Original Article

Evaluation of Hemoglobinopathies in Cases of Microcytic Hypochromic Anemia by High-Performance Liquid Chromatography

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Abstract

Background: Anemia remains a widespread public health problem with major consequences for human health as well as social and economic development. Although estimates of the prevalence of anemia vary widely and accurate data are often lacking, it can be assumed that significant proportions of young children and women of childbearing age are anemic. Hemoglobinopathies are the most common inherited disorders worldwide. These hereditary disorders are a major public health problem in many parts of the world, including India.

Materials and Methods: The cross-sectional study was conducted over one year on 126 cases of microcytic hypochromic anemia. EDTA samples were analyzed using an automated 3-part cell counter (HORIBA) to obtain hemoglobin values and RBC indices. High-Performance Liquid Chromatography (HPLC) was performed using the HbA2-HbF program of D10 (BIO-RAD), based on the principle of cation exchange chromatography.

Results: Out of 126 cases of microcytic hypochromic anemia, hemoglobinopathy was detected in 42 (33%) cases. The results show Beta thalassemia trait constituted 20 (47.6%) cases, followed by 6 (14.5%) cases of Hemoglobin D-Punjab heterozygous, Beta thalassemia major in 5 (11.9%) cases, Hemoglobin D-Iran heterozygous in 2 (4.8%) cases, HbE heterozygous in 3 (7.1%) cases, Hemoglobin E/β+ thalassemia in 1 (2.4%) case, Hemoglobin S heterozygous in 2 (4.8%) cases, and Hemoglobin S/β+ thalassemia in 3 (7.1%) cases.

Conclusion: Our study revealed a substantial presence of hemoglobinopathies in cases with microcytic hypochromic anemia. Most of the hemoglobinopathies detected were accurately quantified by High-Performance Liquid Chromatography (HPLC), which emerges as an optimal method for the routine screening of hemoglobinopathies. HPLC offers advantages such as rapid and accurate results, along with the detection and quantification of abnormal hemoglobin, compared to other tests.

Keywords:

Microcytic hypochromic anemia, Hemoglobinopathies, Peripheral blood film, HPLC, Beta thalassemia, Hemoglobin D-Punjab.

Introduction

Anemia is defined as a reduction of total circulating red cell mass or reduced hemoglobin below normal limits with respect to age

and gender. Due to decreased red cell mass or hemoglobin, the oxygen-carrying capacity of the blood reduces, leading to tissue hypoxia. In practice, measurement of red cell mass is not easy; therefore, anemia is usually diagnosed based on the reduction of hematocrit and the hemoglobin concentration of the blood to levels below the normal range [1].

As per WHO data in 2019, global anemia prevalence is 40% in children aged 6-59 months, 37% in pregnant women, and 30% in women 15-49 years of age [2]. In India, according to NFHS-5, 2022 (National Family Health Survey), the prevalence of anemia in pregnant women (aged 15-49) is 52.2%, in children under 5 years is 67.1%, and in women of reproductive age (aged 15-49) is 57.0% [3].

Due to the higher prevalence of anemia and the varied causes leading to anemia, RBC indices and morphology in peripheral blood are crucial for deciding further investigation to reach a proper diagnosis. According to WHO, 5% of the world population is a carrier for hemoglobin disorders, whereas the frequency of different hemoglobinopathies in India ranges from 7% to 15% for β-thalassemia, 5% to 7% for sickle cell trait, and 0.4% to 1% for sickle cell disease in different population groups. The average frequency of HbD is 0.86% in the Indian population [4].

Clinically, hemoglobinopathies can present either as an asymptomatic state or as severe, lifelong, transfusion-dependent anemia, involving multiple organs and significantly reducing life expectancy. Accurate and timely detection of various hemoglobin variants can prevent the occurrence of more serious disorders like thalassemia major in infants and children [5]. Basic investigations for the detection of hemoglobin variants include red cell indices, hemoglobin pattern analysis by High-Performance Liquid Chromatography (HPLC), electrophoresis, and molecular studies.

