Original Research

Characterization of Hemoglobin Variants in the Çukurova Region of Turkey: Prevalence and Clinical Implications

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Abstract

Background: Hemoglobinopathies, the most prevalent single-gene disorders worldwide, are projected to see an increase in prevalence in the future. This study aims to identify abnormal hemoglobin variants in the Çukurova region.

Methods: Blood samples were collected at the Central Laboratory of Çukurova University Balcalı Hospital, and abnormal hemoglobin types were determined using hemoglobin electrophoresis, sickling, and ARMS methods.

Results: The study identified 115 cases of HbAS, 60 cases of HbSS, 3 cases of HbSC, 2 cases of HbAC, and 1 case of HbAE. The findings indicate that the carriage of sickle cell traits is highest in the Çukurova region. The objective is to reduce the number of affected births and mitigate the economic burden on the country by implementing screening programs in regions where hemoglobinopathy is endemic.

Conclusion: The Çukurova Region's high prevalence of HbS and the common practice of consanguineous marriages increase the likelihood of babies being born with HbSS. New screening methods are urgently needed to accurately distinguish carriers from patients and rule out common conditions like iron deficiency anemia. The success of screening programs depends on the population's acceptance, which can be improved through awareness creation and effective genetic counseling post-diagnosis.

Keywords: Hemoglobin Variants, Çukurova, Prevalence, Clinical Implications

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Introduction

Hemoglobinopathies, such as sickle cell disease and thalassemias, represent intricate red blood cell disorders that are prevalent globally and exhibit diverse clinical manifestations (1). Diagnosing these conditions often necessitates molecular DNA analysis due to the presence of copy number variants (CNVs), which can be difficult to identify without specific diagnostic techniques like multiplex ligation-dependent probe amplification (MLPA) (2).

Mutations in globin genes, which alter the oxygen affinity of hemoglobin variants, contribute to the range of hemoglobin disorders by affecting oxygen dissociation curves. Accurate recognition of clinical and laboratory findings is crucial for diagnosis (3). Comprehensive databases like IthaPhen have been developed to enhance the understanding and management of hemoglobinopathies. These databases integrate genotypic and phenotypic data, aiding in genotype-phenotype correlations and standardized variant classification, thus improving clinical care and genetic counseling. Hemoglobin variant analysis, typically conducted through methods such as high-performance liquid chromatography and capillary electrophoresis, helps in identifying specific variants like HbS, HbD, HbE, HbC, and HbH. The technique of DNA isolation and the use of electrophoresis methods have been common practices for analyzing hemoglobins. A thorough understanding of the molecular pathology, clinical manifestations, and treatment options for these disorders is essential for effective management and prevention strategies. For example, hemoglobin variants can impact diagnostic tests like HbA1c measurements, as observed with the North Manchester mutation affecting HbA1c levels (4). The prevalence of hemoglobin variants, including thalassemias and structural variants, varies geographically. Studies underscore the importance of screening in regions with high prevalence, such as Pakistan (5). Advanced techniques like nano LC−MALDI MS/MS have been developed to improve the identification and analysis of Hb variants, highlighting the need for precise methods due to the high sequence homology between normal and variant chains. Accurate diagnosis and management of hemoglobinopathies rely on characterizing hemoglobin variants. Analytical methods such as capillary electrophoresis, gel electrophoresis, and high-performance liquid chromatography are commonly employed for phenotypic detection of hemoglobin variants (6). Molecular genetic analysis, including Sanger sequencing, plays a crucial role in identifying rare variants like Hb Olupona and Hb Liangqing, providing insights into their genetic basis and clinical significance (7). Advancements in sample processing methods, such as the use of trifluoroethanol (TFE) and LC−MALDI MS/MS, have improved sequence coverage and the accurate identification of single substitution mutations in hemoglobin variants. This offers a robust and reproducible approach for variant analysis. To differentiate HbS and HbD, a sickling test is performed. Hemoglobin S is identified by the sickle shape of erythrocytes in an oxygen-free environment or in the presence of sodium metabisulfite. For the separation of HbC and HbE, the ARMS (Amplification Refractory Mutation System) method is employed. Specific primers designed to amplify segments of DNA are used in PCR reactions to identify the mutations associated with HbC, and HbE. The resulting fragments are analyzed through agarose gel electrophoresis. Hemoglobin variants encompass a broad array of mutations with varying clinical implications. For example, the HbS trait with elevated HbS levels (8), Hemoglobin North Manchester causing false HbA1c readings (9), and Hb Dompierre leading to hemolysis and splenomegaly (10), all underscore the importance of accurate detection and characterization. Genetic studies have identified common variants associated with elevated fetal hemoglobin levels, impacting treatment response in conditions like Myeloproliferative Neoplasms and Myelodysplastic Syndromes (11). Moreover,

