Cord Blood Hematological Parameters of Fetuses Detected Different Thalassemia Genotypes in the Second Trimester of Pregnancy

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Background: Hemoglobinopathies are the most common inherited diseases in humans resulting from impaired globin chain synthesis of hemoglobin. The progression of thalassemia rates is prevented with prenatal screening methods.

Aims: This study aimed to retrospectively evaluate the hematological parameters of α- and β-thalassemia and normal fetuses aged 17-25 weeks of gestation. Pregnant women who underwent cordocentesis in the second trimester because of the risk of having a baby with thalassemia were included in the study.

Study Design: A retrospective case-control study.

Methods: Hematological indices and molecular DNA methods were analyzed from the cord blood samples of 129 women who were 17-25 weeks into pregnancy. In total, 112 of the fetuses carry α- and β-thalassemia heterozygous or homozygous, and 17 fetuses had a normal genotype for thalassemias. The fetal hematological parameters analyzed included red blood cells (RBCs), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW). The HPLC method was used for Hb fraction analysis. Amplification refractory mutation system, restriction enzyme analysis, multiplex polymerase chain reaction, and sequencing methods were used for the molecular analysis. Maternal contamination was eliminated by the short tandem repeat method.

Results: In this study, 28.6% (37), 45.0%, 13.1%, and 13.1% of the fetuses had α-thalassemia, β-thalassemia, mixed thalassemia (α-/β-thalassemia with sickle cell anemia), and normal findings, respectively. Significant differences in adult hemoglobin (HbA), fetal hemoglobin (HbF), Hb Barts, MCV, MCH, and RDW were detected in three groups compared with the normal group ($p < 0.001$, except for RBC, Hb, HCT, and MCHC). Differences in HbF, Hb Barts, MCV, MCH, and RDW were observed in the α-thalassemia groups compared with the normal group ($p < 0.001$). Among the five β-thalassemia subgroups, only HbA and RDW were different from the normal group ($p < 0.001$).

Conclusion: This study could be a good reference for future studies and prenatal diagnostic applications in emphasizing the importance of changes in the blood parameters of fetuses before molecular genotyping. These hematological data give valuable information to clinicians about the fetus to enlighten families in making appropriate decisions during prenatal diagnosis. The investigated hematological parameters are useful for the differentiation of thalassemias mid-pregnancy.

INTRODUCTION

Hemoglobinopathies are common inherited disorders caused by mutations or deletions in globin genes that result in abnormal hemoglobin (Hb) formation and are major public health problems affecting 7% of the world population.[1,2] According to the defects of globin genes, hemoglobinopathies may be classified as structural Hb variants and thalassemias. Alpha (α-) and beta (β-) thalassemias are the two major types that are caused by deficient synthesis of α- and β-globin chains.[3-5] Structural Hb variants are developed by single amino acid substitutions in the α- or β-globin chains.[6] One of the most common Hb variants is sickle cell anemia (SCA), and in the South part of Turkey, the prevalence of sickle-cell Hb (HbS) is 10% in the Çukurova region. SCA affects millions of people with serious complications such as hemolysis.[7] Moreover, 1% of pregnancies are at risk for this disease, resulting in 330,000 births affected by SCA (83%) or thalassemia (17%) annually.[8] The incidence of β-thalassemia is 2.1%, which varies in Mediterranean coastal regions: Mediterranean (Adana) with 3.7%, Aegean (İzmir) with 4.8%, and Marmara (İstanbul) with 4.5%.9 α-Thalassemia is another hemoglobinopathy, and the culprit gene is found in 2.0% of the Turkish population.[9-11] Accordingly, preventive programs for hemoglobinopathies have been established worldwide. These preventive programs involve the detection of carriers, diagnosis

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with molecular DNA techniques, genetic counseling, and prenatal diagnosis. Prenatal diagnosis is an important part of the preventive program, and an invasive procedure is preferred in the first trimester for families at risk of having an affected fetus.[10,12] Once parent mutations are identified, fetal DNA analysis is performed by molecular techniques based on polymerase chain reaction (PCR) techniques. Prenatal diagnosis is performed by chorionic villus sampling (CVS) in the 11th week of pregnancy.[13,14] Cordocentesis is another invasive procedure performed after the first trimester, which is a useful method in fetal medicine.[15,16] Cordocentesis is a required option for couples who applied in the second trimester to obtain the prenatal diagnosis as soon as possible. Cord blood analysis is carried out at weeks 18-22 of gestation.[16] At present, cordocentesis has become one of the most commonly used invasive diagnostic techniques in maternal-fetal medicine, especially in the assessment of fetal hematological disorders.[17]

Despite wide knowledge and experience in the prenatal diagnosis of thalassemias today, there is not much knowledge on the distribution of erythrocyte indices of fetuses, especially in different α- and β-thalassemia subgroups and mixed thalassemias with SCA.[3,17,18] Thus, this study aimed to investigate the differences in hematological parameters in fetuses with several thalassemia genotypes in mid-pregnancy.

