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Detection of Thalassemia and Thalassemia Traits by High Performance Liquid Chromatography (HPLC) Among Anemia Patients and Their Family Members in A Tertiary Care Hospital

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Abstract

Background: Hemoglobinopathies result from a structural defect in the globin gene. Thalassemia occurs predominantly in persons of Mediterranean, African, and Asian ancestry. Estimates show that 1.5% of the world population are carriers of β-thalassemia and about 50% of the incidence is from the South East Asian population which mainly includes countries like India. An attempt has been made to detect the different types of hemoglobin pathies prevalent among the anemic patients undergoing blood transfusion for various causes using HPLC in our tertiary care hospital.

Objectives: 1. To determine the occurrence of β-thalassemia and thalassemia traits among the patients undergoing blood transfusion by analyzing haematological parameters and HPLC. 2.To determine the occurrence of thalassemia traits and carriers among the family members of the patients undergoing blood transfusion who will be detected in the current study.

Methodology: A prospective study was conducted in the Department of Pathology, Mandya Institute of Medical Sciences, from March 2023 to May 2024 after obtaining Institutional Ethics Committee approval. A total of 358 participants were selected using a stratified convenience sampling method, including anaemic patients (Hb <10 g/dL) requiring blood transfusion and their first-degree family members when abnormal haemoglobin variants were detected. Patients with a history of blood transfusion within the preceding three months were excluded. Pre-transfusion critical blood samples were analysed using complete blood count, peripheral smear examination, and High - Performance Liquid

Chromatography (BIO-RAD D-10 β-thalassaemia short programme). Serum ferritin estimation, along with relevant clinical history and transfusion history, was obtained wherever indicated to differentiate iron deficiency anaemia from thalassaemia.

Results: A total of 358 anaemic patients underwent HPLC screening. Among them77(21.8%) cases displayed abnormal haemoglobin fractions on HPLC of which 72(19.83%) cases were diagnosed as thalassemia trait, 2 cases (0.56%) as β-thalassemia major. Other variants detected included δβ- thalassemia, HbSβ thalassemia and HbD-Punjabtrait were also identified. β-thalassemia trait was most frequently detected hemoglobinopathy in all age groups and the remaining 281(78.2%) cases had a normal HPLC pattern.

Conclusion: The study population ranged in age from 1 to 90 years, with an overall mean age of 30.19 years (31 years in males and 29 years in females). Adults constituted 68% of the study population, while 32% were paediatric patients. Among the abnormal cases, 75 (71%) were males and 31 (29%) were females, although β-thalassemia trait showed a higher frequency among females.β-thalassemia trait was the most common hemoglobinopathy detected in both paediatric and adult age groups and represented the predominant abnormal hemoglobin variant overall.

The mean HbA_2 level in β-thalassemia trait was 6.47%, confirming the characteristic elevation of HbA_2 in affected individuals and highlighting the utility of HPLC as a reliable screening and diagnostic tool for hemoglobinopathies.

Keywords: Haemoglobinopathy, High Performance Liquid Chromatography, Prevalence, Thalassaemia, Mentzer Index, A-Thalassemia

Introduction

Hemoglobinpathies are one of the most common inherited blood illnesses in India and are one of the country's most serious public health issues(1).The occurrence of hemoglobinpathies has been reported to be the highest in the Middle East and the Indian subcontinent(2). The World Health Organization (WHO) considers thalassemia to be the most common genetic blood disorder in the world, affecting over 60 countries (1). Recently As per WHO, 4.5 to 5% of the world's population are carriers of hemoglobinpathies (3,4). In India, 30 million carriers and a mean prevalence is 3.3%. The incidence of β-thalassemia trait in India ranges from 3 – 17% the possible cause could be because of a higher rate of consanguinity[3,5].

WHO figures estimates the frequency of β-thalassemia in India ranges from 3.5 to 15% in general population(5). Parts of South East Asia and the Mediterranean region have high prevalence of alpha thalassemia[2]. In the Indian subcontinent, the incidence of β-thalassemia major and haemoglobin E is high[2]. Delhi has the greatest prevalence of β-thalassemia, followed by Sindh, Punjab, Tamil Nadu, South India, and Maharashtra[1,3].The prevalence of carrier β-thalassemia gene varies from 1 to 3% in Southern India to 3 to 15% in Northern India[6].Every year 10,000 children with thalassemia major are born in India, which constitute 10% of the total numbers in the world[5,7].Thalassemia can cause significant problems because these are inherited disorders, newborn screening and prenatal diagnosis are important in management of patients. Thalassemia and other haemoglobin variants are restricted to some particular geographical areas, caste, tribes and religion especially where marriages were confined to the same community and regions.

Formerly the distribution of thalassemia had been mainly limited to the areas from the Mediterranean basin through the Middle East and Indian subcontinent up to Southeast Asia so called "thalassemia belt"[8]. The probable explanation to this is following increased migration of people from one place to other. Inter-caste marriages are another reason for increase in the incidence and prevalence rate of hemoglobin pathies[2]. In South India, the prevalence of β-thalassemia trait was between 8.50 and 37.90% and β-Thalassemia Major was reported to be between 2.30 and 7.47%. Northern and Western Indian states had a higher thalassaemic burden. The carrier rate for β-thalassemia varies from 1-17% in India with an average of 3.2%[9]. The purpose of this study is to identify thalassemia and thalassaemia traits in anaemia patients undergoing blood transfusion and also to screen among their family members (first degree family members) who will be detected using HPLC.

Burden of Hemoglobinpathies in India

In India, β-Thalassemia is prevalent across the country, with an average frequency of carriers being 3-4%. A higher frequency has been observed in certain communities, such as Sind his, Punjabis, Gujarat is, Bengalis, Mahars, Kolis, Sara swats, Lohanas and Gaurs. HbS is highly prevalent in the tribal populations of Southern, Central and Western states reaching as high as 48% in some communities. HbE is common in the North Eastern states, and has a carrier frequency as high as 50%, in some areas. The only cure available for these children with thalassemia major is bone marrow transplantation (BMT) more appropriately called hematopoietic stem cell transplant (HSCT). Prevention is thus extremely cost effective rather than treatment of those who are affected[7]. Thalassaemic individuals have reduced MCV and MCH, an MCV of 72fL or less and MCH of less than 27pg are suggestive of a presumptive diagnosis of thalassemia[10].The RBC count in thalassemia is high even in the presence of anemia. This characteristic is useful in differentiating iron-deficiency anemia from thalassemia using the Mentzer Index, based on the MCV and RBC[11].

All β-thalassemia are characterized by an increase in HbA2 concentration[10]. HbF estimation is particularly important in diagnosis of carriers of δβ-thalassemia and hereditary persistence off et al hemoglobin (HPFH) as well as the major thalassemia syndromes[12].However, erroneously low levels may be obtained when HbF levels are very high[12].Patients with homozygous β-thalassaemia, referred to as β-thalassaemia major, are chronically anaemic, are transfusion-dependent, and have a lowlife expectancy when optimal care is unavailable. Persons heterozygous for β-thalassaemia, referred to as carriers, are asymptomatic and mildly anaemic[13].Serum ferritin is higher in β-thalassaemia heterozygote's. Iron overload can occur, particularly as a result of long continued inappropriate administration of iron[14].Doubly heterozygous state of HbE and B-thalassemia clinically characterized by thalassemia major[15].Family studiesalso play a crucial role in clinching the diagnosis in certain Problematic Cases.

Materials and Methods

A prospective cross-sectional study was done in the Department of Pathology at Mandya Institute of Medical Sciences for a period of 1 year 2 months from March 2023 to May 2024 after Institutional Ethical Committee approval adhering to the established protocol for human research. Study population was selected by stratified convenience sampling method with a sample size of 358 mainly including adults and children of both gender with anaemia who require blood transfusion and also among

their family members (first degree family members) who are detected in the current study. The objective of the study is to determine the occurrence of β-thalassemia and thalassemia traits in the patients undergoing blood transfusion and among the family members of the positively screened patients by analysing hematological parameters and HPLC. Inclusion criteria were all patients with anaemia (Hb <10 gm/dl) undergoing blood transfusion with MCV < 80fl and with Mentzer index less than 13 and first-degree family members of the patients who are detected in the current study by HPLC method. Exclusion criteria are patients who had undergone blood transfusion in the last 3 months, all patients with anemia (Hb <10gm/dl) undergoing blood transfusion with MCV >80fl. And all patients with anaemia (Hb <10gm/dl) undergoing blood transfusion with MCV < 80fl and with Mentzer index more than 13.

Methodology

The anticoagulated venous critical blood samples of the anemia patients collected prior to blood transfusion was analysed for Complete Blood Count (CBC) using a Coulter automated cell counter. Mentzer's index was calculated to rule out all Iron deficiency anemia [MI >13]. All samples with low Mentzer's index were subjected to peripheral smear examination and additional special tests such as reticulocyte count, and sickling tests in required cases to support the diagnosis. blood samples are refrigerated at 2-8°C (stable for 4 days) for batch wise BIO-RAD D-10 HPLC analysisusing β-thal short program. Additionally, a comprehensive correlation of clinical history, previous transfusion history, nutritional deficiency status, and biochemical investigations (such as serum ferritin levels) was conducted to differentiate between iron deficiency anaemia (IDA) and confirm the diagnosis. When abnormal haemoglobin was detected in a participant, their first-degree family members were also screened.

Sample Preparation

Stored samples were subjected to HPLC testing within 4 days of collection. Samples were allowed to reach room temperature (15-30°C) prior to analysis. Hemolysate was used in the course of the analysis. Whole blood samples (5µl in 1ml of buffer) are haemolyzed prior to injection. The prepared samples separated by the cation exchange cartridge using a phosphate ion gradient generated by mixing two buffers of different ionic strengths to elute the different haemoglobins. A sample report along with a chromatogram was generated for each sample. The test results were interpreted according to the graph and the acceptance criteria. The sample is then centrifuged at 2000-2500rpm to separate serum from the cells, and the samples were refrigerated at -20°C for subsequent serum ferritin analysis in required cases.

