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KEY WORDS: High performance liquid chromatography (HPLC), Hemoglobinopathies, Haematological indices, Mentzer index

ROLE OF HPLC AS DIAGNOSTIC TOOL IN HEMOGLOBINOPATHIES AT A TERTIARY CARE HOSPITAL

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ABSTRACT

The presence of structurally faulty hemoglobin (Hb) as a result of anomalies in the globin component of the molecule characterizes hemoglobinopathies. High performance liquid chromatography (HPLC) being an automated instrument is highly sensitive and specific, has high resolution and helps in quantification of various hemoglobins. The current study's aim is to examine laboratory components, specifically the hematological profile and HPLC results of the hemoglobin variants found in all patients suspected of hemoglobinopathies. This was a prospective observational study. Total 351 suspected hemoglobinopathy were enrolled in this study with mean age was 34.79 ± 18.22 years. Majority 118 (3.6%) of the patients belonged to age group of 21-30 years. About 258 (73.5%) were female and 265(75.5%) were married. About 153(59.%) female patients receiving ANC care. About 286 (81.7%) had Mentzer index ≥13. All the haematological indices except MCHC had statistically highly significant association with hemoglobinopathies. Also age of the patients, peripheral blood smear findings, Mentzer index & serum iron level had significant association with hemoglobinopathies. From this study we conclude that, β-thalassemia trait is the most prevalent hemoglobinopathy, Significant statistical associations between hemoglobinopathies and parameters like hemoglobin levels, RBC count, MCV, MCH, RDW and peripheral blood smear findings validate the heterogeneity of these conditions. However, the lack of association with serum ferritin and TIBC levels suggests a complex interplay of factors influencing these parameters.

INTRODUCTION:

The primary oxygen-carrying metalloprotein of red blood cells (RBCs) is called hemoglobin. Its structural makeup consists of a tetramer made up of two alpha and two β globin chains covalently connected to four heme groups that bind oxygen. A variety of illnesses known as hemoglobinopathies impact the way hemoglobin (Hb) functions [1] The presence of structurally faulty hemoglobin (Hb) as a result of anomalies in the globin component of the molecule characterizes hemoglobinopathies. The two primary categories of hemoglobinopathies are structural hemoglobin variations (abnormal hemoglobin) and thalassemia syndromes. The production of the α and β-globin sub-units is either absent or reduced in α and β-thalassemia respectively. The cause of sickle cell disease is a point mutation in β-globin at position six [2].

Earlier studies have shown that the overall prevalence of β-thalassemia is 3–4 % with an estimate of around 8,000 to 10,000 new births with major disease each year[3]. Three to four percent of Indians are carriers of β-thalassemia. There is a higher incidence (5–17%) among certain ethnic groups, such as Sindhis, Kutchis, Lohanas, Punjabis, a few Muslim communities, and a small number of tribal people[4]. There may be over 24.7 million carriers of β thalassemia and 16.9 million carriers of sickle cell anemia throughout all age categories, according to estimations. It is predicted that there are 5500 instances of β thalassemia major and 11,000 cases of sickle cell anemia in India each year[5].

Considering the Burden of hemoglobinopathies in India, government of India has now launched a national program to detect and screen hemoglobinopathies. Finding carriers through trustworthy laboratory techniques is essential to preventing this dangerous health issue. The health care system's mass screening initiatives and growing worldwide awareness of the issue have made laboratory staff much more accountable for identifying and preventing this issue. In the past, the preferred technique for identifying and measuring variant Hb has been electrophoresis. However, Hb D and Hb G, as well as Hb E and Hb O, cannot be distinguished using electrophoretic techniques. Furthermore, when it comes to the detection of fast Hb variants (Hb H, Hb Barts) or the quantification of low-concentration Hb variants (e.g., Hb A2), electrophoresis is labour-intensive, slow, and inaccurate. HPLC is becoming the preferred technique for quantifying Hb A2 and Hb F concentrations as well as for the initial screening of Hb variants, including neonatal screening when required. Hb A2, Hb F, Hb A, Hb S have all been quantified using the automated cation-exchange HPLC instrument known as the Bio-Rad Variant[6].