MATERIALS AND METHODS

Subjects

This retrospective study included 129 pregnant women suspected of being carriers of thalassemia admitted to the Third Affiliated Hospital of Zunyi Medical University Hospital for prenatal diagnosis in the second trimester of pregnancy (17-25 weeks of gestation) between 2010 and 2020. Perinatologists recommended cordocentesis to these women for being in the second trimester of pregnancy at the time of admission. Molecular diagnosis was performed in the medical biochemistry department.

Informed consent was collected from all participants. Ethical approval was obtained from the Ethics Committee of the Third Affiliated Hospital of Zunyi Medical University.

In total, 129 fetuses were included in the study and were divided into four groups: α- thalassemia (n = 37), β-thalassemia (n = 58), mixed thalassemia (n = 17), and normal fetus (n = 17). Fetuses that did not inherit the mutation from thalassemia carrier mothers and fathers were defined as "normal." The experimental processes were performed as in the flow chart provided in Figure 1.

The inclusion criteria were as follows: pregnant women who are at high risk of having a child with thalassemia major and who applied to the hospital for prenatal diagnosis in the second trimester of pregnancy. The exclusion criteria were as follows: (1) mutation analysis of the subjects was not completed at the time of admission, (2) failure of taking cord blood, and (3) informed consent was not collected.

Cordocentesis

Cord blood samples were taken by experienced perinatologists using GE Voluson 730 Pro-ultrasound equipment with a convex volumetric transabdominal (RAB 4-8 MHz) probe in the department of obstetrics and gynecology. Approximately 2.0 ml of fetal cord blood was collected from each pregnant

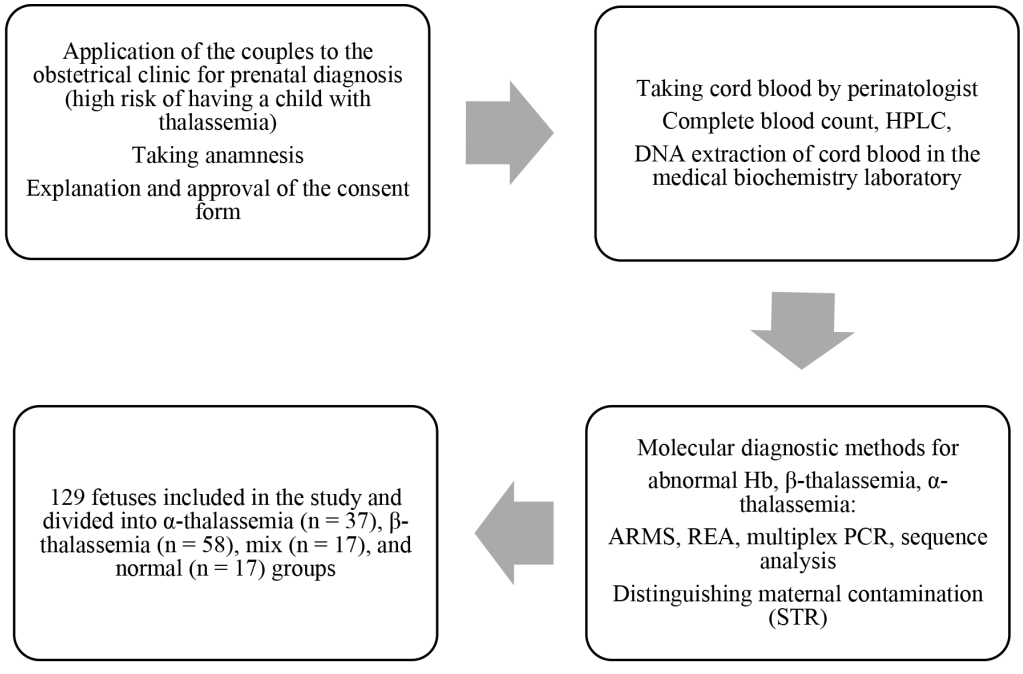

FIG. 1. Flow of the working process in the prenatal diagnosis laboratory for the study participants.

woman and transferred into 5-ml vacutainer tubes containing ethylenediaminetetraacetic acid (5 mg/ml) for hematological and DNA analysis.

