GMR
The Original

HAEMOGLOBINOPATHIES: PREVALENCE IN A TERTIARY CARE POPULATION, ROLE OF HPLC IN DIAGNOSIS, AND THE IMPORTANCE OF EARLY DETECTION AND RECENT ADVANCES

Dr. Sandesh Thorat

Associate Professor, Department of Biochemistry, Dr. D. Y. Patil Medical College, Hospital & Research Centre, Pune – 411018, India.
*Corresponding Author: Dr. Sandesh Thorat, MD (Biochemistry), ORCID: https://orcid.org/0000-0002-1992-2908

ABSTRACT

Background: Haemoglobinopathies are among the most common inherited blood disorders, ranging from asymptomatic carriers to severe transfusion-dependent anaemia. High-Performance Liquid Chromatography (HPLC) is widely used for screening and quantifying hemoglobin variants. However, its utility may be limited in cases with borderline HbA_2 levels or co-eluting peaks.

Objectives: To determine the prevalence of haemoglobinopathies in a tertiary care population, evaluate the diagnostic performance and limitations of HPLC, and emphasize the importance of early detection and recent diagnostic advances.

Materials and Methods: A cross-sectional study was conducted on 200 patients attending a tertiary care hospital. Hemoglobin variant analysis was performed using the Bio-Rad D-10 HPLC system. Chromatograms were interpreted per manufacturer guidelines and correlated with clinical and hematological findings.

Results: Among the 200 samples, 41% showed normal hemoglobin patterns. Abnormal variants included β-thalassaemia trait (38%), β-thalassaemia major (1%), sickle cell trait (4%), sickle cell anaemia (5%), and HbE trait (1%). Additionally, 35% had borderline HbA_2 levels (3.2–4.0%), 28% had HbA_2 <2.1%, and 6% exhibited co-eluting peaks.

Conclusion: β-thalassaemia trait was the most common hemoglobinopathy observed. A significant proportion of cases with borderline or low HbA_2 values highlights the limitations of relying solely on HPLC. Incorporating molecular diagnostic methods and AI-assisted interpretation tools may enhance diagnostic accuracy, particularly in ambiguous or co-eluting cases.

KEYWORDS: Haemoglobinopathies; HPLC; Beta-thalassaemia; Early Detection; Borderline HbA_2; Diagnostic Advances.

INTRODUCTION

Haemoglobinopathies are a group of inherited disorders resulting from structural abnormalities or reduced synthesis of globin chains, giving rise to abnormal hemoglobin variants. These conditions encompass a spectrum from asymptomatic carrier states to severe transfusion-dependent anaemias such as β-thalassemia major and sickle cell disease. Early and precise diagnosis is critical for optimal clinical management, genetic counselling, and prevention of affected births, particularly in high-prevalence countries like India[1].

A wide range of diagnostic modalities are available for hemoglobin variant detection. Initial screening includes peripheral smear, sickling test, and solubility test. Confirmatory methods involve alkaline and acid electrophoresis, isoelectric focusing, and High-Performance Liquid Chromatography (HPLC), which is currently the frontline method owing to its automation, rapidity, reproducibility, and ability to quantify hemoglobin fractions such as HbA_2 and HbF[2]. However, HPLC has limitations including co-elution of variants, borderline HbA_2 levels, and inability to identify rare or silent mutations. To overcome these challenges, newer technologies such as capillary electrophoresis, molecular testing, and AI-assisted interpretation are increasingly being used, particularly in cases with ambiguous HPLC findings[3].

Globally, hemoglobinopathies pose a significant public health challenge. Recent advances in screening strategies, including integrated approaches combining HPLC with molecular diagnostics, have substantially improved diagnostic accuracy and public health interventions[4]. According to the World Health Organization (WHO), these disorders affect nearly 71% of countries and contribute to about 3.4% of childhood mortality in those under five years of age[5]. In India, the prevalence is particularly high due to its ethnically diverse population with varying carrier rates[1]. Early detection

through school and antenatal screening programs is now considered essential to reduce the burden of severe hemoglobinopathies[2,4].
Therefore, the present study was undertaken to evaluate the prevalence and spectrum of hemoglobinopathies in a tertiary care population, with special emphasis on the utility and limitations of High-Performance Liquid Chromatography (HPLC) in variant detection, the importance of early diagnosis, and the relevance of recent diagnostic advances. The specific objectives were:

1. To determine the prevalence of hemoglobinopathies in a tertiary care setting.
2. To assess the diagnostic utility and limitations of HPLC.
3. To emphasize the importance of early detection.
4. To discuss recent advances in hemoglobinopathy diagnostic techniques.

MATERIALS AND METHODS

Ethical Clearance

This was a retrospective observational study using anonymized patient data. As per institutional policy, formal ethical approval was not required.

Study Design and Duration

This was a cross-sectional observational study conducted over a one-year period (2023–2024) at a tertiary care teaching hospital.

Study Population

A total of 200 patients were enrolled from the Medical Outpatient Department (OPD), presenting with nonspecific symptoms or mild anaemia. The study cohort comprised 66 males (33%) and 134 females (67%), reflecting a broader population spectrum for hemoglobinopathy screening.

Exclusion Criteria: Patients who had received blood transfusions within the past three months were excluded from the study.

HPLC Methodology

Haemoglobin variant analysis was conducted using the Bio-Rad D-10 Ion-Exchange High-Performance Liquid Chromatography (HPLC) system. This analyzer quantifies hemoglobin fractions based on retention time and peak area. The specific fractions measured included HbA_0, HbA_2, HbF, HbS, and HbE. Interpretation was based on retention time windows provided by the manufacturer and supported by published guidelines.
Chromatographic profiles were analyzed using system-integrated software, and variant identification was cross-verified with peak characteristics. All results were interpreted in the clinical context.

