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# HPLC in Characterization of Hemoglobin Profile in Thalassemia Syndrome and Hemoglobinopathies: An Experience in Pediatric Tertiary Care Hospital

**Fauzia Aamer[1], Muniba Shair[2], Neelam Mazhar[3], Rabia Ahmad[4]**

1 *Associate Professor, Department of Hematology, The Children Hospital / The University of Child Health, Lahore Pakistan*
*Contribution in the study*

2 *MLT Student, University of Child Health Sciences and Children's Hospital, Lahore Pakistan*
*Contribution in the study*

3 *Medical Officer, University of Child Health Sciences and Children's Hospital, Lahore Pakistan*
*Contribution in the study*

4 *Associate Professor, Department of Hematology, Allama Iqbal Medical College, Jinnah Hospital, Lahore Pakistan*
*Contribution in the study*

*CORRESPONDING AUTHOR*
***Dr. Fauzia Aamer***
*Associate Professor, Department of Hematology, The Children Hospital / The University of Child Health, Lahore Pakistan*
*Email: drfauzia.haem@gmail.com*

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## ABSTRACT

**Background:** Hemoglobinopathies constitute an increasing global health burden as they are the most common genetic disorders across the globe. Identification of these disorders is important for epidemiological purposes and the prevention of Thalassemia's and clinically severe hemoglobinopathies. **Objective:** To characterize the hemoglobin profiles of patients with thalassemia and hemoglobinopathy using cation exchange HPLC. **Study Design:** Cross-sectional study. **Settings:** Department of Hematology and Transfusion Medicine, University of Child Health Sciences and Children's Hospital, Lahore Pakistan. **Duration:** August 2024 to November 2024. **Methods:** Samples were analyzed on the BIO-RAD VARIANT II for the HPLC, and CBC was performed on an automated cell counter, BECKMAN COULTER(US). Data was analyzed with a chi-Square test with IBM SPSS Statistics 23 software. Continuous variables were expressed as mean ±SD, and Categorical variables as frequencies and percentages. **Results:** A total of 105 individuals were studied, 62 were normal cases, showing normal hemoglobin profiles, and the most common diagnosis obtained on chromatograph by HPLC was thalassemia trait (21.9%), followed by beta-thalassemia major (10.5%), having an abnormal hemoglobin profile. However, only one case of Hb-S trait, Hb-D trait, Hb D disease, and Hetero Hb-E was found. Mean RDW-CV is raised in all Thalassemia's. **Conclusion:** This study confirms the diagnostic utility of cation exchange HPLC as a reliable and sensitive tool for identifying thalassemia and hemoglobinopathies in pediatric populations and underscores the importance of HPLC in distinguishing these conditions through distinct hemoglobin fraction patterns.

*Keywords:* *Hemoglobinopathies, HPLC, Thalassemia.*

## INTRODUCTION

Hemoglobin is the protein present in red blood cells responsible for the transport of gases to and from the body tissues. The genetic defects of hemoglobin are the most common genetic disorder worldwide.[1] Inherited abnormalities of hemoglobin include various disorders ranging from thalassemia syndrome to structurally abnormal hemoglobin variants.[2] Thalassemia's are groups of autosomal recessive inherited blood disorders characterized by quantitative decreased or absence of production of globin chains of the hemoglobin (Hb) molecule.[3] Haemoglobinopathies are characterized by the production of structurally defective hemoglobin due to abnormalities in the formation of globin molecules as a result of altered amino acid sequence.[4] These abnormal hemoglobin variants include Hb C, Hb S, D, and HbE, and in certain situations, they can potentially result in serious clinical symptoms. Every ethnic group has a unique set of common and rare mutations, and the range of hemoglobinopathies varies greatly among communities around the world.[5] To effectively screen for and diagnose hemoglobinopathies, a thorough evaluation is needed. This should combine clinical assessments, family history, blood counts, red blood cell indices, and molecular tests. HPLC is an accurate and sensitive technique for abnormal hemoglobin, Hb-F, and Hb A2 detection.[6] Due to its reliability and speed, it is considered a technique of choice for screening thalassemia. Using HPLC, percentage levels of Hb F, Hb A2, Hb Ao, Hb S, and Hb E were estimated to diagnose

the cases.[7] The different Hb variants, like Hb E, S, D, and C, are identified based on percentage and retention time windows.[8] Each hemoglobin has a characteristic retention time, which makes HPLC specific for diagnosis. A printed chromatograph is delivered by the software, where the different peaks are identified in different windows with relevant retention time, relevant percentage, and area.[7]