Hb fraction analysis

The cord blood samples HPLC analysis was performed by Agilent 1100, a cation-exchange column was used with combined pH and NaCl gradient for quantification and separation using the Hb variant analysis (β-thalassemia short program). HbA levels were calculated automatically in 6 min by this program.[19,20]

Hematological parameter analysis

Red blood cells (RBCs), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW) were analyzed from 1 mL of fetal cord blood samples. A complete blood count of couples and cord blood samples were analyzed in a Sysmex KX-21N hematology analyzer (Sysmex Corp. Kobe, Japan).

Molecular Methods

DNA Extraction

A high pure template DNA isolation kit (Roche, Germany) was used for DNA extraction following the manufacturer's instructions.[21]

Maternal Blood Contamination

Maternal contamination was eliminated by short-tandem repeat (STR) analysis.[21] We defined STR in individuals with STR analysis. In the STR analysis, 15 loci, and the amelogenin gender-determining marker gene were identified. This test is used to distinguish the loci that the fetuses have received from their parents. Thus, if the cord blood sample has the same genotype as the mother, maternal contamination is excluded by determining loci different from the mother based on the STR analysis. The AmpFlSTR® Identifiler PCR Amplification Kit (Applied Biosystems, MA, USA) was used according to the manufacturer's instructions.[11]

Mutation Analysis

Mutations that included 95% of β- and α-thalassemias in the Çukurova region were analyzed based on PCR techniques: amplification refractory mutation system (ARMS) and restriction enzyme analysis (REA) for β-thalassemia. The multiplex PCR used with mutation-specific primers was described in the reference article for deletional mutations of α-thalassemia.[22] Sanger sequencing was used for unknown α- and β-thalassemia mutations.[11,23,24] The primer sequences used in these methods are shown in Table 1.

ARMS

A total of 350 ng of genomic DNA was amplified in 25 µl containing 25 mM of each deoxynucleotide triphosphate, 2 M KCl, 1M Tris-HCl (pH 8.3), 1 M $MgCl_2$, 1 M spermidine, 0.5 units of Taq DNA polymerase (Sigma), and 5 pmol of each primer (Table 1). The amplification reaction was carried out in a thermal cycler (Eppendorf) for 25 cycles at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 1.5 min, and one cycle of extension at 72 °C for 1.5 min. The amplified product was analyzed on 3% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining.[23]

TABLE 1. Primers Used in ARMS and Sequencing Methods for β-thalassemia Mutations.

ARMS primers	β-thalassemia
Primer 15 F	5′CAA TGT ATC ATG CCT CTT TGC ACC 3′
Primer 16 R	5′ GAG TCA AGG CTG AGA GAT GCA GGA 3′
Primer 30 F	5′ ACC TCA CCC TGT GGA GCC AC 3′
Primer 31 F	5′ CCC CTT CCT ATG ACA TGA ACT TAA 3′
Hbs*	5′CCC ACA GGG CAG TAA CGG CAG ACT TCT GCA3′ (mutant) 5′CCC ACA GGG CAG TAA CGG CAG ACT TCT GCT3′ (normal)
IVSI-110*	5′ACC AGC CTA AGG GTG GGA AAA TAC ACT 3′ (mutant) 5′ACC AGC CTA AGG GTG GGA AAA TAC ACC 3′ (normal)
Sequencing primers	β-thalassemia
Primer 108F	5′GCCAAGGCACGGTACGGCTGCATC-3′
Primer 109R	5′CCCTTCCTATGACATGAACTTAACCAT-3′
Seq primer 410F	5′TGTGGAGCCACACCCTAGGGTTGG-3′
Seq primer 16R	5′GCAGGTTGGTATCAAGGTT-3′
Primer 143F	5′CAGTGTGGAAGTCTCAGG-3′
Primer 230R	5′GTATTTTCCCAAGGTTTGAACTAGCTC-3′
Seq primer 229F	5′ATACAATGTATCATGCCTCTTTGCACC-3′
Seq primer 169R	5′GTCTGTGTGCTGGCCCATC-3′

*Two of the specific ARMS PCR primers given for two mutations that were used for mutant and normal sequences.
ARMS, amplification refractory mutation system; PCR, polymerase chain reaction.

