bilimleri Üniversitesi Kanuni Sultan Süleyman Eğitim ve Araştırma Hastanesi'nde Ekim 2015 - Aralık 2018 tarihleri arasında anti-D antikorları ve anti-D ile kombine D dışı (anti-c, -C, -e, -E, ve -Kell) Rh antikorları olan hastaların perinatal ve neonatal sonuçlarını geriye dönük olarak araştırdık. D dışı antikor pozitifliğinin varlığı için bağımsız risk faktörlerini tanımlamak için, tek değişkenli ve çoklu lojistik regresyon analizleri ve bunların güven aralıklarıyla ayarlanmış olasılık oranlarını kullandık.

**Bulgular:** Şiddetli fetal hidrops oranı anti-D ile kombine D dışı grupta (3/25, %12) anti-D grubundan (1/128, %0,08) anlamlı olarak daha yüksekti ( $p<0,001$ ). Anti-D ile kombine D dışı gruptaki (16/25, %64) intrauterine transfüzyon (IUT) gereksinimi anti-D grubundan (5/128, %7,46) önemli ölçüde daha yüksekti ( $p<0,001$ ). Anti-D ile kombine D dışı grupta neonatal kan değişimi, tamamlayıcı transfüzyon ve postnatal fototerapi sıklığı anti-D grubuna göre anlamlı olarak daha yüksekti.

**Sonuç:** Anti-D ile kombine D-dışı Rh alloantikoru ile oluşan gebelikler anti-D alloimmünize gebeliklerden önemli ölçüde daha yüksek FYHH oranları ile sonuçlanmıştır. Ayrıca, D-dışı Rh antikorları ile birlikte anti-D antikor varlığı, İUT, neonatal kan değişimi ve tamamlayıcı transfüzyon da dahil olmak üzere invaziv prosedürleri gerektiren daha ciddi FYHH ile sonuçlandı.

**Anahtar Kelimeler:** Fetal anemi, fetüs ve yenidoğanın hemolitik hastalığı, D dışı antikorlar, Rh alloimmünizasyonu

**PRECIS:** Anti-D combined with non-D Rh antibodies significantly influence the severity of fetal anemia compared with anti-D alone.

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## Introduction

Red blood cell (RBC) alloimmunization occurs if an Rh-negative pregnant woman is exposed to Rh-positive fetal blood cells. This exposure leads to Rh-antibody development during pregnancy or delivery. RBC alloimmunization also happens when an Rh-negative woman undergoes an Rh-positive blood transfusion<sup>(1)</sup>. The minimal fetal blood volume required to cause alloimmunization varies from 0.1 mL to 1 mL and is possibly associated with the Rh-positive RBCs' immunogenic capacity and the patient's immune responsiveness<sup>(2)</sup>. Fetomaternal hemorrhage adequately induces alloimmunization. It occurs most commonly at parturition, known as the most vulnerable period, from 15% to 50% of deliveries<sup>(3)</sup>. When fetomaternal hemorrhage occurs, ectopic pregnancy, threatened abortion, spontaneous or induced pregnancy termination, invasive intrauterine procedures, blunt abdominal trauma, any antepartum bleeding episode and external cephalic version<sup>(2,3)</sup>. It was determined that if the prevention with anti-D prophylaxis is not performed during the antepartum and within 72 hours of delivery, approximately 14% of these patients will develop anti-Rh antibodies within six months or during their subsequent pregnancy<sup>(4)</sup>. Hemolytic disease of the fetus and newborn (HDFN) remains a severe pregnancy complication that continues to be a major cause of adverse perinatal outcomes. HDFN is caused by maternal immunoglobulin G (IgG) red cell alloantibodies that are actively transported across the placenta, bind to fetal erythrocytes via the involved antigen, and cause immune-mediated hemolysis and anemia. If left untreated, they may cause fetal heart failure, fetal hydrops, and fetal death<sup>(5)</sup>. The use of anti-D prophylaxis has led to a decrease in the incidence of Rh alloimmunization in developed countries. About 1.8% of Rh-negative women develop anti-Rh antibodies following only postpartum prophylaxis, and 0.2% of Rh-negative patients develop these antibodies following both antepartum and postpartum prophylaxis<sup>(4,6)</sup>. However, no immunoprophylaxis has been produced to inhibit non-D alloimmunizations<sup>(7)</sup>.

