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The spectrum of thalassemia syndromes and abnormal haemoglobins in adult patients based on HPLC in a tertiary care centre of Punjab, India

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Abstract
In the present study adult patients with anemia were analysed for thalassemia syndromes and other haemoglobino pathies by using high performance liquid chromatography. A total of 102 Patients' blood samples were evaluated for complete blood count, RBC indices, reticulocyte count and peripheral blood film for morphology. For the confirmation and characterization of hemoglobinopathy, 'BIORAD VARIANT II' of HPLC instrument with CDM software was utilized. 60.8% of the patients were females while the remaining 39.2% were males. The results of this study showed that 79.4% of patients were having a normal hemoglobin while 17% patients showed the prevalence of thalassemia trait. Two cases of hereditary persistence of fetal hemoglobin (HPFH) were seen and one case each of sickle cell-beta thalassemia (HbSThal) and hemoglobin D-Iran (HD-Iran) were seen. It was observed that 65.4% of the patients without thalassemia trait had normocytic normochromic type of anemia. In contrast, all the patients with thalassemia trait and HbSThal had microcytic hypochromic anemia. This association was statistically significant with hemoglobinopathies mostly having microcytic hypochromic. The present findings show high performance liquid chromatography forms a rapid, accurate, and reproducible tool for the early detection and management of hemoglobinopathies and variants.

Keywords: β-Thalassemia, hemoglobinopathy, double heterozygous for HbS and β-Thalassemia, HbD-Iran

Introduction
About 698 genetically different haemoglobin (Hb) variants are found scattered all over the world causing various haemoglobinopathies [1]. Globally, it is estimated that there are 270 million carriers with abnormal haemoglobins and thalassemias, of which 80 million are carriers of β-thalassemia. The frequency of beta-thalassemia trait in India has been reported to vary from 1 to 17% depending on the region studied, with an average of 3.3% [2]. Haemoglobin E has been reported as the most common haemoglobin variant in Southeast Asia and the second most prevalent worldwide. However, in India it is commonly seen in the north-eastern region with a prevalence of 10.9% but is relatively uncommon in other parts [3, 4]. A characteristic feature of thalassaemia syndromes is their marked phenotypic heterogeneity. For over 20 years now, the molecular basis of thalassaemia has been studied and understood in great detail. The number of mutations identified as causing thalassaemia is large and continues to grow. Clinically, it has been seen that in rare β-thalassemia patients with elevated production of HbF resulted in a milder clinical course and infants with β-thalassemia only began to show symptoms after the expression of HbF declined in the months following birth [5]. Their clinical severity widely varies, ranging from asymptomatic forms to severe or even fatal entities. The heterogeneous variant of moderate severity is called beta-thalassemia intermedia. Hemoglobinopathies showed highly variable clinical signs, ranging from mild hypochromic anemia to moderate hematological disease to severe, lifelong, transfusion-dependent anemia with multiorgan involvement. Plethora of hemoglobin variants are prevalent in India owing to ethnic diversity of its population with minimal to major clinical significance. Detection of asymptomatic carriers by reliable laboratory methods is the cornerstone of prevention of this serious health problem [6].

With increasing global awareness and mass screening programs undertaken at various levels by health care system, the responsibilities of a medical professional have greatly enhanced in detection and prevention of thalassemia. Awareness about the diagnostic problems as well as their solutions is very important so that one does not miss a single case.

Thus taking in account the increasing burden of hemoglonipathies in Indian subcontinent, in this study adult patient with anemia will be analysed for thalassemia syndromes and other haemoglobinopathies by using HPLC.

Materials and Method

The present study was conducted in the Department of Pathology, Government Medical College, and Amritsar after approval from the Institutional Ethics Committee. This study included 102 adult patients with anemia who did not responded to the conventional treatment and were suspected of haemoglobinopathy. Patients with nutritional anemia, inflammatory disorder, bleeding disorders and any hematological malignancy were excluded.

Patients' blood samples were evaluated for complete blood count, RBC indices, reticulocyte count and peripheral blood film for morphology and were compared with reference ranges as depicted in supplementary material [Table S1].

For the confirmation and characterization of hemoglobinopathy, 'BIORAD VARIANT II' of high performance liquid chromatography (HPLC) instrument for Beta Thalassemia screening with CDM software (Clinical Data Management) was used by comparing with manufacturer assigned retention time window [Table S2].

Results

Out of a total of 102 patients with anemia, maximum patients 47.1% were in age group 21-30 years, followed by 25.5% in the age group 31-40 years, 16.7% below 20 years of age. The age group of 41-50 years and 51-60 years had 6.9% and 3.9% patients respectively. 60.8% of the patients were females while the remaining 39.2% percent were males. 96.1% of the patients had no comorbidity while 2% had diabetes, 1 case each of Hypertension and hyperthyroidism.

