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Research Article

Association of Xmn-1 Polymorphism with HbF Levels in Patients Presenting with Sickle Cell Disease at Tertiary Care Hospital, Karachi

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Abstract: Background: Sickle cell anemia is a common hereditary disease in Pakistan, which still maintains significant inter patient variation in disease manifestations and the reasons behind such variations remain uncertain. Among other genetic modifiers the XmnI polymorphism has been noted to influence HbF levels, response to HbF augmenting drugs and milder phenotype of disease as observed in studies conducted in various parts of world and subcontinent. XmnI polymorphism frequency and its association with HbF levels have not been investigated in Pakistani patients of sickle cell disorder. This study aims to determine different genotypes of XmnI polymorphism, and association with high HbF levels in patients of homozygous sickle and sickle/β thalassemia.

Objective: This study aimed to ascertain the association of XmnI polymorphism with HbF levels among patients with sickle cell disorders treated at Karachi's Tertiary Care Hospital.

Materials and Methods: A cross-sectional investigation was conducted at the National Institute of Blood Diseases & Bone Marrow Transplant in Karachi after obtaining approval from the Ethical Review Board bearing number NIBD/RD-208/09-2020. Data collection spanned twelve months from January 01 to December 31 2021. Prospective data collection involved obtaining verbal consent from 150 patients who were diagnosed on the basis of DNA mutation analysis and fulfilled eligibility criteria. 66 sickle homozygous (HbSS) and 84 sickle β thalassemia patients (Hb S/ß Th) were enrolled. XMN1 polymorphism analysis was performed through Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and both groups were stratified by presence of XMN-1 polymorphism i.e. homozygous (+/+), heterozygous (+/-) and negative (-/-) to compare frequencies of demographic factors while one-way and Welch's ANOVA tests were applied to analyze variance among these groups.

Result: The study reported a mean age of 11.43±7.19 years and hemoglobin levels averaging 8.53±1.50 mg/dL. Gender distribution was slightly male-skewed, and ethnicity showed a predominance of individuals from Baluchistan. The frequency of XmnI polymorphism was higher in sickle homozygous patients as compared to sickle ß-thalassemia patients, significantly affecting HbF, MCV, and MCH levels. HbF levels differed across XmnI polymorphism categories, with +/+ having the highest mean. The family history of thalassemia and consanguineous marriages were more common in those with the +/- and -/- genotypes. The most common β-globin mutation in sickle ß-thalassemia patients was found out to be IVSI-5.

Conclusion: This study concludes that XmnI polymorphism influences HbF levels of sickle cell patients in Pakistan, emphasizing the need for further comprehensive studies.

Keywords: Xmn-1 polymorphism, Sickle cell disorders, HbF level, Sickle cell anemia and β globin polymorphism, BCL11A, HBS1L-MYB.

INTRODUCTION

Sickle cell disease (SCD) represents a group of inherited red blood cell disorders with a global distribution and a significant health burden and is characterized by presence of an abnormal hemoglobin called HbS. HbS is structurally different hemoglobin caused by a mutation in HBB gene. This mutation causes substitution of glutamic acid with valine at position 6 of β globin chain. The resulting abnormal hemoglobin HbS polymerises under hypoxic conditions and causes red blood cells to form characteristic sickle shape. The sickled erythrocytes then cause blockages in blood vessels leading to complications such as vaso-occlusive crisis. In Pakistan, a country with a diverse genetic background, SCD prevalence and its variable clinical manifestations present a healthcare challenge that is accentuated by a scarcity of local data on genetic modifiers that influence disease phenotype [1-8].

The clinical course of SCD is highly variable and determined by several factors, including presence of coexisting conditions such as α thalassemia and the level of HbF. HbF is considered as a key ameliorating factor due to its anti-polymerizing effects on HbS

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[9-11]. HbF is regulated by several genetic factors, among them haplotypes of β globin gene cluster are comprehensively studied. Senegal and Asian-Indian haplotypes, are associated with higher expression of HbF, resulting in a milder clinical phenotype as compared with the other African haplotypes. Moreover, genetic polymorphisms at other regulatory loci that affect HbF levels have been identified such as Xmn1-polymorphism (XMNI-HBG2), HBS1L-MYB intergenic region on chromosome 6q23, and BCL11A on chromosome 2p16 [3-6]. The Xmn-1 polymorphism, located at position -158 upstream of the Gγ-globin gene, has been known to augment HbF levels in individuals with hemoglobinopathies [10, 11]. The role of XmnI polymorphism has been studied in various parts of world as a key modifier, however its prevalence and impact on patients with SCD in Pakistan remain under investigated [6-8].