HPLC is a powerful and excellent diagnostic tool to directly detect the hemoglobin variants in the quantification of abnormal and normal hemoglobin fractions with high precision.[9]

## METHODS

This cross-sectional study was conducted at the Department of Hematology and Transfusion Medicine in the Children's Hospital and University of Child Health Sciences, Lahore, from August 2024 to November 2024. A total of 105 patients (Cochran's Formula)[16] aged 1-15 years attending the Children's Hospital, who are suspected of thalassemia with abnormal CBC findings and have anemia, were included in our study. Patients who received a recent blood transfusion and were diagnosed with iron deficiency anemia were excluded. After approval of the IRB (No. 762/SAHS Dated; 25-7-2024), 1-2 ml of venous sample was drawn into an EDTA tube and mixed gently to avoid hemolysis. All samples were tested within 24 hours of collection. Tests were performed on Beckman Coulter for CBC and HPLC on BIO RAD VARIANT II. Data included patient demographics (Name, Age, Gender, Ward, Family history, and Transfusion history), complete blood count, and hemoglobin profile. In this context, a self-designed proforma was used.

The data was entered and analyzed using IBM SPSS 23.0 software. The continuous variables (Age, CBC, Hb profile, etc.) were expressed as Mean ± SD, whereas categorical variables were expressed in the form of frequencies and percentages. Chi-square was applied to determine the association between variables. A p-value less than 5% was considered statistically significant.

## RESULTS

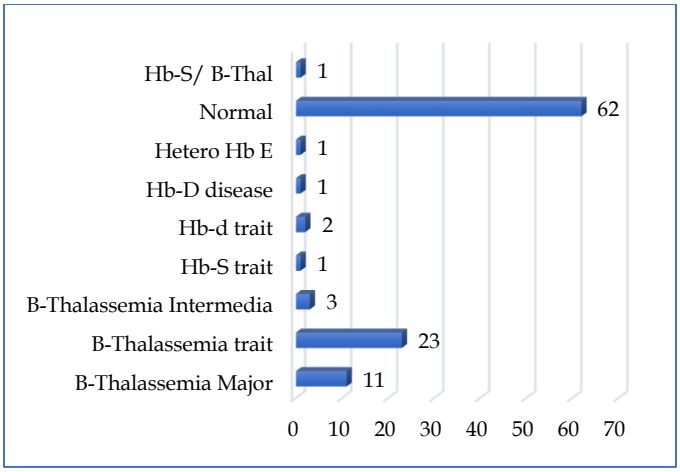
A total of 105 individuals suspected of having thalassemia or hemoglobinopathies were included in this study. This study showed that 73 (70%) were male, while 32 (30%) were female, resulting in a male-to-female ratio of approximately 2.3:1. This highlights a slight predominance of male patients in the dataset. The study primarily involved pediatric individuals, ranging in age from as young as 4 months to 15 years. The average age of the participants was 5.04 years, with a standard deviation of 4.22 years, indicating a relatively broad age range within the group. In this study, 12 individuals (11.4%) had a documented family history of thalassemia, and 76 (72.4%) reported a history of consanguineous (cousin) marriage within their families. Regarding transfusion history, 29 individuals (27.6%) had undergone blood transfusions. Of these, 13 (44.8%) had received a single transfusion, 8 individuals (27.6%) had been transfused 2–3 times, and another 8 individuals (27.6%) had undergone multiple transfusions. This distribution underscores the significant role of familial factors and the varying transfusion needs in patients with thalassemia and hemoglobinopathies, which helps in differentiation between β-TM and β-TI.

**Table 1: History profile of patients**

| History of Patients | Frequency | | |
|---|---|---|---|
| **Family history of Thalassemia** | 12 | | |
| **History of Cousin Marriage** | 76 | | |
| **History of Transfusion** | 29 | One Time | 13 |
| | | 2-3 Times | 8 |
| | | Multiple | 8 |

The study analyzed the frequency of hemoglobinopathies in 105 participants. The majority had normal hemoglobin (n = 62, 59.0%), followed by beta-thalassemia trait (n = 23, 21.9%) and beta-thalassemia major (n = 11, 10.5%). Beta-thalassemia intermedia was observed in 3 individuals (2.9%), while Hb-D trait was identified in 2 individuals (1.9%). Hb-S trait, Hb-D disease, heterozygous Hb E, and Hb-S/B-thalassemia were rare, with one case each (1.0%). These findings highlight the predominance of normal hemoglobin and beta-thalassemia trait within the population, with other hemoglobinopathies being less common.