High performance liquid chromatography (HPLC) being an automated instrument is highly sensitive and specific, has high resolution and helps in quantification of various hemoglobins. When used systematically can achieve laboratory diagnosis of thalassemia syndromes and hemoglobinopathies[7,8]. When it comes to quantifying Hb F and Hb A2 and identifying additional variants in a single screening test, HPLC is superior to electrophoresis. Furthermore, HPLC takes less time, manpower, and is sensitive, specific, and repeatable. It is therefore perfect for a typical clinical laboratory with a heavy workload. To prevent false negative diagnoses in daily practice, one must be aware of the shortcomings and issues related to the diagnostic techniques[9].

The current study's aim is to examine laboratory components, specifically the hematological profile and HPLC results of the hemoglobin variants found in all patients suspected of hemoglobinopathies.

METHODOLOGY:

This was a prospective observational study. The permission for conducting the study was obtained from the head of the Department and ethical approval from the institutional ethical committee. Participants who are suspected by clinicians and who come for screening purpose in OPD and IPD of all age

groups were enrolled for the study. Data collection was done by one-to-one interview using semi structured questionnaire after obtaining informed consent from the study participants. Detailed history was taken including age, sex, caste, religion was recorded. The study include EDTA blood sample that came to PATHOLOGY department in Jehangir Hospital, Pune. EDTA anti-coagulated fresh blood samples used for investigation.

Hemoglobinopathy studies done on automated BIORAD D-10 analyzer with CBC for RBC indices on Sysmex XN series 5 part differential automated hematology analyzer including serum iron, ferritin and TIBC studies. The suspected cases in our study were ones who may have family history or persistent unexplained anemia but clinically asymptomatic.

Sample size was determined by using the effect size from the previously published study and with the help of following formula: $n = z^2[pq/me^2]$. Prevalence was 0.1121 (11.21%) (Published estimate of prevalence of most common Hb disorder being Beta-thalassemia trait)[10]. Thus, the minimum sample size required according to this formula is 351.

The data on categorical variables was shown as n (% of cases) and the data on normally distributed continuous variables is presented as mean and standard deviation (SD). The inter group statistical comparison of distribution of categorical variables is tested using Chi-Square test or Fisher's exact probability test if more than 20% cells have expected frequency less than 5. The inter-group statistical comparison of distribution of means of continuous variables is tested using ANOVA. The underlying normality assumption was tested before subjecting the study variables to ANOVA. All results are shown in tabular as well as graphical format to visualize the statistically significant difference more clearly. Details of one CBC sample did not found so excluded from analysis. In the entire study, p-values less than 0.05 are considered to be statistically significant. The entire data is statistically analyzed using Statistical Package for Social Sciences (SPSS ver 24.0, IBM Corporation, USA) for MS Windows.

OBSERVATION:

Total 351 suspected hemoglobinopathy patients referred for High-Performance Liquid Chromatography (HPLC) analysis were enrolled in this study with mean age was 34.79 ± 18.22 years. Majority 118 (3.6%) of the patients belonged to age group of 21-30 years. out of total, about 258 (73.5%) were female and 265(75.5%) were married. About 153(59.%) female patients receiving ANC care. About 286 (81.7%) had Mentzer index ≥13. (Table 1)

About 153 (86%) patients had RBC count <6 million cells/µL. About 205 (71.2.%) patients had Hb <12 g/dL. About 301 (86.0%) patients had <40% hematocrit. About 176 (50.3%) has < 80 fL MCV & 231(66%) had <27 MCH g/dL. About 250(71.4%) had <32g/dL MCHC. About 345 (99.1%) patients had ≥11.5 RDW. (Table 2)

Fig 1 shows, majority of cases, 232 (66.1%) had a normal chromatogram followed by 19.40% had beta thalassemia trait. Table 3 shows, all the haematological indices except MCHC had statistically highly significant association with hemoglobinopathies. Table 4 shows age of the patients, peripheral blood smear findings, Mentzer index & serum iron level had significant association with hemoglobinopathies.

