

The Incidence and Effects of Alloimmunization in Pregnancy During the Period 2000–2013

Die Häufigkeit und Effekte der Immunisierung während der Schwangerschaft im Zeitraum 2000–2013

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ABSTRACT

Introduction The objective of the analysis was to examine the epidemiological aspects of maternal alloimmunization and to determine the most common antibody specificities resulting in hemolytic disease of the newborn (HDN).

Materials and Methods The retrospective epidemiological study encompasses all pregnant women who underwent immunohematological screening and the newborn treated for HDN in the period from 2000 to 2013 in the Herzegovina region.

Results The indirect Coombs test (ICT) detected antibodies against antigens in 384 (2.4%) pregnant women of the 18800 who were tested at the Department of Transfusion Medicine. The direct Coombs test (DCT) detected antibodies against antigens in 160 (0.8%) newborn treated for HDN. The results of our 13-year study, in which 60% of the pregnant women had non-RhD antibodies, confirms this finding.

Conclusion However, we have reached the 21st century and the burden of alloimmunisation in pregnancy is still on our backs. The problem of immunization and HDN is still present in our region, which is a neonatal and public health problem.

ZUSAMMENFASSUNG

Einführung Das Ziel dieser Analyse war die Untersuchung von epidemiologischen Aspekten der häufigsten Antikörperspezifitäten, die zu einem Morbus haemolyticus neonatorum (MHN) führen können.

Methode Die retrospektive Studie umfasste alle Schwangeren, bei denen im immunhämatologischen Screening ein erythrozytärer Antikörper entdeckt wurde, sowie Neugeborene, die im Zeitraum von 13 Jahren (2000–2013) in der Region Herzegowina wegen MHN behandelt wurden.

Ergebnisse Der indirekte Coombs-Test (ICT) war positiv bei 384 (2,4%) von insgesamt 18800 an der Abteilung für Transfusionsmedizin getesteten Schwangeren. Der direkte Coombs-Test (DCT) war positiv bei 160 (0,8%) der Neugeborenen, die wegen MHN behandelt wurden. Das Ergebnis der 13-jährigen Untersuchung zeigte, dass 60% der Schwangeren Anti-Rh-D-Antikörper haben.

Schlussfolgerung Das Immunisierungsproblem und MHN sind weiterhin erheblich vorhanden in der Region Herzegowina, was ein neonatales und öffentlich-gesundheitliches Problem darstellt.

Introduction

Hemolytic disease of the fetus and newborn (HDFN) is a direct consequence of the immunological breakdown of fetal erythrocytes caused by the mother's antibodies that have passed through the placenta [1]. Alloimmunization in pregnant women has been extensively studied in different parts of the world, with the frequency of incidence found to range from 0.4 to 2.7% worldwide, as indicated by the results of a study conducted in 2015 [2]. Owing to the introduction of immunoprophylaxis and mandatory immuno-hematological screening three times during pregnancy, the number of fatalities caused by RhD (Rhesus D) sensitization and the severe consequences on the child and pregnant woman have decreased significantly [3, 4]. However, we have reached the 21st century and the burden of alloimmunization in pregnancy is still on our shoulders. Besides the D antigen, other blood group antigens of the Rh system (C, c, E, e, Cw) and other blood group systems have come to light [5, 6]. Although progress has been made over the past few decades in terms of identifying blood group antigens and in predicting fetal anemia through the use of noninvasive monitoring, many questions remain in terms of understanding red blood cell alloimmunization risk factors, preventive therapies and treatment strategies [7]. Immuno-hematological tests should be conducted on all pregnant women three times during pregnancy in order to detect the presence of antibodies, which, unfortunately, is not the case in many developed countries of the world due to the high costs to the health systems [8]. The labor and delivery of a sensitized pregnant woman should be closely monitored, so that diagnostics and treatment of the newborn can begin immediately upon birth. The frequency of hemolytic disease and its consequences for the mother and child have not been reduced, even thirty years upon the introduction of immunoprophylaxis, because preventive measures for non-RhD sensitization have not yet been introduced [9, 10]. The objective of the analysis was to examine the epidemiological aspects of maternal alloimmunization against erythrocyte antigens of fetuses (ABO, Rhesus, Lewis, Kell, Duffy and others) and to determine the most common types of hemolytic disease of the newborn (HDN) in the period from 2000 to 2013 in the West Herzegovina region.