**Figure 1: Frequency of disorders**




In this study, Beta-thalassemia major was observed in 11 participants (9 males and 2 females), while beta-thalassemia trait accounted for 23 cases (17 males and 6 females). Beta-thalassemia intermedia was identified in 3 males only. Hb-S trait was reported in 1 male, and Hb-D trait was present in 2 females. Hb-D disease was found in 1 male, and heterozygous Hb-E was also identified in 1

male. The majority of the sample exhibited normal hemoglobin (62 participants: 31 males and 31 females). Additionally, 1 female exhibited Hb-S/B-thalassemia. These findings highlight the male predominance in most hemoglobinopathies except for Hb-D trait and Hb-S/B-thalassemia. Normal hemoglobin distribution showed relatively balanced gender representation.

**Table 2: Gender distribution in disorders**

| Hemoglobinopathies | Male | Female | Total | Percentage |
|---|---|---|---|---|
| **Normal** | 31 | 31 | 62 | 59.05% |
| **B-Thalassemia Major** | 9 | 2 | 11 | 10.4% |
| **B- Thalassemia trait** | 17 | 6 | 23 | 21.9% |
| **B- Thalassemia Intermedia** | 3 | 0 | 3 | 2.85% |
| **Hb-S trait** | 1 | 0 | 1 | 0.9% |
| **Hb-D trait** | 0 | 2 | 2 | 1.9% |
| **Hb-D disease** | 1 | 0 | 1 | 0.9% |
| **Hetero Hb E** | 1 | 0 | 1 | 0.9% |
| **Hb-S/ B- Thalassemia** | 0 | 1 | 1 | 0.9% |

Among 105 participants, normal hemoglobin was the most prevalent (59.0%, mean age 5.2 years), followed by beta-thalassemia trait (21.9%, mean age 6.0 years) and beta-thalassemia major (10.5%, mean age 3.1 years). Less common conditions included beta-thalassemia intermedia (2.9%), Hb-D trait (1.9%), Hb-S trait, Hb-D disease, heterozygous Hb E, and Hb-S/B-thalassemia (each 1.0%). Ages were generally lower for beta-thalassemia intermedia (mean 1.4 years) and higher for Hb-S/B-thalassemia (mean 11.0 years). These findings indicate that beta-thalassemia trait and normal hemoglobin were the most prevalent conditions, with affected individuals generally older than those with beta-thalassemia major or intermedia.

**Table 3: Age distribution in disorders**

| Hemoglobinopathies | Age of Patients | | |
|---|---|---|---|
| | Mean | Total Patients | Percentage |
| **B-Thalassemia Major** | 3.1 | 13 | 10.5% |
| **B- Thalassemia trait** | 6.0 | 23 | 21.9% |
| **B- Thalassemia Intermedia** | 1.4 | 3 | 2.9% |
| **Hb-S trait** | 2.0 | 1 | 1.0% |
| **Hb-D trait** | 3.1 | 2 | 1.9% |
| **Hb-D disease** | 6.0 | 1 | 1.0% |
| **Hetero Hb E** | 4.0 | 1 | 1.0% |
| **Normal** | 5.2 | 62 | 59.0% |
| **Hb-S/ B- Thalassemia** | 11.0 | 1 | 1.0% |

A chi-square test of independence was conducted to examine the relationship between gender (male, female) and disease diagnosis (thalassemia, hemoglobinopathies, normal). The results indicated a significant association between gender and diagnosis ($\chi 2=8.04$, $p=0.018$), suggesting that the distribution of diagnoses (thalassemia, hemoglobinopathies, and normal) differs significantly between male and female patients. The findings suggest that gender is associated with the type of disease diagnosis, with male patients (29 males) more likely to have thalassemia compared to female patients (8 females), who showed a more even distribution across the disease categories.

**Table 4: Association of gender with diagnosis**

| | Thalassemias | Hemoglobinopathies | Normal | P-value |
|---|---|---|---|---|
| **Male** | 29 | 3 | 31 | **0.018*** |
| **Female** | 8 | 3 | 31 | |

**P-value<0.05 is significant.*

The analysis of hematological parameters demonstrated distinct patterns in red blood cell (RBC) count, hemoglobin (Hb) levels, and red cell indices across various hemoglobinopathies, highlighting their diagnostic significance as shown in Table 4.