Table 1: Distribution of patients according to demographic parameter

Demographic Parameter		Frequency	Percentage
Age Group	**<10**	1	8.8
	10-20	15	4.3
	21-30	118	33.6
	1-40	99	28.2
	41-50	2	6.6
	>50	65	18.5
Gender	**Male**	9	26.5
	Female	258	73.5
Marital status	**Married**	265	75.5
	Unmarried	86	24.5
ANC Status	**ANC**	153	59.3
	Non-ANC	105	40.7
Mentzer index	**<13**	64	18.3
	≥13	286	81.7

Table 2: Distribution of patients according to Haematological Indices

Haematological Indices		Frequency	Percentage
RBC	**<4.52**	20	48
	4.52-5.9	133	38
	≥6	14	4
HB	**<12**	250	71.2
	≥12	101	28.8
Hematocrit	**<40**	204	85.7
	≥40	34	14.3
MCV	**<80**	124	51.9
	80-100	108	45.4
	>100	6	2.5
MCH	**<27**	163	68.5
	≥27	75	31.5
MCHC	**<32**	237	99.6
	≥2	1	0.4
RDW	**<11.5**	1	0.4
	≥11.5	235	99.6

RBC: Red blood cell, Hb : Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, RDW: Red Cell Distribution Width)

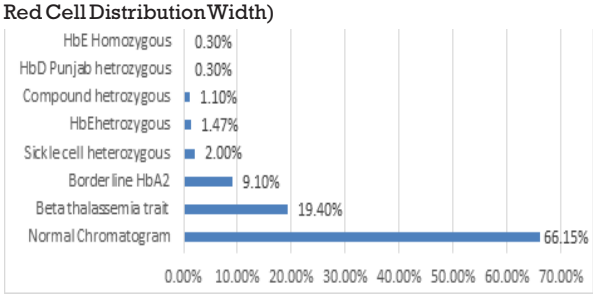

Fig 1: Distribution of patients according to hemoglobinopathy

Table 3: association between hemoglobinopathies and haematological Indices

Hemoglobinopathies		Normal Chromatogram	Beta thalassemia trait	Borderline HbA2	Sickle cell heterozygous	HbE Homozygous	HbE Homozygous	HbD Punjab hetrozygous	Compound hetrozygous	p Value
Age Group	<10	26(8.9%)	5(16.1%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0.001
	10-20	9(80%)	4(2.7%)	0(0%)	0(0%)	0(0%)	1(6.7%)	0(0%)	1(6.7%)	
	21-30	77(65.3%)	19(16.1%)	17(14.4%)	2(1.7%)	0(0%)	2(1.7%)	0(0%)	1(0.8%)	
	31-40	75(75.8%)	7(7.1%)	1(33.3%)	2(2%)	0(0%)	0(0%)	0(0%)	2(2%)	
	41-50	11(47.8%)	10(4.5%)	0(0%)	1(4.3%)	0(0%)	1(4.3%)	0(0%)	0(0%)	
	>50	34(52.3%)	23(3.4%)	2(3.1%)	2(.1%)	1(1.5%)	2(3.1%)	1(1.5%)	0(0%)	
Peripheral Blood Smear	Normocytic normochromic anaemia	131(79.8%)	2(1.8%)	24(14.6%)	3(1.8%)	0(0%)	3(1.8%)	0(0%)	0(0%)	0.001
	Microcytic hyperchromic anemia	92(52.6%)	65(37.1%)	6(3.4%)	4(2.3%)	1(0.6%)	3(1.7%)	1(0.6%)	3(1.7%)	
	Microcytic anaemia	8(80%)	0(0%)	2(20%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	
Mentzer index	<13	17(26.6%)	42(65.6%)	2(.1%)	0(0%)	1(1.6%)	1(1.6%)	0(0%)	1(1.6%)	0.001
	≥13	215(75.2%)	26(9.1%)	30(10.5%)	7(2.4%)	0(0%)	5(1.7%)	1(0.3%)	2(0.7%)	
Serum iron (mg/dl)	<50.00	42(71.2%)	13(22.0%)	3(5.1%)	0(0%)	0(0%)	1(1.7%)	0(0%)	0(0%)	0.002
	50.00-170.00	21(47.7%)	18(40.9%)	1(2.3%)	0(0%)	1(3%)	2(4.5%)	0(0%)	1(2.3%)	
	>170.00	0(0%)	2(66.7%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(33.3%)	
Serum ferritin (ng/mL)	<4.63	8(88.9%)	0(0%)	1(11.1%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0.139
	4.63-204.00	46(61.3%)	21(28%)	4(5.3%)	2(2.7%)	1(1.3%)	0(0%)	0(0%)	1(1.%)	
	>204.00	10(47.6%)	8(8.1%)	0(0%)	0(0%)	0(0%)	2(9.5%)	0(0%)	1(4.8%)	
TIBC	<265.0	7(43.8%)	8(50%)	1(6.3%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0.168
	265.0-497.0	39(58.2%)	20(29.9%)	1(1.5%)	2(3%)	1(1.5%)	2(3%)	0(0%)	2(3%)	
	>497.0	11(84.6%)	0(0%)	2(15.4%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	