High Performance Liquid Chromatography

HPLC: BIORAD-D10

HPLC is one of the most important tools for accurate diagnosis of hemoglobinpathies and thalassemia.

Principle

High-Performance Liquid Chromatography (HPLC) separates positively charged hemoglobin (Hb) fractions based on their ionic interactions with a negatively charged stationary phase in a chromatography column. The β - thalassemia Short Program utilizes the principles of cation-exchange high pressure liquid chromatography in which a mixture of molecules with a net positive charge is separated into their components by absorption onto a negatively charged stationary phase in a chromatography column, followed by their elution on the with a mobile phase. Specifically, the β-thalassemia short program uses a 3cm×0.46cm cartridge packed with

a 5µm silica-based weak cation exchange material(10). Reference ranges provided by the manufacturer were used to determine normal values: HbA2 (2.0-3.9%) and HbF (up to 1.3%). Haemoglobins are classified into defined windows based on retention times. Peaks with retention times outside these windows are detected as unknown. This program has been designed to separate and quantify the hemoglobin in a 6 min run. When HbA1cis measured by HPLC using standard mode (including separation of individual hemoglobin fragments via ion exchange in a column of chromatography followed by its photometric quantification and calculation), variant hemoglobin can be detected if present, along with falseHbA1c result in most of the cases. This false estimation is due to the co-elution of hemoglobin variant with haemoglobin fraction other than HbA1c. Hemoglobin variants thus interfere with the quantification of HbA1c, HbA2, and HbF, thus generate abnormal chromatograms. Many cases with variant hemoglobin are asymptomatic and often remain undetected throughout life. Nowadays an increasing number of people are being detected to have hemoglobin variants especially when their blood is tested for HbA1cby the HPLC method [16].

During the interpretation of chromatograms, nutritional anaemia was taken into account. A low level ofHbA2 may be induced by iron deficiency, thus masking β-thalassaemia trait. Similarly, cobalamin or folate deficiencymay raise HbA2 level leading to a false diagnosis of thalassaemia trait[17].

HbA2 values between 3.5 and 3.9% are considered equivocal, need careful assessment, detailed evaluation and should be repeated before a diagnosis of β-TT can be made. Subjects with HbA2 level between 4.0 and 9.0 % were diagnosed as β-TT. Haemoglobin A2 values in α-thalassaemia trait are usually below 2.5%. Some types of β thalassaemia trait have normal haemoglobin A2 values.

Table 1: Manufacturer Assigned Windows - Hbf Values

Hbf Values	Interpretation
Upto 5%	Normal variation, pregnancy,
5-15%	β-thal trait
15-45%	β-thal homozygous, HPHF heterozygous
>90%	HPHF Homozygous, β-thal homozygous

Table 2: Manufacturer Assigned Windows-Hba2 Values

Hba2 Values	Interpretation
< 3.5%	Normal, alpha thal
3.5 – 3.9%	Beta-thal, Iron deficiency
3.9 – 9%	B-thal trait
> 9%	HbE, Hb Lepore, HbD Iran

Table 3: Manufacturer Assigned Windows For Co-Eluting Hemoglobin Variants In Retention Time For Bio - Rad D10

Peak Name	RT (min)	Hemoglobin
Pre- integration	< 0.63	Hb H, Hb Barts
F	0.96-1.20	Hb F

P2	1.20-1.40	HbA1C, Hb hope
P3	1.40-1.90	Hb J Meerut
A0	1.93-3.10	Hb A
A2	3.3-3.90	HbA2, Hb Lepore, Hb D- Iran, Hb E
D	3.90-4.30	Hb D Punjab, G-Philedelphia
S	4.3-4.7	Hb Q, A2’
Unknown	4.7-4.9	Hb Q
Q	4.9-5.30	Hb C, Hb Constant

Analysis

Data were entered in MS Excel worksheet and analysed with Statistical Package for the Social Sciences trail version 20 statistical software. The descriptive statistics of qualitative data was measured using frequency and percentage and quantitative data was analysed using Mean, Median and Standard deviation. Data will be represented in the form of tabulations and graphical representation such as pie chart and bar diagrams.

Statistics and Results

Table 4: Frequency distribution of age in study population

Age	Male(yrs)	Frequency	Female(yrs)	Frequency	Total
Minimum	1	16	1	15	31
Maximum	80	1	90	1	2

The distribution of age in the study participants ranges from 1year to 90 years. The overall mean age of study participants was 30.19years.

Table 5: Mean and standard deviation of study population

Age	Male	Female	Total
Mean	31	29	45.5
Standard deviation	24.58	19.16	21.21

The mean age of study participants among males and females were 31years and 29years respectively. The mean age of the study population was 45.5±21.21 years.

Table 6: Distribution of Study Population According to Age and Sex

Age (yrs)	Male	Female	Total (%)
1-10	38 (10.61%)	39 (10.89%)	77 (21.51%)
11-20	14 (3.91%)	32 (8.94%)	46 (12.85%)
21-30	10 (2.79%)	62 (17.32%)	72 (20.11%)
31-40	19 (5.31%)	42 (11.73%)	61 (17.04%)
41-50	14 (3.91%)	21 (5.87%)	35 (9.78%)
51-60	8 (2.23%)	21 (5.87%)	29 (8.10%)

61-70	15 (4.19%)	11 (3.07%)	26 (7.26%)
>70	8 (2.23%)	4 (1.12%)	12 (3.35%)
Total	126 (35.2%)	232 (64.8%)	358 (100 %)

Majority of the study participants were within 21-30yrs in females 62(17.32%) and within 1-10yrs 38(10.61%) in males. The distribution of subjects among different age groups is slightly larger proportion in the paediatric age group of 1–10 years (21.51%).

The anaemia prevalence was notably higher among women compared tomen in all age groups except in elderly age group of more than 60yrs. The anaemia in elderly age group of more than 60yrs was 23 (6.42%) in males and 15(4.19%) in females respectively.

The lines converge in older ages, as the prevalence rises in males and falls in females after middle-ages. In women, the anaemia prevalence peaked in the 21–30 years, whereas in men the prevalence peaked at 1-10years of age.

Figure 1: Distribution of study population according to age and sex

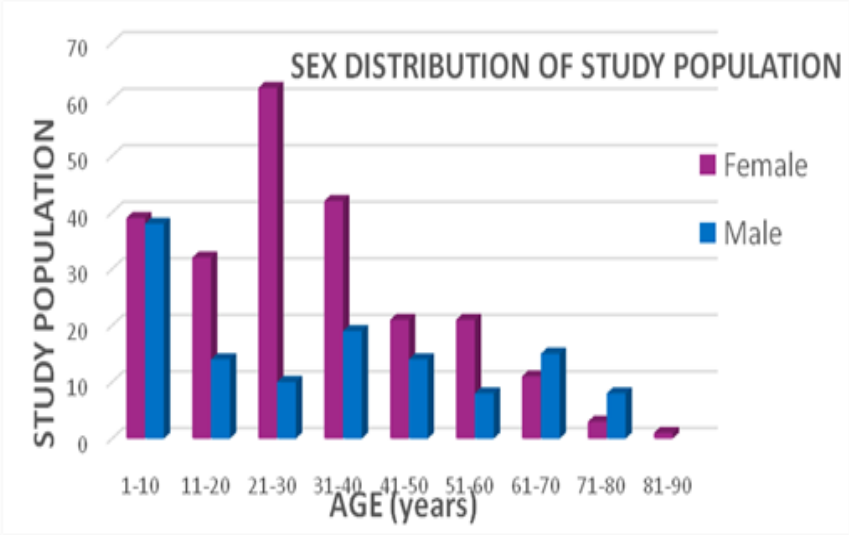
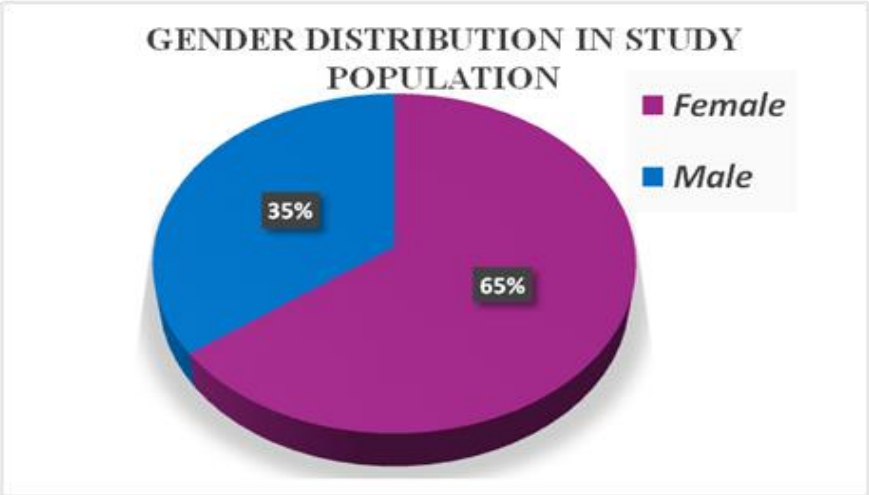


Figure 2: Sex distribution of study population



Male to Female ratio: 1:1.8

Table 7: Distribution of study population according to age group

Age group	Paediatric (1-18yrs)	Adult (>18yrs)
Males	(13.41%)	(21.79%)
Females	(18.44%)	(46.37%)
	(31.85%)	(68.16%)

Figure 3: Gender distribution of study population into adult group.