compound heterozygous sickle cell trait and Hb-Ferrara can complicate diabetes management, emphasizing the need for comprehensive hemoglobin evaluation and appropriate therapeutic adjustments (12). Understanding the prevalence and clinical implications of these hemoglobin variants is crucial for accurate diagnosis, effective management, and genetic counseling. Hemoglobin variants exhibit significant geographical diversity, impacting prevalence and clinical implications. Genetic variation in hemoglobin concentration is complex and influenced by factors like altitude, nutritional status, and genetic diversity, with malaria-endemic regions showing high frequencies of variants associated with anemia (14). Research on hemoglobin variants in Thailand identified 23 variants, underscoring the importance of DNA sequencing for accurate identification due to similar elution patterns (15). Additionally, studies in Sri Lanka highlighted the variable distribution of hemoglobin disorders over short distances, influenced by factors like malaria, altitude, and ethnic origin, emphasizing the need for precise heterozygote mapping for effective prevention and management (16). In Turkey, studies have identified 60 hemoglobin variants, including 15 alpha, 37 beta, 1 gamma, 2 delta, 2 hybrid hemoglobins, and variants with structural changes due to deletion/insertion, with 10 of these variants being first identified in the Turkish population. The frequency of HbS in Turkey ranges between 0.5-44.2%, Hb E between 0.16-2.4%, Hb D at 0.2%, and Hb C and Hb O-Arab at 3.9% (17, 18). Table 1 shows the distribution of abnormal hemoglobins in Turkey (17). Extensive studies in the Çukurova region of Türkiye have identified mutations such as Hb S IVS-I-110 (G > A) in β-thal and the 3.7 kb deletion in α-thal (19). Additionally, prenatal diagnosis studies have revealed novel mutations like a homozygous 5 nt deletional mutation [α2 IVS-1 134.-138. nt (TGAGG)] and Hb Stanleyville II [α2 78 (EF 7) Asn→ Lys (AAC→AAA)] (20). Molecular characterization in individuals with low Hb A2 levels has led to the identification of new δ-globin variants like Hb A2-Canakkale (21). These findings underscore the importance of comprehensive genetic analyses in understanding and managing hemoglobinopathies in the Çukurova region of Türkiye.

Methods

In this research, blood samples were collected from individuals who visited Çukurova University Balcalı Hospital. A total of 5 mL of blood was drawn into EDTA tubes and transported to the laboratory while maintaining the cold chain. Hemoglobin electrophoresis was performed on these samples to identify HbS, HbD, HbC, and HbE variants. DNA was then extracted for further analyses, and the genomic DNA samples were stored at +4°C for future use.

The blood samples were analyzed for hemoglobin (Hb), erythrocyte count, hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) using a Beckman DxH 800 device. The samples were washed three times with physiological serum. Subsequently, 200 µL of blood and 1000 µL of physiological serum were placed in Eppendorf tubes and mixed using a vortex.

Plasma was separated by centrifugation at 3000 rpm for 1 minute to sediment the erythrocytes. After adding 1 mL of physiological serum to the sedimented cells, the mixture was centrifuged at 3000 rpm for 10 minutes, and the supernatant was discarded. This washing process was repeated, and after the final centrifugation, the washed erythrocytes remained at the bottom of the tube. Then, 200 µL of hemolysis solution was added to an Eppendorf tube, followed by 50 µL of washed erythrocytes, resulting in a 1/5 dilution of the erythrocytes. The hemolysate obtained from this process was used for Hb electrophoresis.

Hemoglobin electrophoresis was conducted to separate hemoglobin variants based on their migration rates in an electric field at an alkaline pH. The SAS-1 AlkHb Kit components included

agarose gels, acid blue stain, hemoglobin lysing agent, and washing solutions.