**Table 5: CBC parameters in hemoglobinopathies / thalassemia**

| Diagnosis | RBCs | Hb | MCV | MCH | MCHC | RDW-CV |
|---|---|---|---|---|---|---|
| **Normal** | 3.97±2.26 | 7.50±2.83 | 69.87±18.3 | 21.7±6.58 | 29.96±5.2 | 20.4±5.28 |
| **B-Thalassemia Major** | 2.44±0.59 | 5.51±1.75 | 73.69±10.9 | 23.46±4.0 | 31.49±3.0 | 26.9±8.5 |
| **B-Thalassemia trait** | 4.42±1.41 | 8.69±2.96 | 63.50±11.3 | 19.91±4.1 | 30.07±7.3 | 20.7±5.5 |
| **B-Thalassemia Intermedia** | 3.62±2.79 | 5.10±1.85 | 74.47±4.5 | 32.17±14.4 | 36.40±3.6 | 24.9±3.2 |
| **Hb-S trait** | 4.45 | 9.10 | 57.10 | 20.4 | 35.8 | 15.3 |
| **Hb-D trait** | 4.20±0.42 | 8.55±6.15 | 61.25±19.6 | 22.5±8.1 | 30.1±10.5 | 10.75±0.64 |
| **Hb-D disease** | 8.04 | 10.80 | 50.80 | 16.5 | 82.8 | 12.9 |
| **Hetero Hb E** | 3.24 | 5.00 | 53.70 | 15.4 | 28.7 | 22.5 |
| **Hb-S/ B-Thal** | 6.0 | 8.0 | 59.0 | 24.0 | 32.0 | 17.0 |

RBC indices were analyzed against diagnosis, results were expressed as mean ± SD. Mean values of RBC indices were normal in normal subjects. Mean value of RDW was markedly increased in beta thalassemia major along with low mean values of Hb, MCV, MCH and RBC count. RDW was low in Hb D trait. Mean RBC count was

high in Hb D disease and BTT. Mean Hb was low in all diagnosis indicating all subjects are anemic.

The findings reveal distinct hemoglobin fraction profiles for each condition, demonstrating the precision and utility of HPLC in clinical practices Beta-thalassemia major showed significantly reduced Hb A levels (26.7 ± 31.2) and markedly elevated Hb F (70.2 ± 31.1), alongside a mild increase in Hb A2 (3.1 ± 1.6). These findings reflect a severe deficiency in beta-globin chain production with compensatory fetal hemoglobin synthesis. Beta-thalassemia trait exhibited a mild increase in Hb F (6.3±19.8) and elevated Hb A2 (5.1 ± 1.1), with predominantly high Hb A levels (88.4±19.1), consistent with heterozygous mutations.

Beta-thalassemia intermedia presented intermediate Hb A levels (65.2 ± 14.5) with elevated Hb F (34.3 ± 18.9) and mildly increased Hb A2 (3.9 ± 0.8), indicative of a less severe defect in beta-globin production

Hemoglobinopathies involving structural variants displayed distinct profiles on different windows. Hb-S trait was characterized by a mixture of Hb A (63.2), Hb S (32%), and small amounts of Hb A2 (2.3) and Hb F (2.6), confirming heterozygosity for sickle hemoglobin. Hb-D trait presented with moderate Hb A (52.4 ± 15.6), increased Hb D (30 ± 4.1), and low Hb A2 (1.6 ± 0.1) and Hb F (1.4 ± 1.1). In Hb-D disease, Hb D was predominant (90%), with minimal Hb A (2.1%), reflecting homozygosity. Heterozygous Hb E showed a distinct pattern with significant Hb A2 elevation (21.8%), reduced Hb A (76.7%), and minimal Hb F (1.5%), alongside the presence of Hb E, as Hb E coelutes in Hb A window. Normal hemoglobin profiles exhibited high Hb A (96.7 ± 1.1), normal Hb A2 (2.3 ± 1.0), and minimal Hb F (1.0). Hb-S/beta-thalassemia displayed high Hb S (68%) and Hb F (27.8%), with minimal Hb A (0.4%) and slightly elevated Hb A2 (3.8%), reflecting the compound heterozygous state.

