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"DETECTION AND CHARACTERIZATION OF HEMOGLOBINOPATHIES IN FAMILY MEMBERS OF HEMOGLOBINOPATHY PATIENTS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY"

Pathology

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ABSTRACT

Background: Hemoglobinopathies are inherited blood disorders with significant public health implications. In India, the prevalence of Beta-Thalassemia Trait (BTT) and sickle cell hemoglobinopathies varies considerably, making screening essential for early detection and management. High-Performance Liquid Chromatography (HPLC) is a highly sensitive tool used for detecting and characterizing abnormal hemoglobins in at-risk populations. **Aims & Objectives:** This study aimed to detect and characterize hemoglobinopathies in family members of hemoglobinopathy patients using HPLC, providing insights into genetic risk and aiding in early intervention strategies. **Methodology:** A cross-sectional study was conducted from August 1, 2022, to January 31, 2024, at a Tertiary Care Centre of Central India. The study included 130 family members of hemoglobinopathy patients (parents and siblings) who had no history of blood transfusion in the last three months. Blood samples were collected and analyzed using HPLC, along with complementary tests such as Complete Blood Count (CBC), peripheral smear examination, and hemoglobin F (HbF) estimation. **Results:** Of the 130 participants, 6 (4.6%) were normal, 6 (4.6%) had Beta-Thalassemia Major (BTM), 35 (26.9%) had BTT, 35 (26.9%) had sickle cell disease (SCD), 45 (34.6%) had sickle cell trait (SCT), and 3 (2.3%) had sickle-thalassemia trait (STT). A significant age distribution was observed, with most individuals with BTT and SCD aged between 20-40 years. Clinical presentations varied, with fever and joint pain being common in the SCT and SCD groups, respectively. CBC parameters showed statistically significant differences across groups ($p<0.001$ for most parameters). **Conclusion:** HPLC proved to be an effective tool for detecting and characterizing hemoglobinopathies in family members of affected patients. Early detection through familial screening allows for timely genetic counseling and better disease management. The findings highlight the importance of targeted screening programs in regions with high hemoglobinopathy prevalence.

KEYWORDS

INTRODUCTION

Hemoglobinopathies and thalassemia are among the most common genetic disorders worldwide, with significant variation in prevalence based on geography. According to the World Health Organization (WHO), approximately 5% of the global population carries a pathological haemoglobin gene. In India, the incidence of the thalassemia trait ranges from 3-17%, while the prevalence of sickle cell hemoglobinopathy varies between 1-44%, reflecting a substantial public health burden. Screening and management of these disorders are critical for reducing their impact on affected families and communities.

Hemoglobinopathies are inherited blood disorders that involve structural or functional abnormalities in haemoglobin, with sickle cell disease (SCD) and thalassemia being the most common forms. Given their genetic inheritance, screening family members of individuals diagnosed with hemoglobinopathies is essential for early detection of carriers and individuals at risk. High Performance Liquid Chromatography (HPLC) is a highly sensitive and specific tool for detecting and characterizing abnormal haemoglobins, offering clear advantages over traditional screening methods like electrophoresis. HPLC enables the accurate identification of haemoglobin variants by separating haemoglobin molecules based on their physicochemical properties, thus facilitating comprehensive family screening and risk assessment.

Familial screening through HPLC allows for the detection of carriers, including those with mild or asymptomatic forms of hemoglobinopathies. Early identification plays a crucial role in genetic counseling, helping families make informed reproductive decisions and manage the risk of disease transmission. Additionally, the quantitative data provided by HPLC enhances the ability to assess complex genetic traits such as heterozygosity and compound heterozygosity, which are pivotal for personalized patient care.

This study aims to assess abnormal hemoglobin in family members of patients diagnosed with hemoglobinopathies using HPLC, with the goal of improving early diagnosis and management strategies for at-risk individuals.

Methodology

Study Design: This cross-sectional, hospital-based study was conducted at the Department of Pathology, Tertiary Care Centre of Central India from August 1, 2022, to January 31, 2024.

Study Population: The study included family members (father, mother, and siblings) of patients diagnosed with hemoglobinopathies. Only those who provided consent and had no history of blood transfusion in the last three months were included.

Sample Size: All eligible family members of hemoglobinopathy patients attending the hospital during the study period were included. A total of 130 participants met the inclusion criteria and were included in the study.

