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"Characteristics of Erythrocyte Blood Group Antigen and Antibody Synthesis in the Prenatal and Postnatal Periods"

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A u t o r e f e r a t

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INTRODUCTION

Relevance of the Research Topic. Blood group antigens play a crucial role in the adaptation of the biological species *Homo sapiens* to its environment [1], [2], [3]. Currently, the International Society of Blood Transfusion (ISBT) recognizes 45 blood group systems, encompassing 360 antigens on human red blood cells [4]. Most of these antigens are polysaccharides, although they can also be proteins or protein-carbohydrate-lipid complexes. Blood group antigens primarily participate in the trophic and regulatory functions of blood cells. They play a crucial role in the circulatory system, facilitating the transport of hormones, vitamins, enzymes, and other biologically active proteins. Moreover, they often serve as fundamental structural elements in cellular membrane adhesion [5], [6], [7].

The ABO blood group is determined by the presence or absence of A and B antigens on the surface of erythrocytes and A and B antibodies in the serum [8]. ABO naturally IgM antibodies are typically absent in newborns but appear during the first year of life. They may arise in response to dietary and environmental antigens similar to A and B antigens [9], [10].

To study of the enzymatic and structural characteristics of A and B transferases is essential to various branches of medicine. For instance, the ABO system plays a vital role in genomic medicine**,** neurobiology, and the development of universal/artificial blood [11]. Several factors can influence the expression of ABO antigens on the erythrocyte surface, including low antigenic expression**,** frequent transfusions**,** hematologic disorders - particularly hematologic malignancies and other cancers - and surgical history. These factors can lead to discrepancies in ABO typing results [12], [13].

Compared to adult red blood cells, newborn erythrocytes exhibit significantly weaker expression of A and B antigens**.** This reduced reactivity serves as an essential protective mechanism against maternal antibodies that may cross the placental barrier [8]. From this perspective, the fetus is partially protected from maternal immune reactivity due to the low expression of ABO antigens on fetal erythrocytes. However, this also complicates blood group typing**.** Furthermore, newborn serum may lack the corresponding antibodies, leading to incorrect blood group identification when immunoserological techniques - especially reverse blood typing - are used. Such inaccuracies in neonates may result in post-transfusion complications**,** including fetal immune sensitization. This is particularly critical in emergency transfusion settings, where identifying a compatible donor for newborns with complex medical histories (e.g., hemolytic disease or prematurity) may be challenging.

Rh antigens demonstrate similar expression levels in both newborns and adults. Clinical experience and research have shown that newborns with complex medical histories often present difficulties in blood group identification. Commonly, an AB(IV) group is initially detected, which later converts to A(II) or B(III) within a week. In some instances, different blood groups are identified in the same neonatal sample when alternative testing methods are applied.

According to the available literature, antigenic profiling of erythrocytes in adults has received considerably more attention compared to similar analyses in newborns. It is also noteworthy that information on A_1 and A_2 subgroups remains limited. Among the most significant scientific advances is the discovery of the association between the ABO blood group system and various diseases [13]. Genomic sequencing has not only enabled the identification of this association but has also enhanced our understanding of the evolutionary history of the ABO gene and its related genes across different species. This achievement was made possible through the identification of orthologous genes and paralogous genes [1].

Hemolytic disease of the fetus and newborn (HDFN) represents one of the primary pathological conditions characterized by the destruction of erythrocytes. The primary underlying mechanism involves antigen–antibody interactions, typically resulting from maternal–fetal incompatibility of erythrocyte antigens and antibodies [14],[15],[16]. Among the various antigen systems, antibodies directed against ABO and Rh group-specific antigens are most frequently associated with HDFN. This condition often requires intensive clinical intervention, including phototherapy**,** exchange transfusion, and passive immunization with IgG immunoglobulin [17].

Globally, HDFN remains a significant cause of perinatal morbidity and mortality**,** particularly in cases and regions where preventive diagnostic measures are not implemented on time [17]. Although the introduction of anti-D immunoglobulin prophylaxis has markedly reduced the incidence of Rh(D) related hemolytic disease in developed countries, cases continue to occur that are associated with sensitization to ABO and Kell, Duffy, and MNS systems [18], [19]. The relevance of this topic is determined by both its pathophysiological complexity and the ongoing need to update and refine clinical management strategies to improve fetal and neonatal health outcomes and ensure survival [16].

Research Aim and Objectives. Modern perinatal research relies on comprehensive analyses that encompass both clinical and laboratory parameters to assess the severity of hemolysis. Key diagnostic and prognostic indicators include bilirubin concentration**,** antiglobulin tests (DAT and IAT), and anthropometric measurements**,** all of which play a critical role in predicting disease severity and guiding appropriate clinical management [16], [18].

In light of these considerations, the primary objective of our study was to conduct an in-depth investigation of blood group characteristics in newborns. This involved a comprehensive analysis of the presence of A_1, A_2, B, H, and D antigens on red blood cells (RBCs), as well as the detection of anti-A and anti-B antibodies in neonatal plasma. Furthermore, we considered it particularly interesting to explore the expression of these biological markers in infants under one year of age to elucidate the features of their postnatal antigen expression patterns**.** Additionally, this study aimed to evaluate the clinical and laboratory profiles of newborns with hemolytic anemia and to compare them with those of healthy neonates. Such an approach is expected to contribute to the early diagnosis and improved management of this pathology. Based on the objectives of the study, the following tasks were defined:

1. To perform screening of ABO and Rh system antigens in neonatal blood using various immunoserological methods;
2. To conduct screening for A1 and A2 antigens in neonatal blood;
3. To perform screening for naturally occurring anti-A and anti-B antibodies in neonatal blood and to evaluate their quantitative characteristics;
4. To obtain DNA samples from the blood of newborns diagnosed with hemolytic anemia;
5. To perform genotyping of the ABO system using the extracted DNA samples;
6. To collect clinical data from patient medical records.
7. To identify the causes of hemolysis**;**
8. To determine the severity of hemolysis based on bilirubin levels**;**
9. To assess anthropometric parameters in neonates with hemolysis and compare them with those of healthy newborns**;**
10. To perform statistical analysis and processing of the obtained data.

Research Methods and Material-Technical Base. The study employed immunoserological methods in different combinations, including reverse grouping procedures for blood typing. The analysis was conducted using monoclonal anti-A, anti-B, and anti-D antibodies, as well as anti-A_1 lectin and anti-H antibodies. Standard A and B group erythrocytes and neonatal plasma were used to detect the naturally occurring anti-A and anti-B antibodies of the ABO system. In the "weak" agglutination cases EUROMEX digital microscope was used for detailed studies. Upon detection of specific antigens and antibodies, their quantitative characteristics were also evaluated. To assess the distribution characteristics of the corresponding genotypes, the Hardy–Weinberg equilibrium law was applied. In addition, questionnaire-based data analysis was conducted. The questionnaire included demographic parameters, clinical indicators, as well as biological parameters.

The study involved 3,774 newborns and infants under 1 year of age, conducted between 2020 and 2024 at two clinical centers in Batumi. Initially, blood samples from 208 newborns younger than one month**,** collected from the *Iris Borchashvili Health Center "Medina"* laboratory, were studied. At the next stage, blood samples from 202 infants aged 28 days to 12 months were analyzed. For the study, these were categorized into two groups: infants aged 28 days to 6 months and infants aged 6 to 12 months. The biological materials were provided by the *M. Iashvili Batumi Maternity and Child Central Hospital*, and analyses were conducted at the Biosafety Laboratory and the A. Diasamidze Immunogenetics Laboratory of *Shota Rustaveli State University of Batumi*. Additionally, questionnaire data from 3,364 newborns were reviewed, including 1,425 who were admitted to the intensive care unit of the Iris Borchashvili Health Center "Medina" with various diagnoses. Among these, 86 cases of hemolytic anemia were identified, and their data were compared with those of 1,939 healthy newborns**.** Furthermore, genetic testing was performed on 34 newborns with hemolytic anemia to investigate the prevalence of the G deletion in SNP rs8176719, located at position 261 within the ABO gene locus**,** which encodes the ABO system antigens.

The severity of hemolysis was evaluated based on the concentration of total bilirubin in serum (data obtained from physician-documented diagnoses recorded in the medical questionnaires). Statistical analysis was performed using the SPSS software and the **https://www.socscistatistics.com** platform. The study adhered to ethical standards and was approved by the Bioethics Committee (Decree No. BIH-24-0215-01).

Scientific Novelty and Originality of the Study. The originality of this research lies in its comprehensive analysis of blood group antigens and antibodies in newborns, as well as its investigation of postnatal antigen expression in infants up to one year of age. Comparing clinical and laboratory characteristics between infants with hemolytic anemia and the healthy control group enables an in-depth evaluation of the results. This approach represents a novelty in both its study of the local population and its broader scientific context. The dissemination of the study's findings will be valuable to the international scientific community, as it will expand theoretical knowledge while fostering the development of new, practically significant perspectives to improve early diagnostic and clinical management strategies. Notably, this problem has been studied for the first time in our region. Of particular importance is the introduction of ABO genotyping in newborns and its integration into transfusion medicine and neonatology. Through molecular genetic analysis, it is possible to identify rare or subtype alleles whose phenotypic expression is often undetectable by conventional serological methods.

Analysis of Research Results
Distribution of ABO Blood Groups in the Studied Newborns

Determination of a newborn's blood group is an essential laboratory test that is performed shortly after birth. The procedure involves collecting a biological sample from the newborn's heel, umbilical cord, or peripheral vein. However, particular caution must be exercised when obtaining blood from the umbilical cord to prevent potential contamination with maternal blood. Such contamination may adversely affect the serological expression of blood group antigens, leading to false agglutination or nonspecific reactions and ultimately to incorrect interpretation of test results.

Our study included blood samples from 208 newborns. The ABO system's phenotypic groups are unevenly distributed among the newborns. The majority of the studied newborns (43.75±3.4%) have blood group O (I) (n=91). A little law distribution characteristic has the A (II) blood group. 41.35±3.4% of the studied newborns have A (II) blood group phenotypic characteristics (n=86). B (III) blood groups have 21 studied newborns (10.10±2.0%), and only 10 studied newborns' blood samples show both A and B antigens specifications, and 4.8±1.4% of the studied newborns have AB (IV) blood group. There are four categories: O (I), A (II), B (III), and AB (IV), and, based on these degrees of freedom (df), the value is 3. One variable Chi-square (χ^2) equals 89.65, which is 11.4 times more than the Critical Values (CV=7.815). The result is significant at $p < 0.05$. This statistical characteristic shows a unique distribution of ABO blood groups in the studied newborns (Table 1).

Table 1. ABO blood group distribution in the studied newborns

Blood group	**Number (n)**	**Percent (%)**	**df***	**CV***	**χ^2**
O (I)	91	43.75±3.4	3	7.815	89.65
A (II)	86	41.35±3.4			
B(III)	21	10.10±2.0			
AB (IV)	10	4.8±1.4			
Total	208	100			

Table 2. Frequency of distribution of the genes of the ABO system in the studied newborns

Three-allelic genetic system	Distribution
r = √O*	0.6
p = 1- √A+O*	0.3
q = 1- √B+O*	0.1

The ABO blood group system's gene distribution frequency in the newborns under study was also studied. The formula used to analyze the three-allelic genetic system was applied to determine the frequencies of the three alleles. The r allele was detected at the highest frequency in the studied samples (0.6), while the prevalence of the p allele lags significantly behind at 0.3, and the frequency of the q allele is the lowest at 0.1 (Table 2). The total frequency of the r, p, and q

alleles equals 1 in our studied cohort. Where O, A, and B are the ratios of newborns carrying O, A, and B phenotypes to the total number of research subjects.

Characteristics of ABO and Rh Blood Group Antibody Synthesis in Newborns

In addition to screening newborns for group antigens, we were also interested in the characteristics of this target group to identify naturally occurring group-specific anti-A and anti-B antibodies. In adults, individuals with blood group O (I) typically have both group-specific anti-A and anti-B antibodies in their plasma. However, it is essential to note that antibody expression in newborns differs from that in adults.

38.46±5.0% of the studied newborns with the O (I) blood group carried both anti-A and anti-B antibodies (n=35). In contrast, none of the antibodies were detected in 30.77±4.8% (n=28) of cases, and 20.88±4.2% (n=19) of newborns carried only anti-A antibody, while 9.89±3.1% (n=9) carried only anti-B antibody. The degrees of freedom (df) are equal to 3 in this particular case. Chi-square (χ^2) equals 16.6, which is 2.12 times more than the Critical Values (CV=7.815). The P-value is < 0.00001. The result is significant at $p < 0.05$ (Table 3).