Materials and Methods

Location and time study

The research was conducted as a retrospective epidemiological analysis at the Department of Transfusion Medicine and the Neonatal Intensive Care Unit (NICU) of the UCH Mostar in the period from 2000 to 2013. Prenatal immunohematological screening was conducted at the Department of Transfusion Medicine for all pregnant women who were permanent residents in the West-Herzegovina County and the Herzegovina-Neretva County, both within the jurisdiction of the Maternity Hospital of the UCH Mostar. 18800 pregnant women were tested at the Department for Transfusion Medicine.

Patients and parameters study

The study encompasses all the pregnant women whose immunohematological tests, conducted at the Department for Transfusion Medicine, detected antibodies against any of the antigens in ABO, Rh (anti-D, anti-C, anti-c, anti-E, anti-e), Kell, Duffy, Kidd and/or other blood group systems. If the ICT tested negative three times during the screening, or if sensitization of mothers occurred, the pregnant women were excluded from the study. The mandatory immunohematological screening of all pregnant women (RhD positive and RhD negative) three times during pregnancy was introduced at the Department for Transfusion Medicine of the UCH Mostar in 2003. The immunohematological tests were conducted at 12 to 16 weeks of pregnancy, then at 24 to 26 weeks and after 35 weeks. If the anti-erythrocyte antibody test was positive, the test was performed once a month, and once every two weeks after week 28. This enabled the monitoring of both the antibody titer increase and the pregnancies. Thus, each reported titer was monitored and repeated in the pregnant women. These recommendations and guidelines were implemented in the Herzegovina region. For the pregnant women included in the study, data on the type of antibodies and antibody titers in pregnancy were retrospectively extracted from the archived documents at the Department of Transfusion Medicine. For other data (course of pregnancy, type of delivery), medical documentation in the form of the letter of discharge for newborns treated at the NICU was used. The following parameters were analyzed in the mothers included in the study: parity, mode of delivery, blood type, RhD factor, the type, specifics and antibody titer.

In the study period from 2000 to 2013, 18914 children were born alive at the Clinic for Gynecology and Obstetrics UCH Mostar. The study included all the newborn that developed HDN due to some of the antigens in the period under analysis. The study included all neonates with HDN who had been moved from local maternity wards (Mostar, Livno, Konjic, Brankovac) to our Neonatal Intensive Care Unit (NICU) Mostar. The newborn who were treated for cholestatic jaundice or physiological jaundice were not included in the research. The data was collated on the basis of medical history, documentation and protocols and letters of discharge for the period 2000 to 2013, which are available in the medical archives of the NICU of the UCH Mostar. The following parameters were analyzed in the newborn: gestational age, gender, DCT, bilirubin, type and length of treatment depending on the severity of the disease during the period of hospitalization in the NICU. The relation between incompatibility type and the clinical features of HDN (mild, severe, very severe) was also analyzed. For the treatment of newborn with HDN, supportive therapy and phototherapy (albumin, plasma, immunoglobulin) were used and where necessary, exchange transfusions (ETR) were performed.

Laboratory and immunohematological examinations

The pregnant women gave samples of venous blood in order to determine blood groups, Rh-D antigen and the type, specifics and antibody titer at the Department of Transfusion. The optimal blood sample for erythrocyte blood group testing for the presence of antibodies in serum and to determine the specificity of antibodies is obtained by taking 5–10 ml of blood into a sterile tube with no anticoagulant. Any newborn whose mother had a

► **Table 1** The average concentration of individual laboratory parameters and gestational age of newborns with HDN who underwent ETR in the period from 2000 to 2013.

Newborn with HDN	Gestational age newborn 37 + 6/7–40 weeks	Concentration of bilirubin (normal values 0–200 µmol/L) Mean ± standard deviation	Number of erythrocytes (normal values $3.9\text{--}5.5 \times 10^{12}/\text{L}$) Mean ± standard deviation	Concentration of hemoglobine (normal values 136–199 g/L) Mean ± standard deviation	Reticulo-cytes (%)
Anti-A*	12	379 ± 14.9	4.19 ± 0.48	121 ± 5.3	47
Anti-B*	4	391 ± 5.8	4.57 ± 0.31	122 ± 5.0	70
Anti-D*	6	221 ± 13.4	3.20 ± 0.17	115 ± 3.9	35
Anti-K*	1	342	3.70	108	9

* antibodies

positive ICT had umbilical cord blood samples taken upon birth, and were tested for blood type, Rh factor and DCT. In the event of the development of jaundice and hemolysis, the infant was transferred to the NICU for further treatment and control. The newborn with HDN treated at the NICU had further blood samples taken for blood count and bilirubin. For the blood count test, a 2-ml sample was taken in a test tube with anticoagulant (EDTA). The blood count and bilirubin tests were conducted by the Department of Laboratory Diagnostics UHC Mostar. The bilirubin concentration test was constantly repeated until discharge. The recommendations of the American Academy of Pediatrics, issued in 2004, were followed for the treatment of newborns with HDN depending on age and the concentration of bilirubin.