Ethical Considerations: The study was approved by the Institutional Ethics Committee. IEC protocol No : 32210/MC/IEC/2022. Letter No: 32210/MC/IEC/2022 Informed consent was obtained, and confidentiality was maintained.

Sample Collection: 2 mL of venous blood was collected in K_3EDTA vacutainers from each participant during routine visits by venipuncture. No additional costs were incurred by the participants.

Laboratory Analysis

The following laboratory investigations were carried out on each blood sample:

1. Complete Blood Count (CBC):

CBC was performed using automated hematology analyzers (Mindray BC5300 and BC3600) to measure hemoglobin levels, red blood cell (RBC) count, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW). These parameters helped in the initial screening for anemia and other red cell abnormalities.

2. Peripheral Smear Examination:

A peripheral blood smear was prepared, air-dried, and stained using

Leishman stain. Microscopic examination was performed to assess red cell morphology, identifying features like sickle cells, target cells, microcytosis, and anisocytosis. Red cell morphological abnormalities provided clues to underlying hemoglobinopathies.

3. Sickling Test:
The sickling test was conducted by mixing a drop of the participant's blood with a 2% sodium metabisulfite solution on a clean glass slide. The mixture was covered with a cover slip, sealed with petroleum jelly, and incubated for 1-2 hours. Microscopic examination was performed to detect sickling of red cells, indicative of sickle cell disease.

4. Hemoglobin F Estimation (Modified Betke Method):
Hemoglobin F (HbF) levels were estimated using the modified Betke method, a chemical denaturation technique. Blood samples were lysed, and the hemoglobin was treated with sodium hydroxide to denature adult hemoglobin, leaving HbF intact. The remaining hemoglobin was quantified spectrophotometrically, and the percentage of HbF was calculated. This method was particularly useful for detecting elevated HbF levels in conditions like β-thalassemia.

5. High-Performance Liquid Chromatography (HPLC):
HPLC was performed to identify and quantify different types of hemoglobin present in each sample. An analytical C18 column and a UV detector were used to separate hemoglobin variants based on their retention times. Hemoglobin variants such as HbA, HbA2, HbS, and HbF were identified and quantified based on their respective peaks in the chromatogram. This technique allowed precise detection and characterization of hemoglobinopathies.

Data Collection
Demographic data, clinical history, transfusion history, and family history were recorded for each participant using a structured patient proforma. All laboratory findings were documented and regularly monitored.

Statistical Analysis
Data were compiled and analyzed using Microsoft Excel and IBM SPSS software (version 25). Descriptive statistics were used to summarize the demographic characteristics, clinical history, and laboratory findings of the participants. Categorical variables were expressed as frequencies and percentages, while continuous variables were presented as means with standard deviations (SD). The Chi-square test was used to compare categorical variables, and one-way ANOVA was applied to compare the means of continuous variables across different groups. A p-value of less than 0.05 was considered statistically significant. The results were compared with those from similar studies for validation and further interpretation.

RESULTS

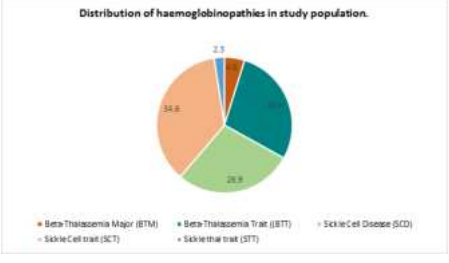

Figure 1: Distribution of haemoglobinopathies in study population.

The study examined the distribution of haemoglobinopathies among 130 individuals, comprising both parents and siblings. Of these, 60 (46.15%) were parents, 64 (49.23%) were siblings, and 6 (4.61%) were categorized as normal.

Table 1: Distribution of haemoglobinopathies in family members.

