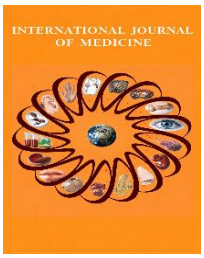

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Original Article

Hematological indices and hemoglobin high-pressure liquid chromatography for diagnosis of hemoglobinopathies in a secondary care center in North-East India

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Abstract

Background: Hemoglobinopathies occur globally and are often presented with anemia. This study was performed in a secondary care center in Sonitpur District, Assam, to study the usefulness of hematological indices and hemoglobin High-Pressure Liquid Chromatography (HPLC) for characterization of hemoglobinopathies and to quantify the prevalence and types of anemia in a resource-poor setting of North-East India.

Methods: Data of 9936 hemoglobin estimations and 708 peripheral blood smear examinations performed over a year were retrieved. Complete blood count for 170 patients was performed by XS 800i Five-Part Sysmex Cell Counter whereas hemoglobin (Hb) HPLC was outsourced. Serum iron estimation was done for 100 samples by dry chemistry and serum ferritin assay was tested by direct chemiluminescence for 13 patients. The number of hospital visits, hospitalization duration, blood transfusions, demographic profile, and unusual features in some patients was recorded.

Results: Anemia (Hb < 11.0 g/dl) was present in 79.6% of 9936 samples tested. Microcytic hypochromic anemia was present in 278/534 (52%) patients. The mean age of 170 subjects was 15.4 years (3 months – 56 years) with a slight female preponderance 87/51.2%. Erythrocytosis was observed in 32/18.8% of samples, of which 18 were females. Microcytosis and low mean corpuscular hemoglobin (MCH) were observed in 143 (84.1%) and 153 (90%) samples, respectively. The number and percentage of conditions identified by HPLC are no abnormality in 53 (31.2%), hemoglobin E disease 31 (18.2%), hemoglobin E trait 25 (14.7%), β-thalassemia minor 11 (6.5%), β-thalassemia major 4 (2.4%), compound hemoglobinopathy 15 (8.8%), sickle cell trait 12 (7%) and sickle cell disease 8 (4.7%), and inconclusive in 11 (6.5 %) patients. Serum iron was low in 39 (34.5%), normal in 65 (57.5%), and high in 9 (8%) of the 113 subjects tested.

Conclusions: Prevalence of anemia and hemoglobin E abnormality was high with unexpected severe anemia in some heterozygotes for HbE, HbS, and β- thalassemia. Hemoglobin HPLC was useful in arriving at a presumptive diagnosis and must be used as a frontline investigation even in resource-poor settings.

Keywords: anemia; chromatography; hemoglobinopathies; India; iron

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1. Introduction

Hemoglobinopathies are the commonest single-gene disorders in the world with over 5% of the world population carrying a potentially abnormal hemoglobin gene [1]. The spectrum of hemoglobinopathies differs in North East (NE) India from other parts of the country as shown by Mohanty et. al [2]. This partly prospective study was performed in a secondary care 130 bedded hospital in NE India without a blood bank to study the usefulness of hematological indices and hemoglobin High-Pressure Liquid Chromatography (HPLC) for characterization of hemoglobinopathies and to examine the frequency of anemias and identify morphological types among patients visiting the hospital for outdoor (OPD) or indoor (IPD) treatment. It caters to clientele from very low socioeconomic strata. Ethical clearance was obtained from the institutional ethics committee and informed written consent from the patients or either parent of children.

2. Methods

Information Technology Department extracted hemoglobin estimation and peripheral blood smear (PBS) examination data for one year from 01 Jan 2019 to 31 December 2019 and shared it with the Pathology Department. This information was used to determine the prevalence of anemias (hemoglobin < 11 g/dl) which was graded as severe, moderately severe, and mild as per WHO standards [3]. Anemia identified on PBS examination was categorized on basis of morphology. Clinical history was obtained from all participants and ethnicity was recorded. The number of blood transfusions given to the patients as to available in records was noted.