HPLC is intended for the percent determination of hemoglobin A2, F, A1C, and for the detection of abnormal hemoglobin in human whole blood using ion-exchange chromatography. HPLC offers a reliable tool for the early detection of hemoglobin variants, thereby aiding in the prevention and management of various hemoglobinopathies. This study is being conducted to evaluate hemoglobinopathies in cases of microcytic hypochromic anemia by HPLC.

Materials and Methods

This cross-sectional study was carried out in the Department of Pathology at Bhagat Phool Singh Government Medical College for Women in Khanpur Kalan, Sonepat. The study was conducted over one year and received ethical approval from the Institutional Ethics Committee, with registration number BPSGMCW/RC/798/IEC/22 dated 11/10/2022.

The study examined 126 cases of microcytic hypochromic anemia. EDTA samples were analyzed using an automated 3-part cell counter (HORIBA) to obtain hemoglobin values and RBC indices based on electric impedance principles. Red cell morphology was assessed in peripheral blood films stained with Leishman’s stain. Reticulocyte counts were performed using new methylene blue, and sickling tests were conducted as necessary.

HPLC was performed using the HbA2-HbF program of D-10 (BIO-RAD), based on the principle of cation exchange chromatography. On the D-10, samples were automatically diluted and injected into the analytical cartridge. The D-10 administered a programmed buffer gradient with increasing ionic strength to the cartridge, causing separation of hemoglobins based on their interactions with the cartridge material. Separated hemoglobins then flowed through the filter photometer's flow cell, measuring absorbance changes at 415 nm.

The D-10 software processed raw data collected from each analysis. Quantitation of HbA2/F/A1c values employed a two-level

calibration. Each sample generated a sample report and chromatogram. Data processing included generating a report displaying the chromatogram, identifying different peaks within defined windows, and providing relevant information such as retention, relative percentage, and area [7].

Results

A total of 126 cases of microcytic hypochromic anemia were included in the present study. The age of cases ranged from 0 to 70 years, with 95.2% of cases belonging to the age group of 0 to 40 years. In our study, we found that 64.3% of cases of microcytic hypochromic anemia were reported in females, while males accounted for the remaining 35.7%.

In the present study, cases were distributed with respect to the grading of anemia according to WHO criteria for anemia [8]. We observed that severe anemia constituted 87 (69%) cases, followed by 24 (19%) cases of mild anemia and 15 (12%) cases of moderate anemia. Hemoglobinopathies were detected in 42 (33%) cases, with the majority of hemoglobinopathy cases occurring in the age group of 0 to 10 years (35.7%), followed by 30.9% of cases in the age group of 21 to 30 years. The mean age of cases was 20.07 years. Hemoglobinopathies were detected in 69% (29 cases) of females and 31% (13 cases) of males [Table 1].

Table 1: Demographic and lab findings in cases of microcytic hypochromic anemia (N=126)

Parameter	**No. of Cases (n)**	**Percentage (%)**		
Total cases	126	100		
Age				
<40 Years	120	95.2		
>40 Years	6	4.8		
Mean age:	14.5	-		
Median age:	40	-		
Range:	0-70	-		
Gender				
Male	45	35.7		
Female	81	64.3		
Grading of Anemia				
Mild	24	19.1		
Moderate	15	11.9		
Severe	87	69		
Hemoglobinopathy detected	42	33.3		
Age-wise Distribution of Individual Hemoglobinopathies (n)				
Hemoglobinopathy	<40 years	%	>40 years	%
Beta thalassemia trait (20)	19	45.2	1	2.4
Beta thalassemia major (5)	5	11.9	0	0
Hemoglobin D-Punjab heterozygous (6)	6	14.2	0	0
Hemoglobin D-Iran heterozygous (2)	1	2.4	1	2.4
Hemoglobin E heterozygous (3)	3	7.1	0	0
Hemoglobin E/β+ thalassemia (1)	0	0	1	2.4
Hemoglobin S heterozygous (2)	2	4.8	0	0
Hemoglobin S/β+ thalassemia (3)	3	7.1	0	0
Gender-wise Distribution of Individual Hemoglobinopathies				
Hemoglobinopathy	Male (n)	%	Female (n)	%
Beta thalassemia trait (20)	7	16.7	13	30.9
Beta thalassemia major (5)	3	7.1	2	4.8
Hemoglobin D-Punjab heterozygous (6)	0	0	6	14.3
Hemoglobin D-Iran heterozygous (2)	0	0	2	4.8
Hemoglobin E heterozygous (3)	0	0	3	7.1
Hemoglobin E/β+ thalassemia (1)	1	2.4	0	0
Hemoglobin S heterozygous (2)	0	0	2	4.8
Hemoglobin S/β+ thalassemia (3)	2	4.8	1	2.4