REA analysis

Each 25 µl of PCR reaction contained 400 ng of genomic DNA and 2 M KCl, 1 M Tris-HCl (pH 8.3), 1 M $MgCl_2$, 1 M spermidine, 25 mM of each deoxynucleotide triphosphate, 0.5 units of Taq DNA polymerase (Sigma), and 20 pmol of primers (forward 5V-GGC CAA TCT ACT CCC AGG AG-3V, reverse 5V-ACA TCA AGG GTC CCA TAG AC-3V). The amplification program was initiated by first heating the samples for 2 min at 95 °C. After the denaturation step, 30 cycles of amplification were carried out by denaturation for 30 s at 94 °C, followed by annealing for 30 s at 65 °C and extension at 72 °C for 1.5 min. The PCR products were digested by 10 U of Dde1 (New England Biolabs) for 16 h at 37 °C, separated by agarose gel electrophoresis (2%), and examined by UV transillumination. REA was performed for HbS (HBB:c.20A>T)and FSC 22-24 (HBB:c.68_74delAAGTTGG) with *DdeI* enzyme digestion.[11]

Multiplex PCR

This method was applied to identify known inherited deletional α-thalassemia mutations according to the method described by Chong et al.[22] Common deletions screening with single-tube multiplex PCR include -α3.7 (HBA2:c.1A>G), MED-I (NG_00 0006.1:g.24664_41064del16401), -(alpha) 20.5 (NG_000006.1: g.15164_37864del2270), and 4.2 kb deletion.[11,24]

Sanger Sequencing

Mutations in couples that were not identified by classic PCR were analyzed by the Sanger sequencing (ABI 3130 automatic sequencer) method. The cord blood samples of these parents were also analyzed by sequencing. The big Dye Terminator v3.1 Cycle Sequencing kit (ABI) and primers that cover all three exons, introns, and exon-intron boundaries of the β-globin gene were used for the sequencing analysis,[11] as shown in Table 1. SeqScape® Software v.3.0 was used for analyzing sequencing results. Data were evaluated according to the NCBI reference sequence: NC_000011.10 (https://www.ncbi.nlm.nih.gov/genome/gdv/browser).

Statistical Analysis

Variables are summarized as mean and standard deviations. The Shapiro-Wilk or Kolmogorov-Smirnov (Lilliefors significance correction) test was used to test normality depending on the sample size (< 50 or > 50). For the comparison of variables between the groups, a one-way analysis of variance or Kruskal-Wallis test was used depending on whether the statistical hypotheses were fulfilled or not. For normally distributed data, regarding the homogeneity of variances, the Tukey, and Games-Howell tests were used for comparisons of multiple groups. For non-normally distributed data, Bonferroni-adjusted Mann-Whitney U test was used for comparisons of multiple groups. For the comparison of continuous variables between two groups, the Student's t-test was used. Continuous variables were summarized as mean and standard deviation and as median and interquartile range (IQR) where appropriate. All analyses were performed using the IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA). The statistical level of significance for all tests was considered 0.05.2[5]

RESULTS

Features of the study group

Within the scope of this study, hematology, and genotype of 129 fetuses of 129 pregnant women were evaluated. The age and gestational age of the pregnant women were 24.76 ± 3.06 years and 19.41 ± 1.72 weeks, respectively. The mutation types of fetuses are shown in Table 2.

α-Thalassemia has three subgroups: trait (with two defective α-genes), intermedia (with three defective α-genes), and mixed (combination of α- and β-genes) groups (Table 4). β-thalassemia has six subgroups, as shown in Table 5. In this study, 28.7% (37/129), 45.0% (58/129), 13.2% (17/129), and 13.2% (17/129) of the fetuses had α-thalassemia, β-thalassemia, mixed thalassemia, and normal findings, respectively.

Comparison of Hb fractions between the groups

Hb fractions were detected as HbA, HbF, and Hb Barts (only in α-thalassemia and some mixed samples). No HbA expression was detected in samples with β-thalassemia major. Among all groups, HbA levels were decreased in β-thalassemia cases ($p < 0.001$, Table 3).