The erythrocyte blood group of the pregnant women and newborns was determined using test serum that causes visible agglutination of red blood cells at the Department of Transfusion. Monoclonal antibodies or the polyclonal human antibodies of IgM or IgG class were used. In addition to detecting antigens on erythrocytes, the presence of anti-A or anti-B antibodies in serum was also tested using erythrocyte phenotypes A1, A2, B or O. ABO blood typing was conducted via slides, test tubes, micro methods and cards. Rh testing was conducted on a slide and in a test tube using monoclonal or polyclonal test serum containing IgM or IgG anti-Rh antibodies so that agglutination was visible.

By using the column agglutination test, the so-called cards, the presence of irregular antibodies, DCT and ICT was detected. The test was performed by dripping red blood cells or serum onto the column containing agarose or glass beads.

The antibody titer defines the volume levels of antibodies in serum. Titer indicates the inverse ratio of diluted serum which results in a visible, that is a positive, reaction with corresponding erythrocyte tests. The antibody titer can only give an approximation of the concentration of the antibody as it only measures antibodies bound to erythrocyte antigens, and not the total concentration of an antibody in serum.

Statistical data processing

The measurement scales for the results were nominal, ordinary and interval. The measure of central tendency was Mod. For testing statistical significance, χ^2 -test was used with the aid of the PASW Statistics 17 programme (version 17.0.2 from 2009).

Results

18800 pregnant women were tested at the Department for Transfusion Medicine at the UCH Mostar. The indirect Coombs test (ICT) detected antibodies against antigens in the various blood group systems in 384 women. The incidence of sensitization during pregnancy was 20.6 in 1000 examined pregnant women. Of the total 384 sensitized women, antibodies were detected in 157 (40.3%) during their second pregnancies, which is statistically significant in relation to the presence of antibodies in other parities ($\chi^2 = 11.01$, d. f. = 3, $p = 0.02$).

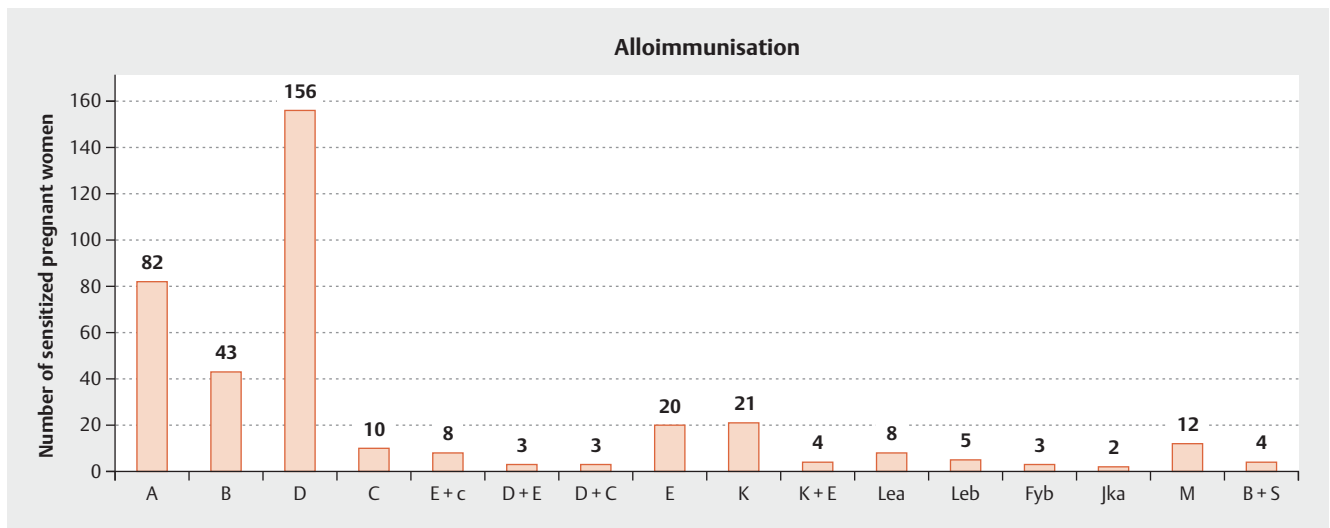
There were 156 pregnant women with Rh D immunization. In 34 of them (21%), antibodies were detected in the third immunohematology test at 34–35 weeks of pregnancy. 69 pregnant women tested positive for anti-D-antibodies in the first ICT at 12–16 weeks of pregnancy. Approximately 50% of the other antibodies were detected in the third test at 34 to 35 weeks of pregnancy. All women with ABO immunization had a positive ICT at the time of delivery or shortly after delivery. The DCT of the newborn was positive in cord blood.