In the present study, the most common hemoglobinopathy detected was beta thalassemia trait, comprising 20 (47.6%) cases, followed by 6 (14.3%) cases of hemoglobin D-Punjab heterozygous and 5 (11.9%) cases of beta thalassemia major. It was observed that 3 (7.1%) cases each of hemoglobin E heterozygous and hemoglobin S/beta thalassemia, 2 (4.8%) cases each of hemoglobin D Iran heterozygous and hemoglobin S heterozygous, and 1 (2.4%) case of hemoglobin E/beta thalassemia were also detected.

In our study, 45% of cases of beta thalassemia trait belonged to the age group of 21 to 30 years, whereas 80% of cases of beta thalassemia major were diagnosed in the 0 to 10 years age group, and 66% of cases of hemoglobin D-Punjab heterozygous detected belonged to the age group of 21 to 30 years. We observed that hemoglobinopathies were more frequently detected in females compared to males. The majority of heterozygous hemoglobinopathies presented with mild anemia, including 50% of cases of beta thalassemia trait, 50% of cases of hemoglobin D-Punjab, 66% of cases of hemoglobin E heterozygous, and 50% of cases of hemoglobin S heterozygous. In contrast, homozygous and double heterozygous hemoglobinopathies more commonly presented with moderate and severe anemia. In the present study, we reported that 60% of cases of beta thalassemia major, 100% of cases of hemoglobin E/β+ thalassemia, and 60% of cases of hemoglobin S/β+ thalassemia presented with severe anemia [Table 2].

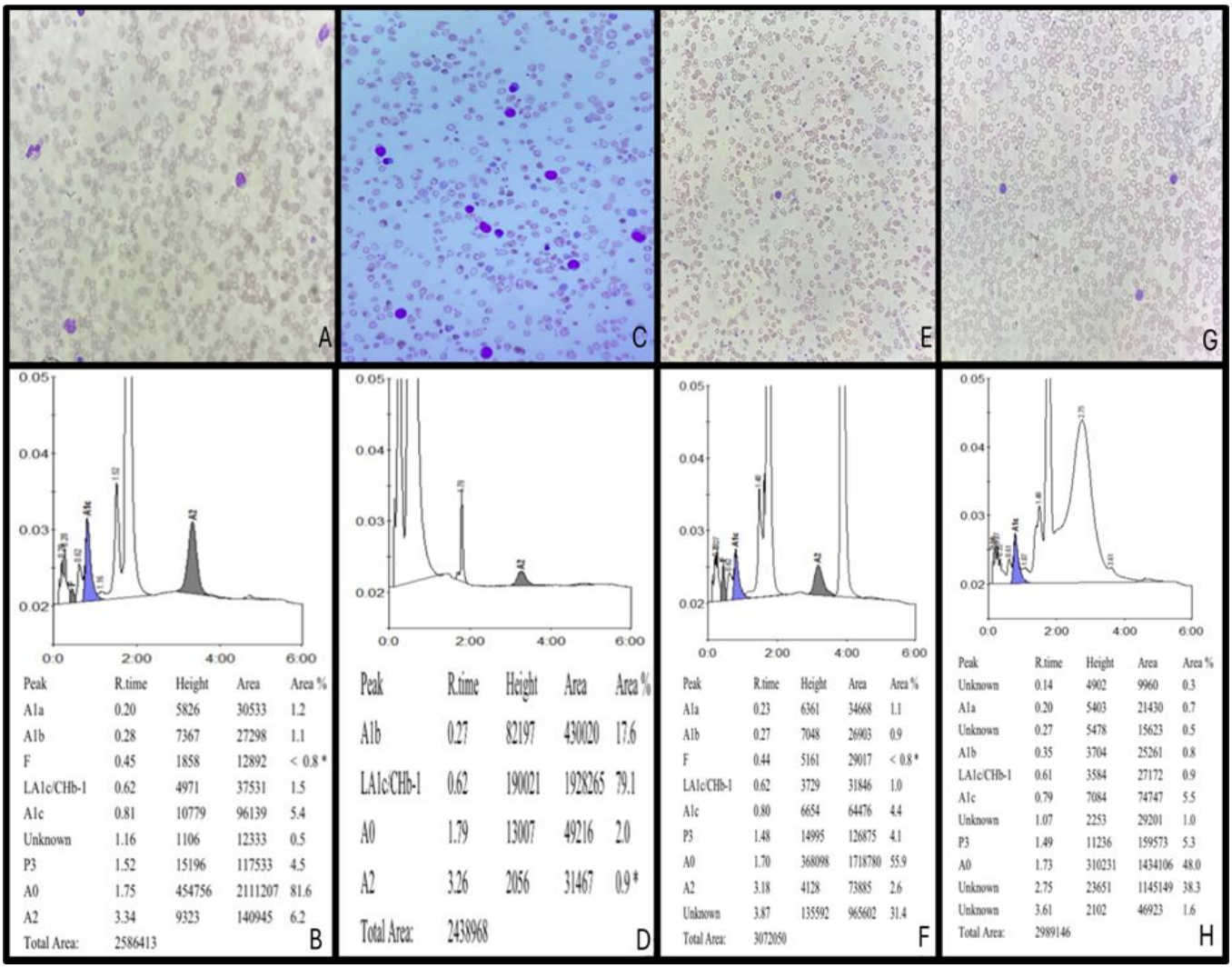
Table 2: Distribution of hemoglobinopathy cases with respect to grading of anemia (N=42)