Table 3. Anti-A and anti-B antibody expression characteristics in the O (I) blood group newborns

ABO blood group antibodies	Number (n)	Percent (%)	df*	CV*	χ^2
Both anti-A and anti-B antibodies	35	38.46±5.0	3	7.815	16.6
Only anti-A antibodies	19	20.88±4.2			
Only anti-B antibodies	9	9.89±3.1			
None of them	28	30.77±4.8			
Total	91	100			

Table 4. Anti–B antibodies expression in the A(II) blood group newborns

Anti–B antibodies	Number (n)	Percent (%)	df*	CV*	χ^2
Present	35	40.7 ±3.4%	1	3.841	44.4
Don't present	51	59.3 ±5.2%			
Total	86	100			

We have 86 newborns with blood group A(II). Adult individuals with A(II) blood group in plasma have naturally occurring anti-B antibodies. We find that 40.7±3.4% (n = 35) of the newborns studied have anti-B antibodies in their plasma as adults. Still, the majority of them (59.3±5.2%; n=51) did not show any agglutination reaction with the standard erythrocyte mass of the B blood group, indicating that anti-B antibodies had not yet developed (Table 4). The degrees of freedom (df) are 1 in this case, because there are two categories. χ^2 is 44.4, while the Critical Values (CV) are 3.841. The P-value is < 0.00001. The result is significant at $p < 0.05$.

We also analyze the frequency of natural anti-A antibodies in the studied newborns. We have 21 newborns with blood group B (III). Adults with blood group B (III) have naturally occurring anti-A antibodies in their plasma. 61.9±3.4% (n=13) of our studied newborns had anti-A antibodies in the plasma, similar to adults; 38.1±3.3% (n=8) did not show agglutination with standard erythrocyte mass in A(II) blood group, indicating that no anti-A antibodies were present yet (Table 5). The degrees of freedom (df) are equal to 1 in this particular case. Chi-square (χ^2) equals 11, which is higher than the Critical Values (CV=7.815). The P-value is 0.000911. The result is significant at $p < 0.05$.

AB(IV) blood groups lack plasma antibodies. None of our 10 AB blood group samples showed agglutination with standard erythrocytes.

Information about the synthesis of these antigens at an early stage of embryogenesis can be found in the literature [23]. Erythrocyte antigens A and H are found in the blood of 5-week-old embryos. According to one hypothesis, group-specific antibodies are synthesized in two- to three-month-old embryos, which is associated with the influence of intestinal microflora. This phenomenon is considered a result of bacterial immunization.

Table 5. Anti-A antibodies expression in the B(III) blood group newborns

Anti-A antibodies	**Number (n)**	**Percent (%)**	**df***	**CV***	**χ^2**
Present	13	61.9 ±3.4	1	3.841	11
Don't present	8	38.1 ±3.3			
Total	21	100			

Table 6. ABO system antibody synthesis frequency in the studied newborns

ABO blood type	Normal expression	Partially expression	without expression	Total
O (I)	35	28	28	91
A(II)	35	-	51	86
B(III)	13	-	8	21
Total	83	28	87	198
%	41.92± 3.5	14.14±2.47	43.94±3.5	100

In our study, we suggest that, in the majority of cases (43.94±3.5%) of newborns, naturally occurring anti-A and anti-B antibodies were not detected (n=87); in the same instances (14.14±2.4%) of O (I) blood group individuals, they were partially synthesized (n=28). 41.92±3.5% of our studied newborns expressed natural antibodies as adults. The chi-square statistic is 35.5725. The p-value is < 0.00001. The result is significant at $p < 0.05$ (Table 6).

We also study the distribution of the Rhesus system in the newborns studied. It was typing the D antigens in the 148 samples. The majority of our samples (87.84±2.6%) exhibit Rh^+ phenotypic expression (n=130). The remaining 12.16±2.6 belong to the Rh^- group (n=18). We didn't encounter any difficulties typing the Rh blood group in the newborns, because in all Rh^+

samples the D antigen was well agglutinated. The P-value is < 0.00001. The result is significant at $p < 0.05$ (Table 7).

Table 7. Rh^+ and Rh^- phenotypes in the studied newborns

Rh Phenotypes	Number (n)	Persent (%)	df*	CV*	χ^2
Rh^+	130	87.84 ±2.6	1	3.841	84.6
Rh^-	18	12.16±2.6			
Total	148	100			

A_1/A_1B and A_2/A_2B Subgroups in Newborns

We used a serological plate test to identify neonatal blood groups. Each newborn's sample was subjected to an extra investigation using anti-A1 and anti-H lectins. Based on serological tests, we studied subgroups A_1/A_1B and A_2/A_2B. The four variations found in the newborn study samples are shown in the table below (Table 8).

Table 8. Four variations of A antigen subgroup presence in the newborn samples (A_2, A_1, A_2B, A_1B)

Variation	Anti-A	Anti-B	Anti-A1 lectin	Anti-H lectin	Interpretation of results
1	+	-	-	+	A_2
2	+	-	+ (weak agglutination)	+	A_1
3	+	+	-	+	A_2B
4	+	+	+ (weak agglutination)	+	A_1B

Table 9. A_1, A_2, A_1B, and A_2B subgroups distribution characteristics in the studied A (II) and AB (IV) blood group newborns

ABO Phenotypes	Subgroups	Number (n)	Persent (%)	χ^2
A (II)	A_1 A_2	12 74	12.5±3.3 77.08±4.2	140.76
AB (IV)	A_1B A_2B	2 8	2.09±1.4 8.33±2.8	
Total	4	96	100	

There are a total of 96 blood samples with A(II) and AB (IV) specifications. Among them, 86 newborns have A(II) blood group, and the rest of them (n=10) AB (IV) blood group. We have two subgroups (A_1 and A_2) with blood groups A(II) and AB(IV), also found in two categories: A_1B and A_2B. It is an interesting case for our studied newborns. The majority of newborns (77.08±4.2%) have A_2 subgroups (n=74). In the donor population, this subgroup is very rare. The majority (8.33±2.8%) of AB (IV) blood group is also found in the A_2B subgroup, which is highly underrepresented in the donor population (Table 9).

In our current study, we observed that, unlike in adults, the expression of A and B antigens of the ABO blood group system was weak on the surface of red blood cells in some of the newborns

we studied. In most cases, we used an optical microscope with low- and/or high-magnification lenses (10×4, 10×10, or 10×100) to detect so-called "weakly" agglutinated erythrocytes.

As mentioned above, in 43.94±3.5% (n = 87) of the studied newborns, naturally occurring anti-A and anti-B antibodies were not detected. In certain specific cases (14.14±2.4%), these antibodies were partially synthesized in individuals with blood group O(I) (n = 28). In 41.92±3.5% of the newborns studied, the natural antibodies were fully expressed, similar to the adult pattern. The P-value is < 0.00001. The result is significant at $p < 0.05$ (Table 9).

Some studies have shown that pneumococcal polysaccharide vaccines contaminated with A-like substances can stimulate the prolonged production of anti-A antibodies in individuals with blood groups O or B [20], [21]. We did not find research directly comparable to our study to align our current findings with the existing literature. Although all studies agree that newborns possess specific ABO antigens and initiate antibody synthesis, the detailed distribution of antibodies among newborns with blood groups O, A, and B, and their comparison with those in adults, has not been extensively described.

Suppose antibody synthesis is indeed influenced by diet and environmental antigens. In that case, we believe our current findings may differ from those observed in newborns from other regions, since each region has its own specific diet and environmental conditions.

Analysis of Blood Group Characteristics in Newborns and Blood Donors

Our study included blood samples from 208 newborns. The blood group distribution among the studied newborns was O > A > B > AB, similar to that of blood donors in the Adjara region [22]. The majority of both the studied newborns and blood donors had blood group O (43.75±3.4% and 50.2±1.83%, respectively; n=91/208 and n=373/743) (Table 10).

Table 10. Distribution of ABO Blood Groups in Studied Newborns Compared to Blood Donors

ABO blood group	**Newborns (n)**	**Newborns (%)**	**Blood donors (n)**	**Blood donors (%)**	**χ^2**
O	91	43.75 ±3.4	373	50.2±1.83	4.0219
A	86	41.35 ±3.4	281	37.8±1.77	
B	21	10.10 ±2.0	68	9.15±1.04	
AB	10	4.8 ±1.4	21	2.82±0.6	
Total	208	100	743	100	

Table 11. A_1, A_2 subgroups distribution characteristics in the studied A (II) and AB (IV) blood group newborns compared with the donor population

ABO Phenotypes	**subgroups**	**Blood donors (n=368)**	**Newborns (n=96)**	**χ^2**
A(II) and AB (IV)	A_1	91.3±0.4 (n=336)	14.58±3.5 (n=14)	241.8203
	A_2	8.7±1.4 (n=32)	85.42±3.5 (n=82)	
Total	2	100	100	

Blood group A was found in 41.35±3.4% of newborns and 37.8±1.77% of blood donors (n=86/208 and n=281/743, respectively). Blood group B was present in 21/208 newborns and 68/743 blood donors (10.10±2.0% and 9.15±1.04%, respectively). Meanwhile, 10/208 newborn samples and 21/743 blood donor samples carried both A and B antigens (AB) (4.8±1.4% and 2.82±0.6%, respectively). The χ^2 statistic is 4.0219, indicating a comparable distribution of blood groups between the studied newborns and regional blood donors (Table 10).

In our previous work, we studied the distribution of the A_1 and A_2 blood group subtypes among blood donors in our region (Table 11). As shown below, the frequency of A_2 subgroups is very low in the blood donor population (8.7±1.4%), whereas in newborns it is 85.42±3.5%. The chi-square statistic is 241.8203. The p-value is < 0.00001. Significant at $p < 0.05$.

It must be highlighted that a deficient synthesis of blood group antigens is linked to the predominance of the A_2 and A_2B phenotypes found by serology in the newborns. Since the postnatal period of development is required for the full expression of ABO system antigens, as we have already indicated in the literature section, neonates' group A_1 erythrocytes fail to exhibit a serological response to anti-A_1 lectin due to incomplete synthesis. As a result, the A_2 subgroup exhibits serological mimicry of the A_1 subgroup, a trait that shifts in the following stage of ontogenesis.

Postnatal development of blood group antigens and antibodies in the first year of life

It is exciting to study the expression characteristics of ABO system antigens from an ontogenetic perspective. As is well known, these antigens are oligosaccharides and do not represent a direct gene product. The expression of these antigens is a multistage process; therefore, their complete expression requires the passage through specific stages of ontogenesis. The characteristics of antigen expression in newborns have been investigated, and studies have shown that their expression is weak. Regarding the synthesis of group-specific antibodies, the majority of the studied newborns do not produce them [23].

Our study aimed to investigate the expression characteristics of the ABO system during the postnatal period. This chapter focuses on both the qualitative and quantitative features of the manifestation of group-specific antigens and antibodies.

In infants aged 28 days to 12 months, the distribution of antigens and antibodies of the ABO system was studied and compared with that in newborns from the same region (Adjara, Georgia Republic), which were investigated in our previous work [22].

As mentioned above, we studied biological material from infants under 1 year old (n=202). The ABO system's phenotypic groups are represented at varying frequencies in the study group. The majority of the studied infants (43.08±3.4%) have the O (I) blood group (n=87), which is slightly lower than the prevalence of the A (II) blood group; in particular, 41.57±3.4% of the studied infants have this group (n=84). 11.39±4.9% of the studied cases of infants belong to the B (III) blood phenotypic group (n=23). The AB (IV) phenotypic group has the lowest distribution. In particular, only 3.96±1.8% of the studied infants have the mentioned phenotype (n=8) (Table 12). χ^2 equals 147.03, which is 18.8 times more than CV (critical value) for df (degrees of freedom) 3, which equals 7,815. In this case, the P-value is < 0.00001. The result is significant at $p < 0.05$.

We also analyzed the distribution of phenotypic groups of the ABO system by gender. 38.30±5.0% of female infants under one year old have blood group O (I) (n=36). 42.55±5.0% have A (II) blood group (n=40), and 14.89±3.6% have B (III) blood group (n=14). The lower phenotypic rate is 4.26±2.0% for AB (IV) blood group (n=4). As for the characteristics of the phenotypic distribution of the ABO system in male infants under one year of age, its distribution is as follows: 47.22±4.8% of the studied male infants have O (I) blood group (n=51), followed by 40.74±4.7% of A (II)) blood group (n=44), followed by 8.33±2.6% B(III) blood group (n=9). As in the case of female infants, we also observe the lowest value in the phenotypic group, 3.71±1.8% for AB (IV) blood group (n=4) (Table 12). No significant difference was observed in the male-to-female ratio or in the blood group distribution. The chi-square statistic is 2.9073. The p-value is 0.820388. The result is not significant at $p < 0.05$.