HbA_2 Interpretation Criteria

Based on prior literature and population-specific diagnostic trends, HbA_2 values in our study were categorized as follows:

- <2.2% (Low HbA_2): May indicate δ-thalassaemia, iron deficiency anemia, or technical assay interference. Iron deficiency is particularly known to suppress HbA_2 levels, potentially masking β-thalassaemia trait[6,7].
- 2.2–3.1% (Normal Range): Commonly observed in individuals without thalassaemia traits or interfering hematologic conditions.
- 3.2–4.0% (Borderline): This range is diagnostically ambiguous. It may represent β-thalassaemia trait, especially when coexisting with iron deficiency or α-globin gene triplications. Further confirmatory molecular testing is recommended in such cases[8-10].
- >4.0% (Elevated HbA_2): Strongly suggestive of heterozygous β-thalassaemia, as levels above this threshold are less likely to be caused by secondary factors.

This classification aligns with published diagnostic criteria and was specifically chosen to account for variations observed in iron-deficient individuals and mixed-ethnicity populations. Borderline HbA_2 values can yield false positives or false negatives if not interpreted in the context of ferritin levels, red cell indices, and clinical background[6-10].

Specimen Collection and Processing

Specimen type: Venous whole blood
Anticoagulant: EDTA
Volume: Venous whole blood specimens were collected in EDTA anticoagulant tubes (minimum volume 1.3 mL). Dilution protocols were followed as per Bio-Rad's standard operating procedures when sample volumes were below

1.5 mL. Internal quality control (QC) samples were analyzed at the beginning and end of each HPLC run to ensure result accuracy and reproducibility.

RESULTS

Gender-wise Distribution of Haemoglobinopathy Cases

Out of the 200 patients enrolled in the study, 66 (33%) were male and 134 (67%) were female

Table 1: Gender-wise Distribution of Haemoglobinopathy Cases

Gender	Total Patients	Haemoglobinopathy Cases	% with Abnormal HPLC
Male	66	40	**60.6%**
Female	134	78	**58.2%**

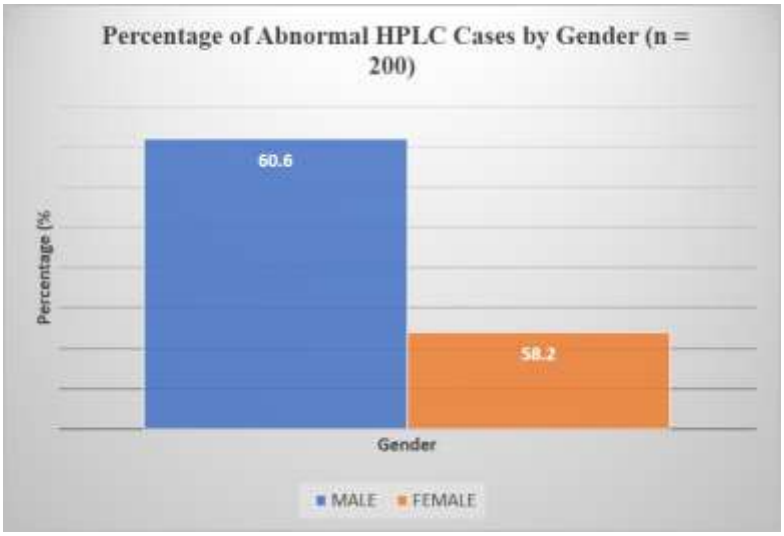


Figure 1: Gender-wise Percentage of Abnormal HPLC Cases (n = 200).

Abnormal hemoglobin patterns were detected in 118 individuals, representing 59% of the total study population. Among these, 40 out of 66 males (60.6%) and 78 out of 134 females (58.2%) exhibited abnormal HPLC profiles. Although the within-group prevalence of haemoglobinopathy was slightly higher in males compared to females, the difference was not statistically significant ($\chi^2 = 0.74$, df = 1, p = 0.39).

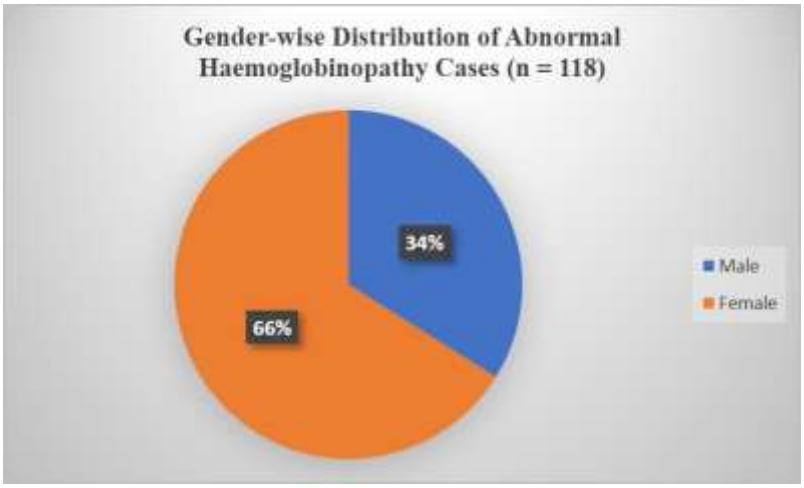


Figure 2: Gender-wise Distribution of Abnormal Haemoglobin Cases (n = 118).

This pie chart represents the gender-wise distribution among all 118 patients with abnormal HPLC profiles.
When analyzing the gender distribution among the 118 abnormal HPLC cases, females accounted for 66.1% (78/118) and males for 33.9% (40/118). This reflects the larger proportion of females in the study population rather than a higher individual risk.