HPLC(n)	**Anemia**		
	Mild (n)	Moderate (n)	Severe (n)
Beta thalassemia trait (20)	10	7	3
Beta thalassemia major (5)	0	2	3
Hemoglobin D-Punjab heterozygous (6)	3	1	2
Hemoglobin D-Iran heterozygous (2)	0	0	2
Hemoglobin E heterozygous (3)	2	0	1
Hemoglobin E/β+ thalassemia (1)	0	0	1
Hemoglobin S heterozygous (2)	1	1	0
Hemoglobin S/β+ thalassemia (3)	0	1	2
Total (42)	16	12	14

The most common hemoglobinopathy, i.e., beta thalassemia trait, showed hemoglobin levels in the range of 5 to 11.5 g/dl and reduced MCV in the range of 46 to 79 fl. In contrast, cases of beta thalassemia major revealed severely reduced hemoglobin in the range of 3 to 8.5 g/dl and raised RDW ranging from 28.6 to 35.3%. Cases of hemoglobin D-Punjab showed hemoglobin levels in the range of 3.5 to 11.5 g/dl and MCV ranging between 54 to 76 fl [Table 3].

Table 3: Hematological parameters assessment in various hemoglobinopathies (N=42)

HPLC(n)	**Hematological parameters**					
	Hb (g/dl) Mean±***SD***	MCV (fl) Mean±***SD***	MCH (pg) Mean±***SD***	MCHC (g/dl) Mean±***SD***	RDW (%) Mean±***SD***	RC (%) Mean±***SD***
Beta thalassemia trait (20)	9.3±1.76	63.9±8.26	19.4±2.45	29.8±1.99	18.5±2.38	2.44±1.83
Beta thalassemia major (5)	5.8±2.33	66.9±10.8	16.6±5.95	25.4±9.58	32.1±3.10	2.7±1.58
Hemoglobin D-Punjab heterozygous (6)	8.9±3.06	68.6±7.79	26.1±6.24	31.6±3.30	23.7±7.52	1.8±1.25
Hemoglobin D-Iran heterozygous (2)	6.0±2.12	52.5±13.8	15.5±5.02	29.3±1.91	20.9±2.33	3.2±0.57
Hemoglobin E heterozygous (3)	9.2±2.31	70.0±2.52	21.6±1.30	30.9±1.81	16.7±3.72	1.2±0.39
Hemoglobin E/β+ thalassemia (1)	5.0	65.0	20.1	26.0	25.1	0.5
Hemoglobin S heterozygous (2)	9.2±1.06	66.2±15.41	19.8±3.39	28.5±0.35	17.7±2.05	2.75±0.21
Hemoglobin S/β+ thalassemia (3)	4.2±4.48	53.1±30.1	22.2±5.81	23.8±4.91	20.1±3.47	2.6±0.35