As a consequence of extended use of anti-D prophylaxis in developed countries, non-D antibodies account for a relatively higher proportion of alloimmunized pregnancies<sup>(8)</sup>. Previous data indicated that RBC transfusion is the most significant independent risk factor for non-D Rh alloimmunization, followed by delivery, major surgery, and hematological diseases<sup>(9)</sup>. A limited number of studies examined the management and neonatal outcome of maternal alloimmunization based on the antibody types. This is especially concerning since middle cerebral artery (MCA) peak systolic velocity is the measurement used in routine practice to evaluate fetal anemia. Some patients have multiple RBC antibodies, which might lead to a more complicated state and require additional interventions, including intrauterine transfusion (IUT), during HDFN management in pregnancy than the presence of a single RBC antibody<sup>(10)</sup>.

This study investigates the distribution of antibodies that cause HDFN and compares the clinical outcomes of pregnancies affected by anti-D and anti-D combined with non-D Rh alloimmunization in a Turkish tertiary referral center.

## Materials and Methods

This retrospective case-control study was performed in the Kanuni Sultan Süleyman Training and Research Hospital from October 2015 to December 2018. All Rh-negative pregnant women with RBC alloimmunization confirmed by Rh titers, aged between 18 and 40 years, who managed and delivered in this hospital were included in this study. We searched and obtained the perinatal and neonatal data of patients with anti-D antibodies and anti-D combined with non-D Rh antibodies [anti-c, -C, -e, -E, and -Kell (K)] during the study course from the hospital's electronic database and medical files of both the mother and the newborn. The ethics committee of the hospital approved the study (2019/04/86).

Of the 153 pregnant women included in the study, we enrolled 128 patients with anti-D antibodies as the anti-D group and 25 patients with anti-D combined with non-D Rh antibodies as the anti-D combined non-D group. Patients were enrolled only if non-D Rh antibodies occurred in conjunction with an anti-D antibody during the pregnancy course. Patients with multiple pregnancies, any major structural fetal abnormality on the ultrasound scan (US), who delivered at another institution, with unavailable or incomplete medical records, and were unwilling to participate in this study, were excluded. Patients were excluded if fetal or neonatal death occurred for reasons other than alloimmunization. Also, alloimmunized patients were excluded if the antibodies identified were deemed clinically insignificant, including passive anti-D, anti-HLA, anti-N, Ig-M class anti-M, and anti-Le<sup>(11)</sup>.

The following protocol was used to investigate and manage the Rh-sensitized pregnancies in our hospital. All Rh-negative pregnant women were routinely screened with an Rh-positive father for antibodies during the first trimester. The maternal antibody titer was determined utilizing the Indirect Coombs test (ICT). Maternal antibody detection and titrations were conducted by the indirect gel antiglobulin technique. Titers were obtained in the same laboratory since variations in titer results from different laboratories are common. Titrations were determined every 2 to 4 weeks with the exception of anti-K. Anti-K is demonstrated to suppress fetal erythropoiesis, and therefore, antibody titers are not predictive of fetal outcome in HDFN. When anti-K was detected, no more titers were conducted<sup>(12)</sup>. A titer  $\geq 1:16$  indicates a significant risk for HDFN. If the cut-off value was reached, the laboratory follow-up was discontinued. In patients with an Rh-titer of  $\geq 1:16$ , antenatal fetal monitoring by color Doppler US was performed to determine the MCA peak systolic velocity. Pregnancies complicated by HDFN were managed by weekly monitoring with MCA Doppler US until anemia is suspected and IUT is