These findings highlight the importance of interpreting both within-group prevalence and total case contribution to accurately assess gender-based patterns in haemoglobinopathies.
While the prevalence of hemoglobinopathy was similar in males and females, the majority of cases were seen in emales due to their higher representation in the screened group. There was no significant gender difference in risk, but population composition influenced total case distribution.

Significance
This study highlights the high burden of hemoglobinopathies in a routine clinical setting, with nearly 60% of patients showing abnormal HPLC patterns. By comparing gender-wise distribution, we show that both males and females are equally at risk, reinforcing the need for universal screening regardless of gender. Our findings also emphasize the importance of using HPLC as a frontline diagnostic tool for early detection, which can guide timely genetic counseling and intervention.

Table 2: Age-wise Distribution

Age Group (Years)	Number of Patients	Percentage (%)
0–10	42	21.0%
11–20	30	15.0%
21–30	60	30.0%
31–40	33	16.5%
41–50	21	10.5%
51–60	8	4.0%
61–70	4	2.0%
71–80	1	0.5%
81–90	1	0.5%

Age-wise distribution revealed that a majority of the study participants were in the 21–30 years age group (30%), followed by 0–10 years (21%) and 31–40 years (16.5%). Notably, 82.5% of the total patients were under 40 years of age. This skewed distribution towards the younger population highlights the need for early and systematic screening strategies, especially among children and individuals of reproductive age.

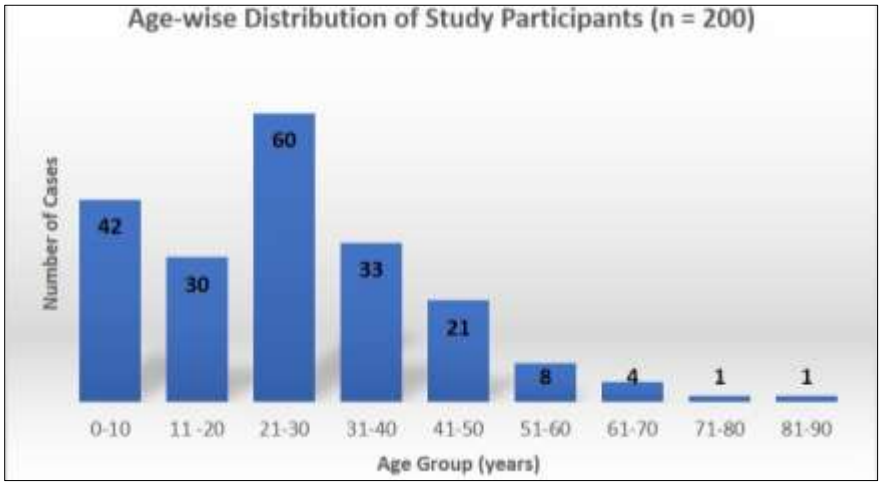


Figure 3. Age-wise Distribution of Study Participants (n = 200)

This bar chart illustrates the age distribution of the 200 individuals evaluated for haemoglobinopathies. The highest representation was observed in the 21–30 years age group (30%), followed by 0–10 years (21%) and 31–40 years (16.5%). A majority of the participants (82.5%) were under 40 years of age, emphasizing the relevance of early detection strategies in younger and reproductive-age populations

Table 3: HPLC findings were categorized as follows:

Category	Cases (n)	Prevalence (%)
Normal	82	41.0%
Beta-thalassaemia trait	38	19.0%
Borderline HbA_2 (3.2–4.0%)	35	17.5%
$HbA_2 < 2.1\%$	28	14.0%

Sickle cell anaemia	5	2.5%
Sickle cell trait	4	2.0%
Co-eluted/Unknown peaks	6	3.0%
Beta-thalassaemia major	1	0.5%
HbE trait	1	0.5%

These findings indicate that beta-thalassaemia trait was the most common abnormality. Borderline HbA_2 levels (3.2–4.0%) and low HbA_2 (<2.1%) were also common, warranting further investigation in suspected carriers. Sickle cell disorders and HbE trait were less frequent.

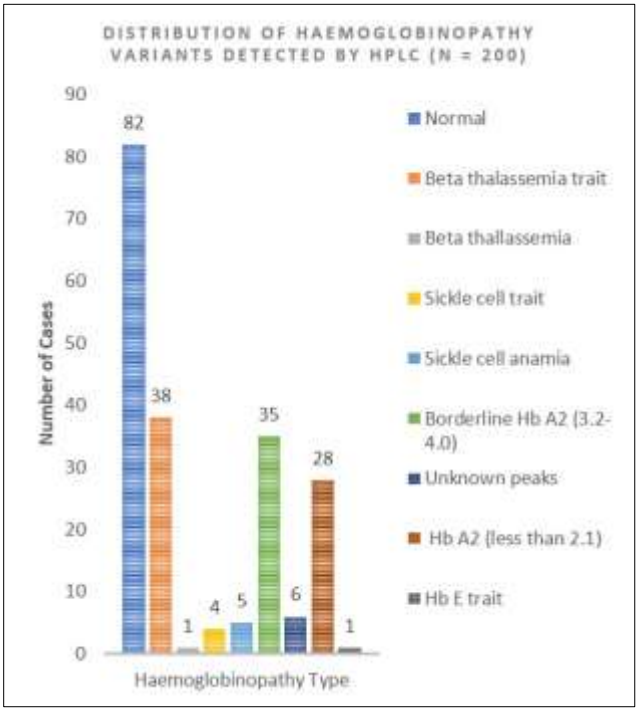


Figure 4: Hemoglobinopathy Pattern Distribution Based on HPLC (n = 200)

The chart displays the frequency and percentage of various hemoglobinopathy patterns identified through HPLC. Normal profiles were found in 41% of cases. β-thalassaemia trait was the most common abnormal variant (19%), followed by borderline HbA_2 values (17.5%) and low HbA_2 levels (<2.1%) (14%). Rare variants included HbE trait (0.5%), β-thalassaemia major (0.5%), and co-eluting peaks (3%).
Note: Some percentages reflect overlapping categories (e.g., borderline HbA_2 may be seen in beta-thalassaemia traits). Final interpretation was based on combined clinical and HPLC data.
Representative HPLC chromatograms used to highlight normal and abnormal hemoglobin profiles are included and discussed in detail under the Discussion section.