Our study aimed to determine the frequency of the XmnI polymorphism in Pakistani patients presenting with sickle cell disorders at a tertiary care hospital in Karachi, and ascertain its association with high HbF levels, which lacks regional context regarding genetic profile of SCD in Pakistan. This is the first study of its kind in Pakistan as it is being done on homozygous sickle and sickle/β patients as compared to previous studies done on β thalassemia patients. The information gained from the study will be useful in understanding of the disease's diversity and predicting the course and phenotype of SCD. This investigation is a start towards applying genetic testing in clinical assessment for individuals with SCD in an attempt to improve and estimate directions regarding treatment of patients with SCD and ultimately the comprehensive management of patients.

MATERIALS AND METHODS

This study, conducted at the National Institute of Blood Diseases & Bone Marrow Transplant in Karachi, employed a cross-sectional design over a twelve-month period from January 01, 2021 to December 31, 2021. Ethical approval was obtained from the Ethical Review Board bearing number NIBD/RD-208/09-2020. A sample of 150 patients was determined using the WHO calculator, based on a 31.67% expected prevalence [12], a 9% margin of error, and a 95% confidence level, with participants recruited through non-probability consecutive sampling.

Participants included individuals with homozygous sickle cell and sickle/β-thalassemia(diagnosed on DNA mutation analysis), ranging from 01 year to 35 years of age, irrespective of gender. Exclusions applied to those with hereditary fetal hemoglobin persistence, heterozygous sickle cell forms, and other hemoglobinopathies (as evident on DNA mutation analysis) and those who were taking hydroxyurea.

After obtaining ethical approval and informed consent, demographic data and blood samples for DNA analysis targeting the Gγ gene's C-T polymorphism at position -158 were collected. PCR products were digested with the Xmn1 enzyme for gel electrophoresis.

STATISTICAL ANALYSIS

Data analysis was performed on SPSS Version 20. Exploratory data analysis was conducted to note the frequency distributions. In order to analyze the variance between groups (XmnI polymorphism: +/+, +/- & -/-), one-way and Welch's ANOVA tests were applied, with a P-value <0.05considered as statistically significant. Further, comparison of demographic factors in homozygous (HbSS) and heterozygous (HbSß) thalassemia groups, stratified by carrier and non-carrier XmnI polymorphism was performed.

RESULT

In this study, the baseline characteristics of 150 individuals reveal a mean age of 11.43±7.19 years, with a broad age range of 1 to 35 years, indicating a sample that spans from children to adults. Hemoglobin levels average at 8.53±1.50 mg/dL. The hematocrit percentages average at 25.50±4.53%, with the mean corpuscular volume (MCV) at 74.23±9.98 fL, mean corpuscular hemoglobin (MCH) at 24.50±3.70 pg, and mean corpuscular hemoglobin concentration (MCHC) at 33.07±1.53 mg/dL. The mean HbF level was 20.10±7.90 mg/dl, with a broad range (Min-Max: 0.1 to 36) and median HbF level was 21.1 mg/dL.

Out of 150 subjects in the study, 55.33% (83 individuals) were male. In contrast, females represent 44.67% (67 individuals), reflecting a somewhat disproportionate representation between the two groups (Fig. **1**). Further, Fig. (**2**) shows the ethnicity-wise distribution of our study population, wherein the highest number of individuals belonged to Baluchistan (49.3%) followed by Urdu-speaking individuals (23.33%).

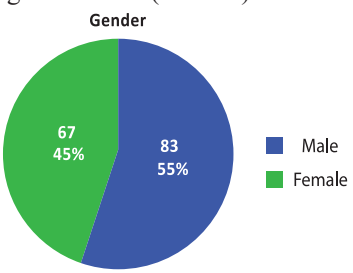

Fig. (1). Distribution of Gender in Our Study Population (N=150).