2.1. Inclusion criteria

- Patients with hemoglobin (Hb) < 11 g/dl with a microcytic hypochromic smear.
- Unexplained gallstones.
- Erythrocytosis (>20%) of normal for age and gender.
- Age <30 years at presentation for the prospective component.

2.2. Exclusion criteria

- Patients with a history of transfusion in the last three months.

The prospective study involved the processing of 100 samples obtained in K_2EDTA Becton Dickinson vacutainers for complete blood count (CBC) by Sysmex XS 800i 5 Part Hematology Analyzer, preparation of PBS, sending a sample in EDTA vacutainer for hemoglobin HPLC to Kolkata Reference Laboratory of Dr. Lal Path Labs Ltd. where it was performed on the D-10™ fully automated Hemoglobin Testing System (Bio-Rad Laboratories, USA). Test results of additional 70 samples that were originally not a part of the project were included after obtaining ethical clearance. Serum iron estimation was done for 90 samples on VITROS® 250 Chemistry System – (Ortho Clinical Diagnostics) by dry chemistry. Serum ferritin assay was carried out on Siemens-Advia Centaur cp by direct chemiluminescence for 13 patients. The iron assay was not done for 11 patients with erythrocytosis that had normal hemoglobin and in 46 patients where it was presumed that the level was likely to be normal or due to poor patient compliance. The number of hospital visits, hospitalization duration, unusual features of some patients, and blood transfusion details of all patients except those that were on regular supportive transfusion therapy were studied.

3. Results

Anemia was present in 79.6% of 9936 samples tested; 1061/10.7 % were severely anemic (Hb < 7.0 g/dl), 2017/20.3% had moderately severe anemia (Hb: 7-8.9 g/dl) and 4832/48.6% had mild anemia (Hb: 9.0-10.9 g/dl).

Types of anemia in one year: 708 peripheral blood smears were examined of which 125 (18%) were normocytic normochromic, 278 (39%) were microcytic hypochromic, 46 (6%) were macrocytic, 85 (12%) were dimorphic or normocytic hypochromic, 103 (15%) were not tested for anemia, and 71 (10%) were normal smears. So essentially microcytic hypochromic anemias comprised 278 /534 (52%) of anemic smears as depicted in Figure 1.

The mean age of patients was 13.4 years (3 months – 30 years) with a female preponderance of 95/56%. Hematological indices showed microcytosis with low MCH in 127 (74.7%) of which 67 (48.8%) had low MCHC. The numerical mean and range for hematological indices was as follows MCV 64.4 fl (43.2-113.4), MCH 19.6 pg (9.8-30.4), MCHC 30.5 g/dl (23-35.9), and RDW CV 21.9% (13.3-48). The number and (%) of conditions identified by HPLC are: - no abnormality 53 (31.2%), Hb E abnormalities 56 (32.9%), β-thalassemia 15 (8.8%), sickle cell abnormality 20 (11.8%), compound heterozygotes 15 (8.8%), and inconclusive 11 (6.5%). Table 1 summarizes demographic features, iron levels, and relevant erythrocyte indices for all nine categories. The demography of subjects was representative of the local clientele and included Bodos, Adivasis, Ahoms, Muslims, Orangs, Mompahs, Nepalis, Bengalis, Biharis, and Oriyas.

Table 1. Demographic profile, erythrocyte indices, and iron status of 170 subjects with suspected hemoglobinopathies: mean values and (range) for all parameters are depicted