Peak	R.time	Height	Area	Area %
A1a	0.20	5826	30533	1.2
A1b	0.28	7367	27298	1.1
F	0.45	1858	12892	< 0.8 *
LA1c/CHb-1	0.62	4971	37531	1.5
A1c	0.81	10779	96139	5.4
Unknown	1.16	1106	12333	0.5
P3	1.52	15196	117533	4.5
A0	1.75	454756	2111207	81.6
A2	3.34	9323	140945	6.2
Total Area:	2586413			

Peak	R.time	Height	Area	Area %
A1b	0.27	82197	430020	17.6
LA1c/CHb-1	0.62	190021	1928265	79.1
A0	1.79	13007	49216	2.0
A2	3.26	2056	31467	0.9 *
Total Area:	2438968			

Peak	R.time	Height	Area	Area %
A1a	0.23	6361	34668	1.1
A1b	0.27	7048	26903	0.9
F	0.44	5161	29017	< 0.8 *
LA1c/CHb-1	0.62	3729	31846	1.0
A1c	0.80	6654	64476	4.4
P3	1.48	14995	126875	4.1
A0	1.70	368098	1718780	55.9
A2	3.18	4128	73885	2.6
Unknown	3.87	135592	965602	31.4
Total Area:	3072050			

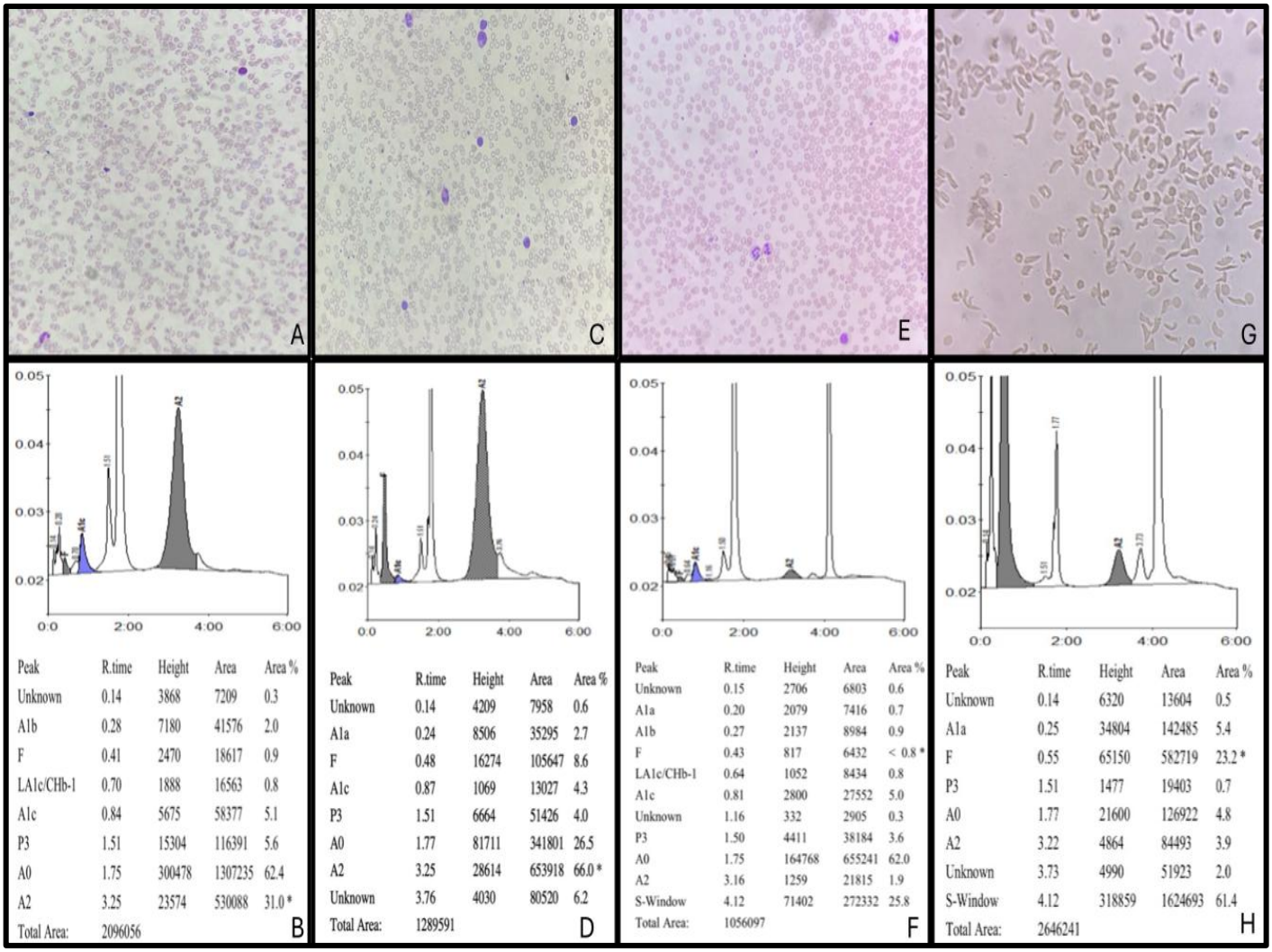
Peak	R.time	Height	Area	Area %
Unknown	0.14	4902	9960	0.3
A1a	0.20	5403	21430	0.7
Unknown	0.27	5478	15623	0.5
A1b	0.35	3704	25261	0.8
LA1c/CHb-1	0.61	3584	27172	0.9
A1c	0.79	7084	74747	5.5
Unknown	1.07	2253	29201	1.0
P3	1.49	11236	159573	5.3
A0	1.73	310231	1434106	48.0
Unknown	2.75	23651	1145149	38.3
Unknown	3.61	2102	46923	1.6
Total Area:	2989146			