Table 4: association between hemoglobinopathies and clinic-social findings

Hemoglobinopathies	Normal Chromatogram	Beta thalassemia trait	Borderline HbA2	Sickle cell heterozygous	HbE Homozygous	HbE Homozygous	HbD Punjab hetrozygous	Compound hetrozygous	p Value
Age (Years)	32.12±17.01	42.2±22.25	33.91±11.33	43.43±12.15	57	45±2.98	62	26.50±9.98	0.001
Hemoglobin (mg/dL	10.48±2.34	9.69±1.95	11.93±1.71	10.30±0.90	11.4	10.78±2.41	7.6	9.7±1.9	0.001
RBC Count	4.27±0.75	4.98±1.06	4.32±0.64	4.28±0.28	6.11	4.56±0.62	3	4.27±1.11	0.001
MCV	80.53±11.57	65.19±7.04	86.02±10.43	78.23±5.35	57.1	75.45±10.75	79.3	69.17±5.82	0.001
MCH	24.81±4.9	19.72±2.68	27.91±3.98	24.11±2.05	18.7	23.70±4.32	26.3	22.13±1.08	0.001
MCHC	30.47±2.48	30.18±1.43	32.03±2.33	30.81±1.05	32.7	31.20±1.94	31.9	32.03±1.29	0.093
RDW	16.37±4.13	17.95±3.54	13.84±2.79	16.64±1.64	16.6	16.68±3.04	14.7	16.86±1.35	0.001

DISCUSSION:

The mean age of cases was 34.79 ± 18.22 years, spanning from infancy to advanced age. The highest prevalence in the 21–30 and 31–40 years age groups reflect the impact of hemoglobinopathies during the reproductive and working-age group. This aligns with the age when individuals are most likely to seek medical care for anemia or undergo routine health evaluations. Additionally, this age group is more likely to undergo screening during pregnancy or pre-marital health consultations. The relatively lower prevalence in pediatric and geriatric populations could be due to the asymptomatic nature of some hemoglobinopathies in early life and limited healthcare-seeking behavior in older populations. This trend is consistent with the findings from the study by Pathak et. Al [11]. The sex distribution reveals a significant gender disparity: 73.5% of the cases were female, while only 26.5 % were male. This finding contrasts with the study by Singh et. Al [12]. The predominance of females in this study may reflect several factors, including cultural practices where females might be more frequently screened for instances during ANC checkup or referred for medical evaluation due to reproductive health issues or family history concerns. Additionally, social norms may lead to increased healthcare access for women in certain communities, resulting in higher detection rates among females. The marital status distribution indicates that a significant majority (75.5%) of the cases were married. This finding is consistent with the study by Singh et. Al [12] The high percentage of married individuals may also suggest that awareness and education about hemoglobinopathies are crucial for family planning decisions, particularly in regions where consanguinity is common and increases the risk for genetic disorders. The study indicates a notable prevalence of hemoglobinopathies among patients, aligning with findings from Kumar et al [13] who reported a 4.55% prevalence of β Thalassemia Trait in antenatal women. The underutilization of ANC services may result from socioeconomic barriers, lack of awareness, or limited accessibility to maternal health services in the study population as similar studies emphasize the importance of early screening in reducing the burden of hemoglobin disorders.