HbA levels in the α-thalassemia intermedia subgroup were higher than those in α-thalassemia trait and mixed subgroups ($p = 0.005$, Table 3). In all group comparisons, HbF levels were low in the α-thalassemia group ($p < 0.001$, Table 3). HbF levels were low in the α-thalassemia intermedia subgroup according to the α-thalassemia trait ($p = 0.002$, Table 4). No difference was found among β-thalassemia subgroups (Table 5).

The Hb Barts fraction was higher in the α-thalassemia intermedia subgroup than in the mixed (α- and β-thalassemia) and normal groups.

Comparison of hematological parameters between the study groups.

Data on peripheral blood parameters of thalassemia carriers in all groups are shown in Table 3. Differences in hematological data between α- and β-thalassemia subgroups are indicated in Tables 4 and Table 5, respectively. No significant difference in RBC and HCT was found between the groups.

Comparison between α-thalassemia groups

Hb, MCV, MCHC, and MCH levels were significantly decreased in the α-thalassemia group compared with those in other groups ($p < 0.001$ for Hb, MCV, MCH, $p = 0.003$ for MCHC; Table 3). The RDW levels were higher in the α-thalassemia group than in other groups ($p < 0.001$, Table 3). Among α-thalassemia subgroups, the Hb and MCV values were significantly decreased in the α-thalassemia intermedia subgroup compared with those in other subgroups ($p < 0.001$, Table 4). RDW values were higher in all α-thalassemia subgroups than in other groups ($p < 0.001$, Table

3). The RDW value of the α-thalassemia intermedia subgroup was higher than those in other α-thalassemia subgroups ($p < 0.001$, Table 4).

Comparison between β-thalassemia groups

Hb values were decreased in β^0/β^0 samples compared with other subgroups ($p < 0.001$, Table 5). The β^0/β^0 group had higher RDW values than the β^+/β^N group ($p = 0.010$, Table 5). MCV levels were higher in the β^0/β^0 group than in the β^+/β^N, β^0/β^N, and β-mixed groups ($p = 0.002$, Table 5).

DISCUSSION

The most important result of the study was the difference in Hb levels and hematological parameters (Hb, MCV, MCH, and RDW) in mutations specific to our region between normal, α-, and β-thalassemia fetuses aged 17-25 weeks of gestation.

TABLE 2. α- and β-Thalassemia Mutation Types of Fetuses.

Subgroup	β-thalassemia genotypes		α-thalassemia genotypes	
β^+/β^+ (n = 12)	$\beta^{Cap+22}/\beta^{IVSII-848}$ (1) $\beta^{IVSII-848}/\beta^{IVSII-745}$ (1) $\beta^{IVSII-848}/\beta^{IVSII-848}$ (3) $\beta^{IVSII-745}/\beta^{Cap+22}$ (1) $\beta^{IVSII-726}/\beta^{IVSI-110}$ (1)	$\beta^{-88}\beta/^{-88}$ (1) $\beta^{-88}/\beta^{Cap+22}$ (1) $\beta^{IVSI-110}/\beta^{-30}$ (1) $\beta^{IVSI-110}/\beta^{IVSII-745}$ (1) $\beta^{-30}/\beta^{IVSI-6}$ (1)	**α-thalassemia Silent carrier (one α-gene defect) (n = 4)**	$\alpha\alpha^{3.7}/\alpha\alpha$ (4)
β^0/β^0 (n = 12)	$\beta^{Cd\ 15}/\beta^{Cd\ 9-10}$ (2) $\beta^{Cd22-24}/\beta^{Cd\ 36-37}$ (1) $\beta^{Cd\ 17}/\beta^{IVSI-130}$ (1) $\beta^{Cd22-24}/\beta^{Cd\ 36-37}$ (1) $\beta^{Cd22-24}/\beta^{Cd\ 15}$ (2)	$\beta^{Cd15}/\beta^{Cd36-37}$ (1) $\beta^{Cd\ 15}/\beta^{IVSI-130}$ (1) $\beta^{IVSI-110}/\beta^{Cd36/37}$ (3)	**α-thalassemia carrier (two α-gene defects) (n = 14)**	$\alpha\alpha^{3.7}/\alpha\alpha^{PolyA}$ (3) $\alpha\alpha^{4.2}/\alpha\alpha^{4.2}$ (1) $\alpha\alpha^{4.2}/\alpha\alpha^{5nt}$ (2) $\alpha\alpha^{3.7}/\alpha\alpha^{3.7}$ (8)
β^+/β^0 (n = 8)	$\beta^{IVSI-110}/\beta^{Cd\ 15}$ (2) $\beta^{IVSI-110}/\beta^{Cd\ 36-37}$ (2) $\beta^{IVSI-110}/\beta^{Cd\ 9-10}$ (3) $\beta^{IVSII-848}/\beta^{Cd\ 17}$ (1)		**α-thalassemia intermedia (three α-gene defects) (n = 20)**	$--^{MEDI}/-\alpha^{4.2}$ (4) $--^{MEDI}/-\alpha^{3.7}$ (9) $--^{20.5}/-\alpha^{3.7}$ (7)
β^S/β^0 β^S/β^+ (mixed) (n = 8)	$\beta^S/\beta^{Cd22-24}$ (2) β^S/β^{-101} (6)		**α-thalassemia mixed (α-thalassemia with sickle cell anemia) (n = 14)**	$\alpha\alpha^{3.7}/\beta^S$ (10) $\alpha\alpha^{5nt}/\beta^S$ (4)
β^0/N (n = 13)	$\beta^{FSC22-24}/\beta^N$ (7) $\beta^{Cd\ 9-10/}\beta^N$ (6)			
β^+/N (n = 14)	$\beta^{IVSII-848}/\beta^N$ (2) $\beta^{IVSII-726}/\beta^N$ (1) β^{IVSI-6}/β^N (3)	β^{-30}/β^N (2) β^{-101}/β^N (4) β^{Cap+22}/β^N (2)		