Figure 1: (A) PBF showing microcytic hypochromic anemia and target cells [Leishman's stain; 400x], (B) beta thalassemia trait, (C) PBF showing microcytic hypochromic anemia, marked anisopoikilocytosis, polychromasia, and nucleated red blood cells [Leishman's stain; 400x], (D) beta thalassemia major, (E) PBF showing microcytic hypochromic anemia with mild anisopoikilocytosis [Leishman's stain; 400x], (F) HbD-Punjab heterozygous, (G) PBF showing microcytic hypochromic anemia [Leishman's stain; 400x], (H) HbD-Iran heterozygous.

Discussion

Hemoglobinopathies are significant contributors to inherited blood disorders, presenting as haemolytic anemias. Thalassemia is characterized by reduced biosynthesis of globin chains, while structural hemoglobinopathies involve alterations in the amino acid sequence of globin. These conditions can lead to various degrees of anemia depending on the severity of globin chain imbalance [9]. These conditions collectively represent some of the most common Mendelian genetic diseases worldwide. Among hemoglobinopathies, sickle cell disease and hemoglobin E (HbE)-associated syndromes are predominant.

In our study, hemoglobinopathies were observed in 42 (33%) cases out of 126 cases of microcytic hypochromic anemia. The results of our study were comparable with Bhokare et al. [10], Raman et al. [11], and Ghosh et al. [12], where the overall frequency of hemoglobinopathy cases was reported as 37.4%, 37.2%, and 44.5%, respectively.