Table 12. ABO blood groups in the studied Infant

ABO blood groups	**Total studied materials**		**χ^2**	**CV for df 3**	**P-value**	**Gender**				**χ^2* P-value**
						Female♀		**Male♂**		
	(n)	**(%)**				**(n)**	**(%)**	**(n)**	**(%)**	χ^2 - 2.9073
O (I)	87	43.08±3.4	147.03	7.815	P-<0.00001.	36	38.30±5.0	51	47.22±4.8	P-0.820388
A (II)	84	41.57±3.4				40	42.55±5.0	44	40.74±4.7	
B (III)	23	11.39±4.9				14	14.89±3.6	9	8.33±2.6	
AB(IV)	8	3.96±1.8				4	4.26±2.0	4	3.71±1.8	
Total	202	100				94	100	108	100	

In addition to the above, the phenotypic distribution of the ABO system across age groups and genders was also analyzed. We divided the age group into two categories: 1. infants from 28 days to 6 months, and 2. the infant category from 6 to 12 months.

In the age category from 28 days to 6 months, 46.67±7.4% A (II) blood group dominates in the order of phenotypic distribution in female infants (n=21), followed by

28.89±6.75% O (I) blood group (n=13), followed by 22.22± 6.1% B (III) blood group (n=10) and only one individual has AB (IV) blood group (2.22±4.8%). 46.67±7.4% A (II) blood group (n=21), followed by 28.89±6.75% O (I) blood group (n=13), followed by 22.22± 6.1% B (III) blood group (n=10), and only one individual has AB (IV) blood group (2.22±4.8%). Regarding male infants aged 0-6 months, the phenotypic variations of the ABO system are presented in the following order: The majority of the studied infants 52.27±7.5% have O (I) blood group (n=23), according to the characteristics of distribution, 31.82±7.0% A (II) blood group (n=14) comes next, then there are 11.36±4.7% B(III) blood group (n=5). The lowest distribution of blood group characteristics is AB (IV), with 4.55±3.1% (n=2).

The distribution of ABO blood group phenotypic characteristics among female individuals up to 6-12 months is as follows: 46.94±7.1% are representative of O (I) blood group (n=23), (here the number for female individuals is the same as in the category up to 0-6 months and up to 6-12 months category), the following are 38.78±6.9% A (II) blood group (n=19), 8.16±3.9% have B(III) blood group (n=4) and and 6.12±3.4% have blood group AB (IV) (n=3). As for the phenotypic distribution characteristics of the ABO system in the studied male infants under one year old, in the 6 to 12 months category, the A (II) blood group dominates at 46.87±6.2% (n=30). Approximately 43.75±6.2% of individuals have O (I) blood group (n=28), followed by 6.25±3.0% for B (III) blood group (n=4), including female individuals in the same category. The lowest rate, 3.13±2.1%, is for AB (IV) blood group (n=2), including male individuals aged 28 days to 6 months (Table 13). The chi-square statistic is 4.7658. The p-value is 0.189775. The result is not significant at $p < 0.05$. The statistical data show that the distribution of blood groups does not depend on age or gender. Therefore, no direct relationship was found between the qualitative variables.

Table 13. ABO blood groups in different age categories of infants

ABO blood groups	**28-day to 6-month age category**				**6-12 month age category**				**χ^2, p-value**
	Female♀		**Male♂**		**Female♀**		**Male♂**		
	(n)	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	
O (I)	13	28.89±6.7	23	52.27±7.5	23	46.94±7.1	28	43.75±6.2	χ^2 -4.7658
A (II)	21	46.67±7.4	14	31.82±7.0	19	38.78±6.9	30	46.87±6.2	
B (III)	10	22.22±6.1	5	11.36±4.7	4	8.16±3.9	4	6.25±3.0	P- 0.189775
AB (IV)	1	2.22±4.8	2	4.55±3.1	3	6.12±3.4	2	3.13±2.1	
Total	45	100	44	100	49	100	64	100	

We analyzed the distribution of antigens A and B in the studied infants. Antigen A was found to be prevalent in 74.80±3.9% (n=92) of the studied infants, which is more common than B antigen. B antigen was present in 25.20±3.9% of the studied infants (n=31).

We analyzed the relationship between the occurrence of A and B blood group antigens and gender. Here, it was revealed that the prevalence of A antigen in female infants under one year of age is less (70.97±5.5%) n=44, and the prevalence of the antigen in male infants under one year of age is higher (78.69±5.2%) n=48. And the prevalence of B antigen, on the contrary, is higher in female infants n=18 (29.03±5.7%) than in male infants n=13 (21.31±5.2%) (Table 14). The chi-square statistic is 30.24. The P-value is <0.00001. The result is significant at $p < 0.05$.

Table 14. A and B antigens in the studied infants

ABO blood groups antigens	**Total studied materials**		**χ^2**	**CV for df 1**	**P-value**	**Gender**				**χ^2**	**P**
						Female♀		**Male♂**			
	(n)	**(%)**	30.24	3.841	<0.00001	**(n)**	**(%)**	**(n)**	**(%)**	0.9723	0.324108
A antigen	92	74.80±3.9				44	70.97±5.5	48	78.69±5.2		
B antigen	31	25.20±3.9				18	29.03±5.7	13	21.31±5.2		
Total	123	100				62	100	61	100		

In the studied infants, we analyzed the distribution characteristics of ABO system group-specific anti-A and anti-B antibodies. Mentioned antibodies were detected in 195 cases out of 202 studied blood samples. 7 studied samples were not analyzed due to a lack of plasma. Only Anti-A antibody was detected in 16.92±2.6% (n=33); only Anti-B antibody was detected in 27.69±3.2% cases (n=54); 24.11±3.0% of infants had both anti-A and anti-B antibodies (n=47). In contrast, no antibodies were detected in 31.28±3.3% of cases (n=61) (Table 15). χ^2 equals 8.77, which is more than the CV (critical value) for df (degrees of freedom) 3, which equals to 7,815. The P-value is 0.03251. The result is significant at $p < 0.05$.

Table 15. Group-specific antibodies in the infant

ABO blood group antibodies	**Number (n)**	**Percent (%)**	**χ^2**	**CV for df 3**	**P-value**
Anti - A antibody	33	16.92 ±2.6	8.77	7.815	0.03251
Anti-B antibody	54	27.69 ±3.2			
Both/ Anti–A and Anti-B antibodies	47	24.11±3.0			
None of them	61	31.28±3.3			
Total	195	100			

74.80±3.9% of the 202 studied infants carry the A antigen, with 41.57±3.4% belonging to the A(II) group (n=84) and 3.96±1.8% belonging to the AB (IV) group (n=8). It is important to note that we simultaneously screened subgroups of the A antigen within the A (II) and AB (IV) phenotypic groups. Around 52.17±5.2% of infants with A(II) blood group phenotype carry the A_1 subgroup (n=48), and about 39.13±5.0% have the A_2 subgroup (n=36). In individuals with the AB(IV) blood group phenotype, both the A_1B and A_2B subgroups were found to occur at approximately 4.35±2.1% (Table 16). χ^2 equals 65.71, which is 8 times more than the CV (critical value) for df (degrees of freedom) 3, which equals to 7,815. The P-value is <0.00001. The result is significant at $p < 0.05$.

We also analyzed the distribution of A antigen subgroups according to age category. Where, in the age category from 28 days to 6 months, the prevalence of the A_1 subgroup is less 4.35±2.1% (n=17), and in the age category up to 6-12 months, the prevalence of A_1 antigen is higher than 56.36±6.6% (n=31). The prevalence of the A_2 antigen is higher in the age category from 28 days to 6 months 48.65±8.2%; (n=18) and lower in the age category from 6 to 12 months 32.73±6.3% (n=18).

The presence of A antigen in AB (IV) blood groups varies by age. In the 28-day to 6-month category, the A_1B subgroup is found in 5.40±3.7% of cases, while the A_2B subgroup is not detected (n=0) in the studied group. In the 6 to 12 months category, the A_1B subgroup is found in 3.64±2.5% of cases (n=2), and the A_2B subgroup is found in 7.27±3.5% of cases (n=4) (Table 16).

Table 16. An antigen subgroup in the studied infant

ABO Phenotypes	**Subgroups of A antigen**	**Total studied materials**		**χ^2**	**CV for df 3**	**P-value**	**28-day to 6-month age category**		**6-12 month age category**	
		(n)	**(%)**	65.71	7.815	<0.00001	(n)	(%)	(n)	(%)
A (II)	A1	48	52.17±5.2				17	45.95±8.1	31	56.36±6.6
	A2	36	39.13±5.0				18	48.65±8.2	18	32.73±6.3
AB (IV)	A1B	4	4.35±2.1				2	5.40±3.7	2	3.64±2.5
	A2B	4	4.35±2.1				0	0	4	7.27±3.5
Total	4	92	100				37	100	55	100

We conducted a study on the prevalence of the Rh system (n=202). The Rh^+ phenotypic variation was predominant in the samples (n=166), representing the majority of individuals studied (82.18±2.6%). The Rh^- phenotypic manifestation was observed in only 17.82 ±2.6% of the cases. χ^2 equals 83.66, which is 21 times more than the CV (critical value) for df (degrees of freedom) 1, which equals to 3.841. The P-value is < 0.00001. The result is significant at $p < 0.05$.

If we compare the Rh phenotypic manifestation in individuals from 28 days to 6 months, the picture in individuals aged 6-12 months does not differ significantly from that in individuals

aged 28 days. In both cases, within the 28-day to 6-month age category, Rh^+ phenotypic expression prevails at 82.02±4.0%, whereas Rh^- phenotypic expression is 17.9 ±4.0%. A similar pattern is observed in the 6-12 month age category, where 82.30±3.5% are carriers of the Rh^+ phenotype and 17.70±3.5% are carriers of the Rh^- phenotype (Table 17).

Table 17. Rh^+ and Rh^- phenotypes in the studied infants

Rh Phenotypes	**Total studied materials**		**χ^2**	**CV for df 1**	**P-values**	**28-day to 6-month age category**		**6-12 month age category**		**χ^2 P-value**
	(n)	**(%)**	83.66	3.841	<0.00001	**(n)**	**(%)**	**(n)**	**(%)**	χ^2-0.0026. P-0.95906.
Rh^+	166	82.18±2.6				73	82.02 ±4.0	93	82.30±3.5	
Rh^-	36	17.82 ±2.6				16	17.98 ±4.0	20	17.70±3.5	
Total	202	100				89	100	113	100	

We were also interested in studying ABO and Rh system combinations in the studied infants. Accordingly, we identified eight phenotypic categories: O(I),Rh^+; O(I),Rh^-; A(II),Rh^+; A(II),Rh^-; B(III),Rh^+ ; B(III),Rh^-; AB(IV),Rh^+; A (IV),Rh^-. O(I),Rh^+ and A (II),Rh^+ phenotypic variations, with 34.65±3.3% (n=70), are shown to occur with equal frequency across the studied phenotypic groups. Among the combinations of ABO and Rh system, the next phenotypic category is O(I) Rh^- 8.42±1.9% (n=17). B (III) phenotypic expression in the studied infants is 10.40±2.1% (n=21), and B(III),Rh^- phenotype is 0.99±0.6% (n=2). AB (IV),Rh^+ combination is prevented in 2.48±1.0% (n=5) cases, and AB(IV),Rh^- has only 1.49±0.8% (n=3) distribution rate (Table 18). χ^2 equals 247.61. The P-value is < 0.00001. The result is significant at $p < 0.05$.

Table 18. ABO and Rh system combinations in the studied infants

Phenotypic variations of the ABO and Rh systems	**Total studied materials**		**χ^2**	**P-value**
	(n)	**(%)**	247.61	<0.00001
O (I) Rh^+	70	34.65±3.3		
O (I) Rh^-	17	8.42±1.9		
A (II) Rh^+	70	34.65±3.3		
A (II) Rh^-	14	6.93±1.7		
B (III) Rh^+	21	10.40±2.1		
B (III) Rh^-	2	0.99±0.6		
AB (IV) Rh^+	5	2.48±1.0		
AB (IV) Rh^-	3	1.49±0.8		
Total	202	100		

We also analyzed quantitative characteristics of natural anti-A and anti-B antibodies. Of course, their titer was not visible where antibodies were not detected. In cases where antibodies were detected, the following result was revealed: In the vast majority of infants from 28 days to 6

months, the titer was very low, in most cases it is equal to 1:2. In the blood samples of infants from 6 to 12 months, the quantitative characteristics of the antibody are presented with different titers. The lowest titer among the infants in this category was 1:2, and the highest was 1:16.

We were interested in studying the quantitative index (titer) of antigens A and B of the ABO blood group system in infants aged 28 days to 6 months and 6 months to 12 months. We performed the titration in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256. For both categories, A and B antigens were well synthesized in infants from 6 to 12 months—the majority cases with a high titer of 1:256. In the next section (Discussion) of the manuscript, we will try to analyze the results of our research.