Haemoglobinopathy	Parents		Siblings	Total	Percent
	Mother	Father			
BTM	0	0	6	6	4.6
BTT	11	11	13	35	26.9
SCD	5	5	25	35	26.9
SCT	14	11	20	45	34.6
STT	1	2	0	3	2.3
No abnormality	2	4	0	6	4.6
Total	33	33	64	130	100

*Beta-Thalassemia Major (BTM), Beta-Thalassemia Trait (BTT), Sickle Cell Disease (SCD), Sickle Cell trait (SCT), Sickle thalassemia trait (STT)

Among the parents, 33 were mothers and 33 were fathers. The mothers' group included 2 normal cases, 11 with Beta-Thalassemia Trait (BTT), 5 with Sickle Cell Disease (SCD), 14 with Sickle Cell Trait (SCT), and 1 with Sickle-Thalassemia Trait (STT). Similarly, the fathers' group comprised 4 normal cases, 11 with BTT, 5 with SCD, 11 with SCT, and 2 with STT. In the siblings' group, there were no normal cases, 6 with Beta-Thalassemia Major (BTM), 13 with BTT, 25 with SCD, and 2 with SCT.

When analyzing the entire study population, the distribution was as follows: 6 normal individuals (4.6%), 6 with BTM (4.6%), 35 with BTT (26.9%), 35 with SCD (26.9%), 45 with SCT (34.6%), and 3 with STT (2.3%).

Table 2: Comparison Of Age Distribution Between Groups

		Age of patients				Total	P value
		1-20	20-40	40-60	> 60		
Interpretation	Normal	4	2	0	0	6	0.005*
	BTM	4	2	0	0	6	
	BTT	11	24	0	0	35	
	SCD	17	16	2	0	35	
	SCT	12	20	6	7	45	
	STT	3	0	0	0	3	
Total		51	64	8	7	130	

Table 2 shows the age distribution varied across groups. Notably, most individuals with BTT and SCD were aged between 20 and 40 years. In contrast, the SCT group had a broader age range, including individuals over 60 years old. Regarding sex distribution, there were no significant differences between groups, with both males and females represented across all categories.(Fig.2)

Religious background showed significant variation. The majority of individuals in the SCD group were Hindu, while the BTT group had a higher proportion of Muslims(Fig.2).

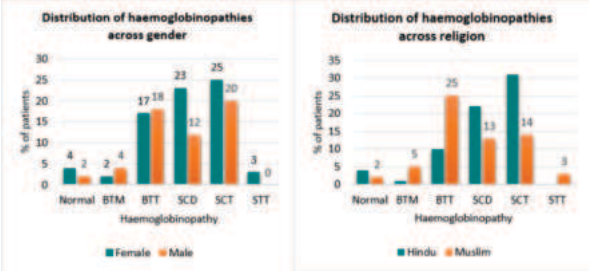

Figure 2: Distribution of haemoglobinopathies across gender and religion

*Beta-Thalassemia Major (BTM) ,Beta-Thalassemia Trait (BTT), Sickle Cell Disease (SCD),Sickle Cell trait (SCT),Sickle thalassemia trait (STT)

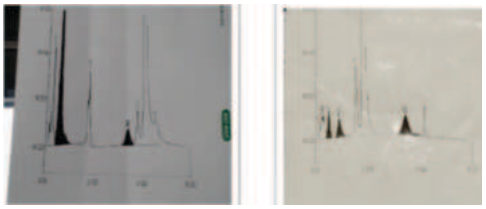

Fig 3a : HPLC report - sickle disease
Fig 3b : HPLC report -thalassemia trait

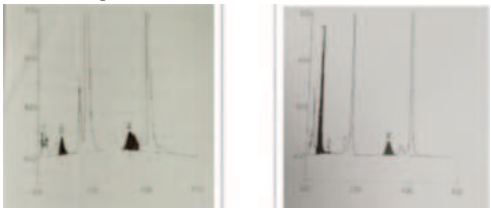

Fig 3c : HPLC report - sickle cell trait
Fig 3d : HPLC report - sickle cell disease
Figure : 3 HPLC Report Images

Table 3: Comparing complaints between groups

Complaints	BTM	BTT	SCD	SCT	STT	No abnormality
Fever	1	2	10	12	1	2
Weakness	4	11	10	9	1	4
Bleeding	0	1	0	2	0	0
Generalized body pain	0	5	0	1	0	2
Abdominal pain	0	0	1	0	0	1
Breathlessness	0	1	0	1	0	0
Pedal edema	0	0	1	1	0	0
Yellowish discoloration of skin	0	0	2	1	0	2
Irritation	0	0	3	0	0	3
Lack of concentration	0	1	1	0	0	0
Loss of appetite	0	1	1	0	0	2
Vomiting	1	0	0	0	0	0
Avascular Necrosis (AVN}	0	0	1	0	0	0
Crisis	0	0	4	0	0	0
Acute chest syndrome	0	0	1	0	0	0
Joint Pain	0	1	7	5	1	1
Pallor	0	1	0	0	0	0
None	1	21	10	28	1	1