The procedure involved:

1. Adding 35 µL of hemolysate to each well of the SAS-1 sample tray.
2. Preparing and placing the SAS-1 AlkHb Gel in the electrophoresis chamber.
3. Conducting electrophoresis at 250°C for 30 minutes at 200 volts.
4. Staining, washing, and drying the gel according to a specific protocol.
5. Analyzing the stained gel to determine the hemoglobin types and percentages.

To differentiate HbS and HbD, a sickling test was performed. Hemoglobin S was identified by the sickle shape of erythrocytes in an oxygen-free environment or in the presence of sodium metabisulfite. Reagents included 0.2 g $Na_2S_2O_5$ dissolved in 10 mL water. A sample of 20 µL whole blood was placed on a slide and mixed with a drop of 2% sodium metabisulfite. This mixture was then covered with a coverslip, sealed with adhesive, and incubated for 5-30 minutes. Under a microscope, the presence of sickled erythrocytes indicated HbS, whereas the absence of sickling indicated HbD.

For the separation of HbC and HbE using the ARMS (Amplification Refractory Mutation System) method, DNA was first isolated from blood samples. Using a kit from Roche (Cat No: 11796828001, USA), reagents were prepared accordingly: Proteinase K dissolved in 4.5 ml distilled water, Inhibitor Removal Buffer with 20 ml absolute ethanol, and Wash Buffer with 80 ml absolute ethanol.

The DNA isolation procedure involved:

1. Mixing 200 µL Binding buffer, 40 µL Proteinase K, and 200 µL whole blood, followed by incubation at 70°C for 10 minutes.
2. Adding 100 µL isopropanol, vortexing, filtering, and centrifuging at 8000 rpm for 1 minute.
3. Discarding the solution under the filter, adding 500 µL Inhibitor Removal Buffer, and centrifuging.
4. Repeating wash steps with 500 µL Wash Buffer twice, followed by centrifugation.
5. Adding 200 µL of 70°C Elution Buffer and centrifuging to collect the genomic DNA, which was stored at +4°C until use.

In the research, specific primers were crucial for the molecular analysis of hemoglobin variants. These primers were short, synthetic sequences of nucleotides designed to bind to complementary sequences in the DNA. They were selected based on their ability to flank the regions of the genes encoding for hemoglobin, ensuring precise annealing to the target DNA sequences.

The primers used in this study were carefully designed to amplify segments of DNA that included the common hemoglobin variants HbC, and HbE. This process involved:

1. Primer Design and Selection:

- The primers were designed to be specific to the regions of the β-globin gene where mutations associated with HbC, and HbE occur. This specificity was essential to differentiate these variants accurately.
- The sequences of the primers were chosen to ensure high specificity and efficiency. This involved selecting sequences that would not bind non-specifically to other regions of the genome.
- The common primers used were:

Common primer 1: 5’ ACC TCA CCC TGT GGA GCC AC 3’

Common primer 2: 5’ CCC CTT CCT ATG ACA TGA ACT TAA 3’

- The constant primers used were:

- 5’Constant primer: 5’ CAA TGT ATC ATG CCT CTT TGC ACC 3’
- 3’ Constant primer: 5’ GAG TCA AGG CTG AGA GAT GCA GGA 3’

2. ARMS Primers:

- For the identification of HbE, the ARMS (Amplification Refractory Mutation System) method was used, employing specific primers:
  - Hb E (T→C)1 (Mutant primer): 5’ TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT 3’
  - Hb E (T→C)2 (Normal primer): 5’ TAA CCT TGA TAC CAA CCT GCC CAG GGC GTC 3’

These carefully designed primers ensured the precise amplification of the target DNA sequences, allowing for accurate differentiation and analysis of the hemoglobin variants present in the samples.

2. PCR Amplification:

The designed primers were used in a Polymerase Chain Reaction (PCR) to amplify the target DNA regions. Each PCR reaction included the genomic DNA extracted from the blood samples, the designed primers, nucleotides, DNA polymerase, and other necessary reagents.

During the PCR cycles, the primers annealed to their complementary sequences on the DNA template, allowing the DNA polymerase to extend from the primer and replicate the target DNA region.

3. Detection and Analysis:

- The amplified DNA fragments were then subjected to further analysis, such as gel electrophoresis, to confirm the presence and size of the amplified products.

- Sequencing or restriction enzyme digestion might have been used to identify the specific hemoglobin variants present in each sample, based on the known mutations.