Peak	R.time	Height	Area	Area %
Unknown	0.14	3868	7209	0.3
A1b	0.28	7180	41576	2.0
F	0.41	2470	18617	0.9
LA1c/CHb-1	0.70	1888	16563	0.8
A1c	0.84	5675	58377	5.1
P3	1.51	15304	116391	5.6
A0	1.75	300478	1307235	62.4
A2	3.25	23574	530088	31.0 *
Total Area:	2096056			

Peak	R.time	Height	Area	Area %
Unknown	0.14	4209	7958	0.6
A1a	0.24	8506	35295	2.7
F	0.48	16274	105647	8.6
A1c	0.87	1069	13027	4.3
P3	1.51	6664	51426	4.0
A0	1.77	81711	341801	26.5
A2	3.25	28614	653918	66.0 *
Unknown	3.76	4030	80520	6.2
Total Area:	1289591			

Peak	R.time	Height	Area	Area %
Unknown	0.15	2706	6803	0.6
A1a	0.20	2079	7416	0.7
A1b	0.27	2137	8984	0.9
F	0.43	817	6432	< 0.8 *
LA1c/CHb-1	0.64	1052	8434	0.8
A1c	0.81	2800	27552	5.0
Unknown	1.16	332	2905	0.3
P3	1.50	4411	38184	3.6
A0	1.75	164768	655241	62.0
A2	3.16	1259	21815	1.9
S-Window	4.12	71402	272332	25.8
Total Area:	1056097			

Peak	R.time	Height	Area	Area %
Unknown	0.14	6320	13604	0.5
A1a	0.25	34804	142485	5.4
F	0.55	65150	582719	23.2 *
P3	1.51	1477	19403	0.7
A0	1.77	21600	126922	4.8
A2	3.22	4864	84493	3.9
Unknown	3.73	4990	51923	2.0
S-Window	4.12	318859	1624693	61.4
Total Area:	2646241			

Figure 2: (A) PBF showing microcytic hypochromic anemia [Leishman's stain; 400x], (B) HbE heterozygous, (C) PBF showing microcytic hypochromic anemia, mild anisopoikilocytosis, and target cells [Leishman's stain; 400x], (D) hemoglobin E/β+ thalassemia, (E) PBF showing microcytic hypochromic anemia with occasional sickle cells [Leishman's stain; 400x], (F) sickle cell heterozygous, (G) sickling test demonstrating sickling, (H) hemoglobin S/β+ thalassemia.

However, other studies conducted by Singh et al. [13], Joneja et al. [14], Jain et al. [5], and Nayak et al. [15] observed a much higher prevalence of hemoglobinopathy—51%, 67.9%, 65.3%, and 58.5% of cases, respectively. In contrast, Bajaj et al. [16], Bhagora et al. [17], and Singh et al. [18] observed a lower frequency of hemoglobinopathies—19.8%, 17%, and 20.1%, respectively. This variation may be attributable to different geographical areas of various studies.

In our study, the maximum number of hemoglobinopathy cases, i.e., 35.7%, were in the age group of 0-10 years, followed by 30.9% in the 21-30 years age group. Similar findings were seen by Jha et al. [19] and Nayak et al. [15]. The majority of hemoglobinopathies were detected in younger age groups, as these patients commonly presented with anemia and were screened for the same. In our study, hemoglobinopathies were detected in 69% of females and 31% of males. Our findings compare well with the studies conducted by Singh et al. [13], Jain et al. [5], and Saha et al. [25], where hemoglobinopathy cases were more frequently detected in females—62.7%, 63.3%, and 72%, respectively. However, Bajaj et al. [16] reported male preponderance

in their study, i.e., 52.3% of cases. The reason could be that different groups of patients were studied by different researchers, for example, pediatric, antenatal, or anemic patients.

In our study, we observed a higher prevalence of anemia among female patients compared to males, likely due to the multifactorial causes of anemia in females. Clinicians also recommended investigating all antenatal cases of anemia, including HPLC analysis. As a result, our study recorded a greater frequency of hemoglobinopathies.

In the present study, Beta thalassemia trait was the most prevalent hemoglobinopathy, constituting 20 cases (47.6%) out of 42 cases. Our results were comparable with Bajaj et al. [16] and Singh et al. [20], who observed Beta thalassemia trait as the most common hemoglobinopathy in 40.5% and 36% of cases, respectively. The prevalence of hemoglobinopathies varies among different studies of anemia patients depending on the geographical region. However, due to population migration and admixture, the entire spectrum of thalassemia and Hb variants is now found across the country.