Category	Age in years	Sex M/F	Iron N/L/H/ND	RBC x 10^6/ µl	Hb g/dl	HCT (%)	MCV fl	MCH pg	MCHC g/l	RDW CV (%)
Normal (n=53)	13.8 (0.25-30)	21/32	15/20/0/18	4 (1-6.6)	7.1 (2.3-14.9)	24.5 (10.3-48.5)	63.2 (47.6-113)	18.2 (9.8-28.3)	28.2 (23-34.3)	23 (14.9-43.4)
HbEE (n=31)	15.3 (0.8-52)	19/12	18/6/0/7	5 (2.3-6.9)	9.2 (5-12.8)	27.9 (17.6-39.4)	56.7 (44.9-81.2)	18.7 (14.5-25.4)	32.9 (27-35)	22.2 (15.9-39.9)
HBAE (n=25)	10.8 (0.5-32)	12/13	10/7/1/7	4.46 (1.82-6.1)	9.3 (4.1-15.1)	29.9 (13.8-54.2)	66.3 (56.7-97.8)	20.4 (16.2-26.9)	31.4 (26.2-33.9)	19.2 (14.3-38.1)
β-thal trait (n=11)	21.7 (3-48)	3/8	6/1/0/4	4.66 (1.16-6.44)	9 (2.9-13.4)	29 (9.5-41.1)	64.1 (52.3-83.6)	19.7 (16.8-25)	31.6 (27.6-33.5)	20.1 (19.6-29.7)
β-thal major (n= 4)	7 (1.8-9)	0/4	1/0/3/0	2.44 (0.82-3.61)	4.8 (1.5-6.9)	15.8 (5.1-21.3)	64.1 (59-70.7)	19.3 (18-21)	29.9 (26.9-32.4)	35.7 (31.3-41.6)
HbSS (n=8)	19.3 (4-38)	5/3	2/0/0/6	2.46 (1.66-3.86)	6.3 (3.3-9)	20.7 (11.8-30.4)	79 (65-103)	26.7 (21.7-30.4)	33.7 (31.4-35.4)	21 (16.9-32.5)
HbAS (n=12)	24.9 (1.75-42)	7/5	2/2/1/7	4.16 1.59-5.61	10 (2.9-14.4)	31.6 (23.3-46.2)	77.4 (56.4-95.3)	23.9 (17.1-29.1)	31.7 (26.9-34.3)	19.3 (13.1-40.8)
Compound hetero (n=15)1*rpt	9.9 (0.8-30)	8/6	6/1/4/4	3.0 (0.9-5.2)	6.2 (2.1-10.9)	19.7 (7.5-29.6)	67.1 (47.4-83.4)	21.5 (16-27.2)	31.5 (26.8-35.9)	25.4 (17.5-36.7
Inconclusive (n=11)	7.7 (0.2-27)	8/3	5/2/0/4	3.7 (1.7-5.5)	6.8 (2.2-10.4)	23.3 (9.1-32.8)	66.7 (43.2-98.3)	19.3 (13.1-28.8)	28.6 (23.7-32.5)	22.1 (14.5-30.7)

AE: Hemoglobin E trait; AS: Hemoglobin sickle cell trait; EE: E homozygous; F: Female; Hb: Hemoglobin; HCT: Hematocrit; Hetero: Heterozygous; H: High; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; N: Normal, RBC: Red blood cell; RDW CV: Radial distribution width; SS: Sickle cell disease; Thal: Thalassemia.

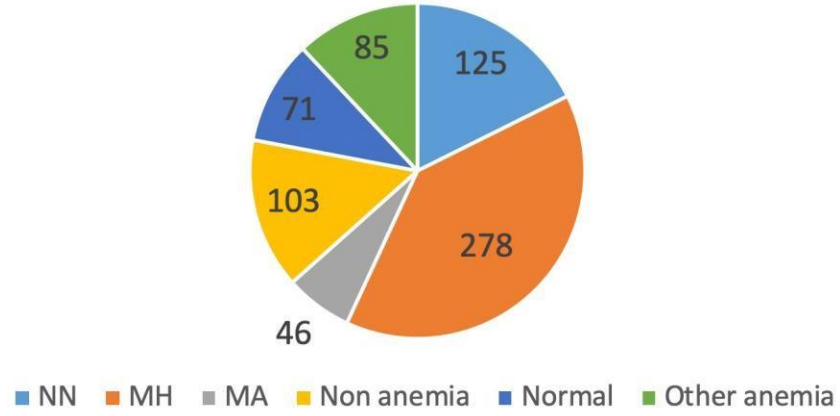

Figure 1. Results of PBS performed from 01 January 2019 to 31 December 2019.
NN: Normocytic normochromic; MH: Microcytic hypochromic; MA: Macrocytic.