Common clinical complaints included fever, weakness, and joint pain, varying in prevalence across the different groups. For instance, fever and weakness were more prevalent in the SCT group, while joint pain was notably higher in the SCD group.(Table 3)

Table 4: Comparison of CBC parameters between groups

Interpretation		HB	TRBC	Hct	MCV	MCH	MCHC	RDW	RETICCOUNT
Normal	Mean	5.63	2.54	16.93	67.01	23.70	32.95	20.60	4.41
	SD	2.76	1.26	8.18	14.84	4.01	1.22	4.25	4.92
BTM	Mean	6.01	2.32	18.11	65.38	22.66	29.48	24.90	3.08
	SD	2.42	.95	7.41	7.97	3.38	4.03	6.34	1.02
BTT	Mean	9.65	4.31	29.57	68.13	22.91	31.29	18.70	2.25
	SD	2.12	1.29	6.94	10.96	5.36	2.92	4.18	1.68
SCD	Mean	8.51	3.07	25.06	77.53	26.63	31.90	18.97	2.85
	SD	2.69	1.10	7.71	13.29	4.78	2.83	5.10	2.23
SCT	Mean	9.93	3.71	29.91	80.84	27.36	32.99	17.31	2.39
	SD	2.60	1.13	7.83	12.76	4.70	2.12	3.74	2.05
STT	Mean	7.80	3.32	24.66	72.13	25.16	32.23	20.96	2.83
	SD	.95	.57	1.53	9.94	2.92	2.20	1.48	1.25
P value		<0.001	<0.001	<0.001	<0.001	0.003	0.006	0.005	0.009

Table 4 compares the Complete Blood Count (CBC) parameters across different diagnostic groups. Significant differences were observed in haemoglobin (HB), total red blood cell count (TRBC), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), and reticulocyte count (Retic Count) between the groups ($p<0.001$ for most parameters). The SCT group had higher MCV and MCH values compared to others. The hemoglobin variant analysis showed elevated HbF levels in the BTM group and higher HbAo levels in the SCD group.

Table 5: Comparison of haemoglobin values between groups

INTERPRETATION		HbAo	HbA2	HbF	S window
Normal	Mean	84.91	2.06	1.76	0.35
	SD	1.62	.708	1.66	0.85
BTM	Mean	8.76	2.60	86.95	0.00
	SD	6.60	1.84	8.190	0.00
BTT	Mean	83.97	4.88	3.10	0.46
	SD	9.61	.93	8.43	1.83
SCD	Mean	17.89	2.54	13.75	65.21
	SD	13.44	.94	6.76	10.16
SCT	Mean	52.56	3.18	2.80	33.18
	SD	13.31	.79	2.77	6.77
STT	Mean	45.86	4.63	6.53	34.56
	SD	.90	.30	3.67	11.72
P value		<0.001	<0.001	<0.001	<0/001

Table 5 compares the hemoglobin variant values (HbAo, HbA2, HbF) across different diagnostic groups. Significant differences were noted in all three parameters ($p<0.001$). The BTM group had a high mean HbF (86.95), while the SCD group had a higher mean HbAo (17.89). The SCD group had a mean S window of 65.214, indicating a high presence of hemoglobin S.

Table 6: Comparison of Peripheral smear (PS) comment between groups

Peripheral smear	BTM	BTT	SCD	SCT	STT	No abnormality	Total
Dimorphic blood picture (DBP)	0	0	0	3	0	0	3
Microcytic hypochromic blood picture (MHBP)	6	26	15	12	1	5	65
Normocytic hypochromic blood picture (NHBP)	0	6	16	15	2	1	40
Normocytic normochromic (NN) & (MHBP)	0	0	0	2	0	0	2
Normocytic normochromic blood picture(NNBP)	0	3	4	12	0	0	19
Oval macrocytes	0	0	0	1	0	0	1

Table 6 compares the Peripheral Smear (PS) comments across different diagnostic groups. The comments included descriptions such as dimorphic blood picture (DBP), microcytic hypochromic blood picture (MHBP), normocytic hypochromic blood picture (NHBP), and others. The BTT group had the highest occurrence of MHBP (26 out of 35), while the SCD group had the highest occurrence of NHBP (16 out of 35).