Studies conducted in northeastern India show hemoglobin E as the most detected hemoglobinopathy. The higher prevalence of HbE hemoglobinopathies in eastern India results from a combination of genetic, historical, and evolutionary factors that have shaped the region's population over time. In contrast, in the eastern belts of Orissa, Jharkhand, parts of Rajasthan, Gujarat, Maharashtra, and southern India, Hemoglobin S is more prevalent. India has among the highest hemoglobin S allele frequencies globally and the third-highest birth rate for babies born with HbSS [25]. In northern and central India, the prevalence of Beta thalassemia trait is more frequent [21-23].

The North Central region has distinct population groups with specific genetic profiles. Thus, studies by Tambse et al. [24] and Jain et al. [5] show sickle cell heterozygous as the most common hemoglobinopathy, i.e., 86.4% and 55.5% of cases, respectively. Saha et al. [25] observed Hemoglobin E heterozygous as the most common hemoglobinopathy, constituting 49.8% of cases.

The prevalence of hemoglobinopathies varies by country. A study by Makkawi et al. [26] in Saudi Arabia observed sickle cell disease as the most common hemoglobinopathy, whereas Zahran et al. [27] in Egypt found sickle cell heterozygous to be the most frequent hemoglobinopathy [Table 4].

Table 4: Comparative study of common abnormal hemoglobinopathy with previous studies

Study	Place of study	Year	Most common hemoglobinopathy (%)	Second most common hemoglobinopathy (%)
Tambse et al.[24]	Maharashtra	2016	Sickle cell heterozygous (86.4)	Sickle cell disease (9)
Makkawi et al.[26]	Saudi Arabia	2018	Sickle cell disease (80.4)	Beta thalassemia trait (19.6)
Jain at al.[5]	Gujarat	2019	Sickle cell heterozygous (55.5)	Sickle cell disease (19.9%)
Bajaj et al.[16]	Nasik	2020	Beta thalassemia trait (40.5)	Sickle cell heterozygous (9.5)
Saha et al.[25]	Kolkata	2020	Hemoglobin E heterozygous (49.8)	Beta thalassemia trait (41.5)
Zahran et al.[27]	Egypt	2022	Sickle cell heterozygous (16.9)	Beta thalassemia trait (13.1)
Singh et al.[20]	Haryana	2024	Beta thalassemia trait (36)	Beta thalassemia major (16)
Present Study	Haryana	2024	Beta thalassemia trait (47.6)	Hb D-Punjab heterozygous (14.3)

Conclusion

Anemia is prevalent among children, young individuals, and those in the reproductive age group, with females being affected more frequently than males. The multifactorial causes of anemia underscore the importance of thorough diagnostic evaluations to identify hemoglobinopathies. Hemoglobinopathies represent a significant public health challenge in many regions, including

India. Understanding the distribution of various hemoglobinopathies aids in identifying endemic zones, planning effective screening and control programs, and developing healthcare, administrative, and clinical practice guidelines. In resource-limited developing nations like India, a comprehensive strategy combining primary and secondary prevention is essential to address anemia. Routine screening for anemia, along with premarital and antenatal screening for hemoglobinopathies, can significantly raise awareness and prevent homozygous births. There is a critical need for awareness initiatives among the general population. Mass screening programs targeting young and adolescent populations in endemic areas should be introduced. Counseling individuals with heterozygous states can help prevent the occurrence of homozygous and compound heterozygous states in the future. Accurate and timely detection of hemoglobin variants can prevent severe disorders like thalassemia major in infants and children. Our study concludes that High-Performance Liquid Chromatography (HPLC) is the optimal method for the routine screening of hemoglobinopathies in anemia cases. HPLC offers rapid and accurate results and can detect and quantify abnormal hemoglobin more effectively than other tests. As hemoglobinopathies are frequently detected in cases of microcytic hypochromic anemia, all such patients should undergo HPLC testing as part of routine investigations.

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Design, Investigation, and Data Collection: RA, SB, KS, RM, MR

Data Analysis: RA, SB, KS

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