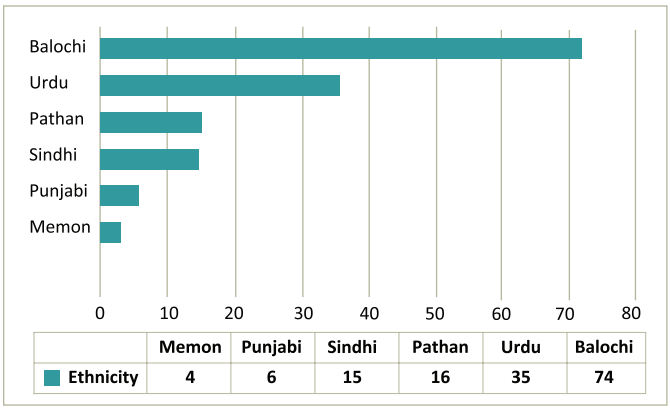

Fig. (2). Distribution of Ethnicities in our Study Population (N=150).

Out of 150 individuals, 84 were sickle ß-thalassemia patients (45 male and 39 females with average age of 13.14±8.13 years) while 66 were sickle homozygous patients (38 male and 28 females with average age of 9.25±4.99 years). XmnI polymorphism was found in 78% of patients.

Among the sickle homozygous patients; 20 (30.3%) were heterozygous (+/-) and 42 (63.6%) were homozygous (+/+), while 4 (6.1%) were normal for XmnI polymorphism. Table **1**(**a**) compares hematological parameters in sickle homozygous patients stratified by XmnI polymorphism status (+/+, +/-, -/-). The statistically significant differences (P-value < 0.05) were observed for MCV, MCH, and hemoglobin-F, indicating variations in these parameters across the different polymorphism groups while hemoglobin and hematocrit levels showed no significant differences, with P-values of 0.341 and 0.392, respectively.

In Table **1**(**b**), frequencies of demographic factors among sickle homozygous patients stratified by XmnI polymorphism have been elaborated. The findings reveal that age distribution is more skewed towards younger population i.e. under 15 years of age with more males across all categories of XmnI polymorphism. The notable frequency was noticed among Balochi and Urdu ethnic groups. A higher occurrence of consanguineous marriages among patients with the +/+ genotype was noted. The family histories of thalassemia are particularly noted to be higher in patients with the +/+ and +/- genotypes. While a higher number of patients belonged to rural areas, particularly those with the +/+ genotype.

Out of 84 sickle ß-thalassemia patients, 44 (52.4%) were heterozygous and 11 (13.1%) were homozygous for XmnI polymorphism, while 29 (34.5%) were negative for XmnI polymorphism. The frequency of the Xmn1 polymorphism was noted to be higher among sickle homozygous than sickle ß-thalassemia patients. Table **2**(**a**) compares hematological parameters in sickle ß-thalassemia patients stratified by XmnI polymorphism status (+/+, +/-, -/-). The statistically significant difference (P-value < 0.05) was observed for hemoglobin-F, while other parameters showed no significant differences.

Table **2**(**b**) shows the frequencies of demographic factors among sickle ß-thalassemia patients stratified by XmnI polymorphism. Majority of patients belonged to younger age group, with dominance of male gender in XmnI categories with +/+ and +/- genotype. The other findings include Balochi and Urdu speaking being the most prominent ethnic group, family history of thalassemia more significant in patients with +/- and -/- genotypes and higher number of patients particularly with +/- genotype belonged to rural areas.

The findings of Tables **1**(**b**) and **2**(**b**) indicate certain demographic pattern among the study population, opening a new arena for further research directed towards studying disease mechanisms keeping in view the demographic influences.

Table **3** shows that the most common β-globin mutation was IVSI-5 which is found in 37(44%) out of 84sickle β-thalassemia patients, followed by Fr 8-9 which was present in 13(15.5%) patients and Fr 41-42 which was present in 9 (10.7%) patients. The rest of the patients showed Cd30, Cd15, Cd5, Del 619, IVSI 1 and Fr 16.

Table 1(a). Comparison of Hematological Parameters in Sickle Homozygous (HbSS), Stratified by XmnI Polymorphism Status (+/+, +/-, -/-), (N=66).

Hematological Parameters	**XmnI Polymorphism (N=66)**			**P-value**
	+/+	+/-	-/-	
	N=42	**N=20**	**N=4**	
Hemoglobin	8.50 ± 1.55	8.79 ± 1.99	7.43 ± 0.98	0.341
Hematocrit	25.11 ± 4.36	26 ± 5.79	22.38 ± 3.14	0.392
MCV	82.67 ± 10.03	75.56 ± 9.36	83.50 ± 10.34	**0.039**
MCH	27.59 ± 3.52	25.24 ± 3.07	28.25 ± 3.78	**0.045**
MCHC	33.69 ± 1.34	33.33 ± 1.08	34.75 ± 1.25	0.133
Hemoglobin-F	24.20 ± 5.09	19.26 ± 9.25	17.63 ± 5.20	**0.045**

Table 1(b). Frequency of Demographic Factors in Sickle Homozygous (HbSS), Stratified by XmnI Polymorphism Status (+/+, +/-, -/-), (N=66).