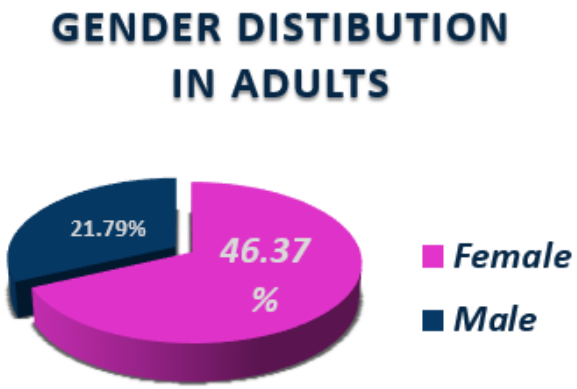


Figure 4: Gender distribution of study population in and group

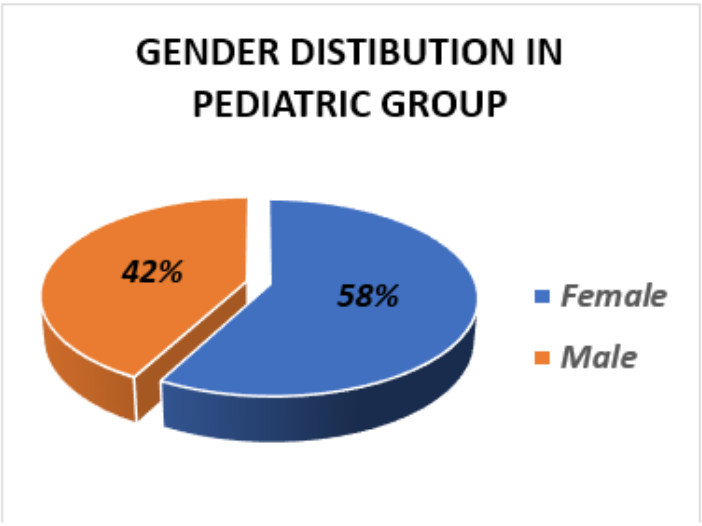


Table 8: Distribution of study population according to religion

Religion	Male	Female	Total (%)
Christians	7(1.96%)	8(2.23%)	15(4.19%)
Hindus	109(30.45%)	201(57.82%)	316(88.27)
Muslims	10(2.79%)	17(4.75%)	27(7.54%)
Total	126(35.20)	232(64.80)	358 (100%)

Hindus accounted for almost majority of the participants (88.27%), followed by Muslims 27 (7.54%) and Christians15 (4.19%).

Figure 5: Distribution of study population in each religion

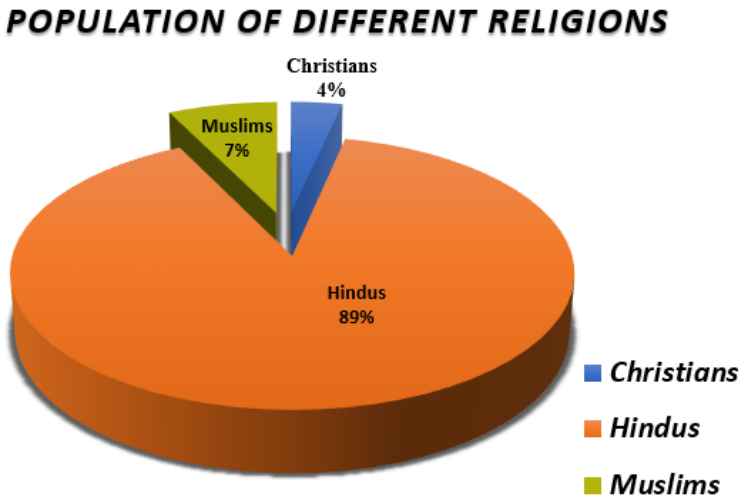


Table 9: Age &Sex-wise distribution of hemoglobin level in study population

Haemoglobin value	Hb < 7g/dl (Severe anaemia)		Hb 7-10g/dl (Moderate anaemia)		Total (%)
Age(years)	M (%)	F (%)	M (%)	F (%)	
1-10	7(1.96%)	6(1.68%)	31(8.66%)	33(9.22%)	77(21.51%)
11-20	2(0.56%)	14(3.91%)	12(3.35%)	18(5.03%)	46(12.85%)
21-30	1(0.28%)	7(1.96%)	9(2.51%)	55(15.36%)	72(20.11%)
31-40	2(0.56%)	8(2.23%)	17(4.75%)	34(9.50%)	61(17.04%)
41-50	1(0.28%)	1(0.28%)	13(3.63%)	20(5.59%)	35(9.78%)
51-60	2(0.56%)	5(1.40%)	6(1.68%)	16(4.47%)	29(8.10%)
>60	7(1.96%)	2(0.56%)	16(4.47%)	13(3.63%)	38(10.61%)
Total	22(6.15%)	43(12.01%)	104(29.05%)	189(52.79%)	358(100%)

The highest value of Hb concentration in the overall study population is 10gm/dl and the lowest value of Hb concentration is 4g/dl. Majority of the study population fall between the age of 1-10years followed by 21-30years of which the female population predominate. The mean Hb concentration of the study population was 8.46±1.34g/dL.

Figure 6: Distribution of hemoglobin level (<7gm/dl) in study population

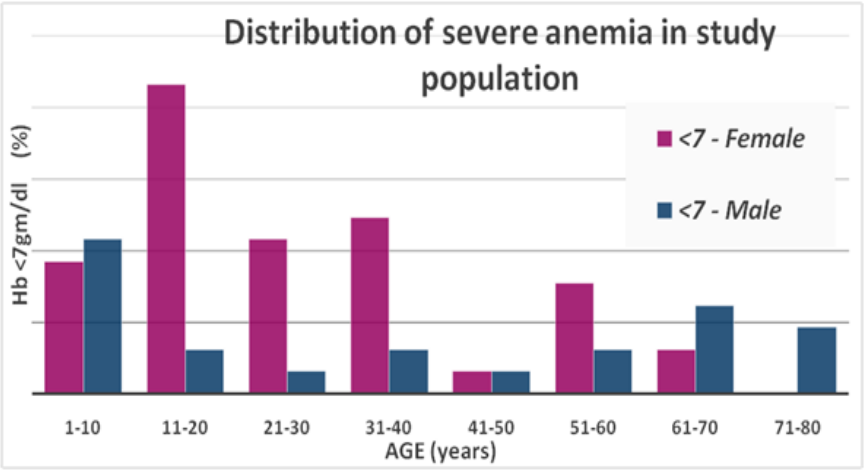


Figure 7: Distribution of hemoglobin level (7-10gm/dl) in study population.