required. Suspected fetal anemia requiring IUT was defined as abnormal MCA Doppler US findings and/or the presence of other anemia signs at US (hydrops, cardiomegaly)<sup>(13)</sup>. We labeled abnormal MCA Doppler US as a peak systolic velocity >1.5 multiples of the median (MoM) value for the gestational age<sup>(14)</sup>. Signs of fetal hydrops on US were described as elevated fluid in higher than two fetal compartments, including pericardial effusion, pleural effusion, ascites, increased amniotic fluid index, and skin edema<sup>(11)</sup>. Fetal hydrops was classified as mild or severe. The presence of a distinct rim of ascites with or without pericardial effusion is described as mild fetal hydrops. Fetal hydrops was considered severe when ascites was abundant with the presence or absence of pleural effusion, skin edema, and pericardial effusion<sup>(13)</sup>. Cordocentesis was performed to confirm fetal anemia if MCA peak systolic velocity exceeded 1.5 MoM and/or if fetal anemia signs were detected on the US. A fetal hematocrit of less than 30% was used as the cut-off for fetal anemia to indicate an IUT<sup>(15)</sup>. After the procedure, antenatal monitoring was performed by weekly MCA peak systolic velocity measurement and fetal biophysical profile. The time interval between the two transfusions depended on the MCA peak systolic velocity measurements during the follow-up and posttransfusion serum hemoglobin concentrations. Since the positive predictive value for a cut-off value of 1.5 MoM decreased significantly from the first IUT to the second and third IUT, a threshold of 1.73 MoM was used to diagnose fetal anemia at the time of the second and third IUT<sup>(16)</sup>.

Data on maternal age, gravidity, parity, alloimmunization type, the presence or absence of fetal hydrops, MCA peak systolic velocity values, the gestational week at birth, and neonatal outcomes were recorded. For fetuses with anemia, data was further recorded on the gestational week at the hemolytic disease of the fetus (HDF) diagnosis, the gestational week at cordocentesis, the gestational week at the first IUT, fetal hemoglobin and hematocrit values before and after IUTs, and the number of IUTs. Neonatal outcomes consisted of birth weight, Apgar scores at 1- and 5-minutes, neonatal intensive care unit (NICU) admission, the requirement for phototherapy, exchange transfusion, and top-up transfusion treatments. Phototherapy, exchange transfusion, and top-up transfusion treatments were performed based on the Turkish Neonatal Society guidelines<sup>(17)</sup>. Neonatal laboratory results were recorded to those collected within 48 hours of birth, including ABO and blood groups, direct antiglobulin (Coombs) test (DAT), hemoglobin and hematocrit values, and serum bilirubin levels (total, direct, indirect). Patients who experienced antibody detection recurring times during the same gestation were enrolled as a single record, and the highest titer was recorded during the pregnancy course. For the anti-D combined non-D group, the titers of all antibody types were recorded and used the highest titer in the analysis. Regarding the women who recorded being pregnant more than

once during the study course, each alloimmunized pregnancy was marked as a separate pregnancy case.

The mode of delivery was determined by standard obstetric indications<sup>(18)</sup>. The primary outcome was the occurrence of HDFN and the overall survival rate of the fetuses. HDFN was defined as fetal hydrops, the need for IUT, intrauterine fetal death, neonatal intensive phototherapy, and neonatal exchange or top-up transfusion. The overall survival rate was based on the live infant number one month after birth.

### Statistical Analysis

Differences between categorical variables were analyzed by chi-square test or Fisher's exact test, where appropriate. The factors that may correlate with the outcome non-D antibody positive or not were analyzed independently (univariate analysis) by either Student's t-test or Mann-Whitney U test where applicable. Variables such as the gestational week at diagnosis, birth week, and Apgar scores also compared groups of patients with anti-D groups were performed using the Kruskal-Wallis test. Multiple comparison tests were used to know which groups differ from which others. Univariate and multiple logistic regression analyses and adjusted odds ratios with their confidence intervals were used to define independent risk factors for non-D antibody positive. Diagnostic powers of variables used to determine non-D antibody positivity are shown with sensitivity, specificity, positive and negative likelihood ratios. The correlation between binary variables was investigated using the Phi correlation coefficient. Statistical analyses were done using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA), and significance was assumed for a p-value of <0.05.

### Results

During the study period from October 2015 to December 2018, a total of 37,344 deliveries occurred at the obstetric unit of the University of Health Sciences Turkey, Kanuni Sultan Süleyman Training and Research Hospital. A total of 178 alloimmunized pregnancies were detected from the medical records of the patients. We excluded 25 patients from this study based on missing medical records or applying the exclusion criteria. Finally, a total of 153 alloimmunized pregnant women and their fetuses were included in this study. None of them were multiple pregnancies. The incidence of pregnancies affected by Rh alloimmunization was 0.40% (153/37344), of which 0.34% (128/37344) of them were alloimmunization with anti-D antibody and 0.06% (25/37344) with anti-D combined non-D Rh antibodies.