In present study maximum cases 68(66.7%) were of moderate anemia followed by 17(16.7%) each of mild and severe anemia. The chromatograms depicting various abnormal hemoglobin are depicted in Figure 1. The distribution of patients according to abnormal Hb & thalassemic trait showed that 81 (79.4%) of patients were having a normal Hb while 17 (16.7%) patients showed the prevalence of thalassemia trait. In 2 (2%) cases HPFH was seen and in 1 case each of HbSThal (HBT) and HbD-Iran were seen.

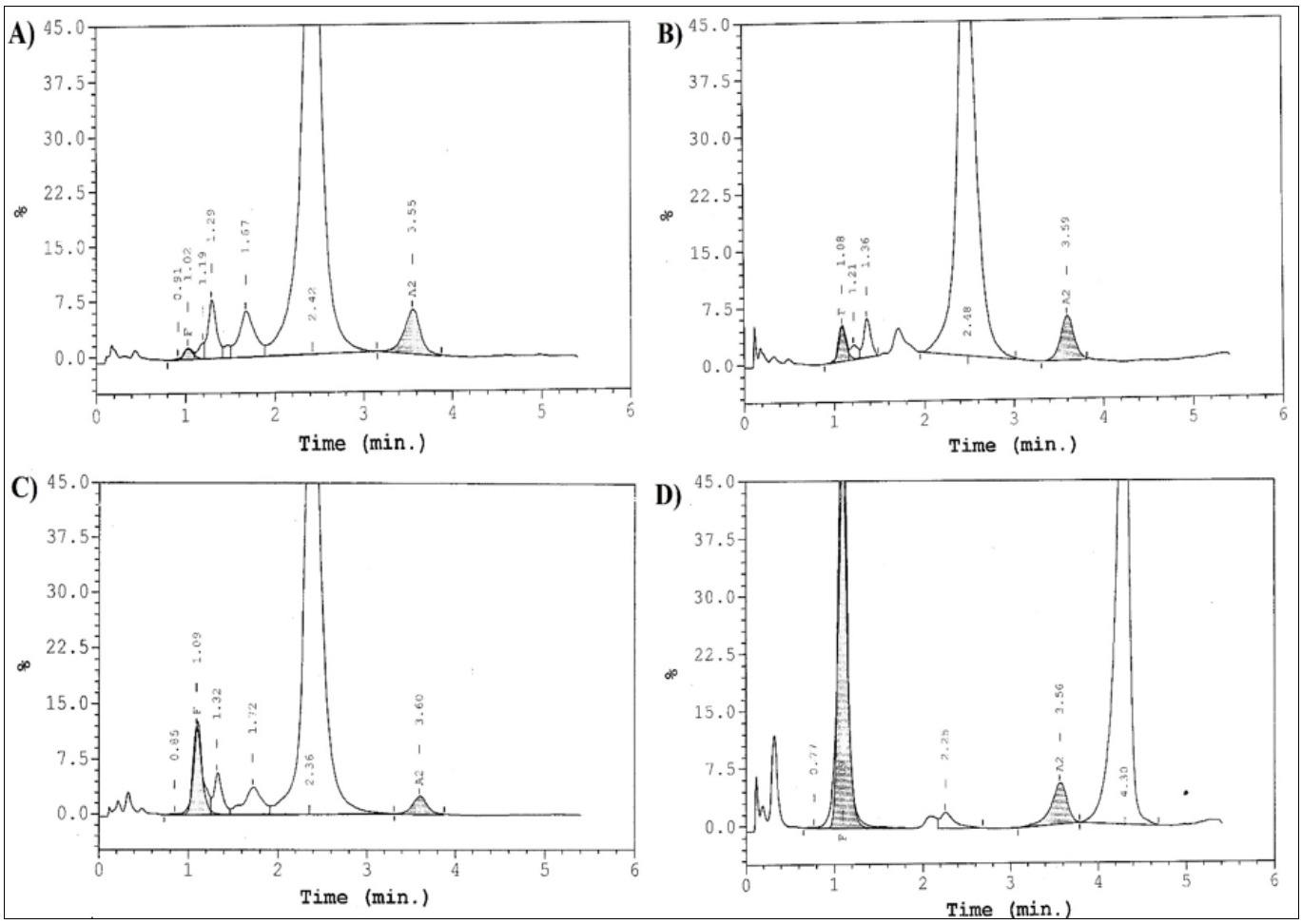

Fig 1: Chromatograms depicting A) Elevated HbA2 in thalassemia trait B) Elevated HbA2 and HbF in thalassemia trait C) Elevated HbF in HPFH D) Elevated HbS, HbF and HbA2 in HbSThal