4. Storage and Subsequent Use:

- The PCR products and remaining genomic DNA samples were stored appropriately, often at +4°C, for any subsequent analyses or validations needed.

By using these primers, the study successfully identified and characterized the prevalence of different hemoglobin variants in the Çukurova region, providing insights into the genetic diversity and clinical implications of these hemoglobinopathies in the local population.

The ARMS method identifies point mutations or small deletions by using mutation-specific primers. For this process, various reagents and primers are used to prepare a PCR mix, which is then placed in a thermal cycler device. The thermal cycler follows a programmed cycle to amplify the DNA, producing a 585 bp fragment, which is analyzed through agarose gel electrophoresis.

The agarose gel electrophoresis process involves preparing a 1% agarose gel, loading the DNA samples mixed with loading buffer, and applying an electric current. After electrophoresis, the gel is stained with Ethidium Bromide, washed, and the DNA fragments are visualized and photographed under UV light. The resulting bands help detect mutations in HbC and HbE, as illustrated in Figure 15 showing agarose gel images for HbAC and HbAE mutations detected using the ARMS method.

Results

This study involved patients with severe anemia whose blood counts were conducted at the Central Laboratory of Çukurova University Balcalı Hospital. The study included blood samples from 181 individuals without iron deficiency. Hemoglobin analysis was carried out using hemoglobin electrophoresis, and abnormal hemoglobins were differentiated using the sickling test and the Amplification Refractory Mutation System (ARMS) method in patients suspected to have HbAS, HbSS, HbAE, HbAC, or HbAD. In our study, hemoglobin electrophoresis, ARMS, and sickling tests identified 115 cases of HbAS, 60 cases of HbSS, 3 cases of HbSC, 2 cases of HbAC, and 1 case of HbAE (Table 2 and Table 3).

During hemoglobin electrophoresis, Hemoglobin S and D migrate to the same position. They were differentiated using the sickling test. Some patients also exhibit Hemoglobin F, as its levels are naturally high from birth until about 2 years of age. As the individual matures, Hemoglobin F is gradually replaced by Hemoglobin A.

The data of 60 hemoglobin patients as a result of hemoglobin electrophoresis and sickling test are shown in Table 4.

Tables 5 and 6 display the data for three hemoglobin cases resulting from hemoglobin electrophoresis and ARMS-PCR.

In our study, 115 HbAS, 60 HbSS, 3 HbSC, 2 HbAC, 1 HbAE hemoglobin types were

determined as a result of hemoglobin electrophoresis, ARMS and sickling tests. It is shown as a percentage in Table 7.

Discussion

Hemoglobinopathies are among the most prevalent genetic abnormalities globally, with the World Health Organization estimating a 5% worldwide prevalence of hemoglobin disorders. These disorders are categorized into two main types: thalassemias and abnormal hemoglobins, the latter resulting from amino acid changes in the globin chains of the hemoglobin molecule (22).

Genetic research, bolstered by the widespread use of recombinant DNA technologies, has greatly advanced the identification of gene clusters and the genetic regulation of hemoglobin synthesis. In Turkey, thalassemia is notably common in the Çukurova, Mediterranean coastline, Aegean, and Marmara regions. Data from the Turkish Ministry of Health and the National Hemoglobinopathy Council indicate that 2.1% of the healthy Turkish population are beta thalassemia carriers, translating to approximately 1,300,000 carriers and 4,000 patients nationwide. Specifically, Adana reports a 3.7% beta thalassemia carrier frequency, with an Hb S frequency of 10% (23).

Geographic and racial variations significantly influence the distribution of abnormal hemoglobins. Besides Hb S, which is the most prevalent abnormal hemoglobin in Turkey and globally, other variants such as Hb D, Hb E, and Hb C are also present (24). For instance, a study by Yüregir et al. (1995) identified an Hb S carriage rate of 8.2% in the Çukurova region (25). Additionally, Arpacı (1991) reported an Hb S frequency of 22.1% and a β thalassemia frequency of 5.1% in Samandağ (26).

Attila et al. (1999) found an Hb S rate of 10%, a β thalassemia rate of 3.7%, and an α thalassemia rate of 3.3% in Çukurova (27). Furthermore, Güler et al. (2007) observed a beta thalassemia carrier rate of 2% and an Hb S carrier rate of 0.05% in premarital screening studies in Konya (29).