Table 1 presents the maternal demographic characteristics, the course of affected pregnancies, management, and treatment outcomes of neonates stratified by anti-D antibody group and anti-D combined with non-D Rh antibodies group. Table 2 summarizes the prenatal and postnatal characteristics of the anti-D combined non-D group. Anti-c<sub>e</sub> was most common (13/25, 52%), followed by anti-C<sub>ce</sub> (5/25, 20%), anti-C<sub>e</sub>

**Table 1.** Maternal demographic characteristics, the course of affected pregnancies, management, and treatment outcomes of neonates stratified by the anti-D antibody group and the anti-D combined with non-D Rh antibodies group

		Anti-D combined non-D group	Anti-D group	p
Gravidity		4 (2-5)	3 (2-4)	0.422
Parity		2 (1-3)	2 (1-3)	0.463
Previous abortion		0.56±0.96	0.54±1.10	0.833
Fetal gender	Male	10 - (40.00)	75 - (58.60)	0.123
	Female	15 - (60.00)	53 - (41.40)	
Gestational week at diagnosis		31.04±4.98	32.16±4.96	0.201
MCA Doppler US	A Zone (>1.5 MoM)	5 - (20.00)	3 - (2.30)	<0.001*
	B Zone (1.29-1.5 MoM)	3 - (12.00)	5 - (3.90)	
	C Zone (<1.29 MoM)	17 - (68.00)	120 - (93.80)	
Fetal hydrops		3 - (12.00)	1 - (0.78)	0.014*
First indirect Coombs test	16-256	9 - (36.00)	83 - (64.84)	<0.001
	512-8192	8 - (32.00)	40 - (31.25)	
	≥16384	8 - (32.00)	5 - (3.90)	
Last indirect Coombs test	16-256	7 - (28.00)	73 - (57.03)	<0.001
	512-8192	7 - (28.00)	47 - (36.71)	
	≥16384	11 - (44.00)	8 - (6.25)	
Intrauterine transfusion		16 - (64.00)	7 - (5.46)	<0.001*
Gestational week at cordocentesis and first intrauterine transfusion		27 (21-33)	30 (25-32)	0.093
Cesarean delivery		20 - (80.00)	75 - (58.59)	0.070
Birth week		34.64±4.27	37.50±1.92	<0.001
Birth weight		2450.80±832.36	3029.49±536.54	<0.001
1-min Apgar score		6.20±2.48	7.24±1.27	0.085
5-min Apgar score		7.52±3.07	9.02±0.91	0.013
NICU admission		17 - (77.2)	53-(43.4)	<0.001
NICU admission, days		16.58±9.89	11.32±9.31	0.014
NICU admission, days	No	8 - (32.00)	75 - (58.59)	0.002
	≤7 days	2 - (8.00)	21 - (16.40)	
	>7 days	15 - (60.00)	32 - (25.00)	
NICU admission indications	RDS	3 - (17.64)	19 - (38.45)	0.173*
	Jaundice	14 - (82.35)	25 - (48.07)	
	Sepsis	0 - (0.00)	5 - (9.61)	
	Hypoglycemia	0 - (0.00)	2 - (3.84)	
Hemoglobin value at birth		14.81±6.62	16.03±2.42	0.007
Hematocrit value at birth		45.87±23.28	47.51±7.02	0.030
Total bilirubin value at birth		9.82±6.17	11.34±7.89	0.727
Phototherapy		15 - (68.18)	30 - (23.62)	<0.001

Neonatal exchange transfusion	4 - (18.18)	4 - (3.14)	0.017*
Top-up transfusion	6 - (27.27)	2 - (1.57)	<0.001*
Direct Coombs test positivity	13 - (59.09)	32 - (25.19)	0.002*
In utero fetal demise	3 - (12.00)	1 - (0.08)	0.014*
HDFN	18 - (72.00)	31 - (24.21)	<0.001

Data were presented as median (minimum-maximum), mean ± standard deviation, and n (%).  
\*represents Fisher's exact test's p-value