TABLE 3. Comparison of Hematological Parameters Between Fetuses of All Groups.

Parameter	Normal (n = 17)	α-thalassemia group (n = 37)	β-thalassemia group (n = 58)	Mixed group (n = 17)	*p value*
Gest. age (week)	20.0 ± 2.2	19.3 ± 1.6	19.2 ± 1.6	19.2 ± 1.6	0.596
HbF (%)	80.6 ± 3.5	73.7 ± 8.7	81.4 ± 4.4	77.5 ± 7.2	< 0.001*
HbA (%)	5.29 ± 0.8	7.4 ± 4.7	2.6 (1.5-3.8)	4.3 ± 3.1	< 0.001*
Hb barts	-	12.5 ± 8.3	-	5.5 ± 8.4	< 0.001*
RBC (10*6/µl)	2.7 (2.3-3.1)	3.0 (2.5-3.4)	2.7 (2.5-3.0)	2.6 (2.4-3.0)	0.184
HGB (g/dl)	11.7 ± 0.6	8.6 ± 1.7	9.6 ± 2.3	11.0 ± 0.9	< 0.001*
HCT (%)	30.1 ± 4.7	29.0 ± 8.8	30.7 ± 8.3	29.7 ± 7.3	0.145
MCV (fl)	122.9 ± 1.6	109.0 ± 9.4	121.4 ± 4.6	119.0 ± 9.9	< 0.001*
MCH (pg)	39.8 ± 6.1	33.4 ± 7.5	40.3 ± 3.1	38.0 ± 4.4	< 0.001*
MCHC (g/dl)	33.9 ± 3.1	31.6 ± 2.9	33.5 ± 2.3	33.6 ± 2.5	0.003*
RDW (%)	15.3 ± 0.4	17.8 ± 1.5	16.2 ± 0.0	15.8 ± 0.9	< 0.001*

Data summarized as mean ± standard deviation and median (25^{th}-75^{th} percentile).
*Statistically significant at $p < 0.05$.
HbF, fetal hemoglobin; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

Previous studies have suggested that hematological data can be used as an important marker in differentiating fetuses with thalassemia.[18] Hematological parameters have been used for years to provide information about the health status of the fetus.[3] The cord blood hematological data provide data on the health status of the fetus in the related period of pregnancy. These indices could be changed according to the mutations identified in the fetus. At this point, these markers could allow clinicians guide couples in genetic counseling to decide on whether to terminate or continue the pregnancy.

However, previous research is limited by the analysis of only finite hematological indices in fetuses with α-thalassemia alone, not β-thalassemia and its different genotype subgroups. Therefore, this study investigated the hematological indices in fetuses with α- and β-thalassemia and their subgroups with varying genotypes between 17 and 25 weeks of gestation, along with how these hematological values differ by age of gestation.