Summary. Anti-A and anti-B antibodies were analyzed in the blood plasma of an infant with the O (I) blood group (n=87). It is recognized that their production is either absent in newborns or occurs in individual cases (14). In infants with blood type O (I), both Anti-A and anti-B antibodies were detected in 49.43±5.3% (n=43) of infants aged 28 days to 12 months, a pattern similar to that seen in adults. Only Anti-A antibodies were found in 24.14±4.5% of the studied samples (n=21), while just Anti-B antibodies were detected in 2.30±1.6% (n=2) of the infants. No antibodies were detected in 24.13±4.5% of infants' blood plasma (n=21). We analyzed the data above by age group. Regarding the expression of anti-A and anti-B antibodies, we observed a difference between the 28-day to 6-month and 6-12-month age groups. According to the literature, there is evidence of a significant increase in the production of group-specific antibodies with age [23].

Based on our research, we found that the synthesis of anti-A and anti-B antibodies in the blood plasma of infants aged 28 days to 6 months with O (I) blood group is 25±7.2% (n=9), while in infants aged 6-12, the synthesis of these antibodies is 74.51±6.1% (n=38). The synthesis of only anti-A antibody in the 28-day to 6-month age category is 19.44±6.5% (n=7), and in the 6-to-12-month category it is 21.57±5.7% (n=11). Anti-B antibody was detected in only 5.56±3.8% (n=2) of cases in the blood plasma of infants aged 28 days to 6 months. In the studied infants aged 6-12 months, no cases of anti-B antibody expression were observed (n=0).

In cases where no antibodies were found, the results were as follows: 50±8.3% (n=18) in the 28-day to 6-month age category, which is relatively high compared to infants studied at 6-12 months. In the mentioned category, the rate of no antibody detection is very low (in some cases) and is 3.92±2.7% (n=2) (Table 19).

We were also interested in comparing these data on the expression of naturally occurring anti-A and anti-B antibodies with the expression data for newborn anti-A and anti-B antibodies

studied in our previous studies [23]. Since people with O (I) blood group have anti-A and anti-B antibodies in their plasma, we studied this characteristic in 91 newborns with O (I) blood group. Where 38.46±5.0% of newborns had anti-A and anti-B antibodies (n=35), as indicated in Table 19, this indicator increased by 1.28 times in infants aged 28 days to 6 months, and by 1.93 times (approximately 2 times) in infants aged 6-12 months. The chi-square statistic is 37.3224. The p-value is 0.000023. The result is significant at $p < 0.05$.

Table 19. Anti-A and anti-B antibody expression characteristics in the O (I) blood group infant compared with newborns

ABO blood group antibodies	**Total studied infants**		**28-day to 6-month age category**		**6-12 month age category**		**Newborns** [23]		χ^2 **P-value**
	(n)	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	χ^2-37.3224 P-<0.000023
Both anti-A and anti-B antibodies	43	49.43±5.3	9	25±7.2	38	74.51±6.1	35	38.46±5.0	
Only anti-A antibodies	21	24.14±4.5	7	19.44±6.5	11	21.57±5.7	19	20.88±4.2	
Only anti-B antibodies	2	2.30±1.6	2	5.56±3.8	0	0	9	9.89±3.1	
None of them	21	24.13±4.5	18	50±8.3	2	3.92±2.7	28	30.77±4.8	
Total	87	100	36	100	51	100	91	100	

Additionally, it is interesting that 30.77±4.8% of newborns lacked antibodies. In infants aged 28 days to 6 months, this rate decreases by 1.27 to 24.13±4.5%, and only two similar cases have been reported in infants aged 6-12 months. This indicates that similar rates are reduced by 7.8 times when compared to infants in the specified age group. This implies that antibody production increases gradually in the postnatal period.

In adults with blood group A (II), anti-B antibody is present in plasma. We studied how this antibody was found in infants with A(II) blood group. We found that 61.90±5.2% (n=52) of the 84 infants with A(II) blood group had anti-B antibody in their blood plasma, while 38.10±5.2% (n=32) did not.

In the 28-day to 6-month age category, the detection of anti-B antibody is 45.71±2.9% (n=16). In the 6-12 month age category, it is 73.47±6.3% (n=36). No anti-B antibody was detected from 28 days to 6 months in 54.29±2.9% (n=19) of cases, and in the 6-12 month age category, it was 26.53±6.3% (n=13).

We compared the obtained results with the data from newborns we studied, in which 86 newborns with A(II) blood group were included. We found that 40.7±3.4% (n=35) of them had expression of anti-B antibodies in their blood plasma samples. On the other hand, the majority of the newborns (n=51) did not yet express anti-B antibodies, with a percentage of 59.3±5.2%.

According to the results, infants aged 28 days to 12 months have approximately 1.5 times higher levels of anti-B antibodies than newborns. In the 28-day to 6-month age category, the increase was only 0.89 times, while in the 6-12 month age group, it was 1.8 times. This indicates that antibody specificity increases with age (Table 20). The chi-square statistic is 16.6728. The p-value is 0.000825. The result is significant at $p < 0.05$.

Table 20. Anti–B antibodies expression in the A(II) blood group infant

ABO blood group anti bodies	**Total studied infants**		**28-day to 6-month age category**		**6-12 month age category**		**Newborns** [23]		**χ^2 P-value**
	(n)	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	**χ^2-** 16.6728. P- 0.000825
Present	52	61.90±5.2	16	45.71±2.9	36	73.47±6.3	35	40.7± 3.4	
Don't present	32	38.10±5.2	19	54.29±2.9	13	26.53±6.3	51	59.3± 5.2	
Total	84	100	35	100	49	100	86	100	

We also investigated the prevalence of anti-A antibodies in infants with blood group B (III) (n=23). 60.87±3.1% of the infant in our study (n=14) have anti-A antibodies in their plasma, while 39.13±3.1% do not have anti-A antibodies in plasma (n=9). Between 28 days and 6 months of age, anti-A antibodies were detected in 53.33± 3.5% (n=8) and not detected in 46.67±3.5% (n=7). In the 6-12 month category, the presence of anti-A antibodies increased to 87.5±3.4% (n=7), while not-detected cases decreased to 12.5±3.4% (n=1).

We compared the expression data for natural anti-A antibodies in one-year-old infants with the B (III) blood group with those of newborns with the B (III) blood group studied in our previous research (12). The majority of the studied newborns, 61.9±3.4% (n=13), had anti-A antibodies in their blood plasma. In contrast, 38.1±3.3% (n=8) did not have natural anti-A antibodies (Table 21). Additionally, it can be noted that in the 6-12 months age category, the detection of anti-A antibodies increased by almost 1.41 times compared to newborns. The chi-square statistic is 2.6183. The p-value is 0.454297. The result is not significant at $p < 0.05$.

Table 21. Anti-A antibodies expression in the B(III) blood group infant

ABO blood group antibodies	**Total studied Infants**		**28-day to 6-month age category**		**6-12 month age category**		**Newborns** [23]		**χ^2 P-value**
	(n)	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	**χ^2-** 2.618 P- 0.454 297
Present	14	60.87 ±3.1	8	53.33±3.5	7	87.5±3.4	13	61.9 ±3.4	
Don't present	9	39.13 ±3.1	7	46.67±3.5	1	12.5±3.4	8	38.1 ±3.3	
Total	23	100	15	100	8	100	21	100	

In our previous chapter, we analyzed the distribution of A_1 and A_2 subgroups in newborns. The frequency of A_1 subgroups was found to be low at 14.58±3.5% (n=14), while the frequency of A_2 subgroups was much higher at 85.42±3.5% (n=82). We then compared this data with the subgroup distribution in the infants we studied (Table 22), in which we found the frequency of A_1 subgroups to be high at 56.52±5.1% (n=52) and the frequency of A_2 subgroups to be low at 43.48±5.1% (n=40). The chi-square statistic is 36.2691. The p-value is < 0.00001. The result is significant at p <0.05. We suggested that A_2 and A_2B phenotypes found by serology in the newborns. Since the postnatal period of development is required for the full expression of ABO system antigens. The A_2 subgroup exhibits serological mimicry of the A_1 subgroup, a shifting trait in the following stage of ontogenesis.

Table 22. A1 and A2 subgroup distribution characteristics in newborns with blood group A (II) and AB (IV) compared to infants

ABO Phenotypes	**Subgroups**	**Total studies infants**		**Newborns** [23]		χ^2	**P-value**
		(n)	**(%)**	**(n)**	**(%)**	36.2691	<0.00001
A(II) and AB (IV)	A1	52	56.52±5.1	14	14.58±3.5		
	A2	40	43.48±5.1	82	85.42±3.5		
Total	2	92	100	96	100		

As indicated in the results section of our study, no differences were found among infant age groups, suggesting that Rhesus system antigens were fully synthesized during the prenatal period. The human Rh blood group system is highly polymorphic, with more than 56 serological specificities, making it particularly important and challenging for biomedical scientists and clinicians [23]**.** In the Rh blood group, the D antigen is the most immunogenic and clinically meaningful epitope [24], [25], [26].

Analysis of Hemolytic Disease of the Fetus and Newborn (HDFN) Cases in Neonatal Intensive Care Unit (NICU) Patients

According to data from 2020–2024, the majority of newborns were male 55.23±1.3%, (n=787) while females accounted for 44.77±1.3% (n=638) of the total population. The χ^2 statistic was 5.7336, with a p-value of 0.016643, indicating a significantly higher proportion of male newborns in both groups. This finding aligns with international studies that suggest biological and immunological factors contribute to the predominance of male births.

Among the pathologies observed in neonates were respiratory distress syndrome, cerebral ischemia, bacterial sepsis, perinatal infections, acute respiratory infections, hemolytic diseases

(ABO and Rh sensitization), unspecified jaundice and other prematurity-related conditions, congenital herpes infection, cardiovascular system pathologies - including congenital heart defects, patent ductus arteriosus, double outlet right ventricle, and major vascular malformations. congenital hydrocephalus, while other congenital anomalies - such as atresia of the esophagus or intestines, anal prolapse, congenital diaphragmatic hernia, and neonatal necrotizing enterocolitis.

We analyzed the number of newborns with HDFN caused by ABO and Rh incompatibility, as well as cases of unspecified jaundice and other hemolytic disorders, according to sex. The results show that among newborns with hemolytic cases due to ABO incompatibility, females slightly predominated at 46.34±7.7% (n = 19) compared to males at 44.44±7.4% (n=20). Among newborns with unspecified jaundice and other hemolytic diseases, the number of female newborns was 31.71±7.2% (n=13), equal to the number of male newborns 31.11±6.9% (n=14). The incidence of HDFN caused by the Rh system was 21.95±6.4% (n=9) of female newborns, which is slightly lower than the number of male newborns 24.45±6.4% (n=11). The χ^2-statistic is 0.0768. The P-value is 0.962329 (Table 23).

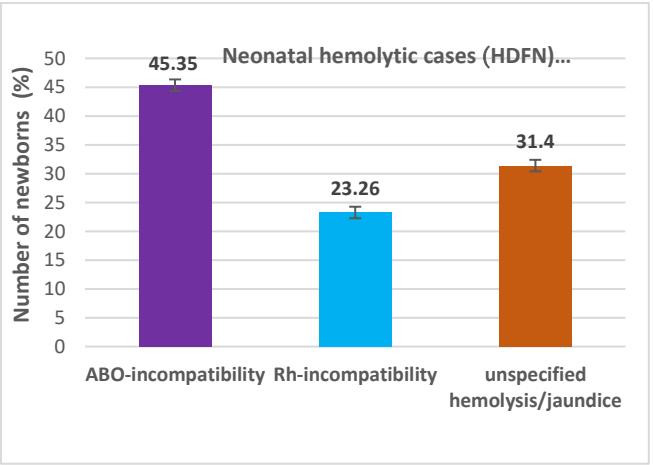
One of the objectives of our study was to investigate cases of hemolytic anemia of the fetus and newborn (HDFN) among neonates admitted to the intensive care unit (NICU).

It is well known that hemolytic anemia in newborns can have various causes, such as bone marrow failure, autoimmune disorders, hereditary blood diseases (including sickle cell anemia and thalassemia), adverse drug reactions, different types of infections, complications following blood transfusions, and immune-conflict pregnancies caused by incompatibility in the Rh or ABO systems, among others [34].