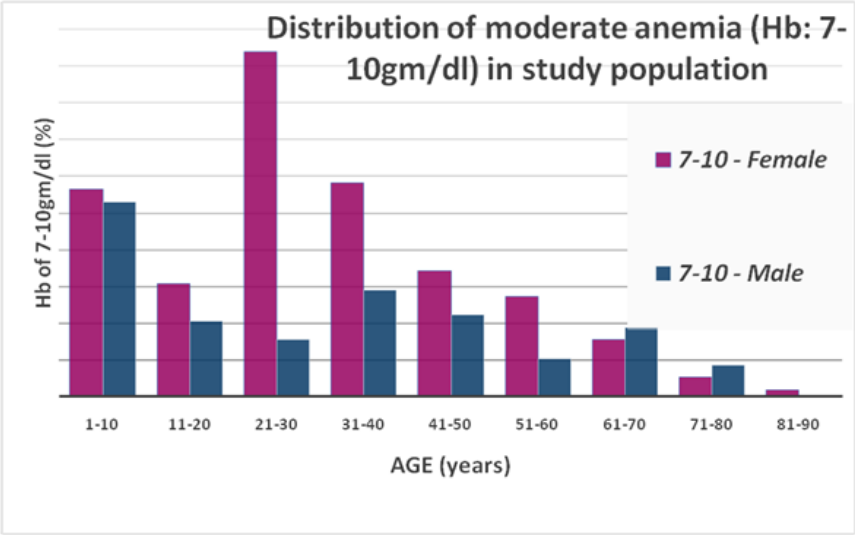


Figure 8: Distribution of severe and moderate anemia in males

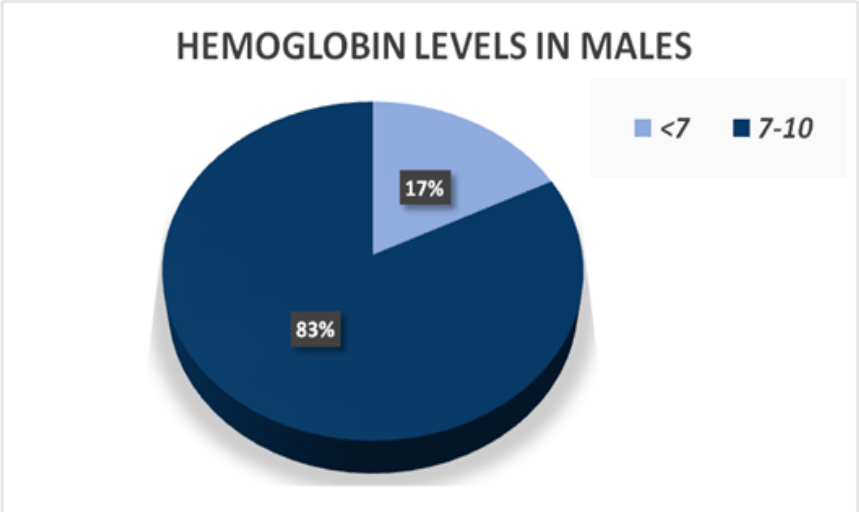


Figure 9: Distribution of moderate and severe anemia in females

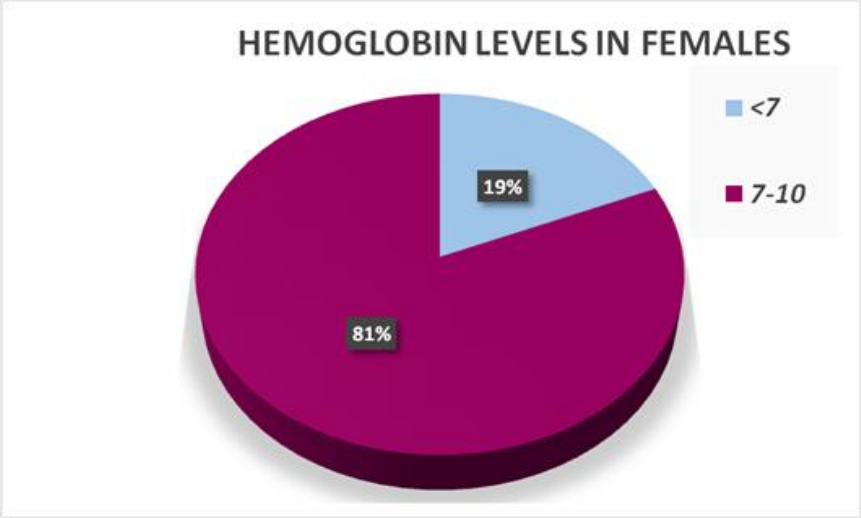


Figure 10: Distribution of severe and moderate anemia in study population.

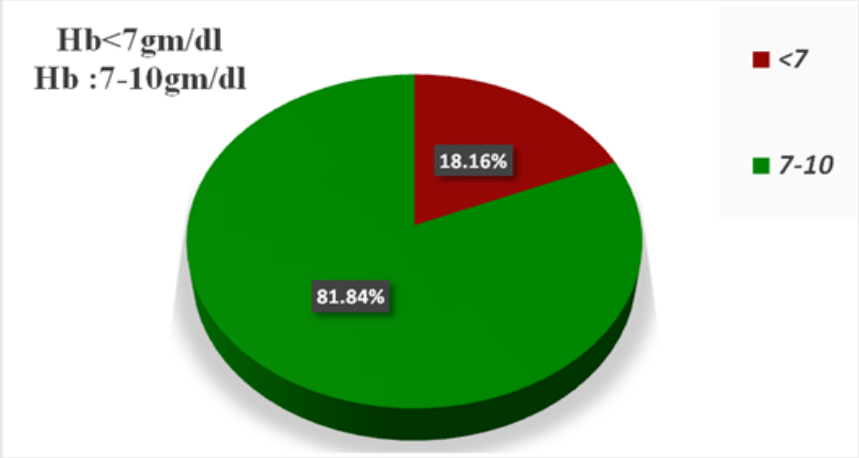


Table 10: RBC count values in study population

RBC Count (x10^6/micro liter)	Frequency		Total (%)
	Males (%)	Females (%)	
<4.00	-	1(0.28%)	1(0.28%)
4.00-5.50	46(12.85)	52(14.53)	98(27.37)
>5.50	80(22.35)	179(50.0)	259(72.3)
Total	126(35.20%)	232(64.8)	358(100)

The RBC count ranged from 3.6 to 10.43 million/ $mm^3$with a meanof5.3million/mm^3. Majority (72.35%) of the participants had increased RBC count of > 5.50x10^6 micro liter followed by normal count 4.00-5.50 x10^6 micro liter (27.37%) and <4.0x10^6 micro liter (0.28%).

Table 11: Age &Sex-wise distribution of Mentzer index in study population

Age (years)	Mentzer Index < 13		Total (%)
	M (%)	F (%)	
1-10	38(10.61%)	39(10.89%)	77(21.51%)
11-20	14(3.91%)	32(8.94%)	46(12.85%)
21-30	10(2.79%)	62(17.32%)	72(20.11%)
31-40	19(5.31%)	42(11.73%)	61(17.04%)
41-50	14(3.91%)	21(5.87%)	35(9.78%)
51-60	8(2.23%)	21(5.87%)	29(8.10%)
>60	23(6.42%)	15(4.19%)	38(10.61%)
Total	126(35.20%)	232(64.80%)	358(100%)

The Mentzer index ranged from 4.6 to 12.9 with a mean value of 11.32±1.36. Majority 42(11.73%) of the female participants belong to the age group of 31-40years. Among male population, majority38(10.61%) belong to paediatric age group ranging from 1-10years.

Figure 11: Sex-wise distribution of Mentzer index in study population

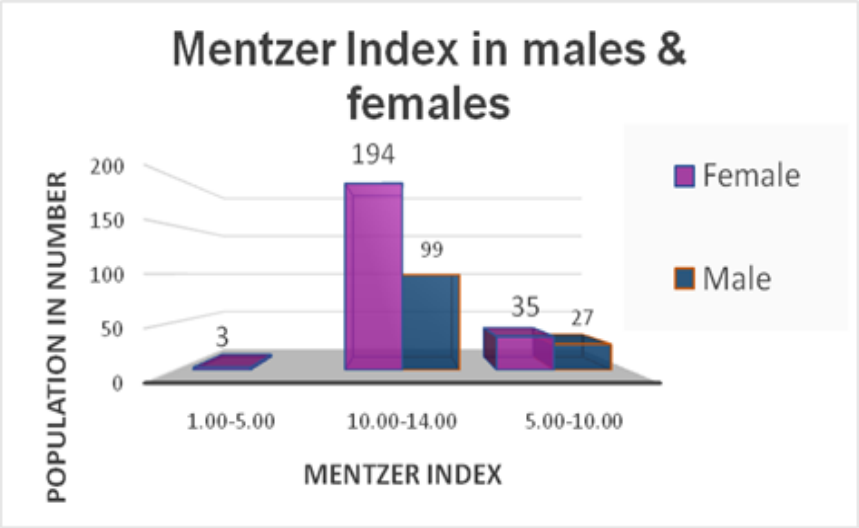
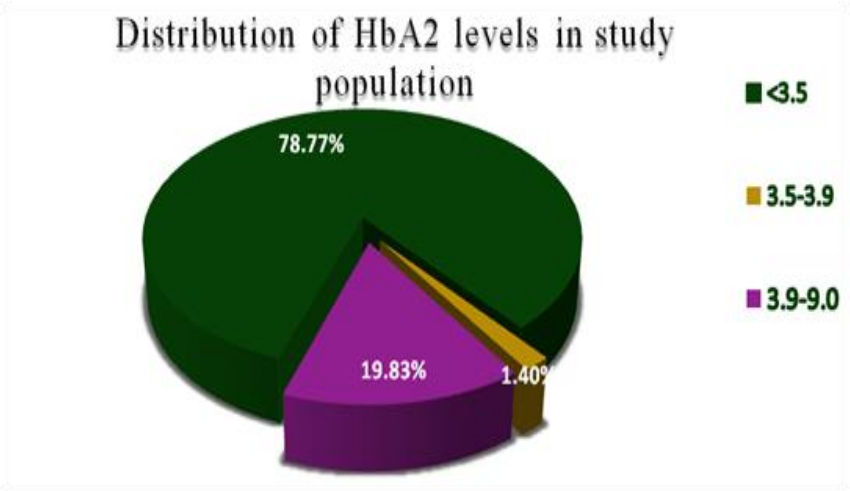


Table 12: HbA2 (%) in study population

HbA2 (%)	Total number		Total (%)
	M	F	
<3.5%	88(24.58%)	194(54.19%)	282 (78.77)
3.5 -3.9%	1(0.28%)	4(1.12%)	5(1.40)
3.9 – 9%	37(10.34%)	34(9.50%)	71(19.50)
>9%	-	-	-
Total	126(35.2%)	232(64.80%)	358

Figure 12: Distribution of HBA2 (%) in overall study population



Majority of the study population 282(78.77%) showed normal HbA2values. Five cases showed equivocal values. Rest 71(19.50%) cases showed increased HbA2 values ranging from 3.9-9%.

Results of HPLC Study

Table 13: Hemoglobinopathies detected in the study

Final Diagnosis	N	(%)
Normal Hb pattern (Nutritional deficiency anemia)	281	78.49%
β thal trait (heterozygous)	72	19.83%
β thalassemia (homozygous)	2	0.56%
Delta β-thalassemia	1	0.28%
HbSβ thalassemia	1	0.28%
HbD-Punjab heterozygous	1	0.28%

Out of 358 cases 281 cases showed normal hemoglobin pattern and rest 77 cases showed abnormal hemoglobin pattern as given in the table above. β thalassemia trait (n=72, 19.83%) was the commonest abnormality detected in the study.

Table 14: Mean & standard deviation of hemoglobin pathies

Age	Male	Female	Total
Mean	33.35	38.05	52.37
Standard deviation	22.61	19.42	21.01

The mean and standard deviation fage among males and females were 33.35±22.61years and 38.05±19.42years respectively. The overall mean and standard deviation fage among study participants showing abnormal hemoglobin pattern is52.37±21.01years.

Male to Female ratio: 1:1.08

Table 15: Age & Sex - wise distribution of various hemoglobin pathies

Age (years)	Male (%)	Female (%)	Total
1-10	7(9.09%)	2(2.6%)	9(11.69%)
11-20	8(10.39%)	2(2.6%)	10(12.99%)
21-30	2(2.6%)	12(15.58%)	14(18.18%)
31-40	7(9.09%)	9(11.69%)	16(20.78%)
41-50	4(5.19%)	6(7.79%)	10(12.99%)
51-60	2(2.60%)	4(5.19%)	6(7.79%)
>60	7(9.09%)	5(6.49%)	12(15.58%)
Total	37(48.05%)	40(51.95%)	77(100%)

Majority of the study participants were within 21-30yrs in females 12(15.58%) and within 11-20yrs 8(10.39%) in males. β thalassemia trait (n=73, 20.11%) was the commonest abnormality detected in the overall female population.