In our study conducted in the Çukurova region, we found the following frequencies: HbAS at 63.53%, HbSS at 33.15%, HbSC at 1.65%, HbAC at 1.12%, and HbAE at 0.55%. No HbH patients were identified. Due to the instability of hemoglobin H, blood must be analyzed promptly after collection.

Reviewing these studies reveals significant regional variations in the distribution of mutant alleles within Turkey.

Conclusion

In this study, the distribution of abnormal hemoglobins in the Çukurova Region was as follows: HbAS at 63.53%, HbSS at 33.15%, HbSC at 1.65%, HbAC at 1.12%, and HbAE at 0.55%. The high prevalence of HbS, coupled with the common practice of consanguineous marriages, increases the likelihood of babies being born with HbSS.

To address this issue, it is crucial to develop new screening methods that can effectively differentiate carriers from patients and exclude common conditions like iron deficiency anemia. The success of these screening programs relies heavily on the target population's acceptance, which can be enhanced by raising awareness and providing comprehensive genetic counseling post-diagnosis. Effective counseling can foster positive attitudes and dispel misconceptions, leading to better understanding and outcomes.

Micromapping studies have indicated a greater diversity in the mutation spectrum among populations in smaller geographic areas, suggesting that the current known prevalence rates of hemoglobinopathies may be underestimated. Gathering more micromapping data will aid in the better planning of future population screening programs.

Advancements in technology and biology are expected to make non-invasive prenatal diagnosis using fetal cells from maternal blood more common in the next decade. Incorporating and validating these new technologies within hemoglobinopathy testing guidelines will streamline future population screening efforts.

Given the significant global prevalence of hemoglobinopathies, various national or regional

programs have been implemented to control the disease burden. The success of these programs is often measured by the reduction in the incidence of affected births. Ensuring the continuous development and application of effective screening and diagnostic methods is vital for the ongoing management and reduction of hemoglobinopathy cases.

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Authors Contributions:

All authors contributed toward data analysis, Drafting and revising the paper and agreed to be Responsible for all the aspects of this work

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Tables& Figures

Table 1. Distribution of abnormal hemoglobins in Turkey (Yıldız, 2019).

No	Name	Chain/Mutation	No	Name	Chain/Mutation
1	Adana*	α59 Gly>Asp	31	J-Antakya*	β65 Lys>Met
2	Ankara*	β10 Ala>Asp	32	J-Iran	β77 His>Asp
3	A2 Yialousa	δ82 Ala>Ser	33	J-Meerut	α120 Ala>Glu
4	Antalya*	Deletion/insertion	34	Knosos	β27 Ala>Ser
5	Başkent*	γ128 Ala>Thr	35	Köln	β98 Val>Met
6	Beograd	β121 Glu>Val	26	Lepore-Boston	Hybrid
7	Brocton	β138 Ala>Pro	37	M-Iwate	α87 His>Tyr
8	Bronova	α103 His>Leu	38	Moabit	α86 Leu>Arg
9	C	β6 Glu>Lys	39	Monthgomery	α48 Leu>Arg
10	City of Hope	β69 Gly>Ser	40	M-Saskatoon	β63 His>Tyr
11	Costant Spring	Prolonged α chain	41	N-Baltimore	β95 Lys>Glu
12	Crete	β129 Ala>Pro	42	Noah	δ143 His>Tyr
13	Çapa*	α94 Asp>Gly	43	O-Arab	β121 Glu>Lys
14	D-Iran	β22 Glu>Gln	44	O-Podova	α30 Glu>Lys
15	D-Punjab	β121 Glu>Gln	45	P-Nilotic	Hybrid
16	D-Ouled Rabah	β19 Asn>Lys	46	Pyrgos	β83 Gly>Asp
17	E	β26 Glu>Lys	47	Q-Iran	α75 Asp>His
18	Ernz	β123 Thr>Asn	48	S	β6 Glu>Val
19	E-Saskatoon	β22 Glu>Lys	49	Sarrebourg	β131 Gln>Arg
20	G-Copenhagen	β47 Asp>Asn	50	Setif	α94 Asp>His
21	G-Cousatta	β22 Glu>Ala	51	Siirt*	β27 Ala>Gly
22	G-Georgia	α95 Pro>Leu	52	South Florida	β1 Val>Met
23	G-Szuhu	β80 Asn>Lys	53	Strumica	α112 His>Arg
24	Hakkari*	β31 Leu>Arg	54	Summer Hill	β52 Asp>His