(3/25, 12%), anti-cE (1/25, 4%), anti-Cc (1/25, 4%), anti-E (1/25, 4%), and anti-K (1/25, 4%). The patients in the anti-D group had a similar number of gravidity, parity, and previous abortions with the anti-D combined non-D group. Also, the two groups were comparable regarding fetal gender and the gestational week at the time of diagnosis. All the pregnant women who underwent cordocentesis had anemic fetuses and experienced intrauterine transfusion. The median gestational week at cordocentesis and the first IUT was similar between the groups. The severe fetal hydrops rate was significantly higher in the anti-D combined non-D group (3/25, 12%) than in the anti-D group (1/128, 0.08%,  $p < 0.001$ ). All fetuses with severe hydrops received an intrauterine transfusion in both groups. In the anti-D combined non-D group, 100% ( $n=3$ ) of severe fetal hydrops cases resulted in fetal death during the pregnancy course. Two of these had anti-D combined with anti-ce and ended in fetal death at the 25<sup>th</sup> and 28<sup>th</sup> weeks of gestation. One had a combination of anti-D and anti-cE and resulted in fetal death in the 22<sup>nd</sup> week of pregnancy. In the anti-D group, a severe fetal hydrops case was born by cesarean delivery at 30<sup>th</sup> weeks of gestation due to non-reassuring fetal heart rate but did not survive after the delivery. The IUT requirement in the anti-D combined non-D group (16/25, 64%) tended to be significantly higher than that in the anti-D group (5/128, 7.46%,  $p < 0.001$ ). No intraperitoneal transfusion was performed. The cesarean delivery rate was not significantly different between the anti-D group (58.59%) and the anti-D combined non-D group (80%,  $p=0.070$ ). The gestational week at birth and birth weight in the anti-D combined non-D group (34.64±4.27 weeks and 2450.80±832.36 g, respectively) were significantly lower than that of the anti-D group (37.50±1.92 weeks and 3029.49±536.54, respectively,  $p < 0.001$ ).

Regarding the management of the neonates, 10.7% (16/149) of infants required treatments for anemia. The incidence of neonatal exchange transfusion in the anti-D combined non-D group (4/25, 18.18%) was significantly higher than in the anti-D group (4/128, 3.14%,  $p=0.017$ ). The top-up transfusion requirement in the anti-D combined non-D group (6/22, 27.27%) tended to be significantly higher than that in the anti-D group (2/152, 1.57%,  $p < 0.001$ ). The postnatal phototherapy frequency was significantly higher in the anti-D combined non-D group (15/22, 68.18%) than in the anti-D group (30/152, 23.62%,  $p < 0.001$ ). In total, 46.9% (70/149) of neonates required NICU admission. The NICU admission rate

was significantly higher in the anti-D combined non-D group (17/22, 77.2%) than in the anti-D group (53/152, 43.4%,  $p < 0.001$ ). The duration of NICU admission in the anti-D combined non-D group (16.58±9.89 days) was significantly longer than in the anti-D group (11.32±9.31 days,  $p=0.014$ ). A DAT was performed on all the neonates; 59.09% (13/22) were positive in the anti-D combined non-D group, and 25.19% (32/149) were positive in the anti-D group ( $p=0.002$ ). A total of 38.5% (59/153) of the fetuses were affected by maternal Rh alloimmunization and developed HDFN. The frequency of HDFN in the anti-D combined non-D group (18/25, 72%) was significantly higher than that in the anti-D group (31/128, 24.2%,  $p < 0.001$ ). All newborns in the anti-D combined non-D group had survived one month after birth.

The requirement of IUT and phototherapy had a high sensitivity to determine the non-D alloantibody positivity (0.64 and 0.68, respectively). However, the presence of fetal hydrops, exchange transfusion, top-up transfusion, and in utero fetal demise had a low sensitivity to determine the non-D alloantibody positivity (0.12, 0.18, 0.27, and 0.12, respectively). Therefore, it is not reasonable to make predictions about the non-D alloantibody positivity with these parameters. However, all parameters had moderate and high specificity values in detecting the absence of non-D alloantibody. Table 3 shows the positive and negative likelihood ratios of these parameters.