Erythrocytosis as defined by absolute erythrocyte count exceeding 4.8 x 10^6/µl for females and 5.5 x 10^6/µl for males was observed in 32 samples (18 females, 14 males) of which 11 had normal hemoglobin. Erythrocytes were microcytic hypochromic in 29 and normocytic normochromic in three patients. HPLC classification, anemia morphology, or absence of anemia in patients with erythrocytosis is depicted in Table 2. In 130 (79%) samples RBC count in 10^6/µl when multiplied by three was more than 30% higher than hemoglobin in g/dl (relative erythrocytosis)- so there was a significant deviation from Rule of Three [4].

3.1. Normal hemoglobin HPLC

This was the largest single category which included 8 (15.1%) infants, 12 (22.6%) adults, and 33 (62.3%) children. Only two individuals were not anemic, but were included in the study because of marked erythrocytosis - counts 6.17 and 6.66 x 10^6/µl with MCV of 60 and 72.8 fl, respectively. The first was a 12-year-old child with nephrotic syndrome and his one earlier report also showed an RBC count of 5.08 x 10^6/µl with Hb of 9.8 g/dl. The second patient was a 29 years old man with lumbar radiculopathy whose Hb was 14.9 g/dl. One or more units of blood transfusion were given to 15/29 severely anemic patients in this category.

Table 2. Hematological indices and HPLC findings in 32 patients with erythrocytosis

HPLC classification	Erythrocytosis	Normal hemoglobin	Anemic	Microcytic hypochromic	Normocytic normochromic
Normal	9	2	7	9	0
Hb EE	12	2	10	12	0
HbAE	5	3	2	3	2
β-thal minor	4	2	2	4	0
HBAS	2	2	0	1	1

HbEE: Hemoglobin E disease; HbAE: Hemoglobin E trait; β thal: Beta-thalassemia; HbAS: Sickle cell trait.

3.2. Inconclusive results

It was not possible to arrive at a diagnosis in 11 patients by HPLC (depicted in Table 3) of which five (45.5%) had received one or more transfusions. Six patients including two adults with severe anemia and three infants with anemia of mild-moderate severity had normal Hb HPLC for age. It was not possible to exclude the presence of β-thalassemia trait in three infants of which two had HbE + A_2 fraction > 78% and the third infant with HbE + A_2 fraction of 13.6% and HbF 52.3% as capillary electrophoresis was not done. Other patients with inconclusive Hb HPLC included a 19-year-old male with unexplained gall stones and chronic anemia, a suspected case of α-thalassemia with slightly reduced HbA and a 14-year-old girl with slightly elevated HbF (1.9%). Due to poor compliance repeat, HPLC was not done for any patients or additional family members.

Table 3. Age, iron profile, RBC count, hemoglobin, MCV, and MCH in inconclusive hemoglobin HPLC

No	Age	RBC x 10^6	Hb g/dl	MCV fl	MCH (pg)	Hb HPLC (HbF/HbA/HbA_2 %)
1	14	3.14	4.4	59.2	14	1.9/86.4/3.4**
2	0.2	1.72	5.5	98.3	32	52.3/31.5/13.6 (A_2+E) ##
3	19	5.49	6.5	43.9	11.8	1/88.1/2.4*
4	5	4.57	10.1	69.1	22.1	5.4/81.3/2.6 *
5	20	1.66	2.2	56.6	13.3	<1/89.8/1.6 *
6	0.25	3.77	9.7	85.1	25.7	5.1/82.9/2.7 Repeat; *
7	1.2	2.12	3.4	66	16	<1/88.6/2.4 *
8	0.25	4.09	8.4	68.9	20.5	18.1/72.4/1.7 *
9	27	3.6	10.4	90.9	28.8	1.5/80.2/3.4 ***
10	1	5.05	6.6	43.2	13.1	9.5/7.2/80.7 (A_2+E) #
11	1	5.16	7.6	53.3	14.7	8/4.4/78.6 (A_2+E) #

*normal for age; **slight increase in Hb F; ***?Alpha thalassemia/ degenerate sample; #HbEE/HbEE + Beta thalassemia, ## Beta thalassemia + HbEe.