25	Hamadan	β56 Gly>Arg	55	Tunis	β124 Pro>Ser
26	Hamilton	β11 Val>Ile	56	Tyne	β5 Pro>Ser
27	Hasharon	α47 Asp>His	57	Ube-2	α68 Asn>Asp
28	Istanbul*	β92 His>Gln	58	Volga	β27 Ala>Asp
29	Izmir*	β86 Ala>Val	59	Yauzi	β79 Asp>Asn
30	J-Anatolia	α61 Lys>Thr	60	Westeinde	α125 Leu>Gln

***First discovered in Turkish patients**

Table 2. Hemoglobin types in percentage groups of all participants

Percentage/patient	0-25%	25-50%	50-75%	75-100%
HbA%	22	24	55	9
HbF%	76	24	1	1
HbS%	11	53	43	36
HbA2%	160	0	0	0
HbC%	-	5	-	-
HbE%	-	1	-	-

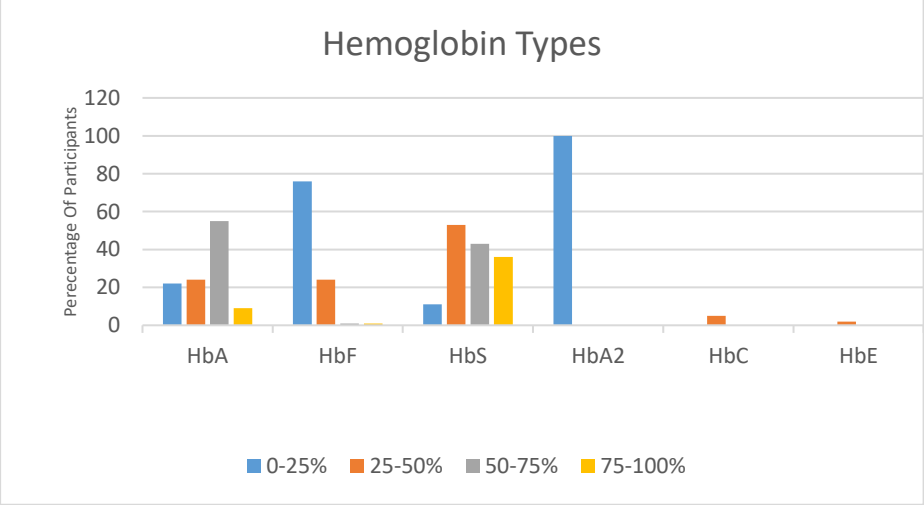

Diagram 1. Hemoglobin types in percentage groups of all participants

Table 3. Hemoglobin types in sickle cell carriers

Percentage/patient	0-25%	25-50%	50-75%	75-100%
HbA%	25	47	5	0
HbF%	22	4	2	3
HbS%	50	0	32	84
HbA2%	31	0	27	21