Table 4 provides the correlation coefficients for non-D alloantibody positivity of key parameters, and Table 5 presents the results of the univariate and multiple logistic regression analysis. Only the requirement of IUT was significant in the final model of logistic regression analysis, which was established with all variables found to be significant in the univariate analysis. In the presence of the IUT requirement, the risk of non-D alloantibody positivity increased 21.4 times and was statistically significant ( $p < 0.001$ ).

## Discussion

The current study evaluates the clinical outcomes of pregnancies affected by anti-D and anti-D combined with non-D Rh antibodies. Our study indicates that anti-D combined with another RBC alloantibody resulted in significantly higher HDFN rates than anti-D alloimmunized pregnancies. Also, anti-D in association with non-D Rh antibodies resulted in more severe HDFN requiring more invasive treatment procedures, including

**Table 2.** The prenatal and postnatal characteristics of the anti-D combined non-D group

Rbc antibody	Gestational week at critical titer reached	Maximum maternal antibody titer	IU Tx, n NICU admission, days	Gestational week at delivery indication for NICU admission	Neonates					
					Phototherapy	Exchange Tx	Top-up Tx			
Cce (n=5)	1	29	1/4096	2	35+4	10	RDS	+		
	2	35	1/8192	-	35+2	15	Jaundice	+		+
	3	29	1/65536	3	37	30	Jaundice	+		+
	4	32	1/2048	4	36	20	Jaundice	+		+
	5	30	1/32768	3	35	3	Jaundice	+		
Ce (n=13)	1	25	1/256	-	38	-				
	2	31	1/32768	3	35	13	Jaundice	+		
	3	32	1/16384	2	25 (IUFD)					
	4	31	1/32768	5	34+1	12	Jaundice	+		+
	5	30	1/65536	2	34	42	Jaundice	+		+
	6	19	1/65536	2	34+2	13	Jaundice	+		
	7	29	1/1024	1	33+3	10	Jaundice	+		
	8	25	1/32768	1	27+6	25	RDS (died)			
	9	33	1/8192	1	33+2	25	RDS			
	10	30	1/32768	3	33+1	25	Jaundice	+		+
	11	35	1/32	-	37+6	-				
	12	32	1/128	5	35+5	9	Jaundice	+		
	13	31	1/4096	-	37+4	7	Jaundice	+		+
Ce (n=3)	1	35	1/256	-	37	-				
	2	29	1/32768	7	34+4	15	Jaundice	+		+
	3	35	1/32768	-	37+2	-				
cE (n=1)	1	22	1/4096	-	22 (IUFD)	-				
Cc (n=1)	1	31	1/65536	4	35+6	9	Jaundice	+		+
E (n=1)	1	33	1/64	-	37	-				
Kell (n=1)	1	32	1/64	-	38	-				

IUT, neonatal exchange transfusion, or top-up transfusion. We detected an RBC alloimmunization incidence of 0.4% (153/37344) that was similar to that reported by previous studies (0.4%-1.1%)<sup>(11,19,20)</sup>. Also, the non-D Rh alloimmunization incidence in our study was 0.06% (25/37344), which is considerably lower than the 0.32% Koelewjin et al.<sup>(21)</sup> found in the Netherlands, the 0.16% Gotvall and Filbey<sup>(22)</sup> found in Sweden, and slightly higher than the 0.04% Healsmith found in Australia<sup>(8)</sup>. The distribution of maternal alloimmunization and HDFN with anti-D and non-D antibodies varies in different populations and countries. This difference can be explained by the geographic variations of Rh antigen frequencies in the populations examined, transfusion practices, and antibody screening frequencies in different countries<sup>(8,11)</sup>. According to the blood transfusion policy in Turkey, ABO and Rh

blood types are routinely identified before blood transfusion. Pretransfusion, an extended Rh antigen phenotyping, is only performed if the patient has previously detected antibodies or a patient who may require a long-term transfusion regimen. Nordvall et al.<sup>(23)</sup> reported that the combination of antibody specificities was more harmful and brought about a more severe form of HDFN than single antibody specificities. They suggested that increased binding of multiple antibodies on target RBCs led to higher hemolysis levels due to a synergistic effect. Markham et al.<sup>(24)</sup> also stated that multiple RBC antibodies are related to an increased risk for significant HDFN development and proposed two possible theories. The first theory is the cumulative effect involving increased hemolysis due to the binding of the multiple antibodies to more fetal RBCs. The second theory is a more aggressive immune response in patients prone to developing