3.3. Hemoglobin E disease (HbEE)

Thirty-one patients were homozygous for HbE of which five were severely anemic, seven had moderately severe anemia, 16 had mild anemia, and three had normal hemoglobin. Three of the four severely anemic patients who required blood transfusion had normal iron levels. Microcytosis was present in 29/31 patients. Moderately severe anemia and MCV <50 fl was present in four patients of which three had low iron levels. Mean value of HbA_2 + E fraction was 81.7% (58.2-90.1), mean HbA was 7.45% (2.8 -20.8), and mean HbF 5.3% (1.3-13.1).

3.4. Hemoglobin E trait (HbAE)

Twenty patients (80%) were anemic of which nine had mild anemia, seven had moderately severe anemia, and four had severe anemia of which three were transfused. On Hb HPLC mean HbA_2 + E, HbA, and Hb F fractions were 25% (18.6-28.6), 63.2% (56.3-70.6%), 3.1 (1 -11.1), respectively. A chronic pyelonephritis patient with serum creatinine 1.75 mg/dl, high iron levels with Hb 6 g/dl, MCV 58.7 fl, MCH 18.6 pg, MCHC of 34.3 g/dl, and RDW CV of 17.6% were refractory to treatment which possibly could be attributed to renal disease. Another patient with Hb 8 g/dl and features suggestive iron deficiency (MCV 60.9 fl, MCH 17.8 pg, MCHC 29.2%, RDW CV of 20.8%) and HbA_2 + E 25.2% showed minimal response after four months of hematinic.

3.5. Beta-thalassemia

Included in this category were 11 patients with β-thalassemia minor and four with β-thalassemia major of which 13 (86.7%) had microcytic anemia. Two patients with trait aged 48 and 18 years unexpectedly presented with severe anemia (Hb 2.9 and 6.3 g/dl, respectively) necessitating blood transfusion. The former presented with pancytopenia while the latter was for antenatal care and had chronic microcytic hypochromic anemia with iron deficiency. One newborn with Hb of 13.8 g/dl, MCV 86.5 fl, MCH 28.2 pg, and 10 nucleated RBCs/100 leukocytes at birth was diagnosed as thalassemia major at 18 months age when the child was brought with severe anemia.

3.6. Sickle hemoglobinopathies

Twenty cases of sickle cell anemia were included in the study of which eight patients had sickle cell disease (HbSS) and 12 with the trait (HbAS) as shown in Table 1. Among the latter five had normal hemoglobin, of which, one was symptomatic with cholelithiasis necessitating cholecystectomy. Patients with HbSS had higher mean HbF 19.3%, mean HbS 74.8%, and mean HbA 2.6 %, while the corresponding values in HbAS were 3.2%, 28.2 %, and 54.9%, respectively. Unusual presentations in HbAS included a child who developed a hemolytic crisis requiring two units of blood transfusion and aplastic crisis in a 56-year-old who presented with pancytopenia, portal hypertension with splenomegaly. In spite of long-standing symptoms, the condition was detected only after the commencement of the project in all 13 adult patients. A 38-year-old female patient with a previous history of cholelithiasis had relatively mild disease with an HbS fraction of 79.2% which was possibly due to the presence of Alpha or Delta Beta-thalassemia as HbA_2 was 3.7% (normal < 3.5%). A three-year-old child who first presented with acute urinary retention showed typical findings on Hb HPLC and had C reactive protein > 250 mg/L (normal <10 mg/L) during admission.