An antibody titer of 1 : 8 was found in the serum of 294 (76.5%) pregnant women, which is statistically significant in comparison to the number of pregnant women with a higher antibody titer ($\chi^2 = 92.3$, d. f. = 1, $p = 0.001$).

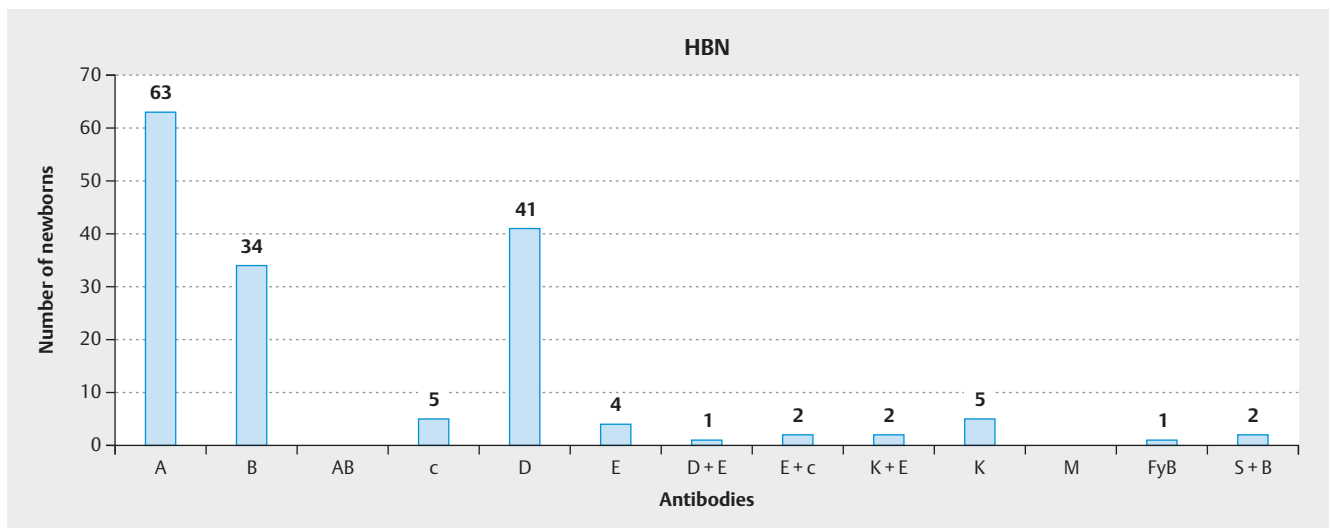
160 (0.8%) newborn with HDN were treated in the NICU at the UCH Mostar during the 13-year-long study. The incidence of HDN was 8.4 per 1000 live births. 81 (50.2%) were male and 79 (49.8%) were female. 135 newborn (82%) were of a gestational age of 38 to 42 weeks. Within the first 24 hours, 75 (46%) of the newborn developed pathological jaundice which required therapy during the first day of life. Of the total 160 infants diagnosed with HDN, 75 (46%) newborn had severe clinical hemolytic disease of the newborn. During the study period, the results indicate a significant correlation between DCT and the severity of the clinical picture of HDN ($\chi^2 = 12.89$, d. f. = 1, $p = 0.001$).

► **Table 1** shows the distribution of the average concentration of individual laboratory parameters and gestational age of newborns with HDN who underwent ETR in the period from 2000 to 2013.