**Table 3.** Diagnostic performance of key parameters for non-D alloantibody positivity

<b>Anti-D combined non-D group vs fetal hydrops</b>	
Sensitivity (95% CI)	0.12 (0.041-0.29)
Specificity (95% CI)	0.99 (0.95-0.99)
Positive likelihood ratio (95% CI)	15.3 (1.66-141.73)
Negative likelihood ratio (95% CI)	0.88 (0.76-1.026)
<b>Anti-D combined non-D group vs intrauterine transfusion</b>	
Sensitivity (95% CI)	0.64 (0.44-0.79)
Specificity (95% CI)	0.94 (0.89-0.97)
Positive likelihood ratio (95% CI)	11.70 (5.36-25.47)
Negative likelihood ratio (95% CI)	0.38 (0.22-0.64)
<b>Anti-D combined non-D group vs phototherapy</b>	
Sensitivity (95% CI)	0.68 (0.47-0.83)
Specificity (95% CI)	0.76 (0.68-0.82)
Positive likelihood ratio (95% CI)	2.88 (1.89-4.40)
Negative likelihood ratio (95% CI)	0.41 (0.22-0.77)
<b>Anti-D combined non-D group vs exchange transfusion</b>	
Sensitivity (95% CI)	0.18 (0.07-0.38)
Specificity (95% CI)	0.96 (0.92-0.98)
Positive likelihood ratio (95% CI)	5.77 (1.55-21.39)
Negative likelihood ratio (95% CI)	0.84 (0.69-1.03)
<b>Anti-D combined non-D group vs top-up transfusion</b>	
Sensitivity (95% CI)	0.27 (0.13-0.48)
Specificity (95% CI)	0.98 (0.94-0.99)
Positive likelihood ratio (95% CI)	17.31 (3.73-80.37)
Negative likelihood ratio (95% CI)	0.73 (0.57-0.95)
<b>Anti-D combined non-D group vs in utero fetal demise</b>	
Sensitivity (95% CI)	0.12 (0.04-0.29)
Specificity (95% CI)	0.99 (0.95-0.99)
Positive likelihood ratio (95% CI)	15.36 (3.56-45.39)
Negative likelihood ratio (95% CI)	0.89 (0.45-0.98)

CI: Confidence interval

multiple RBC antibodies. In the second theory, women prone to developing multiple antibodies have a more aggressive immune response. This theory may also explain the increased risk of significant HDFN in the setting of multiple antibodies, but only one corresponding fetal or neonatal antigen<sup>(24)</sup>.

Previous studies reported that the presence of anti-D combined with another RBC antibody resulted in a significantly increased risk of developing HDFN and receiving invasive treatment procedures, including IUT, top-up transfusion, or exchange transfusion<sup>(10,23,24)</sup>. Also, these studies stated that the majority

**Table 4.** Correlation coefficients for non-D alloantibody positivity of key parameters

	Anti-D combined non-D group (Phi coefficient and p-value)
Fetal hydrops	0.260 (p=0.001)
Intrauterine transfusion	0.606 (p<0.001)
Phototherapy	0.344 (p<0.001)
Exchange transfusion	0.237 (p=0.004)
Top-up transfusion	0.404 (p<0.001)
In utero fetal demise	0.260 (p=0.001)

of alloimmunizations with multiple antibodies included anti-D and the presence of anti-D in multiple antibody combinations was more likely to develop significant HDFN requiring invasive treatment methods than those of the other combinations. However, Sharma et al.<sup>(25)</sup> reported a rare case in which the neonate presented severe hyperbilirubinemia and jaundice due to anti-C and anti-e alloimmunization. They suggested that DCT should be performed in all neonates with severe jaundice even when there is no ABO and Rh isoimmunization. Anti-C can be additive to the hemolytic effects of other antibodies and is more often related to severe outcomes in pregnancies complicated by multiple antibodies or in compound antibodies<sup>(8,25,26)</sup>. All the other non-D Rh antibodies may cause adverse neonatal outcomes<sup>(8)</sup>. However, we only included these antibodies in our study when they are present in conjunction with anti-D to maintain the focus on the additive effects of these antibodies. Currently, all patients with alloimmunization are managed as anti-D alloimmunization based on the various published data about this complication without predicting whether the fetal and neonatal outcomes are similar and whether this approach is correct<sup>(10)</sup>.