**Table 6: Hemoglobin profile obtained in each case in HPLC**

| Disorders | Hb A | Hb A2 | Hb F | Hb S | Hb D | Hb E |
|---|---|---|---|---|---|---|
| **Normal** | 96.7±1.1 | 2.3±1.0 | 1.0±0.7 | - | - | - |
| **B-Thalassemia Major** | 26.7±31.2 | 3.1±1.6 | 70.2±31.1 | - | - | - |
| **B- Thalassemia trait** | 88.4±19.1 | 5.1±1.1 | 6.3±19.8 | - | - | - |
| **B- Thalassemia Intermedia** | 65.2±14.5 | 3.9±0.8 | 34.3±18.9 | - | - | - |
| **Hb-S trait** | 63.2 | 2.3 | 2.6 | 32 | - | - |
| **Hb-D trait** | 52.4±15.6 | 1.6±0.1 | 1.4±1.1 | - | 30±4.1 | - |
| **Hb-D disease** | 2.1 | 0.0 | 1.1 | - | 90 | - |
| **Hetero Hb E** | 76.7 | 21.8 | 1.5 | - | - | Present |
| **Hb-S/ B- Thalassemia** | 0.4 | 3.8 | 27.8 | 68 | - | - |

These findings affirm the diagnostic utility of cation exchange HPLC. The quantitative and qualitative differentiation of hemoglobin fractions on the basis of retention time, windows and elution peak on chromatograph provides accurate identification of thalassemia's and hemoglobinopathies, underscoring HPLC's critical role in diagnosis and clinical decision-making.

## DISCUSSION

This study assessed the utility of cation exchange HPLC in diagnosing thalassemia and hemoglobinopathies in pediatric patients and analyzed the distribution of red blood cell indices. Using data from 105 participants, key observations were made regarding the demographic, clinical, and hematological profiles associated with these conditions.

The analysis revealed distinct patterns in red blood cell indices and hemoglobin fractions among different conditions. For example, beta-thalassemia major showed significantly reduced Hb A levels and markedly elevated Hb F, reflecting a severe deficiency in beta-globin chain production. Beta-thalassemia trait exhibited elevated Hb A2 and mild increases in Hb F, consistent with heterozygous mutations.[10]

Beta-thalassemia major was characterized by low RBC counts, hemoglobin levels and elevated RDW (26.9%). Beta-thalassemia trait exhibited higher RBC counts but lower MCV and MCH, suggesting ineffective erythropoiesis.[11,17]

The findings of this study support the existing literature on the role of HPLC as a reliable diagnostic tool for identifying thalassemia's and hemoglobinopathies, particularly in pediatric patients. A study conducted by Kumar et.al emphasized the effectiveness of HPLC in detecting hemoglobin abnormalities, underscoring its utility in pediatric clinical settings.[12,18] This aligns with the current study, which found beta-thalassemia trait and beta-thalassemia major to be the most common disorders, comprising 21.9% and 10.5% of cases, respectively.[3]

Similarly, Raman *et al.* highlighted the diagnostic significance of distinct hemoglobin fraction patterns, such as elevated Hb F in beta-thalassemia major and increased Hb A2 in beta-thalassemia trait.[13,19] These observations are consistent with the results of this study, further

validating the sensitivity and specificity of HPLC in diagnosing these conditions.[14,15]

An Indian study on pregnant females in antenatal clinic before 14th week of gestation for screening of haemoglobinopathies by HPLC showed that how sensitive this modality is. Fifty percent of females with normal MCV and MCHC were carrier of beta thalassaemia.[20]

An international study conducted in Florida also supports that delta beta ratio determined by HPLC technique is reliable modality to diagnose thalassaemia.[21]

## CONCLUSION

This study demonstrates that cation exchange HPLC is an effective, sensitive and precise method for diagnosis of hemoglobinopathies in children. The results emphasize the utility of HPLC for differentiating hemoglobinopathies based on distinct hemoglobin fraction with the help of RBC indices. HPLC is an essential method for early diagnosis of hemoglobinopathies, especially in populations that are genetically at higher risk.

## LIMITATIONS

The limitation of this study includes that no confirmatory test (Sickling test, molecular studies) was done to confirm the diagnosis due to time constrain. Regional history wasn't taken as thalassemia and hemoglobinopathies depend upon ethnicity.

## SUGGESTIONS / RECOMMENDATIONS

Genetic testing must be incorporated along with HPLC for confirmation.

## CONFLICT OF INTEREST / DISCLOSURE

I have no conflict of interest for this study.

## SOURCE OF FUNDING

All testing's were performed in government hospital free of cost for the patients.

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