Detection of thalassemia and abnormal Hb requires a combination of laboratory tests, including hematological parameters and molecular DNA techniques.[4] Even so, most previous studies investigated hematological parameters in normal fetuses and fetuses with α-thalassemia but rarely those with β-thalassemia with different genotype subgroups.[3,26] Compared with other studies, the present study is unique in that it investigated for the first time the hematological parameters of the fetuses carrying a rich variety of α- and β-thalassemia mutations unique to the Mediterranean

TABLE 4. Distribution and Comparisons of Hematological Parameters Among Fetuses with Different A-Thalassemia Genotypes.

Parameter	α-thalassemia carrier (two α-gene defects) n = 14	α- thalassemia intermedia (three α-gene defects) n = 20	α- thalassemia mixed (α-thalassemia with sickle cell anemia) n = 14	*p value*
Gest. age (week)	19.3 ± 1.2	20.2 ± 4.8	19.7 ± 1.7	0.913
HbF (%)	79.3 ± 3.4	68.6 ± 8.8	76.6 ± 7.6	0.002*
HbA (%)	5.04 ± 2.0	9.74 ± 4.7	4.86 ± 3.1	0.005*
Hb barts	7.96 ± 3.0	17.5 ± 7.8	6.6 ± 8.8	< 0.001*
RBC (10*6/µl)	3.17 ± 0.9	3.0 (2.5-3.5)	2.65 (2.3-3.1)	0.757
HGB (g/dl)	10.1 ± 0.6	7.9 ± 1.4	10.9 ± 0.9	< 0.001*
HCT (%)	29.3 ± 6.1	29.2 ± 10.2	29.4 ± 8.0	0.736
MCV (fl)	116.1 ± 0.9	104.1 ± 10.2	118.4 ± 4.0	< 0.001*
MCH (pg)	32.6 ± 8.8	32.9 ± 6.1	37.8 ± 4.6	0.088
MCHC (g/dl)	32.2 ± 2.8	31.3 ± 3.1	33.9 ± 2.6	0.046*
RDW (%)	16.3 ± 0.2	18.7 ± 1.1	16.1 ± 0.7	< 0.001*

Data summarized as mean ± standard deviation and median (25^{th}-75^{th} percentile).
*Statistically significant at $p < 0.05$.
HbF, fetal hemoglobin; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

TABLE 5. Distribution and Comparisons of Hematological Parameters Among Fetuses with Different β-thalassemia Genotypes.

	β^+/N n = 14	β^0/N n = 13	β^+/β^+ n = 12	β^0/β^+ n = 8	β^0/β^0 n = 12	β/mixed n = 8	*p value*
Gest. age (week)	19.5 ± 1.2	18.6 ± 2.1	19.0 ± 1.6	18.7 ± 0.8	20.1 ± 2.0	19.5 ± 1.9	0.068
HbF (%)	81.1 ± 2.8	82.4 ± 4.4	79.7 ± 5.5	83.4 ± 1.2	80.4 ± 6.1	78.6 ± 5.2	0.113
HbA (%)	2.60 (2.4-2.8)	3.75 ± 0.99	3.63 ± 1.9	2.15(1.8-3.7)	0.48 ± 1.65	4.23 ± 3.55	< 0.001*
Hb barts	-	-	-	-	-	6.21 ± 10.6	-
RBC (10*6/µl)	2.7 (2.7-3.1)	2.6 (2.6-2.9)	2.7 (2.5-2.8)	2.7 (2.6-2.9)	2.4 (1.5-2.9)	3.0 ± 0.7	0.330
HGB (g/dl)	11.5 ± 0.3	10.6 ± 0.5	10.5 ± 1.5	9.2 ± 0.2	5.8 ± 1.5	10.8 ± 0.8	< 0.001*
HCT (%)	31.7 ± 7.5	31.8 ± 8.3	30.3 ± 8.6	33.1 ± 3.0	26.6 ± 10.5	32.5 ± 6.9	0.503
MCV (fl)	118.7 ± 5.1	120.7 ± 2.1	120.2 ± 2.4	120.9 ± 2.1	126.3 ± 5.8	117.3 ± 4.9	0.002*
MCH (pg)	39.0 ± 3.5	40.4 ± 3.4	39.9 ± 3.4	40.8 ± 1.1	41.4 ± 3.5	38.4 ± 5.0	0.299
MCHC (g/dl)	32.9 ± 1.1	34.1 ± 3.9	33.4 ± 1.4	33.0 ± 1.1	33.7 ± 2.3	32.9 ± 3.0	0.698
RDW (%)	15.9 ± 0.4	16.1 ± 0.5	16.3 ± 0.4	16.3 ± 0.3	16.5 ± 0.1	16.2 ± 0.5	0.010*