DISCUSSION

Epidemiology and Prevalence

Hemoglobinopathies are among the most common inherited disorders globally, particularly in tropical and subtropical regions. These autosomal recessive conditions encompass thalassemias and structural hemoglobin variants. To date, over 1,000 hemoglobin variants have been identified, with β-thalassemia and sickle cell disease being the most prevalent worldwide[11]. In India, these disorders pose a significant public health challenge, particularly in tribal and multi-ethnic populations[12].
In our tertiary care center-based study involving 200 patients, 66 (33%) were male and 134 (67%) were female. The higher female representation likely reflects the higher burden of anemia among Indian women, as corroborated by national surveys and clinical studies[13,14].
Participants ranged from 0 to 90 years, with most patients (60%) in the 21–30 years age group, followed by 21% in the 0–10 years group. This age distribution emphasizes the critical need for early screening and prevention programs targeting reproductive-age individuals and children[15].

Variant Distribution and HPLC Interpretation

All samples were analyzed using cation-exchange HPLC, a widely used method for screening hemoglobinopathies due to its automation, reproducibility, and ability to quantify HbA_2 and HbF levels accurately[16]. Chromatograms were interpreted based on retention time and peak characteristics:

In our study the distribution of hemoglobinopathy cases based on HPLC findings (refer Table 4) highlights the predominance of β-thalassaemia trait and borderline HbA_2 patterns. Together, these categories represent over 50% of abnormal findings, underscoring the diagnostic utility of HPLC as well as its limitations in ambiguous or borderline cases.

β-thalassemia trait (19%) was the most frequent abnormality, aligning with findings from studies conducted in high-prevalence zones of India[16,17]. Borderline HbA_2 cases (17.5%) represent a diagnostically ambiguous group that may include early β-thalassemia carriers or iron-deficient individuals[18]. Low HbA_2 (<2.1%), seen in 14%, may suggest δ-thalassemia, iron deficiency, or technical artifacts, and requires correlation with iron profile and clinical background[17,18]. Co-eluted or unknown peaks (3%) necessitate careful interpretation and may require molecular testing for clarification[19,20].

Implication of Statistical Findings

The β-thalassemia carrier rate of 19% observed in this study is significantly higher than the national average of 3–4%, suggesting a local epidemiological cluster and the need for community-based screening and premarital counseling programs[21].

The borderline HbA_2 range (3.2–4.0%), accounting for 17.5%, remains a diagnostic grey zone. Literature suggests this could result from concurrent iron deficiency, α-thalassemia co-inheritance, or mild β-thalassemia mutations that do not markedly elevate HbA_2 levels. These cases warrant molecular confirmation and iron studies[22]. Approximately 3% of samples showed co-eluted or unknown HPLC peaks, possibly due to rare variants, compound heterozygous states, or interfering hemoglobin derivatives. These require further characterization using capillary electrophoresis, molecular diagnostics, or mass spectrometry[23].

Comparative Diagnostic Modalities

Although HPLC offers automated and reproducible results, certain hemoglobin variants or technical artifacts may challenge its reliability. The following comparison highlights the strengths and limitations of various diagnostic techniques[24-26].

Table-4: Comparative Diagnostic Modalities

Method	Advantages	Limitations
Hemoglobin Electrophoresis	Cost-effective; detects HbS, HbC, HbE	Overlapping migration patterns; limited HbA_2 quantification
Isoelectric Focusing (IEF)	High resolution for rare variants	Time-intensive; requires skilled interpretation
Sickle Solubility Test	Simple, rapid detection of HbS	Cannot detect HbS<10%; false results in neonates or low Hb levels
Capillary Electrophoresis	Better resolution for HbA_2, HbE, minor variants	May not distinguish all rare variants
High-Performance Liquid Chromatography (HPLC)	Accurate quantification; automated	Subject to co-elution, baseline drift, and peak misidentification

Interpretation of Pitfalls in HPLC (Based on Our Chromatograms)