Figure 13: Age & Sex distribution of hemoglobinpathies

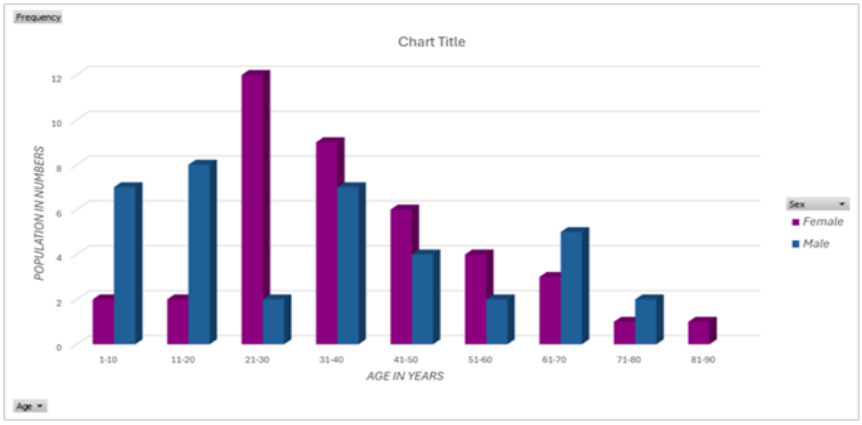


Figure 14: Gender distribution of Hemoglobinopathies

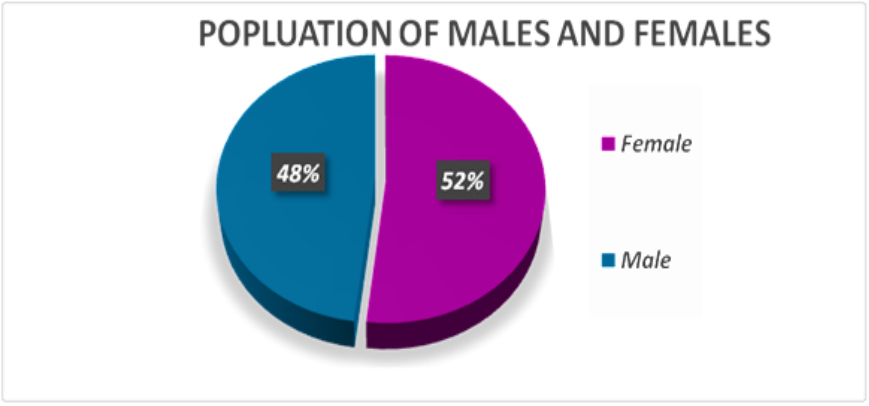


Table 16: Distribution of study population according to age group

Age group	Males	Females
Paediatric (1-18yrs)	26(33.77%)	36(46.75%)
Adult (>18yrs)	11(14.29%)	4(5.19%)
	37(48.05%)	40(51.95%)

Figure 15: Distribution of adult & paediatric Hemoglobino pathies

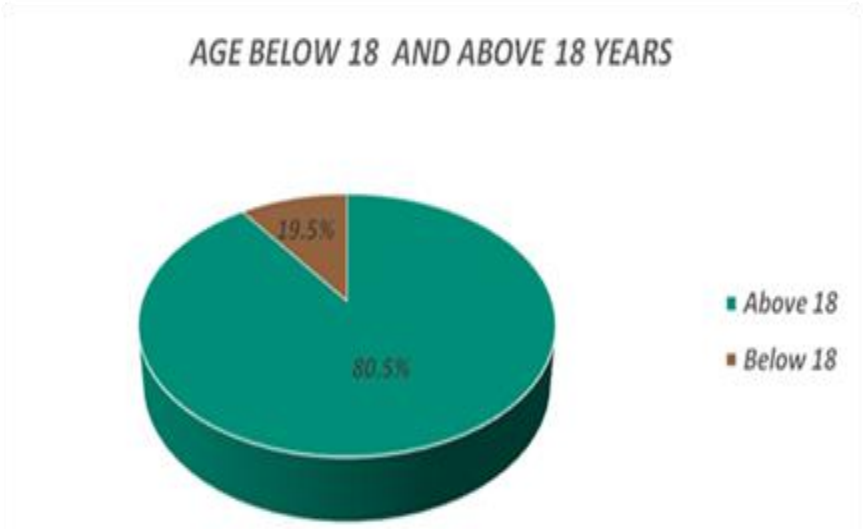


Figure 16: Distribution of adult & paediatric hemoglobinpathies in females

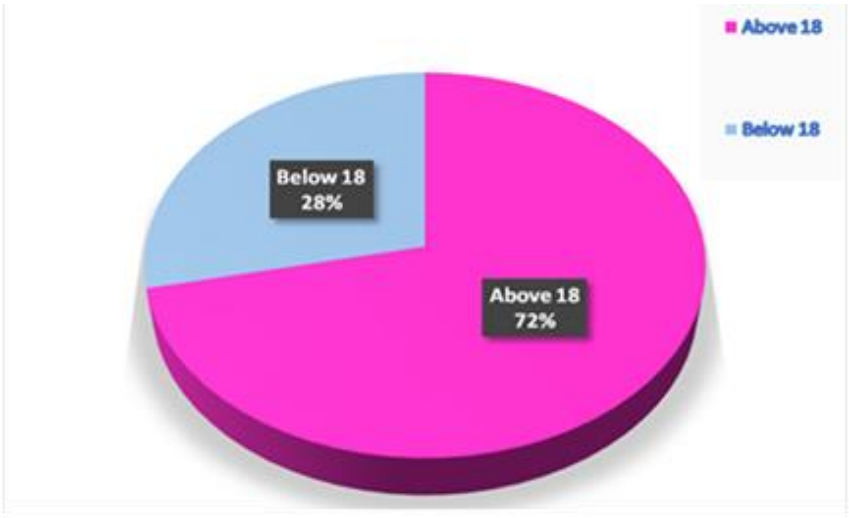


Figure 17: Distribution of adult & pediatric hemoglobinpathies in males

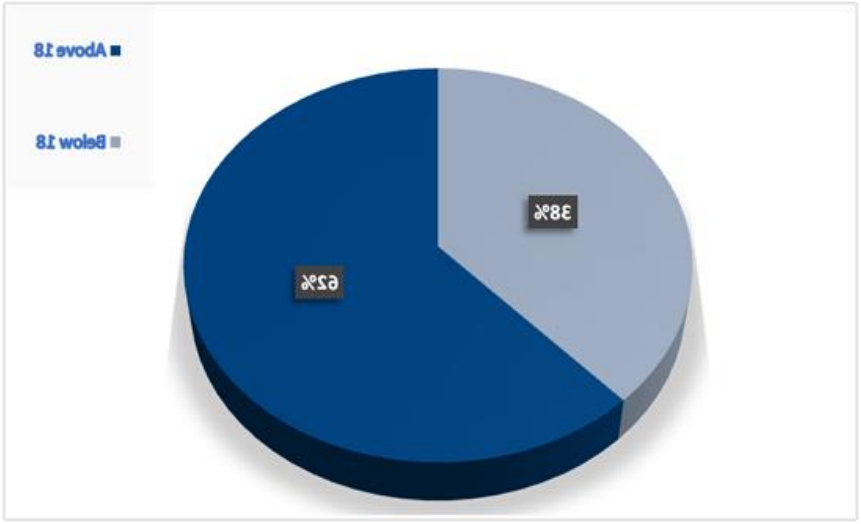


Table 17: Distribution of hemoglobinpathies according to religion

Religion	Male	Female	Total (%)
Christians	1(1.30%)	1(1.30%)	2(2.60%)
Hindus	35(45.45%)	36(46.75%)	71(92.211%)
Muslims	1(1.30%)	3(3.90%)	4(5.19%)
Total	37(48.05%)	40(51.95%)	77

Hindus accounted for almost majority of the cases 71 (92.211%), followed by Muslims 4(5.19%), and Christians 4 (5.19%).

Figure 18: Distribution of hemoglobin pathies in each religion

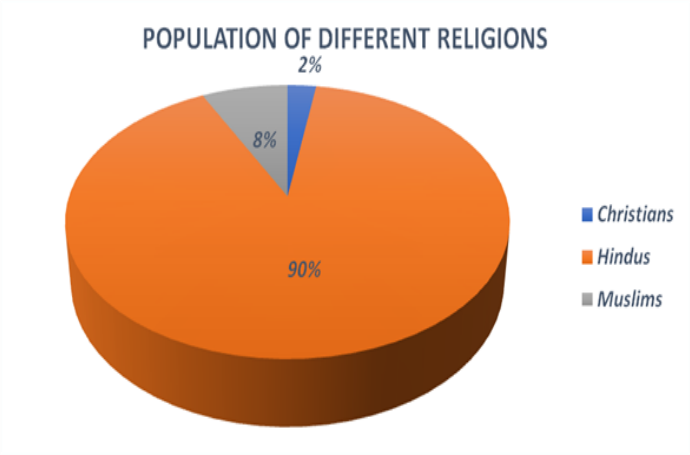


Table 18: Distribution of hemoglobinopathies according to anemia

Hb (g/dl)	Male (%)	Female (%)	Total (%)
<7	-	(3.90%)	(3.90%)
7-10	(48.05%)	(48.05%)	(96.10%)
	(48.05%)	(51.95%)	(100%)