Table 23. Cases of hemolytic disease (ABO and Rh Incompatibility-Induced HDFN) and unspecified Jaundice by Sex *(Based on Data from 2020–2024)*

Cases of hemolytic (ABO, Rh) and unspecified jaundice of newborns	**Gender**				**χ^2 P**
	Female ♀		**Male ♂**		
	(n)	**(%)**	**(n)**	**(%)**	χ^2-0.07 68 P-0.96 2329
ABO	19	46.34 ±7.7	20	44.44 ±7.4	
Rh	9	21.95 ±6.4	11	24.45 ±6.4	
Unspecified hemolytic diseases and jaundice	13	31.71 ±7.2	14	31.1 1±6.9	
Total	41	100	45	100	

Figure 1. Distribution of hemolytic (ABO- and Rh system-related HDFN) and unspecified jaundice cases according to *data from 2020–2024*



Our area of interest focused on studying hemolytic disease of the fetus and newborn (HDFN) caused by erythrocyte blood group antigens in neonates. The development of immune hemolytic anemias is primarily associated with erythrocyte antigen systems that exhibit high immunogenicity. Among them, the ABO system is the most common cause of hemolytic transfusion reactions, particularly in cases of ABO incompatibility.

The Rh system antigens (D, C, E, c, e) play a crucial role in the pathogenesis of hemolytic disease of the fetus and newborn (HDFN) and in the development of immune-mediated hemolysis. The Kell system, in turn, is notable because anti-K antibodies are often associated with severe hemolysis, which can occur even at low antibody titers.

The Kidd antigen system (Jka, Jkb) is frequently linked to the occurrence of delayed hemolytic transfusion reactions. The Duffy (Fya, Fyb) and MNS (M, N, S, s) antigen systems are considered to have moderate clinical significance; however, in certain cases, they can also lead to HDFN or transfusion-induced hemolysis, particularly when anti-S or anti-U antibodies are present in the patient's circulation [35].

The diagnosis of hemolytic disease caused by the ABO system remains a subject of ongoing discussion and debate [33]. The mere presence of ABO incompatibility between the mother and the newborn does not constitute definitive evidence of hemolysis. In the absence of serological confirmation - such as a positive direct antiglobulin test (DAT) or other laboratory indicators - diagnosing hyperbilirubinemia solely based on ABO incompatibility may be misleading.

Therefore, the diagnosis of ABO incompatibility–induced hemolytic disease should be based on a combination of clinical and laboratory findings, rather than on the incompatibility of blood groups alone [33].

In our study group, a total of 86 neonates admitted to the intensive care unit were diagnosed with hemolysis (Fig. 5). The majority of these cases (n=59) were primarily attributed to ABO and Rh system incompatibility, whereas a smaller subset (n=27) included unspecified hemolytic and jaundice conditions.

As illustrated in Figure 5, ABO incompatibility-induced HDFN accounted for 45.35±5.3% (n=39) of all analyzed neonatal cases. Rh incompatibility-induced HDFN represented 23.26±4.5% (n=20) of cases. The remaining 31.40±5.0% (n=27) were classified as unspecified hemolytic states or jaundice of undetermined etiology (Fig. 1).

Hemolytic Disease of the Fetus and Newborn (HDFN) Caused by ABO and Rh Incompatibility

We were interested in studying how these phenotypic combinations were represented specifically in confirmed cases of hemolytic disease of the fetus and newborn (HDFN) caused by ABO and Rh incompatibility. Each case was analyzed separately. In neonates with HDFN caused by ABO incompatibility, we studied the distribution of ABO and Rh blood group combinations.

Within this study group, the most frequent combination among newborns was A(II), $Rh^{+,}$ accounting for 74.36±6.9% (n=29), followed by B(III), Rh^{+} at 15.38±5.7% (n=6), and A(II), Rh^{-} at 5.13 ± 3.5% (n = 2)**.** The combinations AB(IV), Rh^{+} and B(III), Rh^{-} were each observed in only one case, representing 2.5±2.5% (n=1) of the study group. No cases were recorded with O(I), Rh^{+}, O(I), Rh^{-}, or AB(IV), Rh^{-} blood groups among neonates with this type of hemolytic anemia (Table 24).

Table 24. HDFN caused by ABO system incompatibility (according to 2020-2024)

Phenotypic combinations of the ABO and Rh systems of newborns	**HDFN caused by ABO system incompatibility**	
	(n)	**(%)**
O(I),Rh^{+}	0	0.00
O(I),Rh^{-}	0	0.00
A(II),Rh^{+}	29	74.36±6.9
A(II),Rh^{-}	2	5.13±3.5
B(III),Rh^{+}	6	15.38±5.7
B(III),Rh^{-}	1	2.56±2.5
AB(IV),Rh^{+}	1	2.56±2.5
AB(IV),Rh^{-}	0	0.00
სულ	39	100

Table 25. HDFN caused by Rh incompatibility (2020-2024)

Phenotypic combinations of the ABO, Rh system of newborns	**HDFN caused by the Rh system incompatibility**	
	(n)	**(%)**
O(I),Rh^{+}	9	45.00±11.1
O(I),Rh^{-}	0	0.00
A(II),Rh^{+}	10	50.00±11.1
A(II),Rh^{-}	0	0.00
B(III),Rh^{+}	1	5.00±4.8
B(III),Rh^{-}	0	0.00
AB(IV),Rh^{+}	0	0.00
AB(IV),Rh^{-}	0	0.00
სულ	20	100

Separately, we studied phenotypic ABO and Rh blood group combinations in neonates with HDFN caused by Rh incompatibility. The A(II), Rh^{+} blood group combination was the most frequently associated with Rh incompatibility, representing 50.00±11.1% (n=10) of cases, followed by O(I), Rh^{+}, which accounted for 45.00±11.1% (n=9) (Table 25).

Blood Group Combinations of Newborns and Mothers with Hemolytic HDFN (ABO/ Rh/ and other, unspecified hemolytic diseases)

Since the Neonatal Intensive Care Unit (NICU) of the Batumi “Medina” Health Center, named after Iris Borchashvili, admitted not only newborns delivered at the same clinic but also those transferred from various maternity hospitals across the Adjara region (mostly with severe

perinatal histories), it was not possible to determine the maternal blood group for all newborns diagnosed with HDFN. Among the 86 newborns with hemolytic anemia analyzed in our study, detailed information on the mother's blood group was available for 77cases.

Table 26 below presents an analysis of the blood group combinations of mothers and newborns with hemolytic HDFN (ABO, Rh, and other unspecified hemolytic diseases). Theoretically, the highest frequency of hemolytic anemia (both ABO and Rh associated) is observed when the mother's blood group is O(I), Rh^-. This can be explained by the fact that this blood group is generally more prevalent compared to others [27], [28].

Accordingly, we analyzed the following maternal–neonatal combinations: 1. Mother O(I), Rh+ / Newborn O(I), Rh^+ (n=1); 2. Mother O(I), Rh^+ / Newborn O(I), Rh^- (n = 0); 3. Mother O(I), Rh+ / Newborn A(II), Rh+ (n = 31); 4. Mother O(I), Rh+ / Newborn A(II), Rh− (n = 3); 5. Mother O(I), Rh+ / Newborn B(III), Rh+ (n = 8); 6.Mother O(I), Rh+ / Newborn B(III), Rh− (n = 2); 7. Mother O(I), Rh+ / Newborn AB(IV), Rh+ (n = 1); 8. Mother O(I), Rh+ / Newborn AB(IV), Rh− (n = 0).

Among these eight possible mother/newborn combinations, the most frequent was that of A(II), Rh+ newborns born to O(I), Rh+ mothers (n = 31). The next most common were B(III), Rh+ newborns (n = 8), followed by A(II), Rh− (n = 3) and B(III), Rh− newborns (n = 2). Equal distribution was observed among O(I), Rh+ and AB(IV), Rh+ newborns (n = 1 each), while no cases were recorded among O(I), Rh− newborns (n = 0).

This distribution pattern reflects an increased risk of jaundice and hemolytic disease, indicating ABO incompatibility - a condition in which maternally derived antibodies (anti-A or anti-B) can cross the placenta and attack fetal red blood cells, resulting in hemolytic disease [29], [30].

In cases where the mother had an O(I), Rh− blood group, we analyzed the following eight combinations of neonatal blood groups: 1. Mother O(I), Rh− / Newborn O(I), Rh+ (n=10); 2. Mother O(I), Rh− / Newborn O(I), Rh− (n=0); 3. Mother O(I), Rh− / Newborn A(II), Rh+ (n=4); 4. Mother O(I), Rh− / Newborn A(II), Rh− (n=1); 5. Mother O(I), Rh− / Newborn B(III), Rh+ (n=0); 6. Mother O(I), Rh− / Newborn B(III), Rh− (n=0); 7. Mother O(I), Rh− / Newborn AB(IV), Rh+ (n=0); 8. Mother O(I), Rh− / Newborn AB(IV), Rh− (n=0). From these combinations, it can be seen that hemolysis occurred predominantly in newborns with the O(I), Rh+ blood group (n=10); the next most frequent were A(II), Rh+ (n=4), and a single case of hemolysis was observed in a newborn with the A(II), Rh− blood group.

In mothers with the A(II), Rh+ blood group, it is theoretically possible to have newborns with eight different blood group combinations: 1. Mother A(II), Rh+ / Newborn O(I), Rh+ (n=3); 2. Mother A(II), Rh+ / Newborn O(I), Rh− (n=1); 3. Mother A(II), Rh+ / Newborn A(II), Rh+ (n=1); 4. Mother A(II), Rh+ / Newborn A(II), Rh− (n=0); 5. Mother A(II), Rh+ / Newborn B(III), Rh+ (n=0); 6. Mother A(II), Rh+ / Newborn B(III), Rh− (n=0); 7. Mother A(II), Rh+ / Newborn AB(IV), Rh+ (n=0); 8. Mother A(II), Rh+ / Newborn AB(IV), Rh− (n=0). From these combinations, hemolysis was observed only in newborns with the O(I), Rh+ blood group (n=3), and in single cases in newborns with O(I), Rh− and A(II), Rh+ blood groups (n=1). This may be attributed to ABO incompatibility.

In cases where the mother had an A(II), Rh− blood group, analysis of the eight possible neonatal combinations revealed that hemolysis occurred only when the newborn had the A(II), Rh+ blood group (n=9): 1. Mother A(II), Rh− / Newborn O(I), Rh+ (n=0); 2. Mother A(II), Rh− / Newborn O(I), Rh− (n=0); 3. Mother A(II), Rh− / Newborn A(II), Rh+ (n=9); 4. Mother A(II), Rh− / Newborn A(II), Rh− (n=0); 5. Mother A(II), Rh− / Newborn B(III), Rh+ (n=0); 6. Mother A(II), Rh− / Newborn B(III), Rh− (n=0); 7. Mother A(II), Rh− / Newborn AB(IV), Rh+ (n=0); 8. Mother A(II), Rh− / Newborn AB(IV), Rh− (n=0).

When the mother had the B(III), Rh+ blood group, analysis of the eight possible combinations showed the following: 1. Mother B(III), Rh+ / Newborn O(I), Rh+ (n=0); 2. Mother B(III), Rh+ / Newborn O(I), Rh− (n=0); 3. Mother B(III), Rh+ / Newborn A(II), Rh+ (n=0); 4. Mother B(III), Rh+ / Newborn A(II), Rh− (n=0); 5. Mother B(III), Rh+ / Newborn B(III), Rh+ (n=0); 6. Mother B(III), Rh+ / Newborn B(III), Rh− (n=0); 7. Mother B(III), Rh+ / Newborn AB(IV), Rh+ (n=1); 8. Mother B(III), Rh+ / Newborn AB(IV), Rh− (n=0). In this case, hemolysis was observed only once, in a newborn with the AB(IV), Rh+ blood group (n=1).

Similarly, one case of hemolysis was observed among the eight combinations where the mother had a B(III), Rh− blood group and the newborn had a B(III), Rh+ blood group (n=1): 1. Mother B(III), Rh− / Newborn O(I), Rh+ (n=0); 2. Mother B(III), Rh− / Newborn O(I), Rh− (n=0); 3. Mother B(III), Rh− / Newborn A(II), Rh+ (n=0); 4. Mother B(III), Rh− / Newborn A(II), Rh− (n=0); 5. Mother B(III), Rh− / Newborn B(III), Rh+ (n=1); 6. Mother B(III), Rh− / Newborn B(III), Rh− (n=0); 7. Mother B(III), Rh− / Newborn AB(IV), Rh+ (n=0); 8. Mother B(III), Rh− / Newborn AB(IV), Rh− (n=0).

For mothers with AB(IV) and Rh+ blood groups, and with AB(IV) and Rh− blood groups, no cases of hemolysis were recorded (n=0). This may be explained by the fact that individuals with

the AB(IV) blood group lack anti-A and anti-B antibodies. Therefore, this blood group does not induce hemolysis associated with group incompatibility (Table 26).