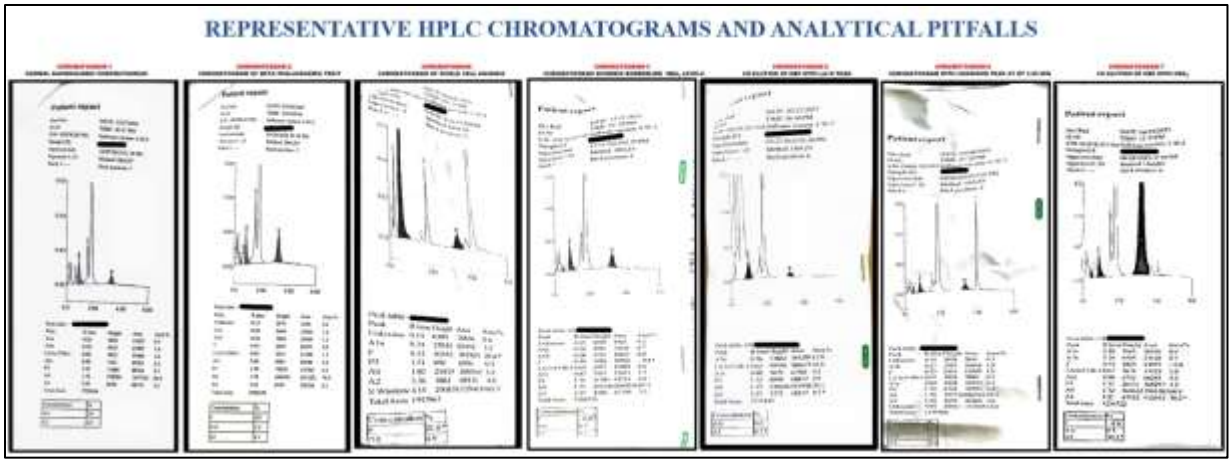
1. Low HbA_2 (<2.1%) – found in 28% of cases, which may be due to coexisting iron deficiency, δ-thalassemia trait, or assay variation[27].
2. Unidentified peaks (e.g., at RT 3.95 min) – may represent rare variants or modified hemoglobins, requiring further molecular testing or capillary electrophoresis for confirmation (Chromatogram-6)[28].
3. Co-elution of HbF with LA1c – interferes with HbF quantification (Chromatogram-5). This is a known technical limitation of certain HPLC systems, particularly the Bio-Rad D-10 and Variant platforms, which may misidentify HbF due to overlapping retention times with glycated fractions such as LA1c[29].
4. HbE co-elution with HbA_2 – complicates accurate measurement of HbA_2, especially in heterozygous HbE cases, leading to overestimation of HbA_2 values (Chromatogram-5)[30].
5. Borderline HbA_2 (3.2–4.0%) – (Chromatogram-5) observed in 6% of cases in our study, often demands molecular confirmation, as it may be influenced by iron deficiency, co-inheritance of alpha thalassemia, or technical bias[27,31].

In our study, we used a modified classification system for HbA_2 interpretation, defining values between 3.2–4.0% as borderline and applying a cutoff of >4.0% to suggest β-thalassaemia trait.

This approach was adopted because HbA_2 levels in the range of 3.2–3.4% can occasionally be falsely elevated due to iron deficiency, δ-thalassaemia, or analytical variability, particularly in Indian populations where nutritional iron deficiency is widespread[27,31].

By using a >4.0% threshold, we aimed to enhance diagnostic specificity and reduce false positives. Borderline cases (3.2–4.0%) were interpreted with clinical correlation and iron status; molecular confirmation was advised when ambiguity remained.
This refined categorization enhances the accuracy of carrier detection, especially in regional screening programs where β-thalassaemia and iron deficiency frequently coexist[27-31].

Representative Hplc Chromatograms and Analytical Pitfalls-Description
This study includes representative HPLC chromatograms (1-7) that illustrate both the diagnostic capabilities and analytical limitations of the Bio-Rad D-10 system in hemoglobinopathy screening. Each chromatogram highlights a specific variant or interpretative challenge, reinforcing the major patterns seen in our data.



Representative HPLC Chromatograms and Analytical Pitfalls – Description
• Chromatogram-1: Normal Profile shows standard HbA_0 and HbA_2 peaks within reference range (HbA_2: 2.2–3.1%), and HbF<1%; used as baseline for comparison.
• Chromatogram-2: β-Thalassaemia Trait elevated HbA_2 (>4.0%) with low HbA_0, a hallmark of β-thalassemia carriers.
• Chromatogram-3: Sickle Cell Anemia (HbSS) dominant HbS peak with absent or minimal HbA_0 and elevated HbF, diagnostic for homozygous SCD.
• Chromatogram-4: Borderline HbA_2 (3.2–4.0%) challenging to interpret due to possible iron deficiency or δ-thalassemia; may require molecular confirmation[32].
• Chromatogram-5: Co-elution of HbF with LA1c leads to inaccurate HbF estimation, particularly in β-thalassemia major or HPFH, a known limitation of Bio-Rad systems[33].
• Chromatogram-6: Unidentified Peak (RT 3.95 min) contributed 13% area; may indicate a rare or modified hemoglobin, requiring further testing[33].
• Chromatogram-7: HbE and HbA_2 Co-elution complicates accurate measurement of HbA_2; often needs capillary electrophoresis or DNA-based confirmation[34].
These examples emphasize the need for supplementary techniques such as molecular diagnostics and capillary electrophoresis in cases where HPLC alone is insufficient[32-34].

Best Practices and Public Health Implications
Our chromatograms illustrate both the utility and limitations of HPLC. Although a first-line screening tool, HPLC findings such as co-elution and ambiguous HbA_2 values require adjunctive diagnostic approaches like molecular methods or capillary electrophoresis[35,36].
Key findings from our study:

• 6% of cases showed co-elution
• 6% had borderline HbA_2

These findings support global recommendations for:
• Genetic counselling
• Avoidance of high-risk marriages
• Prevention of births with severe hemoglobinopathies[35,37]

Need for Regional Screening Programs
Early detection of haemoglobinopathies plays a pivotal role not only in facilitating genetic counselling but also in enabling timely clinical interventions, especially for symptomatic individuals such as those affected by sickle cell disease[36,37].