Newborns with HDN due to RhD antibodies underwent ETR in the first hours of life because they had a high concentration of bil-



► Fig. 1 Distribution by type of sensitization of pregnant women during the period of 2000 to 2013 at UHC Mostar.



► Fig. 2 Distribution of the total number of newborn with HDN during the period from 2000 to 2013 at UCH Mostar.

irubin. Newborns with HDN ABO had a high concentration of bilirubin from 48 to 72 hours of life which required ETR. All newborn who underwent ETR weighed at least 2500 grams and were born at full term. They all developed neonatal anemia at the end of the first month of life.

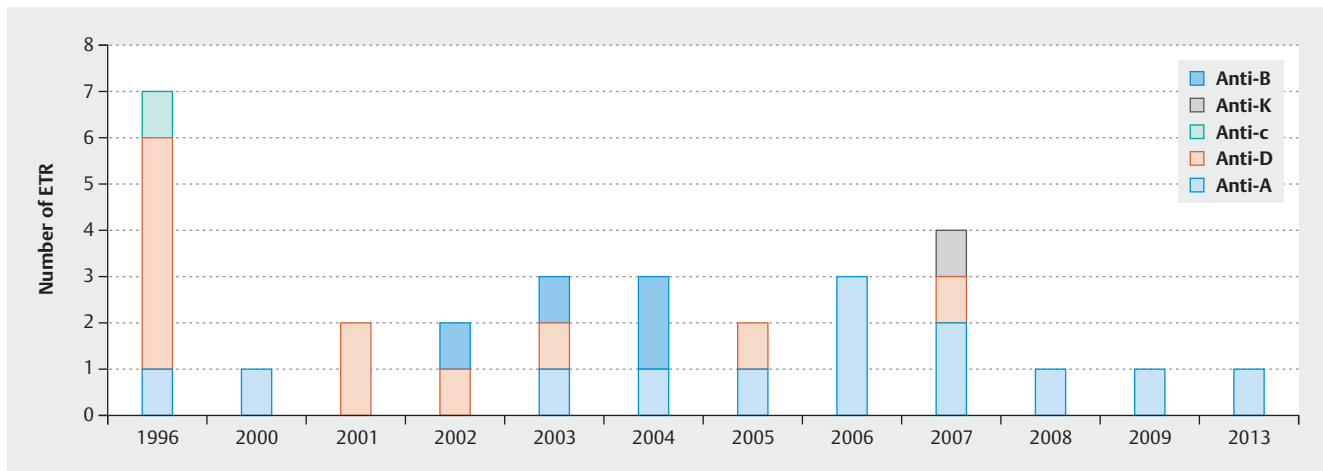
► Fig. 1 shows antibodies against antigens in the various blood group systems ABO, Rhesus, Lewis, Kell, Duffy, Kidd in the pregnant women in the period from 2000 to 2013.

Of the total 384 sensitized women, antibodies against antigen D in the Rhesus system were detected in 156 pregnant women (40.6%), which is statistically significant in relation to the 228 women with other sensitization types ($\chi^2 = 3.69$, d.f. = 1, $p = 0.001$). During the period under analysis, the number of pregnant women with RhD immunization significantly decreased. In the period 2000–2007, 131 cases of RhD immunization were re-

corded, whilst in the last five-year period, there were only 25 cases of RhD immunization. Multiple antibodies (dual) were present in 5.7% (22/384) of the pregnancies. Of the 103 (non-RhD and ABO immunizations) of the total 384 immunizations in this study, antibodies were detected in 67 RhD positive pregnant women. 103 sensitized women (39.7%) developed non-RhD antibodies. Antibodies against the antigen Kell system were detected in 21 pregnant women (5.4%).

► Fig. 2 shows the distribution of the total number of newborn with HDN during the period from 2000 to 2013.

160 infants were treated at the NICU UHC Mostar because of HDN caused by certain types of antibodies. The resulting incidence of HDN was 8 per 1000 live births in our research. A statistically significant number of newborn, 97 (60%), had ABO HDN ($\chi^2 = 908.8$, d.f. = 4, $p = 0.001$). Six (3.7%) newborn with HDN



► **Fig. 3** The distribution of exchange transfusions on HDN cases during the period from 2000 to 2013 and in 1996 at UCH Mostar.

had multiple (dual) antibodies. Children with two antibodies had moderate HDN. 22 (13.7%) infants had non-RhD HDN caused by antibodies (C, F, E, K1, Fyb). The most severe case of HDN was due to Kell antibodies.