Data summarized as mean ± standard deviation and median (25^{th}-75^{th} percentile).
*Statistically significant at $p < 0.05$.
HbF, fetal hemoglobin; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

region. The study showed a great diversity of α-thalassemia and β-thalassemia subgroups according to their genotypes. Contrary to other studies, no α-thalassemia major cases were identified in the present study.[3] α-Thalassemia has 15 types, which included a combination of deletion and point mutations. The most common deletions were 3.7 kb (type II) deletion α-2 and -- (MEDI) and deletion of ~17.5 kb including both α-globin genes α-Thal-1. Moreover, 41 genotype variations were identified in β-thalassemia cases (affected mostly both alleles). Codons 9/10 (+T) (HBB: c.30_31insT) and IVS-II-848 (C->A) (HBB: c.316-3C>A) were the most detected.

Our data confirmed that the levels of HbA, HbF, and RBC indices (MCV, MCH, MCHC, and RDW) were significantly different between the normal, α-thalassemia, β-thalassemia, and mixed groups ($p < 0.001$).[18] In the α-thalassemia group, Hb Barts levels were higher in the α-thalassemia and intermedia groups than in the normal group. This finding was similar to those of Zahn et al.[3] This parameter is useful for estimating three α-gene defects in fetuses. The association of thalassemia groups and hematological parameters was influenced by the number of defective genes in fetuses with α-thalassemia. The MCV and RDW values were higher, but the MCH and MCHC levels were lower in the α-thalassemia intermedia subgroup than in the normal group. These outcomes may suggest that cord blood analysis of hematological parameters of fetuses may be useful as a screening method for the α-thalassemia trait and thalassemia intermedia in the general population.

The HbA fraction in the β-thalassemia group was significantly low, especially between the β^0, β^+ and β mixed subgroups due to the absence of HbA expression in the β0 group. Hb Barts was detected only in fetuses with β-thalassemia with a combination of α-gene mutation (β-mixed subgroup; $p < 0.001$, Table 3). MCV levels were low in the β-mixed group in comparison with those in the β^0 and β^+ subgroup. The severity of β-gene mutation and combination with deletional α-gene mutations was observed. Variations and differences in hematological parameters can be associated with different mutation combinations. The coexistence of different mutations such as deletions, frameshifts in β-thalassemias, combination with abnormal Hb variants, or deletional α-thalassemia mutations affects the levels of RBC indices as seen in previous studies.[3,18,27]

Although the gene combinations varied in the studied fetuses, the limitations of the study were the low number of samples among subgroups, which limited further analysis of the effect on hematological parameters. Prenatal screening for thalassemias and Hb variants should be performed in all couples who carry one of the globin gene defects in the first trimester of pregnancy. CVS is commonly employed in this period. If not in the late stage of pregnancy, cord sampling is less preferred over CVS and amniocentesis for prenatal diagnosis. The variation in gestational age in our samples was not sufficient to see the variation in hematological parameters during the pregnancy, as reported in several studies.[3,16,18,27]

In conclusion, this study is the first to demonstrate the hematological parameters in a wide range of mutations (15 α- and 41 β-thalassemia genotypes) from the cord blood samples of fetuses with thalassemia in the Mediterranean region. For the Hb fractions, results reveal that HbA, HbF, and Hb Barts are good predictors of fetal α-thalassemia. Among RBC indices, low levels of MCV, MCH, and MCHC and high levels of RDW are signs of α-thalassemia in pregnancy. HbA was a predictor of β-thalassemia, showing a defect, or a lack of HbA expression in fetuses. RDW values also rise in fetuses with β-thalassemia. The variability of globin gene defects in thalassemias influences the values of hematological parameters during fetal development. These findings propose that cord blood sampling and RBC may be appropriate as a prenatal screening approach in the general population.

Ethics Committee Approval: Ethical approval was obtained from the Ethics Committee of the Third Affiliated Hospital of Zunyi Medical University.

Informed Consent: Informed consent was collected from all participants.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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