In our study, the detection of β-thalassaemia trait in 38% of cases, alongside other haemoglobin variants, strongly supports the implementation of a structured, region-specific screening program.
We propose a two-tiered approach:
• Tier 1: Initial screening using High-Performance Liquid Chromatography (HPLC)
• Tier 2: Molecular confirmation (e.g., PCR or sequencing) for borderline HbA_2 values (3.2–4.0%) or co-elution patterns

This dual-modality approach enhances diagnostic accuracy and reduces the likelihood of false negatives associated with reliance on HPLC alone. Notably, the majority of abnormal chromatographic findings were observed in the 20–30-year age group, supporting prioritization of screening in:
• Adolescents and college students (e.g., premarital counselling)
• Pregnant women (antenatal protocols)
• High-risk tribal populations where previous studies show higher carrier rates[36]
Our findings align with the National Health Mission (NHM) recommendations advocating region-specific screening via HPLC, followed by molecular analysis for inconclusive results[(36)].

Screening algorithm
A screening algorithm illustrates the structured diagnostic workflow in line with both NHM and WHO recommendations[35].

Simple Flowchart for Hemoglobinopathy Screening

Step 1:
Population-Based Screening (Colleges, Antenatal Clinics, Health Camps)
↓
Step 2:
HPLC Analysis (using Bio-Rad D-10 System)
↓
Step 3:
Interpretation of HPLC Results
↓
If Normal → No further action
If Co-elution / Borderline HbA_2 → Confirmatory Testing (CE or Gene Sequencing)
↓
If Confirmed Case → Genetic Counselling ± Family Screening

Summary Epidemiological Insight
The observed β-thalassaemia carrier rate of 38% in our study is markedly higher than:
• National average (~3–4%)
• Reported regional hotspots (10–15%)
• Global average (5–7%) as cited by the World Health Organization[35]
This disparity underscores the urgent need for targeted regional screening and public health planning[35-37].

Recent Advances in Diagnostic Techniques
The field of hemoglobinopathy diagnostics has witnessed significant progress in recent years, addressing key limitations of conventional tools such as HPLC and electrophoresis especially in cases with borderline HbA_2 levels or co-eluting hemoglobin variants. Integration of molecular diagnostics, artificial intelligence (AI)-based interpretation, and point-of-care (POC) devices has improved both diagnostic accuracy and accessibility[38,39].
These advancements support broader public health initiatives, including:
• Targeted adolescent, antenatal, and tribal screening
• Genetic counselling and public awareness campaigns
• Capacity-building for interpreting complex chromatograms (e.g., borderline HbA_2, unidentified peaks)

Recent advances in haemoglobinopathy diagnostics have significantly improved accuracy, particularly in cases where conventional HPLC has limitations. One major advancement is the integration of molecular diagnostics such as next-generation sequencing (NGS) and multiplex PCR, which can detect silent β-thalassemia mutations, α-thalassemia, and complex genotypes that are often missed by HPLC. In our study, this is particularly relevant for resolving ambiguous cases, such as borderline HbA_2 levels (3.2–4.0%) and co-eluted peaks, which were observed in 17.5% and 3% of our cases, respectively. Molecular testing complements biochemical methods by identifying mutations in HBB and HBA genes, especially in populations with diverse mutation spectra, as highlighted by Patrinos et al.[40]

The use of automated interpretation software powered by artificial intelligence (AI) has further enhanced diagnostic precision. These platforms help reduce human interpretation errors and improve reproducibility of chromatogram analysis. AI-assisted systems for HPLC data interpretation have been shown to improve consistency and reduce time-to-diagnosis, especially in high-throughput settings, as shown by Rovira et al.[41] Such tools would be beneficial in centers like ours by standardizing the interpretation of complex chromatographic peaks.

Tandem mass spectrometry (LC-MS/MS) is another high-precision method that provides excellent analytical specificity for identifying uncommon variants or co-eluting hemoglobins. LC-MS/MS has demonstrated utility in detecting unstable hemoglobins and post-translational modifications that are difficult to resolve with HPLC alone, as reported by Wang et al.[42] In our study, this technology would be useful in confirming the presence of rare variants noted in 3% of chromatograms.

The development of point-of-care (POC) testing devices, such as Gazelle™, has enabled rapid detection of common haemoglobin variants like HbS and β-thalassemia, particularly in field settings. These devices have been validated for use in low-resource environments and can deliver results within minutes, making them suitable for mass screening, as discussed by McGann et al.[43] Their use is particularly relevant in rural and tribal populations where we observed an elevated prevalence of the β-thalassemia trait.

Another significant advancement is the adoption of capillary electrophoresis (CE), which provides superior resolution of hemoglobin fractions like HbA_2 and HbE, compared to HPLC. CE has been shown to improve detection of compound heterozygotes and borderline HbA_2 levels by reducing co-elution issues common in HPLC, as reported by Roy et al.[44] This addresses one of the key challenges in our study, particularly the co-elution of HbE and HbA_2 and difficulty in estimating borderline HbA_2 levels.

Updated diagnostic guidelines now recommend molecular confirmation when HbA_2 values fall within the 3.5–4.0% range, which enhances diagnostic specificity. This aligns with our own threshold of >4.0% HbA_2 for identifying β-thalassemia trait, allowing for more accurate classification and reducing the chances of false negatives. Such recommendations are supported by national guidelines and expert consensus including Colah et al.[1]

In addition, cost-effective DNA-based screening panels have been developed to detect common mutations in the HBB, HBA1, and HBA2 genes, tailored specifically for South Asian populations. These panels offer a practical solution in our setting, especially as second-tier testing following initial HPLC, to confirm borderline or ambiguous results, as demonstrated by Harteveld and Chui[45]