3.7. Compound heterozygous hemoglobinopathies

Fifteen samples on HPLC analysis showed more than one type of genetic abnormality including eight cases of HbE and β-thalassemia, two cases of δβ-thalassemia, three cases of HbS/β-thalassemia, and one case each of HbD/HbE, and HbD/HbS [Table 4]. The percentage of HbD was 61.7 and 48.2 with the corresponding percentage of HbE + A_2 and HbS 26.8 and 32.9, respectively. Additional family members were not evaluated for further confirmation and establishing inheritance as advised by the outsourced laboratory. Cholelithiasis with recurrent jaundice was the chief presentation in three adults 22-30 years age. Eleven patients (73.3%) received one or more transfusions – the maximum being five in a patient with HbE & β-thalassemia with HbA_2 + E of 54.2%.

Table 4. Demography, RBC indices, hospitalization data for compound hemoglobinopathies

No	Age	Sex	HB/RBC g/dl x 10^6/µl	MCV/MCH fl/pg	Diagnosis	Number of visits hospitalization (days)
1	9	M	9.6 /4.5	65.1/21.1	δ β Thalassemia trait	6 (0)
2	21	F	6.8/3.01	73.1/22.6	β Thalassemia - HbS	5 (1)
3	1.4	F	9.1/4.85	59/18.8	δ β Thalassemia trait	2 (3)
4	2	M	2.4 /0.9	83.3/26.7	β Thalassemia - HbS	7 (0)
5	0.8	F	6.9/3.69	57.7/21.3	HBE +βδ Thalassemia	8 (1)
6	25	M	2.98/1.68	66.7/19	HBE- β Thalassemia	1 (12)
7	4	F	7.1/2.97	66.4/ 22.1	HBE - β Thalassemia	10 (5)
8	1.8	M	6.9/2.58	80.3/26.6	HBE - β Thalassemia	21 (14)
9	1	M	6.6/5.05	43.2/13.1	HBE- β Thalassemia	1 (5)
10	0.7	M	4.6 /2.87	47.4/16	β Thalassemia - HBE	1 (10)
11	1	M	7.6/5.16	53.3/14.7	β Thalassemia - HBE	1 (4)
12	23	F	6.7/2.47	83.4/27.2	β Thalassemia - HBE	2 (5)
13	30	F	5.5/2.06	74.3/26.7	HbS- HbD	1 (4)
14	5	M	3.3/1.66	71.1/19.9	HbS- β Thalassemia	1 (0)
15	1.5	M	4.68/7.1	49.8/16.8	HbD- HbE	5(1)

3.8. Hospital visits and duration of hospitalization

A little less than one-third of the patients (31.3%) had received only OPD treatment and 23.5% had visited the hospital only once. Antenatal cases, transfusion-dependent patients and hospital employees accounting for 14.3% of the patients had visited >10 times, because of which the average number of hospital visits was 6.88 and average duration of hospitalization was 3.51 days [Table 5].

Table 5. Nature of treatment, hospital visits, and hospitalization

	Inconclusive	Hb S	β-thal	Hb E	Normal
OPD only (%)	0	17.6	0	46.5	27.9
IPD only (%)	7.8	23.5	33.3	4.6	18.6
Both (%)	92.2	58.9	66.7	48.9	53.5
The average number of visits	7.2	12.5	11.4	5.37	5.85
Single-visit (%)	7.6	33	20	26	35
Mean days of hospitalization	4.4	3.3	3.6	3.06	3.87

4. Discussion

The frequency of anemia was one and a half times higher (79.8%) than that reported in another Indian study from NE India anemia in which 52.5% of 10,173 children were anemic [5]. Erythrocyte counts were higher than expected for hemoglobin value in 69/71 patients (relative erythrocytosis) with HbE and thalassemia which implied that a deviation >40% was observed from Rule of Three [4]. This is because, in contrast to iron deficiency anemia, thalassemia is associated with an increase in RBC number [6]. Low MCH, microcytosis, and "relative erythrocytosis" were also observed in over 80% of patients with normal HPLC, in all cases of HbE disorders and thalassemia minor.