► **Fig. 3** shows the distribution of performed exchange transfusions on HDN causes during the period from 2000 to 2013 and in 1996.

Exchange transfusions were performed on 23 (14.3%) infants from 2000 to 2013, 16 (70%) of whom were newborn with ABO HDN, and 6 (26%) newborn with RhD HDN. In 1996, ETR was performed mainly on children with RhD HDN, while in 2006, ETR was performed only on non-RhD HDN children.

Discussion

Red cell immunization during pregnancy is a challenge that continues to tax obstetricians and blood transfusion specialists even 50 years after the introduction of RhD prophylaxis [3]. Our results show a significant incidence of RhD immunization (131/156) in the analyzed period, especially in the first half of the research. This can be attributed to the lack of implementation of standardised and universal anti-D immunoprophylaxis in our area, caused by demographic changes and the displacement of the population, especially until the end of the nineties of the last century. Since 2003 RhD immunoprophylaxis is required and controlled by gynecologists and transfusion specialists. Better knowledge of the molecular biology of the immune process and better communication between gynecologists and transfusion specialists has significantly improved immunoprophylaxis among pregnant women in the area we cover in the last 10 years. However, besides the D antigen, other blood group antigens of the Rh system (C, c, E, e) and other blood group systems (Kell system) have come to light in Europe [11, 12] and in our region. Of the 384 sensitized pregnant women in our study, the non-RhD antibody was present in 228 women, i.e. 60% of the sample. Our results show an increase in the frequency of Kell antibodies (5.4%) during pregnancy, which

has been confirmed by studies carried out in neighboring countries [13, 14]. Non-Rh D antibodies are a potential constant threat to pregnant women and the incidence of HDN, since effective prevention measures against the non-Rh D sensitization of pregnant women do not yet exist [9]. Universal screening of all antenatal women, including those who are D antigen-positive, has been highly debated and controversial to date [15]. The screening is a good way to detect pregnant women who run the highest risk of severe forms of non-RhD sensitization [16] and it is conducted on all pregnant women who are referred to the Department of Transfusion Medicine UHC Mostar. Our results show that of the 384 antibodies detected in this study, 67 were found in D antigen-positive women. Furthermore, research conducted in Denmark suggests that pregnant women do not obtain enough information from their gynecologists about the possible consequences of non-RhD sensitization and the importance of immunohematological screening [11]. The results of our study, in which 60% (97) of the newborn had ABO HDN, confirm that in today's world ABO incompatibility is the most common individual cause of HDN [17]. In 1996, ETR was performed on 71% of the newborn children due to anti-D antibodies, while in 2006, ETR was reduced by one third and they were all performed on children with anti-A HDN. RhD immunoprophylaxis, immunohematological testing of pregnant women and planned delivery in high-risk pregnancies have significantly reduced the risk of hyperbilirubinemia and thus the need for ETR [18]. The results of our study, in which ETR was performed on 16/97 newborns with HDN ABO, are similar to a study conducted in Turkey where ETR was performed on 10% of the newborn with HDN ABO [19]. The possible reasons for this may stem from the previously held belief that a mild clinical picture of ABO HDN required a less rigorous examination after delivery by pediatricians. Subsequently, the failure to introduce early treatment leads to a rapid increase in bilirubin ($> 380 \mu\text{mol/L}$), especially during the second day of life. It is nevertheless necessary now, and will be in the future, to maintain the existing level of knowledge and skills for performing ETR, because of the potential (ABO, Kidd, Kell system) occurrence of severe forms of non-RhD HDN. How-

ever, preventing immunization in pregnant women is the most important measure to prevent the development of HDN in newborns, because the treatment of HDN is neither simple, nor a financially viable route for treatment, especially in a country such as ours.

The conclusion of this research is that the problem of immunization and HDN is still present in our region. However, we have reached the 21st century and the burden of alloimmunization in pregnancy is still on our shoulders. The introduction of immunoprophylaxis for RhD antigens changed the incidence of maternal isoimmunization. The results of our 13-year-long study, in which 60% of the pregnant women had a non-RhD antibody confirms this finding. Changes in the occurrence of severe HDN in the region are similar to those in other parts of Europe. The problem of immunization and HDN are still present in the Herzegovina region, which renders it a neonatal and public health problem. Awareness of new types of incompatibility (Kell, Kidd) and their immunohematological aspects offers new possibilities for future research in the field of pediatrics.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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