Table 1: Mean Value Analysis of Various Parameters in Different Trait

Trait	No Trait		Thalassemia trait		HPFH		HbSThal		Hb D-Iran		F value	P value
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		ANOVA
RBC count(10^6/ul)	3.55	0.54	4.44	0.82	3.23	1.32	4.12	.	4.60	.	8.59	<0.001
HB(g/dl)	9.59	1.33	9.15	1.20	8.65	3.89	9.20	.	11.50	.	1.12	0.35
MCV	86.53	8.51	70.72	2.16	83.40	0.57	74.50	.	78.00	.	15.02	<0.00
MCH	27.29	3.04	21.03	2.99	26.50	1.23	22.33	.	25.00	.	11.61	<0.00
MCHC	31.58	2.29	29.77	4.17	31.8	1.7	29.97	.	32.50	.	2.92	1.58
RDW	14.17	1.73	15.68	1.08	13.90	0.57	15.20	.	12.60	.	3.38	<0.01
Reticulocyte Count	1.56	0.77	1.68	0.33	1.35	0.35	3.50	.	1.80	.	1.96	0.11
HbA2	2.77	0.42	5.49	0.63	2.60	0.28	5.30	.	45.80	.	2222.21	<0.00
HbF	0.58	0.39	1.59	1.50	6.80	1.27	30.00	.	1.00	.	451.93	<0.00

Table 1 shows the mean value analysis of various parameters in different trait. It was observed that RBC count (p<.001) and RDW (p=.01) was significantly more in hemoglobinopathies than patients without trait. Similarly, the mean value of MCV and MCH was significantly less in patients with Thalassemia syndrome and abnormal hemoglobin. Reticulocytes count and MCHC did not show any significant difference between the different groups.

Table 2: Association of Anemia, Thalassemia Syndromes and Abnormal Haemoglobins

Anemia	Mid		Moderate		Severe		Total		P value
Triat	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**Chi square**
Trait Absent	15	18.50%	53	65.50%	13	16.00%	81	100.00%	0.106
Thalassemia trait	0	0.00%	14	82.40%	3	17.60%	17	100.00%	
HPFH	1	50.00%	0	0.00%	1	50.00%	2	100.00%	
HbSThal	0	0.00%	1	100.00%	0	0.00%	1	100.00%	
Hb D-*Iran*	1	100.00%	0	0.00%	0	0.00%	1	100.00%	
Total	17	16.70%	68	66.60%	17	16.70%	102	100.00%	

Table 2 shows association between anemia and various hemoglobinopathies observed in the present study. No significant association was observed between presence of hemoglobinopathies and anemia (p=0.106).

Table 3: Association of Peripheral Blood Film and Thalassemia Syndromes and Abnormal Haemoglobins

Trait	Trait Absent		Thalassemia Trait		HPFH		Hetero β-thalassemia		*Hb* D-*Iran*		Total	
Peripheral smear	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
NC/NC	53	65.40%	0	0.00%	1	50.00%	0	0.00%	0	0.00%	54	52.90%
NC/HC	12	14.80%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	12	11.80%
MC/HC	13	16.00%	17	100.00%	1	50.00%	1	100.00%	1	100.00%	33	32.40%
NC/NC macro	1	1.20%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	1	1.00%
NC/HC macro	1	1.20%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	1	1.00%
Dimorphic	1	1.20%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	1	1.00%
Total	81	100.00%	17	100.00%	2	100.00%	1	100.00%	1	100.00%	102	100.00%
P value	<.001											

It was observed that majority of the patients without trait had normocytic normochromic type of anemia (65.40%) followed by microcytic hypochromic in 16% (12) and normocytic hypochromic in 14.8% (12). 1.2% cases each were of macrocytic and dimorphic anemia. In contrast all the patients with Thalassemia Trait and HbSThal *had* microcytic hypochromic anemia. This association was statistically significant with hemoglobinopathies mostly having microcytic hypochromic anemia (0<.001).