Figure 19: Distribution of hemoglobin levels in hemoglobino pathies

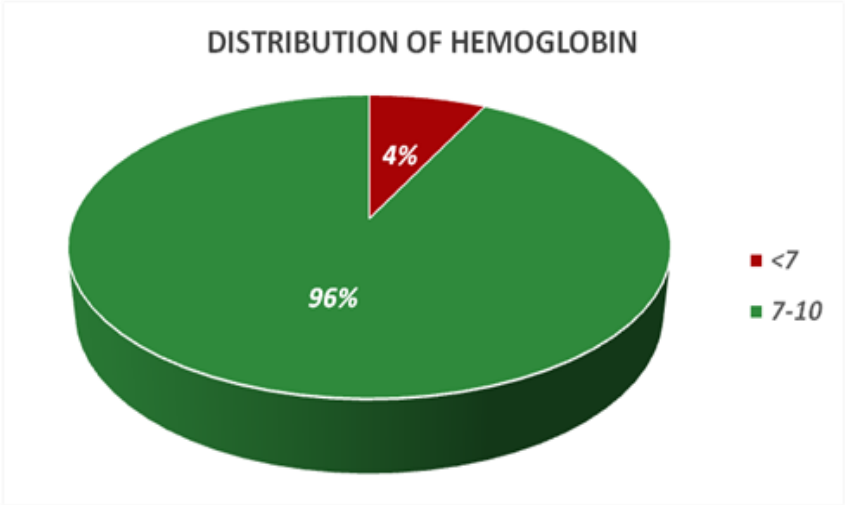


Table 19: Sex-distribution of RDW cv among males and females.

Rdwcv (%)	Males	Females	Total
Low (<11)	37(48.05)	39(50.65%)	76(98.70%)
Normal 11-15	-	1(1.30%)	1(1.30%)
High >15	-	-	-
Total	37(48.05)	40(51.95%)	77

Table 20: Sex distribution of RBC count in hemoglobin pathies

RBC Count (x10^6/ micro liter)	Frequency		Total
	Males (%)	Females (%)	(%)
<4.00	-	-	-
4.00-5.50	12(15.58%)	21(27.27%)	33(42.86%)
>5.50	25(32.47%)	19(24.68%)	44(57.14%)
Total	37(48.05%)	40(51.95%)	77(100%)

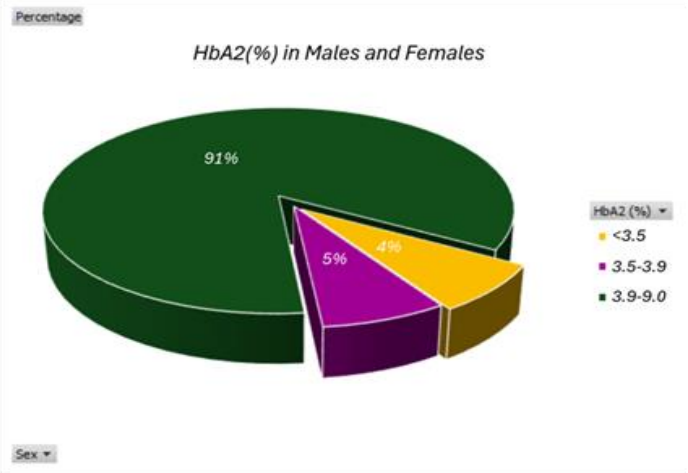
Among the 77 cases, a majority (44, or 57.14%) had high Red Blood Cell (RBC) counts, with females being the predominant group. Normal RBC counts followed, while notably, no cases had low RBC count.

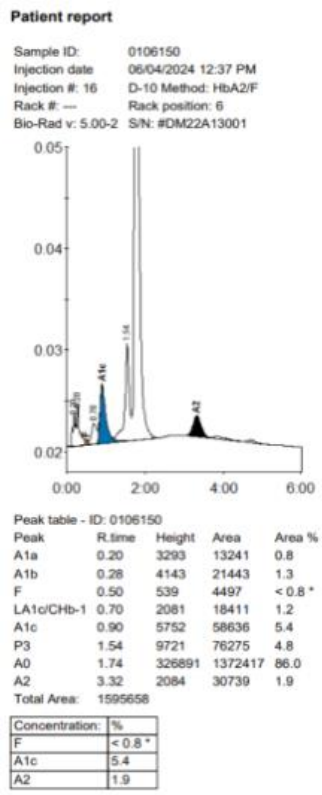
Table 21: Sex-wise distribution of HBA2 (%) in study population

HbA2 (%)	M	F	Total (%)	Interpretation
<3.5%	-	3(3.90%)	3(3.90%)	Iron deficiency anemia
3.5 – 3.9%	1(1.30%)	3(3.90%)	4(5.19%)	beta- TT equivocal
3.9 – 9%	36(46.75%)	34(44.16%)	70(90.91%)	beta-TT heterozygous
>9%	-	-	-	thalassemia, HPFH
Total	48.0%	51.95%	100%	

Among the 77 cases, 3 (3.9%) had normal HbA2 values (<3.5%). The majority, 70 cases (90.91%), exhibited HbA2 values between 3.9-9%, indicative of beta-thalassemia trait. Additionally, 4 cases (5.19%) showed equivocal results, while no cases had HbA2 values greater than 9%.

Figure 20: Distribution of HBA2 (%) in overall study population





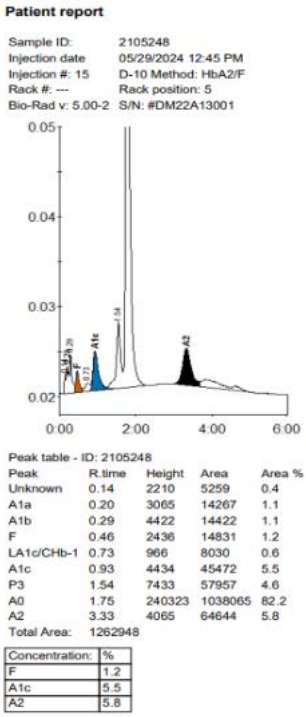
Patient report

Sample ID: 0106150
Injection date 06/04/2024 12:37 PM
Injection #: 16 D-10 Method: HbA2/F
Rack #: --- Rack position: 6
Bio-Rad v: 5.00-2 S/N: #DM22A13001

Peak table - ID: 0106150

Peak	R.time	Height	Area	Area %
A1a	0.20	3293	13241	0.8
A1b	0.28	4143	21443	1.3
F	0.50	539	4497	< 0.8 *
LA1c/CHb-1	0.70	2081	18411	1.2
A1c	0.90	5752	58636	5.4
P3	1.54	9721	76275	4.8
A0	1.74	326891	1372417	86.0
A2	3.32	2084	30739	1.9
Total Area:		1595658		

Concentration:	%
F	< 0.8 *
A1c	5.4
A2	1.9



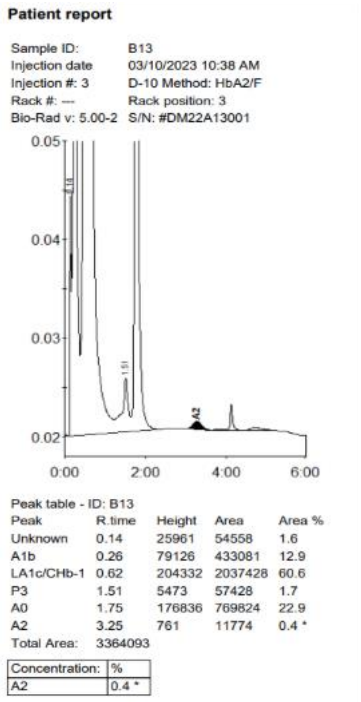
Patient report

Sample ID: 2105248
Injection date 05/29/2024 12:45 PM
Injection #: 15 D-10 Method: HbA2/F
Rack #: --- Rack position: 5
Bio-Rad v: 5.00-2 S/N: #DM22A13001

Peak table - ID: 2105248

Peak	R.time	Height	Area	Area %
Unknown	0.14	2210	5259	0.4
A1a	0.20	3065	14267	1.1
A1b	0.29	4422	14422	1.1
F	0.46	2436	14831	1.2
LA1c/CHb-1	0.73	966	8030	0.6
A1c	0.93	4434	45472	5.5
P3	1.54	7433	57957	4.6
A0	1.75	240323	1038065	82.2
A2	3.33	4065	64644	5.8
Total Area:		1262948		

Concentration:	%
F	1.2
A1c	5.5
A2	5.8



Patient report

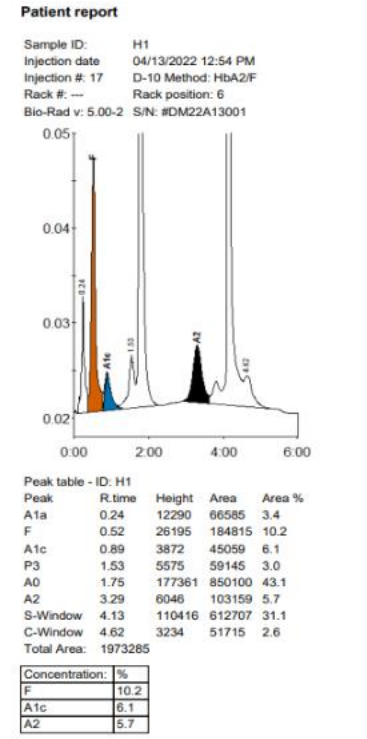
Sample ID: B13
Injection date 03/10/2023 10:38 AM
Injection #: 3 D-10 Method: HbA2/F
Rack #: --- Rack position: 3
Bio-Rad v: 5.00-2 S/N: #DM22A13001

Peak table - ID: B13

Peak	R.time	Height	Area	Area %
Unknown	0.14	25961	54558	1.6
A1b	0.26	79126	433081	12.9
LA1c/CHb-1	0.62	204332	2037428	60.6
P3	1.51	5473	57428	1.7
A0	1.75	176836	769824	22.9
A2	3.25	761	11774	0.4 *
Total Area:		3364093		