The distribution of hemoglobinopathies in the present study provides valuable insights into the prevalence and diversity of these genetic conditions in the population. The findings, including a significant proportion of normal cases and a predominance of β-thalassemia trait, are consistent with existing literature and highlight important considerations for diagnostic and public health strategies. The finding that β-thalassemia trait is the most common hemoglobinopathy in this study aligns with previous research conducted in India. For instance, a study by Mondal B. et al[14] reported that β-thalassemia heterozygous individuals constituted 17.64% of their sample population, supporting the notion that β-thalassemia is a prevalent condition across various regions in India, particularly in states like Gujarat and Punjab where carrier frequencies can reach up to 10-15%. Moreover, the overall prevalence of hemoglobinopathies in this study appears lower than some reports which estimate carrier frequencies for hemoglobinopathies to be between 27% to 40% in certain population. This discrepancy may be attributed to differences in regional demographics, screening methods, or sample sizes. Wendt et al[15], which highlighted the existence of multiple hemoglobin variants across different ethnic groups in India. Sickle cell heterozygous cases were less common. This is because of urban settings like Pune generally exhibit lower sickle cell gene prevalence compared to rural or tribal regions. This reflects differences in migration patterns and genetic drift. The study's findings on the distribution of hemoglobinopathie s across various age groups reveal significant trends, particularly in the prevalence of β thalassemia trait and normal chromatograms. The data indicates that younger age groups exhibit a higher percentage of normal chromatograms, while older age groups show an increased prevalence of β thalassemia trait, suggesting a potential accumulation of genetic traits over time.

Our study shows, about 153 (86%) patients had RBC count <6 million cells/µL. The findings of present study are in line with study by Bhalodia J, et al [16]. RBC indices can be used to screen for thalassemia and other hemoglobinopathies, according to Sadiya S. et al [17] the HPLC method should be used to confirm this. About 205 (71.2.%) patients had Hb <12 g/dL. About 301 (86.0%) patients had <40% hematocrit. About 176 (50.3%) has < 80 fL MCV & 231(66%) indicating microcytic anemia, which is often associated with iron deficiency in the study by Mondal et al[14]. About 250(71.4%) had <32g/dL MCHC, which is frequently linked to iron deficiency in the study by Abdulraheem et al [18]. About 345 (99.1%) patients had ≥11.5 RDW, suggesting a significant variation in red blood cell size and volume, which is often seen in iron deficiency anemia. In contrast, some studies indicate that not all cases of microcytic anemia are due to iron deficiency; conditions like β-thalassemia trait can present similarly [14]. These results highlight a high prevalence of anemia within the population. Low Hematocrit among 86.0% of patients exhibited hematocrit levels below 40%, indicating a high prevalence of anemia, consistent with findings in elderly populations where microcytic hypochromic anemia is common due to iron deficiency in the study by Munesh et al.[19].