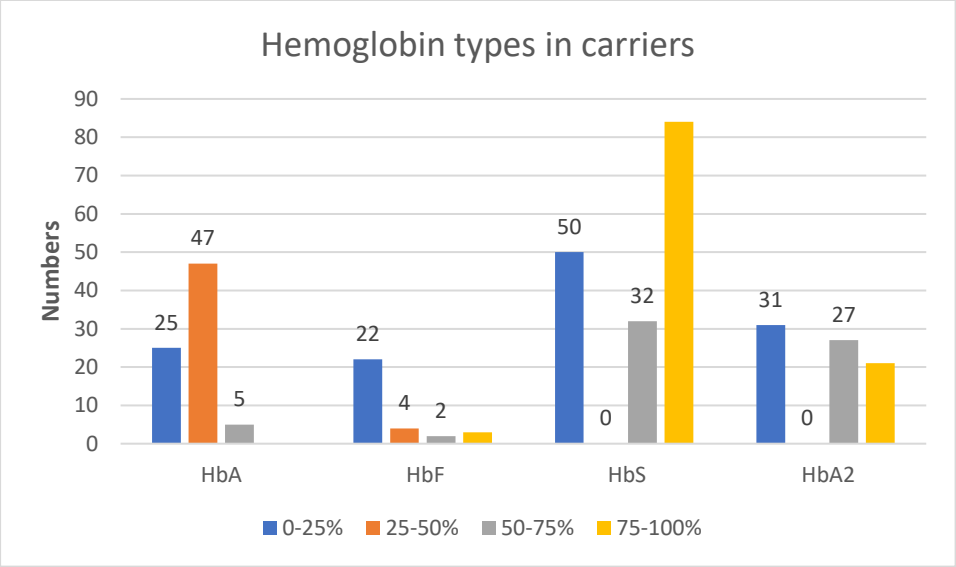

Diagram 2. Hemoglobin types in sickle cell carrier patients

Table 4. Hemoglobin types in percentage groups in sickle cell patients

Percentage/patient	0-25%	25-50%	50-75%	75-100%
HbF%	32	25	1	1
HbS%	1	2	29	28
HbA2%	60	0	0	0

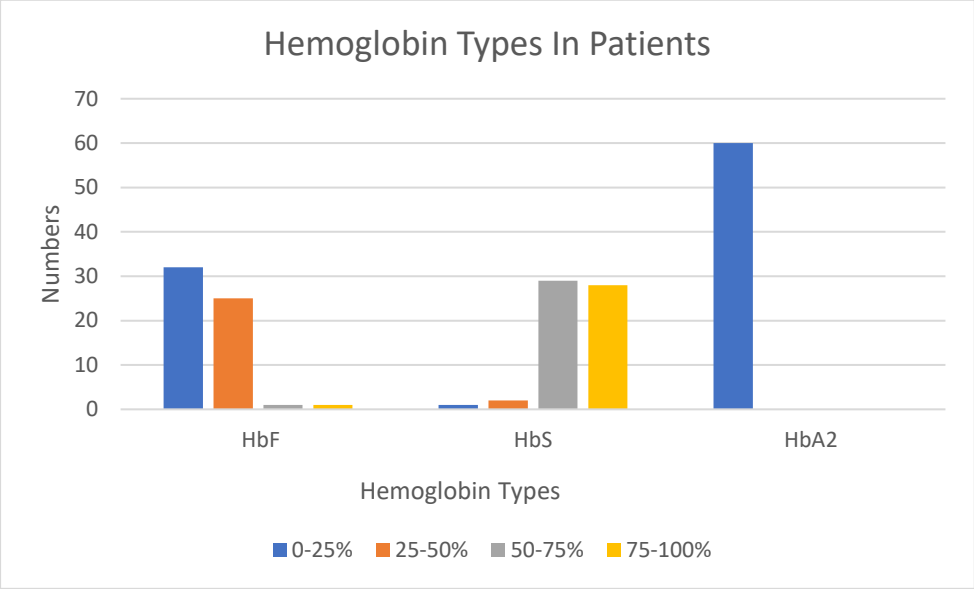

Diagram 3. Hemoglobin types in percentage groups in sickle cell patients

Table 5: Carriers with hemoglobin C

Carriers	HbA %	HbC %	Hb types
86	62.27	35.73	AC
109	67.10	32.90	AC

Table 6. Carriers with hemoglobin E

Carriers	HbA%	HbE%	Hb types
19	65.55	34.45	AE

Table7. Percentage of hemoglobin variants

Hemoglobin Variants	Percentage (%)
HbAS	63.53
HbSS	33.15
HbSC	1.65
HbAC	1.12
HbAE	0.55

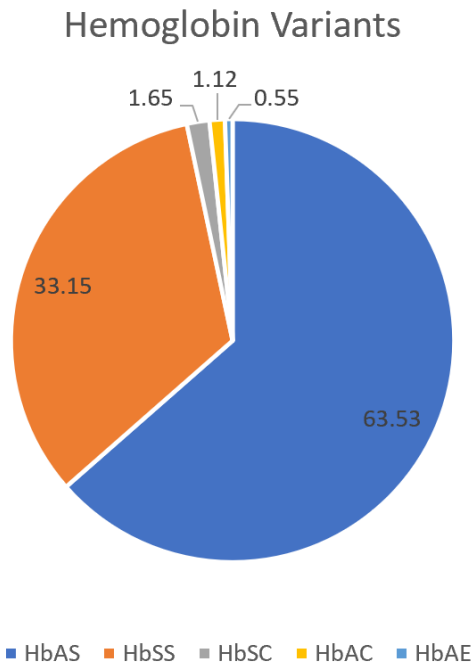

Diagram 4. Percentage of hemoglobin variants