No underlying cause was identified by Hb HPLC in 31.2% of patients, which is lower than 40.8% reported by Baruah et al. on a large study from Dibrugarh and >90% by Khera et al. [7,8]. This percentage could have been reduced further by meticulous history taking and performing iron profile before Hb HPLC. However, in our setting where patients visit from a radius of 80 kms and compliance is poor, Hb HPLC may be used as a frontline test particularly in severely anemic patients, if it is not possible to ensure that the patient shall return three months' post-transfusion.

Inconclusive results on Hb HPLC often require additional tests, or repetition on a second sample, screening of parents and/or siblings. Six of the 11 inconclusive results were on samples from infants, all of whom had high HbF, of which three had additionally increased HbE for which capillary electrophoresis was recommended. The etiology of severe anemia (Hb 2.2, 2.8, and 6.5 g/dl) in three patients could not be ascertained as Hb HPLC was normal in two patients. As a pretransfusion sample from the third patient was not available, a post-transfusion sample was sent, which had slightly raised HbF (1.8%) with normal HbA_2 and HbA. Another adult with slightly low HbA (80.2%) was asked for a repeat sample to exclude α-thalassemia/poor storage which was not provided.

HbE disorders-both trait and disease are far more common in North East India than in other parts of the country [2,7, 9,10]. Normally both heterozygotes and homozygotes states for HbE do not result in severe anemia [11,12]. In a study from North India mean Hb for trait and disease was 11 and 8.97 g/dl respectively with microcytic hypochromic indices in HbEE [10]. In this study mean Hb was slightly lower in the trait (9.3) and disease (9.2) as depicted in table 1. Most (80%) of anemic individuals with HbEE had normal iron levels whereas a patient with HbAE had high serum iron with chronic microcytic hypochromic anemia for one year. In agreement with the study by Sharma et al. and in contrast to that by Kishore et al. no correlation was seen between HbA_2 + E fraction with Hb level, RBC counts, and PCV [10,13]. A positive correlation between HbF and MCH as reported by Sharma et al. was not seen in this study, which could be attributed to a very small number of cases in both studies and a difference in ethnicity of the subjects [10]. According to Ittarat et al., identification of HbAE on basis of hematological indices is sufficient only for initial diagnosis which requires confirmation [14]. Although HbE disease and trait are usually not of much clinical significance, if anemia is clinically significant and refractory in the absence of tritional inadequacies, the patients will require further investigations [15]. The bone marrow was hypoplastic in a HbEE patient from Assam who presented with chronic microcytic anemia, low MCH without nutritional deficiency [16].

Higher frequency of Beta-thalassemia (8.8%) in this study as compared to two studies that reported 4.6% and 4.05% respectively, may be attributed to a small number of subjects and selection bias [17,18]. Six Beta-thalassemia trait patients with normal iron levels had chronic microcytic anemia that did not respond adequately to hematinic. Most general practitioners tend to administer iron for all patients with microcytic hypochromic anemia without evaluating the iron status. As patients with HbE abnormality or Beta-thalassemia often have

microcytic hypochromic anemia with normal iron levels, we recommend evaluation of iron status prior to commencement of therapy, even in resource-poor settings. It is unlikely that some β-thalassemia patients would have been missed due to low HbA_2 in spite of low iron, as reported previously [1,8]. Low MCH, MCV, and hemoglobin at birth if persistent, must be followed up with Hb HPLC at six- or 12-months age, (time for HbF to decline) if DNA testing is not available or unaffordable, to facilitate early diagnosis and provide genetic counseling. Erythrocyte indices are usually normal in patients with sickle cell abnormality [20,21]. One-third of sickle cell trait cases (n=4) had microcytic erythrocytes of which three were anemic and two had low serum iron. Two patients with HbS required blood transfusion. Akinsheye et al. classified sickle cell disease patients as those with low HbF (3.1+/-1.5%) and high HbF (20.2+/-8.2%) [22]. Although higher HbF levels are usually seen to be associated with milder disease because HbF is a modulator of clinical and hematological features, we did not observe this in five out of six cases for whom Hb HPLC was done and HbF values were 16.2-25% [20].