Table 26. Newborn and maternal blood group combinations in anemia, jaundice, and other ABO/Rh-related hemolytic diseases (*2020–2024)*

Newborn / Mother	O(I), Rh+	O(I), Rh-	A(II), Rh+	A(II), Rh-	B(III), Rh+	B(III), Rh-	AB(IV), Rh+	AB(IV), Rh-
O(I),Rh+	1		31	3	8	2	1	
O(I),Rh-	10		4	1				
A(II),Rh+	3	1	1					
A(II),Rh-			9					
B(III),Rh+							1	
B(III),Rh-					1			
AB(IV),Rh+								
AB(IV),Rh-								
Total	14	1	45	4	9	2	2	

Table 27. Newborn and maternal blood group combinations in confirmed (ABO and Rh) HDFN cases *(2020-2024)*

Newborn / Mother	O(I) Rh+	O(I) Rh-	A(II) Rh+	A(II) Rh-	B(III) Rh+	B(III) Rh-	AB(IV) Rh+	AB(IV) Rh-
O(I),Rh+			28	1	6	1	1	
O(I),Rh-	9		4	1				
A(II),Rh+								
A(II),Rh-			7					
B(III),Rh+								
B(III),Rh-					1			
AB(IV),Rh+								
AB(IV),Rh-								
Total	9		39	2	7	1	1	

- **A shaded box indicates the absence of the corresponding combinations in the mother/newborn**

Since hemolysis most frequently occurs in the presence of ABO and Rh system incompatibility, we attempted to analyze the relationship between the newborn's and the mother's blood groups in confirmed cases of hemolytic anemia caused by ABO and Rh incompatibility (n=59). The following table (Table 27) presents the distribution of hemolytic disease among newborns according to the maternal and neonatal ABO and Rh blood group combinations.

When the mother had an O(I), Rh+ blood group, the following combinations were identified: 1. Mother O(I), Rh+ / Newborn O(I), Rh+ (n=0); 2. Mother O(I), Rh+ / Newborn O(I), Rh− (n=0); 3. Mother O(I), Rh+ / Newborn A(II), Rh+ (n=28); 4. Mother O(I), Rh+ / Newborn A(II), Rh− (n=1); 5. Mother O(I), Rh+ / Newborn B(III), Rh+ (n=6); 6. Mother O(I), Rh+ / Newborn B(III), Rh− (n=1); 7. Mother O(I), Rh+ / Newborn AB(IV), Rh+ (n=1); 8. Mother O(I), Rh+ / Newborn AB(IV), Rh− (n=0).

As shown, the highest number of cases was observed among mothers with blood group O(I), Rh+, who had newborns with blood group A(II), Rh+ (n=28). This high frequency clearly indicates hemolytic anemia caused by ABO incompatibility. Hemolysis was also detected in newborns with the B(III), Rh+ blood group (n=6), and single cases were observed in newborns with A(II), Rh−, B(III), Rh−, and AB(IV), Rh+ blood groups (n=1).

In cases where the mother had the O(I), Rh− blood group, the following eight neonatal blood group combinations were analyzed: 1. Mother O(I), Rh− / Newborn O(I), Rh+ (n=9); 2. Mother O(I), Rh− / Newborn O(I), Rh− (n=0); 3. Mother O(I), Rh− / Newborn A(II), Rh+ (n=4); 4. Mother O(I), Rh− / Newborn A(II), Rh− (n=1); 5. Mother O(I), Rh− / Newborn B(III), Rh+ (n=0); 6. Mother O(I), Rh− / Newborn B(III), Rh− (n=0); 7. Mother O(I), Rh− / Newborn AB(IV), Rh+ (n=0); 8. Mother O(I), Rh− / Newborn AB(IV), Rh− (n=0). From these combinations, it is evident that in mothers with O(I), Rh− blood group, hemolysis occurred in newborns with O(I), Rh+ (n=9), A(II), Rh+ (n=4), and A(II), Rh− (n=1) blood groups.

Hemolysis was also observed only in cases where mothers with the A(II), Rh− blood group had newborns with the A(II), Rh+ blood group (n=7). In contrast, in all other combinations, no cases of hemolysis were recorded.

In mothers with the B(III), Rh− blood group, one case of hemolysis was identified in a newborn with the B(III), Rh+ blood group (n=1).

Mothers with A(II), Rh+, B(III), Rh+, AB(IV), Rh+, and AB(IV), Rh− blood groups showed no cases of hemolysis, which may indicate that in mothers with these blood groups, the risk of hemolysis development is relatively low [29], [31] (Table 27).

When we analyzed the relationship between the mother's blood group and the newborn's blood group in cases of HDFN caused only by ABO system incompatibility, out of the eight combinations listed here: 1. Mother O(I), Rh+ /newborn O(I), Rh+ (n=0); 2. Mother O(I),Rh+ /newborn O(I),Rh- (n=0); 3. Mother O(I),Rh+/newborn A(II),Rh+ (n=28); 4. Mother O(I),Rh+ /newborn A(II),Rh- (n=1); 5. Mother O(I),Rh+ /newborn B(III),Rh+ (n=6); 6. Mother O(I),Rh+ /newborn B(III),Rh- (n=1); 7. Mother O(I),Rh+ /newborn AB(IV),Rh+ (n=1); 8. Mother O(I), Rh+ /newborn AB(IV), Rh- (n=0); It was found that mothers with O(I), Rh+ blood group had the highest number of cases when their newborn had A(II), Rh+ blood group (n=28), newborns with B(III), Rh+ blood group (n=6), and there was one case each when the newborn had A(II), Rh-, B(III), Rh- and AB(IV), Rh+ blood groups (n=1). Where the mother has O(I), Rh- blood group, one case of hemolysis was observed in newborns with A(II), Rh+ and A(II), Rh- blood groups (n=1). Whereas, where the mother has A(II), Rh+, A(II), Rh-, B (III), Rh+, B(III), Rh-, AB(IV), Rh+, and AB(IV), Rh- blood groups, no cases of hemolysis were observed in them (n=0) (Table 28).

When analyzing the relationship between the mother's and the newborn's blood groups in HDFN cases (n=20), combinations were identified in which HDFN occurred (Table 29). These combinations are: 1. Mother O(I),Rh- /newborn O(I),Rh+ (n=9); 2. Mother O(I),Rh- /newborn

A(II),Rh+ (n=3); 3. Mother A(II),Rh- /newborn A(II),Rh+ (n=7); 4. Mother B(III), Rh- /newborn B(III), Rh- (n=1). From these combinations it can be seen that in our study more cases of hemolysis were detected when the mother has O(I), Rh- blood group and has newborns with O(I), Rh+ blood group (n=9); we also have cases of hemolysis when mothers have A(II), Rh- blood group, who have newborns with A(II), Rh+ blood group (n=7) and we have one case of HDFN in the combination where the mother has B(III), Rh- blood group and has a newborn with B(III), Rh+ blood group (n=1). This is easily explained because natural hemolysis of group incompatibility does not occur in this case, since the mother and the fetus have the same blood group. The memory cells have time to recognize the Rh antigen, as a result of which the synthesis of immunoglobulin G occurs, which crosses the placenta and causes hemolysis of the newborn's erythrocytes [32].

In combinations where mothers had A(II), Rh+, B(III), Rh+, AB(IV), Rh+, and AB(IV), Rh- blood groups, no cases of hemolysis were observed (n=0) (Table 29).

Table 28. Newborn and maternal blood group combinations in confirmed (ABO) HDFN cases *(2020-2024)*

Newborn / Mother	O(I) Rh+	O(I) Rh-	A(II) Rh+	A(II) Rh-	B(III) Rh+	B(III) Rh-	AB(IV) Rh+	AB(IV) Rh-
O(I),Rh+			28	1	6	1	1	
O(I),Rh-			1	1				
A(II),Rh+								
A(II),Rh-								
B(III),Rh+								
B(III),Rh-								
AB(IV),Rh+								
AB(IV),Rh-								
Total			29	2	6	1	1	

Table 29. Newborn and maternal blood group combinations in confirmed (Rh) HDFN cases *(2020-2024)*

Newborn / Mother	O(I) Rh+	O(I) Rh-	A(II) Rh+	A(II) Rh-	B(III) Rh+	B(III) Rh-	AB(IV) Rh+	AB(IV) Rh-
O(I),Rh+								
O(I),Rh-	9		3					
A(II),Rh+								
A(II),Rh-			7					
B(III),Rh+								
B(III),Rh-					1			
AB(IV),Rh+								
AB(IV),Rh-								
Total	9		10		1			

- A shaded box indicates the absence of the corresponding combinations in the mother/newborn.

This table clearly shows that only mothers with Rh-phenotypic combinations and their newborns with Rh+ phenotypes have HDFN cases, indicating that the Rh system is the leading cause of hemolytic disease in newborns and that it significantly influences its development.

Analysis of Hemolysis Severity and Quantitative Characteristics of Bilirubin in Newborns with Hemolytic Anemia

In all newborns with ABO or Rh incompatibility, bilirubin measurement is essential, as timely assessment facilitates the diagnosis of hemolytic disease. Bilirubin is a compound produced

during hemoglobin catabolism and increases typically in the first days of life. However, elevated levels can lead to hyperbilirubinemia, which in turn increases the risk of neurotoxicity and may result in severe complications such as kernicterus, bilirubin-induced toxic encephalopathy, and other related conditions [33].

The normal range of serum bilirubin (SBR) in newborns is inherently different from that in older children or adults. Functional immaturity of metabolic and hematologic systems contributes to bilirubin accumulation and delayed excretion [34].

Typically, newborns produce bilirubin at a rate of approximately 6–8 mg/dL per day (102.6–136.8 μmol/L), which is roughly twice the bilirubin production observed in adults. This is primarily due to the higher red blood cell count in neonates.

Consequently, contemporary guidelines (AAP, NICE) recommend standardized assessment of serum bilirubin for all newborns, especially those at risk due to hemolysis [35], [36]. Bilirubin production usually declines to adult levels within 10–14 days after birth [37].

In our study of newborns with HDFN admitted to the neonatal intensive care unit (n=86), we analyzed the quantitative characteristics of serum bilirubin (SBR). Each hemolytic newborn exhibited varying serum bilirubin levels. In the studied cohort, SBR ranged from 86.0 μmol/L to 441.0 μmol/L.

It is important to note that clinical studies and visual assessment alone cannot accurately determine the severity of jaundice. For screening purposes, transcutaneous bilirubin measurement is commonly used. If the value exceeds 200 μmol/L, serum bilirubin determination (SBR) is required. In newborns, SBR levels are influenced by gestational age, birth weight, and racial background [29]. However, some authors have criticized the American Academy of Pediatrics (AAP) clinical guidelines for hyperbilirubinemia, arguing that they fail to account for race-based differences and the potential for implicit bias in the assessment of neonatal jaundice [35].

Neonatal hyperbilirubinemia is defined as a serum bilirubin (SBR) level above 5 mg/dL (86 μmol/L) [29]. At such levels, various degrees of health complications may occur. Although clinical jaundice is observed in approximately 60% of term and 80% of preterm newborns during the first week of life, only a small proportion of these infants have a significant underlying disease. However, hyperbilirubinemia in newborns can be associated with severe conditions, such as HDFN, metabolic and endocrine disorders, anatomical liver abnormalities, and infections [29].

Jaundice results from the breakdown of red blood cells, leading to increased SBR levels in the blood. The gold standard for measuring bilirubin is the collection of a blood sample and its laboratory analysis to determine total serum bilirubin (biochemical assessment) [29].

The main risk factors for neonatal hyperbilirubinemia include incompatibility between maternal and fetal blood groups, a history of previous pregnancies, birth trauma (cephalohematoma, skin bruising), delayed meconium passage, and others [34]. The primary goal in managing hyperbilirubinemia is to identify pathological processes early and initiate timely treatment to prevent bilirubin-induced neurotoxicity [34].

Since SBR levels are particularly elevated in cases of ABO and Rh isoimmunization, hemolytic disease, and unconjugated jaundice, we analyzed serum bilirubin (SBR) levels in this group (n=86). According to contemporary classification, there are different degrees of hemolysis [34].

In cases of fetal and neonatal hemolytic jaundice, measuring unconjugated (indirect) bilirubin is crucial for both diagnostic and prognostic purposes. Unconjugated bilirubin is produced during the catabolism of hemoglobin, a process that is particularly pronounced during hemolytic conditions such as ABO or Rh incompatibility. When erythrocytes break down, hemoglobin is first converted to biliverdin, which is then converted to bilirubin. This bilirubin **is** unconjugated**,** meaning it is not water-soluble and cannot be excreted via bile. In neonates, the hepatic enzyme system is still immature, further limiting bilirubin conjugation. As a result, elevated levels of unconjugated bilirubin can cross the blood-brain barrier**,** potentially causing bilirubin encephalopathy (kernicterus)**,** which may lead to neuronal damage, intellectual deficits, and motor impairments [33].

In our study group, some newborns with hemolysis had high SBR levels (342–441.0 μmol/L)**,** considered as severe hemolysis; in some newborns, SBR levels were moderately elevated (206–308 μmol/L)**,** regarded as moderate hemolysis; and in others, SBR levels were low (86–205 μmol/L)**,** considered as physiological hemolysis**.**

Physiological hemolysis indicates SBR levels within the normal physiological range, moderate hemolysis reflects a mild increase in SBR (which may require phototherapy), and severe hemolysis corresponds to high and rapidly increasing SBR levels (which may necessitate intensive phototherapy, immunoglobulin therapy, or exchange transfusion [33].