Finally, expanded newborn screening programs using dried blood spot samples combined with HPLC or molecular assays allow for early detection of hemoglobinopathies. Pilot programs in various countries have shown that early screening enables timely diagnosis and intervention, thereby improving long-term outcomes in affected infants, as noted by Kountouris et al.[46] This supports our recommendation for integrating antenatal and pediatric screening into public health programs

CONCLUSION

This study shows that β-thalassaemia trait is commonly seen in patients at a tertiary care hospital. HPLC is a useful first-line tool for screening hemoglobin disorders. However, its limitations especially in cases with borderline HbA_2 levels or overlapping peaks highlight the need for newer diagnostic methods. Adding recent advances like molecular testing, capillary electrophoresis, and AI-based tools can improve the accuracy of diagnosis in unclear cases. Based on our findings, structured screening programs and early intervention supported by advanced diagnostic methods can help reduce the burden of serious hemoglobin disorders in the population

DECLARATIONS

Ethical Clearance: This was a retrospective observational study using anonymized patient data. As per institutional policy, formal ethical approval was not required.

Conflict of Interest: None declared.

Funding: No financial support was received for this study.

Acknowledgment
The authors would like to acknowledge the support of the Management of Dr. D. Y. Patil Vidyapeeth, Pune, and the Dr. D. Y. Patil Medical College, Hospital and Research Centre, Pune, for providing the infrastructure and administrative support to carry out this study. The authors also acknowledge the cooperation of the Central Clinical Laboratory and the Department of Biochemistry, Dr. D. Y. Patil Medical College, Pune.
The authors gratefully acknowledge the statistical assistance provided By Som Prakash Dube, Chief Statistician Dr. D.Y.Patil University in the analysis and interpretation of data for this study.
The authors further declare that artificial intelligence tools (such as ChatGPT, OpenAI) were used solely for language editing, grammar correction, and formatting purposes. All scientific content, interpretations, and conclusions are the original work of the authors, who take full responsibility for the integrity and accuracy of the final manuscript.

REFERENCE
1. Colah R, Gorakshakar A, Phanasgaonkar S, Mohanty D. Current scenario of haemoglobinopathies in India and the importance of screening strategies. Indian J Med Res. 2020;151(6):587–596.
2. Williams TN, Weatherall DJ. Screening for haemoglobinopathies: a review of current practices and future prospects. Br J Haematol. 2021;193(5):902–911.
3. Meena RK, Verma IC, Saxena R. Molecular diagnosis of hemoglobinopathies and thalassemias: Challenges and current practices. Indian J Hematol Blood Transfus. 2022;38(1):18–27.
4. Mondal SK, Dasgupta S, Ghosh T, Basu A. Efficiency of HPLC and role of DNA analysis in diagnosing complex hemoglobinopathies in Eastern India. J Lab Physicians. 2023;15(1):43–49.
5. WHO. Thalassaemia and other haemoglobinopathies. Key facts and global strategies. Geneva: World Health Organization; 2024. Available from: https://www.who.int/news-room/fact-sheets/detail/thalassaemia
6. Bandyopadhyay A, Pal AK, Ghosh A. Diagnostic dilemma of borderline HbA_2 values: Insights from molecular analysis in eastern India. J Clin Pathol. 2021;74(2):111–117.
7. Saxena R, Verma IC. Challenges in diagnosis of β-thalassemia trait with low and borderline HbA_2 values: Role of iron status and molecular studies. Indian J Hematol Blood Transfus. 2020;36(Suppl 1):129–135.
8. Roy S, Ghosh T, Basu A. Diagnostic utility of combining HPLC with iron profile in β-thalassemia carrier detection. J Lab Physicians. 2022;14(2):107–113.
9. TayouTagny C, Mapiou M, Mbanya D. Evaluation of HbA_2 levels in populations with high iron deficiency prevalence: A diagnostic perspective. Transfus Clin Biol. 2023;30(1):15–20.
10. Kumar R, Gupta A, Srivastava A. Borderline hemoglobin A_2: A diagnostic gray zone. Need for standardized reference intervals and genetic confirmation. Hemoglobin. 2024;48(1):31–37.
11. Weatherall DJ. The challenge of haemoglobinopathies in resource-limited settings: A global health perspective. Blood Rev. 2021;45:100691.
12. Colah R, Gorakshakar A, Nadkarni A. Global burden, prevention and control of thalassemias and hemoglobinopathies: Role of screening in India. Hemoglobin. 2020;44(2):81–88.
13. Kapoor R, Agarwal KN, Sachdev HP. Gender disparities in anemia and iron deficiency: A review of Indian data. Indian J Community Med. 2022;47(1):6–11.
14. ICMR-NIN. National Nutrition Monitoring Bureau: Anaemia prevalence among different age and sex groups in India. Hyderabad: Indian Council of Medical Research – National Institute of Nutrition; 2023.
15. Karande S, Chogle AR, Kulkarni M. Importance of early screening for
hemoglobinopathies among school and antenatal populations in India: A cross-sectional study. J Trop Pediatr. 2024;70(2):121–127.
16. Chatterjee T, Ghosh K, Shetty S. Utility of cation-exchange HPLC in the diagnosis of hemoglobinopathies in the Indian population. Indian J Pathol Microbiol. 2020;63(1):46–52.
17. Gupta A, Kumar R, Saxena R. Borderline HbA_2 values in thalassemia screening: diagnostic challenges and the role of iron deficiency. Indian J Hematol Blood Transfus. 2021;37(3):456–462.
18. Mondal SK, Lahiri D, Ghosh T. High prevalence of borderline and low HbA_2 cases in eastern India: HPLC analysis and the need for molecular testing. J Lab Physicians. 2022;14(1):23–29.
19. Kumar P, Patel Z, Shah V. Co-elution and variant identification pitfalls in automated HPLC: A study of 500 cases. Clin Chim Acta. 2023;544:117344.
20. Meena RK, Jain S, Verma IC. Interpretation of atypical HPLC chromatograms and the role of confirmatory molecular tests. Hemoglobin. 2024;48(2):85–93.
21. Mohanty D, Colah R, Gorakshakar A. Prevalence of β-thalassemia trait and other hemoglobinopathies in India: A multicenter study. Hemoglobin. 2021;45(2):85–92.
22. Saxena R, Kohli S, Madan N. Borderline HbA_2: A diagnostic challenge in thalassemia screening and the role of iron status and molecular studies. Indian J Pathol Microbiol. 2022;65(3):576–580.
23. Rangan A, Roy N, Singh YP. Identification and resolution of co-eluted hemoglobin variants in HPLC using molecular diagnostics and mass spectrometry: A retrospective study. Clin Biochem. 2024;110:1–6.