Concentration:	%
A2	0.4 *

Fig 21: Normal Chromatograph. Fig 22: Chromatograph showing B-thal Trait. Fig 23: Chromatograph showing – Thalassemia



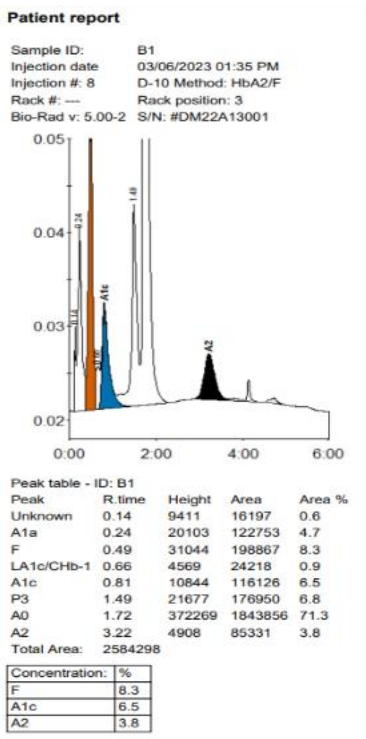
Patient report

Sample ID: H1
Injection date 04/13/2022 12:54 PM
Injection #: 17 D-10 Method: HbA2/F
Rack #: --- Rack position: 6
Bio-Rad v: 5.00-2 S/N: #DM22A13001

Peak table - ID: H1

Peak	R.time	Height	Area	Area %
A1a	0.24	12290	66585	3.4
F	0.52	26195	184815	10.2
A1c	0.89	3872	45059	6.1
P3	1.53	5575	59145	3.0
A0	1.75	177361	850100	43.1
A2	3.29	6046	103159	5.7
S-Window	4.13	110416	612707	31.1
C-Window	4.62	3234	51715	2.6
Total Area:		1973285		

Concentration:	%
F	10.2
A1c	6.1
A2	5.7



Patient report

Sample ID: B1
Injection date 03/06/2023 01:35 PM
Injection #: 8 D-10 Method: HbA2/F
Rack #: --- Rack position: 3
Bio-Rad v: 5.00-2 S/N: #DM22A13001

Peak table - ID: B1

Peak	R.time	Height	Area	Area %
Unknown	0.14	9411	16197	0.6
A1a	0.24	20103	122753	4.7
F	0.49	31044	198867	8.3
LA1c/CHb-1	0.66	4569	24218	0.9
A1c	0.81	10844	116126	6.5
P3	1.49	21677	176950	6.8
A0	1.72	372269	1843856	71.3
A2	3.22	4908	85331	3.8
Total Area:		2584298		

Concentration:	%
F	8.3
A1c	6.5
A2	3.8

Fig 24: Chromatograph showing HbSβ-thal Heterozygous

Fig 25: Chromatograph showing HbD Punjab Trait

Fig 21: Chromatograph shows HbA2 Retention Time at 3.32 and Area Under Curve –1.9%.

Fig 22: Chromatograph shows HbA2 Retention Time at 3.33 and Area Under Curve – 5.8%. Suggestive of β-thalassemia heterozygous.

Fig 23: Chromatograph shows a pre-integration peak with HbF of Retention time 0.62 & AUC-60.6% Suggestive of -thalassemia major.

Fig 24: Chromatograph shows HbA2 Retention Time at 3.37 and Area Under Curve - 2.3%. An unknown peak Retention Time at 3.94 and Area Under Curve - 35.5%. Suggestive HbD Punjab heterozygous.

Fig 25: Chromatograph shows HbA2 Retention Time at 3.33 and Area Under Curve – 5.8%. Suggestive of HbS β-thalassemia heterozygous.

We analysed the various RBC parameters and percentage of Hb fraction in various abnormal hemoglobinopathies encountered in this study. The following tables show haematological profile and Hb fractions in various hemoglobinopathies.

Table 23: RBC indices of various hemoglobinopathies

Hemoglobin pattern (n)	Hb (g/dl) mean ± SD	RBC $x10^{12}L$ mean± SD	M.I (%) mean ± SD	HbA2 (fl) mean± SD	HbF (pg) mean± SD	Unknown peaks
β-thalassemia (2)	9.45±0.07	6.42±0.50	9.4±2.55	3.95±0.21	24.9±23.5	-
β-thalassemia trait (72)	9.25±0.76	5.77±1.08	10.6±1.65	4.97±0.50	1.1±0.52	-
δ β-thalassemia (1)	9.5	5.62	11.4	1.7	19.6	-
HbD Punjab (1)	6.3	4.4	11.8	2.6	<0.8	35.5
HbSβ thalassemia (1)	10	5.4	12	5.7	10.2	31.1

We analysed the various RBC parameters and percentage of Hb fraction in various abnormal hemoglobinopathies encountered in this study. The following tables show haematological profile and Hb fractions in various hemoglobinopathies.

Table 22: Haematological profile with various hemoglobinopathies

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β-thalassemia (2)	9.45±0.07	6.42±0.50	9.4±2.55	3.95±0.21	24.9±23.5	-
β-thalassemia trait (72)	9.25±0.76	5.77±1.08	10.6±1.65	4.97±0.50	1.1±0.52	-
δ β-thalassemia (1)	9.5	5.62	11.4	1.7	19.6	-
HbD Punjab (1)	6.3	4.4	11.8	2.6	<0.8	35.5
HbSβ thalassemia (1)	10	5.4	12	5.7	10.2	31.1

Table 23: RBC indices of various hemoglobinopathies

Hemoglobin pattern	Cases (n)	PCV (%) mean ± SD	MCV (fl) mean ± SD	MCH (pg) mean ± SD	MCHC (g/ dl) mean ± SD	RDW (%) mean ± SD
β-thalassemia	2	38.25±4.71	60±11.31	17.15±2.33	28.8±1.55	23±7.49
β-thalassemia trait	72	33.57±3.70	60.29±4.40	17.25±1.70	28.52±1.65	20.31±3.62
δβ-thalassemia	1	36.3	64.6	18.7	28.9	22.1
HbD Punjab	1	22.7	52	14.4	27.7	25.6
HbSβ thalassemia	1	34.86	65	18.5	28.6	19.2

Discussions

HPLC has been shown to be a sensitive, specific, and reproducible alternative to electrophoresis. With automation and quantitative power, it appears to be a sensitive and accurate technique for direct identification and quantification of normal and abnormal haemoglobin fractions[1].

In this study, apart from β- thalassaemia and β-thalassaemia trait additional variants were encountered such as δβ- thalassaemia trait, HbD-Punjab and HbSβ thalassemia. Along with the cases of homozygous β-thalassaemia, heterozygous β- thalassaemia and β-thalassaemia traitvariants such as HbS-β-thalassemia, HbD-Punjab trait is also seen. The percentage of variant Hb can generally be the initial predictor whether the detected variant is an α-or β-variant. Apart from the various variant haemoglobins, the percentages of Hb F and HbA2 also have important diagnostic implications. There are several causes of borderline HbA2 including β-thalassaemia trait with silent mutations, α-triplication and IDA(1). Careful and detailed complete blood count and peripheral blood smear examination (PBS) is very essential in the diagnosis of various haemoglobin disorders (2).In an α-thalassemia trait when the HPLC can be absolutely normal with perhaps a low normal or low HbA2, careful correlation of the microcytosis which is unexplained by Blood transfusion therapy or iron deficiency can lead us to suspect α-thalassemia disorder which can be confirmed by DNA analysis(2).The patients with IDA (with a normal HPLC) showed significantly lower levels of HbA2 than the patients with normal iron profile as reported by others also[3].

Although previous studies suggest a significant reduction in HbA2 levels in thalassaemia trait patients associated with IDA(1,3). HbD-Punjab produces a significant sickling disorder when present in a double heterozygous HbD-HbS form; whereas HbD-Iran is clinically benign(1). The misdiagnosis of HbD-Iran as HbD-Punjab based solely on Hb- electrophoresis or as HbE based solely on Hb- HPLC, where the subtle difference in %Hb or retention time is disregarded, may lead to incorrect genetic counseling in addition to undue anxiety for the family[1].

Comparison with Other Studies

The present study included 358 anemia patients who was advised to undergo blood transfusion of which 77 abnormal hemoglobin variants were detected.

Table 24: Comparison of Sample Size of Various Studies

Study	Total study population	Total detected Hemoglobinopathies
Present study	358	77
Singh et al(4)	100	51
Shah et al(2)	467	70
Bhalodia et al (5)	500	43
Diane et al(6)	622	181
Rao et al(1)	800	247
Chakma et al(7)	945	618
Mondal et al(8)	958	264
Banerjee et al(9)	1048	604
Garje et al (10)	1260	600
Goswami et al(11)	1872	890
Sachdev et al(12)	2600	327
Shrivastav et al (13)	7261	1615
Mondal SK et al(14)	119336	14532

The study done by Singh et al had the least study population while that by Mondal SK et al had the highest study population (14) (4).