DISCUSSION

High-performance liquid chromatography (HPLC) has proven to be a highly sensitive, specific, and reproducible alternative to traditional electrophoresis for diagnosing hemoglobinopathies. Its automation and ability to directly identify and quantify both normal and abnormal hemoglobin fractions have made it an invaluable tool in the clinical setting. Studies from India have further emphasized the utility of HPLC, particularly in diagnosing thalassemias and other hemoglobinopathies Sinha et al Khera et al[15,16] In our study, conducted among 33 families (130 members), HPLC detected hemoglobin opathies in 95.4% of the individuals, with only 4.6% of the participants being unaffected. The most common findings were Sickle Cell Trait (SCT, 34.6%), Sickle Cell Disease (SCD, 26.9%), and Beta-Thalassemia Trait (BTT, 26.9%), followed by Beta-Thalassemia Major (BTM, 4.6%) and Sickle Thalassemia Trait (STT, 2.3%).

The findings of this study align with other research conducted across India. For example, a study by Khera et al.[16] in Punjab found a high prevalence of β-thalassemia trait (36%), sickle cell trait (27%), and sickle cell disease (22.5%), indicating the significant burden of these hemoglobinopathies in India. Additionally, Bhukhanvala et al[17]. in Gujarat reported similar distributions, with 30.4% SCT and 30.4% BTT in their study population, further highlighting the heterogeneity of hemoglobinopathy prevalence across different regions of India.

Our results underscore the prevalence of β-thalassemia and sickle cell disorders in India, with notable variations depending on geographic and ethnic factors. For instance, the carrier frequency for β-thalassemia is estimated to be between 3-4% in the general population but can rise significantly in communities like the Sindhis, Punjabis, and Gujaratis. Sickle cell disorders are especially prevalent in tribal populations across Southern, Central, and Western India, with carrier frequencies reaching as high as 48%. Hemoglobin E (HbE) is most common in the northeastern states, with carrier frequencies up to 50%, while HbD is prevalent in about 2% of the population in Punjab.

The age distribution of patients in our study revealed significant patterns. Patients with β-thalassemia trait (BTT) and sickle cell disease (SCD) were most commonly within the 20-40 year age group. In contrast, individuals with sickle cell trait (SCT) were found across all age ranges, with a significant portion in the 21-40 year age group. These findings are consistent with other studies. Bhukhanvala et al[17]. found a high prevalence of hemoglobinopathies in younger age

groups, particularly in the 20-30 year range. This age distribution highlights the importance of early screening and intervention, particularly for individuals in their early to mid-adulthood, where the disease is most likely to become symptomatic. The Sickle Cell Trait (SCT) group had the highest representation of both sexes, with 25 females and 20 males, suggesting an equitable distribution of hemoglobinopathies within this group.

Our study found no significant difference in the distribution of hemoglobinopathies between males and females, with 56 male and 74 female patients affected (p=0.355). This equal distribution is consistent with findings from other studies. For example, research by Al-Saqladi et al[18]. in Yemen found no significant gender difference in the prevalence of sickle cell disorders, while Adekile et al[19]. in Benin reported that while the overall distribution was balanced, certain complications such as acute chest syndrome were more common in males. These findings suggest that while hemoglobinopathies affect both sexes equally, some complications may be influenced by sex-specific factors. Similarly, **Serjeant et al**.[20] observed no significant sex differences in the prevalence of sickle cell disease in Jamaica, though they noted variations in clinical severity and outcomes between sexes.

Our study also examined the distribution of hemoglobinopathies across different religious groups. The majority of patients with SCD were Hindu, while a significant number of individuals with BTT were Muslim. This trend mirrors findings from other studies in India. For instance, Kaur et al[21]. reported a higher prevalence of SCD among Hindu populations and a higher prevalence of BTT among Muslims. **Masud et al.**[22] also reported a higher incidence of Beta-Thalassemia among Muslim populations in North India, consistent with our findings in the BTT group. **Shafiquie et al.**[23] conducted research in Pakistan and observed a significant prevalence of Beta-Thalassemia among Muslims, attributing it to higher consanguinity rates in the Muslim population. Similarly, **Mandal et al**.[24] noted a higher prevalence of SCD among Hindu communities in Eastern India This distribution may be influenced by cultural and genetic factors, including consanguinity, which is more common in certain communities.