Management of Rh-isoimmunized pregnancies relies on the regular monitoring of maternal antibody concentration via calculating antibody titration for most antibodies<sup>(12)</sup>. Antibody titration studies evaluate the antibody quantity and serve as a screening test to indicate when MCA peak systolic velocity measurement with Doppler US should be initiated. MCA peak systolic velocity above 1.5 MoM can predict moderate to severe fetal anemia with a sensitivity of 100% and a false positive rate of 12%<sup>(2)</sup>. In pregnancies with Rh alloimmunization, after the occurrence of fetal anemia, the antibody titer should not be used to predict the risk of severe HDFN<sup>(27)</sup>. Nevertheless, previous reports determined a critical titer of  $\geq 16-32$  by conventional tube testing. Below this range, no severe adverse outcomes were observed, including a requirement for IUT, intrauterine fetal demise, or stillbirths<sup>(11)</sup>. Fink et al.<sup>(28)</sup> reported that the indirect gel antiglobulin technique might perform similar to the conventional tube testing in titrating alloantibodies to Rh antigens. Up to now, few studies have evaluated the correlation of HDFN with the gel titer cut-off value. In our study, across

**Table 5.** The results of univariate and multiple logistic regression analysis

Variables	Univariate logistic regression			95% Confidence intervals		P
	B	S.E.	OR	Lower Limit	Upper Limit	
Fetal hydrops	2.852	1.178	17.318	1.722	174.120	0.015
Intrauterine transfusion	3.425	0.570	30.730	10.058	93.891	<0.001
Phototherapy	1.936	0.503	6.929	2.584	18.575	<0.001
Exchange transfusion	1.922	0.751	6.833	1.569	29.765	0.010
Top-up transfusion	3.154	0.859	23.437	4.356	126.107	<0.001
In utero fetal demise	2.852	1.178	17.318	1.722	174.120	0.015
Variables	Multiple logistic regression			95% Confidence intervals		P
	B	S.E.	OR	Lower limit	Upper limit	
Intrauterine transfusion	3.067	0.616	21.488	6.430	71.809	<0.001

all antibody types, 52.2% (n=80) of the patients did not exceed the critical titer of 16 with the gel technique, and no neonates born to these mothers with a titer  $\leq 16$  met the HDFN criteria, suggesting that this cut-off value was clinically suitable.

#### Study Limitations

The main strength of this study was that it was conducted in a well-organized tertiary center with trained medical staff who delivered adequate health care to alloimmunized pregnant patients. Indirect gel antiglobulin testing was used to validate the critical antibody titer. However, there are some limitations to this study. This study was designed retrospectively, with the potential to contain study limitations. Also, patients' previous history of RBC transfusions was not identified due to the lack of data. The rarity of non-D Rh antibodies resulted in the low sample size of this study. The differences in neonatal outcomes between specific single and multiple antibodies could not be identified.

#### Conclusion

The incidence of Rh alloimmunization has decreased notably in recent decades, most probably due to the extended use of anti-D prophylaxis and non-D antibodies. These antibodies represent a relatively higher proportion of alloimmunized pregnancies. Anti-D combined with another non-D Rh alloantibody resulted in significantly higher HDFN rates than anti-D alloimmunized pregnancies. Also, anti-D in association with non-D Rh antibodies resulted in more severe HDFN requiring more invasive treatment procedures, including IUT, neonatal exchange transfusion, or top-up transfusion.

#### Ethics

**Ethics Committee Approval:** The ethics committee of the hospital approved the study (2019/04/86).

**Informed Consent:** Retrospective study.

**Peer-review:** Externally peer-reviewed.

#### Authorship Contributions

Surgical and Medical Practices: Z.G.Ö., Concept: Z.G.Ö., S.C.O., Design: Z.G.Ö., S.C.O., Data Collection or Processing: Z.G.Ö., Analysis or Interpretation: Z.G.Ö., S.C.O., Literature Search: Z.G.Ö., S.C.O., Writing: Z.G.Ö., S.C.O.

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