Based on hemolysis severity, we analyzed the number of newborns in each category. Mild hemolysis (physiological hemolysis) was observed in 25.58±4.7% (n=22) of the newborns, moderate hemolysis was characteristic of the majority, 52.33±5.3% (n=45), and severe hemolysis was present in 22.09±4.4% (n=19) of the newborns (Fig. 2).

We analyzed the number of newborns by hemolysis severity in infants with hemolytic anemia due to ABO and Rh incompatibility. Among newborns with ABO incompatibility, the most

frequent hemolysis severity was moderate, occurring in 61.54±7.7% (n=24) of cases. In contrast, in newborns with Rh incompatibility, severe hemolysis was observed in 55.00±11.1% (n=11) during physiological-grade hemolysis. For ABO-incompatible hemolytic anemia, physiological hemolysis was observed in 23.08±6.7% (n=9) of newborns, and severe hemolysis in 15.38±5.7% (n=6). In contrast, among newborns with Rh-incompatible hemolytic anemia, severe hemolysis accounted for 25.00±9.6% (n=5), which is higher compared to ABO-incompatible cases, while moderate hemolysis was observed in 20.00±8.9% (n=4) of cases (Table 30). The χ^2 statistic was 9.4366, with a p-value of 0.00893.

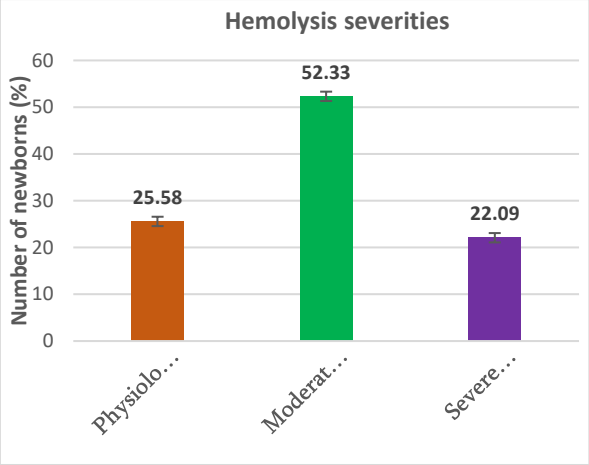


Figure 2. Severity of hemolysis in the studied newborns (*based on 2020–2024 data)*

Hemolysis severity	ABO incompatibility HDFN		Rh incompatibility HDFN		χ^2
	(n)	(%)	(n)	(%)	
Physiological hemolysis (86–205 µmol/L)	9	23.08±6.7	11	55.00±11.1	χ^2-9.4366
Moderate hemolysis (206–308 µmol/L)	24	61.54±7.7	4	20.00±8.9	
Severe hemolysis (>309 µmol/L)	6	15.38±5.7	5	25.00±9.6	
Total	39	100	20	100	

Table 30. Severity of hemolysis in newborns with HDFN Caused by ABO and Rh Incompatibility *(Based on 2020–2024 Data)*

Historically, the most common cause of HDFN was alloimmunization against the Rh(D) antigen. However, the widespread implementation of Rh(D) prophylaxis (anti-D immunoglobulin) has significantly reduced the incidence of HDFN caused by Rh-negative blood. Currently, the most frequent etiological factor for HDFN is ABO incompatibility [38], [39].

Typically, ABO incompatibility arises between O blood group mothers and A or B blood group fetuses. Although ABO incompatibility between mother and fetus occurs in approximately 15–25% of cases, clinically significant hemolysis is observed in only about 1% of newborns. Several immunological and biological factors explain this low frequency. Specifically, ABO antibodies are isohemagglutinins of the IgM class, which cannot cross the placental barrier. Additionally, the expression of ABH antigens on fetal erythrocytes is very weak, further reducing the risk of hemolysis [40].

Nevertheless, the likelihood of developing severe HDFN increases if high-titer IgG antibodies are produced in the maternal circulation, as these antibodies readily cross the placenta and induce hemolysis of fetal erythrocytes. Clinically, HDFN primarily manifests as mild hyperbilirubinemia, occasionally anemia, and only in extreme cases, severe hemolysis. The first-line treatment is usually phototherapy, which reduces bilirubin levels and mitigates the effects of hemolysis. Exchange transfusion is required much less frequently [40].

It is noteworthy that ABO incompatibility may, to some extent, protect the fetus from Rh(D)-dependent sensitization. This is based on the mechanism by which ABO-incompatible erythrocytes are rapidly recognized and neutralized by the maternal immune system, thereby reducing the likelihood of an effective immune response against the Rh(D) antigen. Consequently, the initiation or progression of Rh(D)-specific alloimmunization is significantly delayed [40], [27].

Analysis of our data from 2020–2024 showed that hemolysis caused by ABO incompatibility was the most frequent, accounting for 45.35±5.3% (n=39) of cases, which is significantly higher than the standard values reported in the literature. Globally, HDFN due to ABO incompatibility is more common but usually presents with a mild course. Overall, ABO incompatibility between the mother and fetus is observed in 15–25% of pregnancies, yet clinically significant hemolysis occurs in only 3–4% of cases [41].

As for hemolysis caused by Rh incompatibility, it was observed in 23.26±4.5% (n=20) of cases, a rate that is also relatively high. In developed countries, the use of Rh(D) prophylaxis (anti-D immunoglobulin) has reduced the incidence to less than 1%. Although Rh incompatibility is less common, hemolysis in these cases often follows a severe course [42].

Hemolytic disease and unexplained jaundice were observed in 31.40±5.0% (n=27) of cases. A significant portion of neonatal hyperbilirubinemia remains unexplained, necessitating further investigation, such as testing for antibodies against other erythrocyte antigens (rare causes include the Kell, Duffy, and MNS systems), infections, and other factors [43], [44].

Hemolytic Anemia and Its Association with Selected Anthropometric Characteristics in Newborns

In assessing neonatal health, anthropometric parameters such as gestational age, birth weight, body length, and head circumference are important clinical indicators. These parameters reflect both the quality of intrauterine (prenatal) development and perinatal conditions, as well as potential risks for pathological conditions. Gestational age, birth weight, body length, and head

circumference are particularly significant for the clinical evaluation of hematological disorders, including hemolytic anemia [45], [46].

Gestational age influences both the timing and the pattern of hematopoiesis in the developing fetus. At this stage, any disruption or accelerated destruction of red blood cells may lead to hemolytic anemia, a condition in which erythrocytes are excessively destroyed, and the body is unable to replace them at a sufficient rate [47], [48].

Although some studies classify gestational age in greater detail (e.g., extremely, moderately, and late preterm), clinical practice often employs a simplified categorization. In the present study, data obtained from the M. Iashvili Batumi Maternal and Child Central Hospital were classified into three categories: 1. Very preterm - including newborns with gestational age under 32 weeks (<32 weeks); 2. Preterm- including newborns with gestational age between 32 and 36 weeks (32–36 weeks); 3. Term newborn, including newborns with a gestational age of 37 weeks or more (≥37 weeks).

Accordingly, for this study, newborns were classified into three main categories based on gestational age: very preterm, preterm, and term newborns. This categorization represents a practical approach that is both consistent with international standards and tailored to the study's specific context. Among the studied newborns, the minimum gestational age (GA) was 29 weeks, while the maximum was 41 weeks.

Anthropometric parameters influence both organ maturation and hematological functions in preterm, post-term, and term newborns, including the course of hemolysis. It is well established that preterm newborns have a higher risk of developing hematological disorders and often exhibit incomplete maturation of physiological functions [46].

Therefore, the analysis of neonatal anthropometric indicators is essential not only for assessing growth and development but also for identifying the risk of hemolytic anemia. Accordingly, one of the main objectives of this doctoral dissertation was to compare the anthropometric parameters of newborns with hemolytic anemia to those of healthy newborns.

For comparison, healthy newborns were used. This group was initially composed exclusively of term (gestational age ≥37 and ≤41 weeks) healthy newborns who exhibited no clinically significant pathologies. Preterm or post-term newborns were not included in this group, as their inclusion could compromise the accuracy of data interpretation; in such cases, the variability of anthropometric parameters is often influenced by biological factors independent of gestational age [49].

However, specific analyses did consider other variations. Additionally, data from newborns who were not ethnically Georgian or who were born via surrogacy were excluded due to the unavailability of biographical information and ethical restrictions on confidentiality, which prevented detailed study or processing of personal data (personal data protection standards in force since May 25, 2018) [50], [51].

In total, data from 2,634 newborns born between 2022 and 2024 were available for selection and analysis. For comparison, newborns with hemolytic anemia and healthy newborns (control group) were analyzed by body weight category. Within the scope of our study, newborns were classified into four weight categories: 1**.** Over 4000 g (>4000 g); 2. 2500-4000 g (inclusive); 3. 1500-2500 g (inclusive); 4. under 1500 g (<1500 g).

Since newborns with hemolytic anemia are often born preterm or with birth weights either above or below the gestational age norms, we decided to expand the study group to include healthy newborns who were not classified as term (preterm and very preterm). This approach enables a more accurate and objective analysis of outcomes in newborns with hemolytic anemia. Consequently, all healthy newborns, regardless of gestational age, were included for comparison, yielding a total of 1939 in the healthy group.

Analysis of all healthy newborns and those with hemolytic disease shows that the 2500–4000 g weight range was the most represented in both groups. However, this weight range was more frequent among healthy newborns (92.32±0.5%; n=1790) than among newborns with hemolytic disease (84.89±3.8 %; n=73) (Table 31).

Table 31. Comparison of newborns by weight categories in the hemolytic and all healthy newborn groups

Newborn weight (g)	**Hemolytic newborns**		**Healthy newborns**		χ^2	**P-value**
	(n)	**(%)**	**(n)**	**(%)**	17.9103	0.000459
>4000	7	8.1±2.9	113	5.83±0.5		
2500-4000	73	84.8±3.8	1790	92.32±0.5		
1500-2500	5	5.8±2.5	36	1.86±0.3		
<1500	1	1.1±1.1	0	0		
Total	86	100	1939	100		

Notably, among newborns with hemolytic anemia, those weighing 1500–2500 g were significantly more frequent, 5.81±2.5% (n=5), compared to 1.86±0.3% (n=36) in healthy newborns. Additionally, in the <1500 g category, 1 case was recorded in the hemolytic group (1.16±1.1%; n=1), whereas no newborns in this weight category were observed in the healthy group (n=0).

Regarding the >4000 g weight category, the proportion of newborns was higher in the hemolytic group, 8.14±2.9% (n=7), compared to 5.83±0.5% (n=113) in the healthy group (Table 31). The χ^2 statistic is 17.9103, with a p-value of <0.000459.

Table 31. Comparison of newborns by weight categories in hemolytic and all healthy newborn groups

Newborn weight (g)	Hemolytic newborns		Healthy newborns		χ^2	P
	(n)	(%)	(n)	(%)	17.9103	0.000459
>4000	7	8.14 ±2.9	113	5.83 ±0.5		
2500-4000	73	84.89 ±3.8	1790	92.32 ±0.5		
1500-2500	5	5.81 ±2.5	36	1.86± 0.3		
<1500	1	1.16 ±1.1	0	0		
Total	86	100	1939	100		

Table 32. Comparison of hemolytic and healthy newborns by gestational age

Gestational age of the newborn (Weeks)	Hemolytic newborns		Healthy newborns		χ^2	P
	(n)	(%)	(n)	(%)	94.1394	<0.00001
Full-term (37-41)	75	87.21 ±3.6	1924	99.23 ±0.1		
Premature (32-36)	10	11.63 ±3.4	14	0.72 ±0.1		
Very premature (<32)	1	1.16 ±1.1	1	0.05±0.05		
Total	86	100	1939	100		

We analyzed newborns with hemolytic anemia (n=86) according to gestational age and compared them with corresponding data from healthy newborns (n=1939). Gestational age (GA) in both groups was divided into three categories: term (37–41 weeks), preterm (32–36 weeks), and very preterm (<32 weeks) newborns.

The proportion of term newborns was highest among healthy infants, 99.23±0.1% (n=1924), which is approximately 12% than the corresponding proportion in newborns with hemolytic anemia, 87.21±3.6% (n=75). Conversely, the prevalence of preterm birth was higher in the hemolytic group (11.63±3.4%; n=10) than in the healthy group (0.72±0.1%; n=14). Very preterm cases were rare in both groups, with one case each: 1.16±1.1% (n=1) in the hemolytic group and 0.05±0.05% (n=1) in the healthy group (Table 32). These data indicate that preterm and very preterm births are more frequent among newborns with hemolytic anemia, suggesting a possible influence of hemolytic processes on gestational age. Statistical analysis using the χ^2 test yielded a value of 94.1394 with a p-value of <0.00001, indicating that the difference in the distribution of gestational age categories between the two groups is statistically significant.