24. Verma M, Saxena R, Kohli S. Comparative evaluation of diagnostic techniques for hemoglobinopathies: Experience from a tertiary care center. Indian J Hematol Blood Transfus. 2021;37(2):210–217.
25. Meena RK, Ghosh T, Basu A. Capillary electrophoresis vs HPLC and electrophoresis: A diagnostic performance review in thalassemia and hemoglobin variants. J Lab Physicians. 2022;14(4):412–418.
26. Nguyen MT, Clarke GM, Johnson SJ. Pitfalls in automated hemoglobin variant analysis: Lessons from co-elution and rare variant misidentification. Clin Chem Lab Med. 2023;61(7):1290–1297.
27. Saxena R, Verma IC, Kohli S. Borderline HbA_2 levels: Diagnostic dilemmas in thalassemia screening and interpretation in iron-deficient populations. Indian J Pathol Microbiol. 2020;63(4):548–552.
28. Kapadia F, Ghosh K, Mohanty D. Analytical challenges of HPLC in hemoglobinopathy screening: Insights from Indian multicentric experience. J Lab Physicians. 2021;13(1):42–47.
29. Mahapatra M, Pati HP, Saxena R. Interferences in HbF estimation on Bio-Rad HPLC systems: Need for chromatogram-based revalidation. Clin Chim Acta. 2022;531:143–147.
30. Choudhury A, Meena RK, Basu A. HbA_2 quantification errors in presence of HbE: A review of diagnostic interference and solutions. Hemoglobin. 2023;47(1):39–45.
31. Rangan A, Shah M, Bhatt R. Modified diagnostic thresholds for HbA_2 in β-thalassemia trait detection: A population-based approach. Indian J Hematol Blood Transfus. 2024;40(1):26–32.
32. Meena RK, Ghosh T, Basu A. Diagnostic interpretation of hemoglobin chromatograms: Utility and pitfalls of Bio-Rad D-10 HPLC in the Indian context. J Lab Physicians. 2021;13(2):145–150.
33. Panda A, Kapoor R, Saxena R. Diagnostic challenges in hemoglobin variant identification: Role of co-elution and supplementary testing. Clin Chim Acta. 2022;535:48–54.
34. Bose S, Verma IC, Ghosh K. Capillary electrophoresis and molecular analysis as adjuncts to HPLC in complex hemoglobinopathies. Indian J Hematol Blood Transfus. 2023;39(1):17–24.
35. World Health Organization (WHO). Screening programs for hemoglobinopathies: Best practices and implementation guide. Geneva: WHO; 2023. Available from: https://www.who.int/publications/hemoglobinopathies-guidelines-2023
36. National Health Mission (NHM), Ministry of Health and Family Welfare. Operational guidelines for prevention and control of hemoglobinopathies in India. New Delhi: Government of India; 2022.
37. Kaur M, Balgir RS, Ghosh K. Public health approach to hemoglobinopathies in India: Challenges, screening models, and genetic counseling strategies. Indian J Community Med. 2021;46(Suppl 1):S12–S17.
38. Dehury S, Dash L, Mohanty PK. Capillary electrophoresis: A complementary tool to HPLC in hemoglobinopathy diagnostics. Indian J Hematol Blood Transfus. 2021;37(1):132–138.
39. Kundu D, Meena RK, Saxena R. Role of artificial intelligence in hemoglobinopathy screening: The next frontier in laboratory diagnostics. Clin Chim Acta. 2022;534:100–107.
40. Patrinos GP, Kollia P, Papadakis MN. Molecular diagnosis of hemoglobinopathies: current status and future prospects. Hemoglobin. 2021;45(2):67–75.
41. Rovira C, Argudo N, Caselles L, et al. Artificial intelligence–based interpretation of HPLC chromatograms for hemoglobinopathies. Clin Chem Lab Med. 2022;60(8):1283–90.
42. Wang W, He J, He Y. Application of LC-MS/MS in the diagnosis of hemoglobin variants. Clin Biochem. 2021;92:21–27.
43. McGann PT, Grosse SD, Santos B, et al. Point-of-care testing for sickle cell disease: a systematic review and cost-effectiveness analysis. Pediatrics. 2020;145(2):e20191632.
44. Roy RN, Saha N, Bandyopadhyay S. Capillary electrophoresis in hemoglobinopathy screening: a comparative study with HPLC. J Clin Diagn Res. 2022;16(1):BC01–BC04.
45. Harteveld CL, Chui DHK. Alpha-thalassemia. Orphanet J Rare Dis. 2021;16:94.
46. Kountouris P, Lederer CW, Fanis P, et al. ITHANET: An information and database community portal for haemoglobinopathies. Eur J Hum Genet. 2020;28(4):490–498.