Our study shows, mean hemoglobin levels across various hemoglobinopathies, reveals significant differences among groups, with Borderline HbA2 showing the highest mean hemoglobin level. The findings are consistent with studies indicating that β thalassemia traits typically present lower hemoglobin levels compared to normal individuals. The study by Garg et al[20]. In contrast, the study by Kumar Nath S et al [21] suggests that while hemoglobin levels vary, the clinical implications of these variations may not always correlate with severity, indicating a need for further research into the clinical significance of these findings. Our study shows, mean RBC levels among different

hemoglobinopathies which reveals significant variations, with normal chromatograms showing a mean RBC count of 4.32, while β thalassemia trait presents a higher mean of 4.94 and statistically significant with hemoglobinopathy, aligning with findings from the study by Khachaturian et al [22].Our study shows, mean corpuscular volume (MCV) values across various hemoglobinopathies, reveals significant differences, with borderline HbA2 averaging 86.02 fL, while those with β thalassemia trait show a markedly lower mean of 65.19 Fl, aligning with findings of the study conducted by Yavorkovsky L[23].Our study shows, mean MCH values varied notably among groups, with normal individuals averaging 24.81 pg, while those with β thalassemia trait had a lower mean of 19.72 pg, aligning with the study by Li D et al [24] indicating that MCH is a critical parameter in diagnosing anemia and hemoglobinopathies.Our study shows, normal individuals exhibit a mean MCHC of 30.47 g/dL, those with β thalassemia trait show a slightly lower mean of 30.18 g/dL. Previous study by Zheng Y et al [25] have shown that patients with hemoglobin variants like HbC and HbS often present with elevated MCHC levels, with significant proportions exceeding normal ranges.Our study shows, β thalassemia trait 102 exhibit a notably higher mean RDW (17.95%) compared to those with normal chromatograms (16.37%), suggesting greater red blood cell size and volume variability, This aligns with the study by Keikhaei B et al [26]. Our study shows, prevalence of normocytic normochromic anemia, microcytic hypochromic anemia, and macrocytic anemia, had statistical significant association with hemoglobinopathies, this aligns with findings from Kuppusamy D et al [27]. In this study, 18.3% had <1 mentzer index and significant association with hemoglobinopathies. Mentzer Index and hemoglobinopathy

distribution, supporting its diagnostic relevance aligning with the study by Shah T et. al [28]. The prevalence of low serum iron in hemoglobinopathies is consistent with the findings of Khachaturian A et al [29]., who noted diverse laboratory features in β-thalassemia patients.

This study shows, majority of cases fall within the normal serum ferritin range, while a smaller percentage presents with low and high ferritin levels with a statistically non-significant association with hemoglobinopathy. Study by Jabbar H et al [30], indicate that patients with β-thalassemia often exhibit elevated ferritin levels due to iron overload from frequent transfusions. This study also shows, serum TIBC values among different hemoglobinopathies which reveals a predominance of normal TIBC levels, with a statistically non-significant association between TIBC levels and hemoglobinopathy distribution (p-value = 0.168). This finding aligns with the study by Pathak et al[11], which often highlights the complexity of hemoglobinopathies and their biochemical markers. In contrast, the study by Zheng Y et al [25], suggests that TIBC can be a useful diagnostic tool in specific populations, indicating that regional differences and patient demographics may play a crucial role in these findings.

CONCLUSION:

This study provides a comprehensive analysis of hemoglobinopathies in the population, emphasizing the utility of HPLC in diagnosing and understanding their distribution. The findings highlight that β-thalassemia trait is the most prevalent hemoglobinopathy, with a significant variation in prevalence across different age groups. The study also underscores the high burden of anemia in the population, characterized predominantly by microcytic and hypochromic anemia, as reflected in RBC indices and related parameters. Significant statistical associations between hemoglobinopathies and parameters like hemoglobin levels, RBC count, MCV, MCH, RDW and peripheral blood smear findings validate the heterogeneity of these conditions. However, the lack of association with serum ferritin and TIBC levels suggests a complex interplay of factors influencing these parameters. The study reinforces the importance of routine screening for hemoglobinopathies, particularly β-thalassemia trait, and the role of HPLC as a reliable diagnostic tool. These insights can guide targeted interventions and preventive strategies to address the burden of anemia and hemoglobinopathies in the community.

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