Table 25: Comparison of Gender Distribution with Other Studies

Gender	Present study	Banerjee et al(9)	Goswami et al(11)
Males	35.2%	63.98%	54.4%
Females	64.8%	36.02%	45.4%

The present study consisted of more females (64.8%) than males (35.2%), whereas the studies conducted by J Banerjee et al (9)and Goswami et al(11) showed male predominance of distribution. This can be attributed to the fact that most of the patients in the study are from the Mandya and around the Mandya region are anaemic females.(10)

Table 26: Comparison of Hemoglobinopathies Among Different Studies

Our Study	Our study	Chakma et al.	Garje et al	Bhalodia et al	Shrivastav et al	Singh et al
β-thalassemia	0.56%	-	9.9%	1%	4.02%	4%
β -TT	20.11%	11.0%	38.73%	26%	11.55%	42%
Sickle cell trait	-	36.3%	18.01%	6%	2.95%	-
Sicklecell disease	-	13.1%	6.3%	-	1.17%	-
Sβ heterozygous	0.28%	4.7%	14.41%	-	-	-
HbE	-	-	6.3%	1%	-	2%
HPFH	-	-	-	1%	0.11%	3%

In present study 77 abnormal hemoglobin fractionswere detected on HPLC which account to 21.8% of the total study population of which 71(20.1%) cases were diagnosed as thalassemia trait, 2 cases (0.56%) as β-thalassemia major. Other

variants detected included δβ- thalassemia, HbD-Punjab and HbSβ-thalassemia. β-thalassemia trait was most frequently detected hemoglobinopathy in all age groups and the remaining 281(78.2%) cases had a normal HPLC pattern. Since undiagnosed cases were excluded in the study the prevalence of β-thalassemia is less compared to the study. It also showed that the MCV and MCH levels were lower in hemoglobinopathy cases with coexistent anemia.

Table 27: Comparison of Hemoglobin Levels with Other Studies

Diagnosis	Present study	Mondal et al(8)	Banerjee et al(9)	Mondal SK et al(14)	Khera et al(15)
β -TT	9.25±0.76	9.7±1.8	7.5±3.4	9.7±1.7	9.3±2.7
Thalassemia	9.45±0.07	5.6±1.9	3.4±2.1	5.7±1.9	5.03±1.9

In the present study, patients with β-thalassemia trait had a mean hemoglobin value of 9.45g/dl. This value was concordant with the studies conducted by Mondal et al(8), Mondal SK et al(14)and Khera et al(15) where the hemoglobin levels were slightly lower than the range in the study done by Banerjee et al(9).The mean hemoglobin value of patients suffering from β-thalassemia was 9.45±0.07. Other studies have recorders values ranging from 4.7g/dl to 6.67g/dl. The present study displays a higher value compared to them. This can be attributed the fact that only two cases were diagnosed in the present study which is less to give a standard statistically significant value.

Table 28: Comparison of RBC Count with Other Studies

Diagnosis	Present study	Mondal et al(8)	Banerjee et al(9)	Mondal SK et al	Khera et al(15)
β -TT	5.77±1.08	3.9±1.0	3.5±1.5	4.1±1.3	5.18±2.7
β-Thalassemia	6.42±0.50	2.5±0.7	1.8±1.1	2.3±0.7	2.27±1.2

In the present study, patients with β thalassemia trait had a mean RBC count value of $5.77x10^6$ cells/mm^3. This value was concordant with the studies conducted by Khera et al (15)only. The other studies done by Mondal et al(8), Banerjee et al (9) and Mondal SK et al displayed RBC count values were between $3.9x10^6$ cells/mm^3 and $4.1x10^6$ cells/mm^3.

Table 29: Comparison of MCV with Other Studies

Diagnosis	Present study	Mondal et al(8)	Banerjee et al(9)	Mondal SK et al(14)	Khera et al(15)
β -TT	60.29±4.40	70.4±5.8	71.7±11.1	70.1±5.8	62.7±7.7
Thalassemia	60±11.31	73±7.6	66.2±8.5	72±6.9	64.9±7.9

The present study showed β thalassemia trait having a mean MCV value of 60.29fl. This value was concordant with the study conducted by Khera et al(15) . On the other hand, β thalassemia trait showed a lower value compared to the other studies done by Mondal et al(8), Banerjee et al(9) and Mondal SK et al(14) where the values ranged from 71.7fl to 70.1fl. this is because our study included only the microcytic hypochromic RBCs by ruling out other nutritional causes of microcytosis with the aid of Mentzer index. Normocytic normochromic cause of anemia is not included under the study.

Table 30: Comparison of MCH with Other Studies

Diagnosis	Present study	Mondal et al(8)	Banerjee et al(9)	Mondal SK et al(14)	Khera et al(15)
β -TT	17.25±1.70	21.0±3.2	22.4±4.3	21±3.2	19.56±2.5
Thalassemia	17.15±2.33	22.9±3.4	20.3±3.4	22.7±3.4	20.0±1.9

The mean MCH levels of β thalassemia trait and β thalassemia major calculated in the study were 17.25pg and 17.15pg, respectively. The values were lower when compared to the values obtained by similar studies done Mondal et al(8), Banerjee et al (9), Mondal SK et al(14), and Khera et al(15)where the levels ranged from 19.56.0pg to 21.0pg for β thalassemia trait and 20.0pg to 22.9pg for β thalassemia. The reason for this finding is also due to similar reason stated above for MCV values.

Table 31: Comparison of MCHC with Other Studies

Diagnosis	Present study	Mondal SK et al(14)	Banerjee et al(9)	Mondal et al(8)	Khera et al(15)
β -TT	28.52±1.65	27.8±1.6	32.5±2.7	27.8±1.7	31.4±2.5
Thalassemia	28.8±1.55	30±2.9	32.3±3.5	30±3.4	31.0±1.9

With respect to the MCHC value in β thalassemia trait, the present study showed concordance with values obtained in studies conducted by Mondal SK et al (14)and Mondal et al(8)and a slightly higher values was seen in the study done by Banerjee et al(9) and Khera et al(15).β- thalassemia trait usually have allow mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) with a raised total RBC count. The PBS may show basophilic stippling and target cells which are more characteristic of β-thalassemia than iron deficiency anemia. Iron stores are normal/high in β-thalassemia (2).

Table 32: Comparison of RDW CV with Other Studies

	Present study	Mondal et al(8)	Banerjee et al(9)	Mondal SKet al(14)	Khera et al(15)
β -TT	20.31±3.62	16.9±4.0	18±4.9	33.6±6.9	19.5±4.4
Thalassemia	23±7.49	31.4±7.1	26.2±3.6	56.2±11.5	26.5±6.7

The values of RDW varied in different studies and the value of our study fall within the range of various other studies ranging from 33.6 to 16.9%.

Table 33: Comparison of Abnormal Hemoglobins In Thalassemia Traits With Different Studies

Types of hemoglobin	Present study	Mondal et al(8)	Banerjee et al(9)	Mondal SKet al(14)	Khera et al(15)
HbA2 (%)	2.81±1.16	6.7±1.6	5.1±1.0	2.6±0.4	5.7±0.9
HbF (%)	1.47±3.32	1.2±1	1.5±1.3	1.3±1.0	1.5±1.2

HbA2 is occasionally increased in hyperthyroidism and megaloblastic anemia. It might be increased in sickle cell trait and sickle cell disease, but the increase is only modest and the level stays below 4.0%. Its level decreases in iron deficiency and α-thalassemia[16].

Our study demonstrates that High-Performance Liquid Chromatography (HPLC) is a highly effective diagnostic tool for precisely identifying and quantifying normal and abnormal hemoglobin fractions. The retention time and percentage of variant hemoglobin provide valuable clues for differentiating between variant hemoglobin that elute in the same window. Notably, HbA2 values in the borderline range require further evaluation to rule out silent mutations, α-thalassemia, and co-existing nutritional deficiencies.

The result of this study will also guide and aid future studies, particularly in the application of HPLC in presumptive diagnosis of thalassemia and other hemoglobinopathies in Mandya.

Conclusion

The β-thalassemia trait has a 1.3% prevalence in the anemic population, posing a significant risk of thalassemia major in offspring and substantial treatment costs. To prevent the birth of children with thalassemia major, we recommend universal antenatal screening by HPLC. Raising awareness, educating healthcare workers, antenatal women, and women of reproductive age is crucial. Screening of husbands is also advised. Genetic studies can confirm borderline cases and detect silent carriers of β-thalassemia, alpha thalassemia, and rare variants. Our HPLC-based study highlights the extent of thalassemia and hemoglobinopathies in a small hospital population, which may be just the tip of the iceberg. However, such studies can increase awareness among healthcare providers and the general population.

Advantages

- HPLC offers a reliable tool for early, accurate detection; thereby aiding in prevention and management of various hemoglobinopathies.
- Screening included general population of large sample size which included all ages and both genders.
- The anaemia prevalence was notably higher among women compared to men in all age groups.
- In women, the anaemia prevalence peaked in the 45–49 years, whereas the youngest subjects of 20–24-year group displayed comparably low rates.
- Study also screened first-degree relatives (parents/ children) of the subject.

Limitation of the Study

- Study targeted screening of only microcytic hypochromic anaemia hence other abnormal hemoglobinopathies (Sickle cell anemia) with normocytic normochromic anemia were not included in the study.
- Atypical carriers are not included in the study with MCV >80fl and Mentzer index >13.
- DNA analysis was not done for confirmed cases and persons with β-T Tequivocal results.
- Study before and after iron therapy to know the change in HbA2 level is not evaluated.
- HPLC screening based on RT or different Hb fractions. So known/unknown variants may share a common RT.Hence confirmation is needed by PCR/ β-gene sequencing.

Majority of the centres in India use conventional methods for diagnosis of hemoglobinopathies, which includes clinical and family history, red cell indices, complete blood counts (CBC), HbA2, HbF estimation, sickling test, and Hb electrophoresis. HPLC analysis is expensive and the equipment is not widely available, hence it is not suitable for mass-screening programs in resource-poor country.

Future Scope

- Screening of all antenatal women using HPLC analysis as a preventive measure.
- Screening of infants and children with anemia.
- DNA analysis in thalassemia trait and with β-TT with equivocal result (HbA2 between 3.5-3.9)
- Study before and after iron therapy to know the change in HbA2 level.

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