Diagnosis of compound heterozygous hemoglobinopathies is challenging and may require testing of additional family members or and DNA testing [21]. In this study 11/15 (73.3%) patients had severe anemia at the presentation of which 10 received one or more transfusions. On the basis of Hb HPLC, a diagnosis of compound heterozygous hemoglobin E with Beta-thalassemia was made for eight samples all of which had high HbF (16.2-55.2%), low adult hemoglobin without performing capillary electrophoresis to determine HbA_2 percentage. Patients with more severe condition -HbE + β^0-thalassemia have higher HbE + A_2 >85%, no detectable HbA and HbF 15- 25%, while those with HbE + β^+-thalassemia have HbE + A_2 25-80%, HbA 5-60%, and HbF 6-50% [23]. Surprisingly a 21-year-old patient with HbS/β-thalassemia and HbS fraction 74.2% was not severely symptomatic and was diagnosed in her first hospital visit with fever and anemia (Hb 6.8%). In this study, the frequency of HbD was 1.17%.

In spite of severe anemia at initial presentation in 37/50 adults of which 20 had hemoglobinopathy, Hb HPLC was not considered until the commencement of this project. Mohanty et al. recruited university students and pregnant women for a large multicenter study – which again reflects that many cases are undiagnosed until adulthood in spite of symptoms and also not considered by the physicians at primary health centers [2] There is a pressing need to create awareness in general physicians and public alike to workup such patients, treat early and provide antenatal counseling, and implement Ministry of Health and Family Welfare, Government of India Policy for Prevention and Control of Hemoglobinopathies – Thalassemia, Sickle Cell Disease and variant hemoglobins in 2018 vide a 32-page document [24]. Identification of abnormal hemoglobins, based on electrophoretic mobility or other characteristics in an individual of appropriate family origin is often presumptive and ideally should be based on a minimum of two techniques based on different principles. Definitive identification usually requires DNA analysis, mass spectrometry or protein sequencing. Family studies are also of considerable importance in elucidating the nature of disorders.

A major limitation of this study was poor patient compliance as 40% of the patients had visited the hospital only once or twice, implying that a second sample for additional testing was not provided. Secondly, it was primarily a laboratory-based study in which patients were interviewed by the pathologist with limited clinical input and minimal specialist participation. Capillary electrophoresis was not done for any patients due to limited budget and the fact that a presumptive diagnosis of hemoglobin E disease/trait was available for most patients without the need to exclude coexisting β-thalassemia.

Three patients were suspected to have alpha thalassemia which could not be confirmed as it was beyond the scope of this study. The first was a case of HbSS with HbF 16.2%, HbA 2%, and HbS 79.2% on HPLC. The second case was a one-year-old male child with HbF 8% and HbA_2 + E value of 78.6%, and the third was suspected to have isolated alpha thalassemia as she had low HbA (80.2%) with 1.5% HbF. In India, prevalence of alpha thalassemia is 12.9% and it can confound iron deficiency anemia for which RBC indices and discriminant functions are inadequate, necessitating molecular workup [25]. Gap PCR for common α-thalassemia mutation including -α^{SA} should be done even in the face of low iron stores in subjects who respond incompletely to iron supplementation [26]. Patients with a chronic refractory microcytic anemia with MCH < 23.4 pg having normal HbF and HbA_2 should be worked up for alpha thalassemia [27]. Although the study was performed in a secondary care center with very limited resources, we succeeded in identifying 117 cases of hemoglobinopathies and creating awareness in the center.

4.1. Conclusion

The frequency of anemia and hemoglobinopathies was high in our clientele and we recommend hemoglobin HPLC as a frontline investigation in suspected cases on basis of MCH and MCV along with the evaluation of iron status. Additionally, bone marrow examination should be reserved for unexplained refractory cases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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