Within the scope of our study, the distribution of body length was assessed in newborns with hemolytic anemia and healthy newborns. Body length parameters were divided into three categories: >52 cm, 46–52 cm (inclusive), and 32–46 cm (inclusive). In both groups, the majority of newborns fell within the 46–52 cm range.

Table 33. Comparison of hemolytic and healthy newborns by body length

Newborn Length (cm)	Hemolytic newborns		Healthy newborns		χ^2	P
	(n)	(%)	(n)	(%)		
>52	6	6.98 ±2.7	57	2.94 ±0.3	20.872	0.00 0029
46-52	74	86.05 ±3.7	1855	95.67 ±0.4		
32-46	6	6.98 ±2.7	27	1.39 ±0.2		
Total	86	100	1939	100		

Table 34. Comparative analysis of HC in newborns with hemolytic disease and healthy newborns

Newborn head circumference (cm)	Hemolytic newborns		Healthy newborns		χ^2	P
	(n)	(%)	(n)	(%)		
>36	5	5.81 ±2.5	3	0.15 ±0.08	85.5 18	<0.00 001
34-36	37	43.02 ±5.3	465	23.98 ±0.9		
26-34	44	51.16 ±5.3	1471	75.86 ±0.9		
Total	86	100	1939	100		

Specifically, 95.67±0.4% (n=1855) of healthy newborns and 86.05±3.7% (n=74) of newborns with hemolytic anemia were in this category. Newborns with a body length >52 cm accounted for 6.98±2.7% (n=6) in the hemolytic group, nearly twice the proportion observed in the healthy group (2.94±0.3%; n=57). For the lowest length category 32–46 cm, the proportion was higher among hemolytic newborns, 6.98±2.7% (n=6), compared to 1.39±0.2% (n=27) in healthy newborns (Table 33). The χ^2 statistic is 20.872 with a p-value of 0.000029, indicating an unequal distribution between the two categorical variables and suggesting a potential association.

As noted above, head circumference (HC) is an essential anthropometric parameter in newborns, reflecting not only general brain development but also serving as a critical indicator in newborns with hemolytic disease. It assists neonatologists in identifying various pathologies. Hemolytic conditions (e.g., Rh or ABO incompatibility) cause erythrocyte destruction, leading to severe anemia and hyperbilirubinemia, which can result in neurotoxicity [52], [53], [54].

Anemia resulting from hemolytic disease affects cerebral blood flow. Although a direct association has not yet been fully established, these processes may potentially influence head circumference [33].

Encephalopathy caused by hyperbilirubinemia can significantly impact neuronal development if not promptly managed. Deviations in head circumference from the normal range can be indicative of neurodevelopmental issues, particularly in cases of microcephaly [55].

Analysis of head circumference in newborns indicates that its distribution differs between those with hemolytic disease and healthy newborns, representing an essential aspect in assessing disease manifestation [55], [53].

Given that head circumference is an essential parameter for evaluating newborns with hemolytic disease, we decided to study this anthropometric measure. For analysis, newborns in both groups were divided into three categories: 1. Head circumference >36 cm; 2. Head circumference 34–36 cm (inclusive); 3. Head circumference 26–34 cm (inclusive) (Table 34).

The results for head circumference among newborns with hemolytic anemia and healthy newborns were as follows: 43.02±5.3% (n=37) of newborns with hemolytic anemia had a normal head circumference (34–36 cm), whereas in healthy newborns, the proportion with this head circumference was significantly lower, 23.98±0.9% (n=465). Conversely, the most common head circumference category among healthy newborns was 26–34 cm, 75.86±0.9% (n=1.471), which is considerably higher than in the hemolytic group, 51.16±5.3% (n=44).

In the category where head circumference exceeds 36 cm (>36), 5.81±2.5% (n=5) of newborns with hemolytic anemia were included, whereas only 0.15±0.08% (n=3) of healthy newborns fell into this group.

These findings indicate that increased head circumference is more frequent in newborns with hemolytic disease, which may be associated with cerebral edema or other clinical complications arising from hemolysis [56], [52] (Table 34). A statistically significant difference between the two groups was observed, with a χ^2 value of 85.518 and a p-value of 0.00001.

Study of the c.261delG (O allele) using SNP rs8176719 in Newborns with Hemolytic Anemia

In blood samples from 34 newborns with hemolytic anemia, we investigated one locus (c.261delG) of the ABO genetic system, which was identified using SNP rs8176719 (Table 35).

We did not have the opportunity to perform additional invasive procedures on the newborns. So we used the remaining blood samples collected in the laboratory. However, after conducting multiple immunoserological tests on these samples, the remaining volume was minimal for DNA extraction. This represents the number of cases in which we fully implemented the methodology. SNP rs8176719 allows the investigation of one locus within the ABO gene that determines blood group antigens, commonly referred to as c.261delG or, more rarely, c.260_262insG. This SNP is the primary determinant of blood group O status [57], [58], [59]. Using the specified SNP rs8176719, the results are interpreted according to established criteria (Table 35).

An allele that determines the A or B blood group genotype carries a (G) at the investigated locus, meaning that such an allele is represented as SNP rs8176719 (G). If a single nucleotide is deleted or entirely removed at this position, the corresponding allele is designated as rs8176719 (-;-), which encodes the most common O blood group allele. However, an individual is typically classified as having blood group O only if they carry two copies of this deletion (i.e., are homozygous). In other words, their genotype at SNP rs8176719 would be (-; -). Individuals with

a single copy may have blood group A or B. A person with the SNP rs8176719 (G; G) genotype is likely to have blood group A, B, or AB.

As shown in the table, the study cannot distinguish between blood groups A, B, and AB. However, it can identify c.261delG, which defines the O allele and is found in a homozygous state in individuals with blood group O. When in a heterozygous state with one deleted locus, blood groups A and B are represented as AO and BO genotypes, respectively. When the deletion is absent, three genotypic states are possible: AA, BB, and AB.

Results from polymerase chain reaction (PCR/qPCR) are presented in different graphical plots, which are crucial for analyzing the study and evaluating data reliability. For instance, an amplification plot illustrates how fluorescence signals increase over cycles, showing amplification dynamics. The threshold cycle (Ct), which represents the initial DNA quantity in the sample, is directly obtained from this plot.

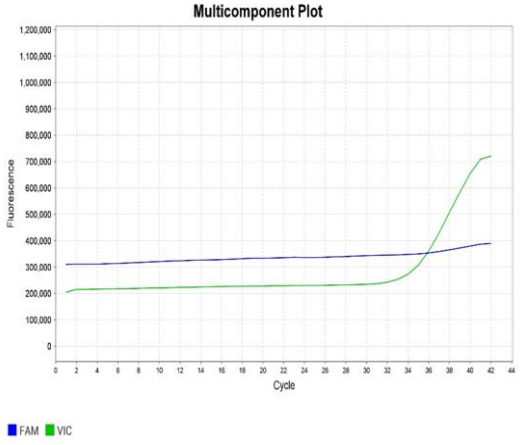


Figure 3. Multicomponent plot for the homozygous G deletion (-; -) case

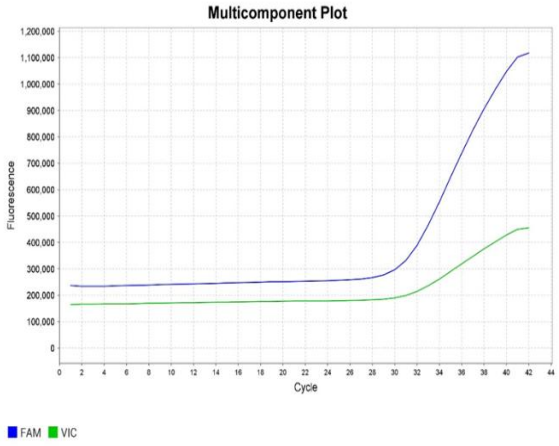


Figure 4. Multicomponent plot for the heterozygous (-; G) case

There is also the multicomponent plot, which displays changes in fluorescence signals across different fluorophore channels. This plot is essential for PCR/qPCR experiments that analyze multiple target genes simultaneously in a single reaction.

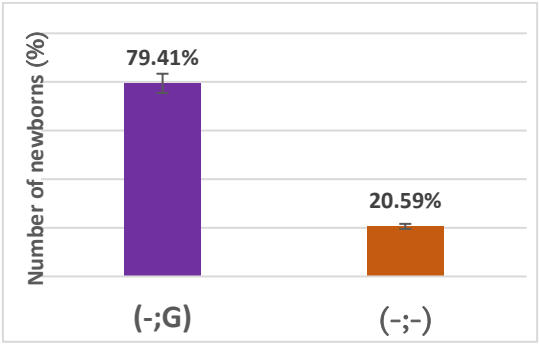
In our study group, two genotypic variants were identified. The first case is shown in Figure 8, where only a single signal is observed, indicating a homozygous (-;-) state (Fig. 3) in which the G nucleotide is completely deleted, corresponding to the O allele. The second case is characterized by two signals, indicating a heterozygous (-; G) state (Fig. 4).

It can be observed that only seven of the analyzed samples carry the complete deletion, meaning that the G nucleotide at position 261 is absent (deleted). This deletion is present in both alleles 1 and 2, allowing us to conclude that these individuals are homozygous at this locus, corresponding to the OO genotype and, consequently, exhibit the O phenotypic blood group.

Table 35. Genotypic and phenotypic correlation of blood groups in the ABO system

Genotype	Analysis
(-;-)	Corresponds to blood group O
(-;G)	Corresponds to blood group A and B
(G;G)	Corresponds to blood group A, B, and AB

Figure 5. Prevalence of the SNP rs8176719 c.261delG deletion in newborns with hemolytic anemia



As the results indicate, the frequency of the O blood group is lower than that of non-O blood groups among the studied newborns with hemolytic anemia. We also analyzed the distribution of the G deletion within the studied blood samples. It was found that its prevalence in the studied samples was 100%, while the G nucleotide at the same locus was detected in 27 cases, representing 79.41±6.9% of the total samples (Fig. 5).

The limited number of samples in this study does not allow for definitive conclusions. However, the analysis conducted on this small cohort demonstrated that hemolytic reactions were more prevalent among newborns with non-O blood groups. Furthermore, the presence of the (-;G) genotype in newborns with non-O blood groups appears to reduce the quantitative expression of group-specific antigens, which may, in turn, decrease the frequency or severity of hemolytic anemia associated with ABO incompatibility.

Conclusions:

- The expression of ABO antigens and antibodies begins prenatally and continues postnatally, particularly during the first six months of life. In some cases, newborns exhibit well-defined natural anti-A and anti-B antibodies of the ABO system. However, in most cases, these antibodies are not yet expressed and therefore cannot be detected through serological methods.
- Serological studies in newborns have revealed a predominance of the A_2 and A_2B phenotypes. Since full expression of ABO system antigens requires postnatal developmental maturation, the A_2 subgroup in the neonatal period shows serological similarity to the A_1 subgroup, which changes as ontogenesis progresses.
- The Rh phenotypic expression in newborns is similar to that observed in children aged 6–12 months and corresponds to its respective expression in adults.
- The development of hemolytic disease is not sex-dependent (as sex-related distribution was not statistically significant).
- Cases of hemolytic anemia in fetuses and newborns were most frequently observed among infants with blood group A(II), Rh^+ (58.14±5.3%). This may reflect a somewhat increased susceptibility to the development of hemolytic disease, particularly when the mother has blood group O(I), Rh^-.
- Hemolytic disease caused by ABO incompatibility generally exhibits a milder course, whereas Rh-mediated hemolysis tends to be more severe. Analysis of hemolysis severity between ABO and Rh-mediated groups revealed a statistically significant difference (χ^2-9.4366; P-0.00893), with moderate hemolysis predominating in cases of ABO incompatibility.
- Anthropometric parameters: gestational age, birth weight, body length, and head circumference are unevenly distributed between newborns with hemolytic disease and healthy newborns.
- Hemolytic disease is associated with prematurity, whereas in the healthy group these proportions are considerably lower (preterm 0.72±0.1%; very preterm 0.05±0.05%; χ^2- 94.13; $p < 0.00001$).
- Hemolytic reactions are more frequent in newborns with non-O blood groups, as evidenced by the relatively rare occurrence of the complete c.261delG deletion (homozygous state) detected using SNP rs8176719 in hemolytic newborns.
- The presence of the **(-; G)** genotype in non-O blood group newborns reduces the quantitative expression of ABO antigens, thereby decreasing the frequency or mitigating the severity of hemolytic anemia caused by ABO incompatibility.

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