Discussion

In present study a total of 102 patients, maximum patients 48 (47.1%) were in age group 21-30 years followed by 26 (25.5%) in 31-40 age group. The mean age of the study population was 29.11±8.7 years. We observed female predominance in the present study with 62 (60.8%) of the patients were females while the remaining 40 (39.2%) were males. The distribution of patient according to severity of anemia, maximum cases 68 (66.7%) were of moderate anemia followed by 17 (16.7%) each of mild and severe anemia. It was observed that out of 102 patients in total, 21 cases of abnormal hemoglobin were detected out of which 17 patients of thalassemia trait, 2 cases of HPFH and 1 case each of HbSThal (HBT) and HbD-Iran were seen. Out of total hemoglobinopathies, thalassemia trait predominated with total of 80.9% patients followed by 9.5% patients of HPFH, 4.8% of HbSThal and HbD-Iran each.

Sachdev *et al.* [7] in their study observed in a total of 2600 out of which 232 cases (8.9%) were of beta thalassemia trait were diagnosed which was less than observed in the present study. In their study they observed cases of Elevated HbF (25 cases), HbE (seven cases including two HbE homozygous and five HbE heterozygous), Double heterozygous HbE-β thalassemia trait (six cases), HbQ India (five cases), Double heterozygous HbQ India-β thalassemia trait (two cases), HbS (total cases three including one HbS homozygous; two HbS-β thalassemia trait) and one case each of HbJ Meerut, HbD-Iran and Hb Lepore trait.

In a study by Gupta *et al.* [8] 955 patients of anemia with suspected haemoglobinopathies were analysed and 137 (14.3%) patients showed different abnormal haemoglobins variants. Of these 91 (66.4%) patients were diagnosed to have heterozygous beta-thalassaemia based on the high level

of HbA_2 (>3.9%). In present study also based on the high level of HbA_2 (>3.9%) the Thalassemia trait was seen in 17% patients, Whereas, HbSThal (HBT) and HbD-Iran was seen in 0.9% patients each while HPFH was observed in 2% cases. Buch *et al*. [9] in their study also reported maximum number of patients to be of Thalassemia trait with the prevalence of 3.7%. Beta Thalassemia trait was diagnosed based on high levels of HbA2 (4-8%) in their study by HPLC. The prevalence of trait was low in their study than observed in the present study. In their study HbSThal (HBT) was detected in 0.28% patients while in present study it was present in 0.9% patients. In a study by Pant *et al*. [10] among 4800 cases screened, 290 (6.04%) cases were detected with abnormal hemoglobin in this study while in present study 20% patients with abnormal hemoglobinopathies were reported.

Khera *et al*. [11] in their study showed that out of total Consecutive 1,500 blood samples 110 cases of Thalassemia syndromes and hemoglobinopathies were detected in which maximum cases were of Thalassemia trait.

The diagnosis of BTT is established by the presence of characteristic red blood cell microcytosis and elevated levels of HbA2. HPLC has the advantage of quantifying HbF and HbA2 along with detecting other variants in a single screening test. Besides HPLC, there are other analytical procedures used for detection of thalassemia and hemoglobinopathies such as alkaline and acid electrophoresis, HbA2 quantification by ion-exchange column chromatography, and HbF quantification by alkali denaturation and radial immunodiffusion [12].

In present study the mean HbA2 was 2.77±0.42 for patients without trait and for beta thalassemia trait it was 5.49 ±0.63 while for HPF it was 2.60±0.28, for HbSThal and HbD-Iran being 5.30 and 45.80 respectively. Similarly, HbF was significantly more in patients with HPF (6.80±1.27) and HbSThal [30].

HPLC also helped us in detecting various heterozygous states. Though these abnormal variants have less clinical significance, when combined with other variants, they can give rise to severe disease. This once again highlights the significance of mass screening of the population. We had 1 case with combination of HbS with thalassemia trait. Although these states can be diagnosed both on HPLC and gel electrophoresis, use of HPLC has an added advantage of further sub-classifying these syndromes based on identification and quantification of various Hemoglobins depending on their retention time. HbD Punjab and HbD Iran exhibit identical electrophoretic mobilities at alkaline pH, but their mean retention time in HPLC is unique and significantly different. This differentiation is in fact quite significant especially in a double heterozygous state with HbS, as HbSD-Punjab is a significant sickling disorder whereas HbSD-Iran is clinically benign [13, 14]. All the cases of heterozygous states should undergo proper genetic counselling to avoid severe hemoglobinopathies in offspring.

Conclusion

The present findings show HPLC forms a rapid, accurate, and reproducible tool for the early detection and management of hemoglobinopathies and variants. This is especially important in view of the high incidence of beta thalassemia trait in the Indian subcontinent. Thus, premarital and antenatal screening should be mandatory to prevent the birth of offspring with β thalassemia major. Moreover, knowledge of common Haemoglobin patterns in a particular region helps to formulate appropriate preventive and therapeutic strategies.

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Disclosure statement

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of this article.

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