The most common clinical complaints in our study included fever, weakness, joint pain, and abdominal pain, with joint pain being particularly prominent in patients with SCD. This symptom pattern is consistent with the vaso-occlusive crises commonly associated with sickle cell disease, which can lead to significant pain and inflammation, particularly in the joints. These findings are consistent with those of Khera et al[16], who reported similar complaints in patients with hemoglobinopathies. A study by **Naaz et al.**[25] reported similar findings. Their study also noted that joint pain was a prominent symptom in SCD patients, supporting our data.

Our study found significant variations in complete blood count (CBC) parameters across different diagnostic groups (p<0.001 for most parameters). For instance, patients with SCT had higher mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) compared to other groups, while patients with BTT showed elevated red cell distribution width (RDW), indicating anisopoikilocytosis. These findings are consistent with those of Bhukhanvala et al.[17]We also found a high prevalence of reduced MCV and MCH in patients with hemoglobinopathies. In β-thalassemia trait cases, increased red cell distribution width (RDW) was observed, consistent with findings by **Bhukhanvala et al**.[17] indicating a degree of anisopoikilocytosis likely due to concomitant iron deficiency present in 12.9% of our cases.

Bhukhanvala et al.[17] found that the majority (80%) of samples showed reduced MCV and MCH, similar to findings in our study. However, only 2.1% were confirmed to have BTT upon further testing with cellulose acetate membrane electrophoresis and HPLC, highlighting the challenge of differential diagnosis in populations with high prevalence of iron-deficiency anaemia. **Mehta and Pandya**[26] indicated that individuals with BTT might maintain iron balance better, which could explain variations in RBC indices.

Our study compared hemoglobin variant values (HbAo, HbA2, HbF) across different diagnostic groups, revealing significant differences (p<0.001) in all three parameters. A study by **Bhukhanvala et al**.[17] observed that in the context of thalassemia and hemoglobinopathies, HbA2 levels could be reliably used to diagnose β-thalassemia trait (BTT) even in the presence of iron deficiency, as most heterozygotes showed elevated HbA2 (>3.5%). **Buch et al.**[27] identified that the accurate identification of hemoglobin variants is essential for diagnosing conditions such as sickle cell disease and thalassemia. Their findings that sickle cell traits and disease can show variable levels of HbAo and HbF.

S Windows: Our study compared the S windows (percentage of hemoglobin S) across different diagnostic groups, revealing significant differences (p<0.001). The Sickle Cell Disease (SCD) group had a mean S window of 65.21, indicating a high presence of hemoglobin S. The high mean S window in the SCD group, indicating a predominant presence of hemoglobin S, is consistent with findings by **Baig et al**.[28], who reported similar high percentages of hemoglobin S in sickle cell disease patients. This highlights the reliability of the S window measurement in diagnosing SCD.

We analysed Peripheral Smear (PS) comments, which included descriptions such as dimorphic blood picture (DBP), microcytic hypochromic blood picture (MHBP), normocytic hypochromic blood picture (NHBP), and others.

The observation of MHBP being most prevalent in the BTT group aligns with **Bhukhanvala et al.**[17], who found a high occurrence of microcytic hypochromic blood pictures in β-thalassemia trait cases due to the characteristic red blood cell morphology.

Similarly, the prevalence of NHBP in the SCD group is consistent with the literature. **Baig et al.**[28] and **Khera et al**.[16] noted that normocytic hypochromic blood pictures are commonly seen in SCD due to the characteristic haemolytic anaemia and compensatory reticulocytosis.

These findings show the diagnostic importance of Peripheral smear comments in differentiating between various hemoglobinopathies. The significant differences in S windows and peripheral smear findings across diagnostic groups in our study are well-supported by other research. High hemoglobin S percentages in the SCD group and the specific blood smear patterns in BTT and SCD groups reinforce the diagnostic utility of these parameters.

Limitations

The reliance on HPLC without DNA molecular studies might have missed certain hemoglobin variants. Iron deficiency in some patients may have affected hematological parameters, complicating result interpretation.

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