Demographic Factors	**XmnI Polymorphism (N=66)**			**Total (N=66)**
	+/+	+/-	-/-	
	N=42 (63.6%)	**N=20 (30.3%)**	**N=4 (6.1%)**	
Age				
Upto 10 years	27 (40.9%)	16 (24.2%)	2 (3%)	45 (68.2%)
>10 to 15 years	8 (12.1%)	1 (1.5%)	1 (1.5%)	10 (15.2%)

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>15 years	7 (10.6%)	3 (4.5%)	1 (1.5%)	11 (16.7%)
Gender				
Male	24 (36.4%)	12 (18.2%)	2 (3%)	38 (57.6%)
Female	18 (27.3%)	8 (12.1%)	2 (3%)	28 (42.4%)
Ethnicity				
Urdu	9 (13.6%)	7 (10.6%)	1 (1.5%)	17 (25.8%)
Balochi	22 (33.3%)	11 (16.7%)	1 (1.5%)	34 (51.5%)
Pathan	5 (7.6%)	1 (1.5%)	1 (1.5%)	7 (10.6%)
Sindhi	5 (7.6%)	1 (1.5%)	1 (1.5%)	7 (10.6%)
Memon	1 (1.5%)			1 (1.5%)
History of Consanguineous Marriage				
Yes	29 (43.9%)	14 (21.2%)	3 (4.5%)	46 (69.7%)
No	13 (19.7%)	6 (9.1%)	1 (1.5%)	20 (30.3%)
Family History of Thalassemia				
Significant	34 (51.5%)	14 (21.2%)	3 (4.5%)	51 (77.3%)
Non-Significant	8 (12.1%)	6 (9.1%)	1 (1.5%)	15 (22.7%)
Residence				
Rural Areas	23 (34.8%)	12 (18.2%)	3 (4.5%)	38 (57.6%)
Urban Areas	19 (28.8%)	8 (12.1%)	1 (1.5%)	28 (42.4%)

Table 2(a). Comparison of Hematological Parameters in Sickle ß-Thalassemia (HbSß), Stratified by XMN-1 Polymorphism Status (+/+, +/-, -/-), (N=84).

Hematological Parameters	**XmnI Polymorphism (N=84)**			**P-value**
	+/+	+/-	-/-	
	N=11	**N=44**	**N=29**	
Hemoglobin	8.75 ± 1.74	8.52 ± 1.52	8.51 ± 0.91	0.865
Hematocrit	27.06 ± 5.26	25.60 ± 4.99	25.62 ± 2.97	0.63
MCV	71.78 ± 5.30	68.29 ± 6.11	69.50 ± 6.52	0.261
MCH	22.38 ± 2.37	22.52 ± 2.38	22.77 ± 2.91	0.89
MCHC	32.20 ± 1.87	32.64 ± 1.53	32.70 ± 1.51	0.675
Hemoglobin-F	25.02 ± 5.67	20.93 ± 6.82	12.03 ± 6.92	**<0.01**

Table 2(b). Frequency of Demographic Factors in Sickle ß-Thalassemia (HbSß), Stratified by XmnI Polymorphism Status (+/+, +/-, -/-), (N=84).

Demographic Factors	**XmnI Polymorphism (N=84)**			
	+/+	+/-	-/-	**Total**
	N=11 (13.1%)	**N=44 (52.4%)**	**N=29 (34.5%)**	
Age				
Upto 10 years	5 (6%)	21 (25%)	13 (15.5%)	39 (46.4%)
>10 to 15 years	2 (2.4%)	6 (7.1%)	8 (9.5%)	16 (19%)
>15 years	4 (4.8%)	17 (20.2%)	8 (9.5%)	29 (34.5%)
Gender				
Male	9 (10.7%)	23 (27.4%)	13 (15.5%)	45 (53.6%)
Female	2 (2.4%)	21 (25%)	16 (19%)	39 (46.4%)

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Ethnicity				
Urdu	3 (3.6%)	10 (11.9%)	5 (6%)	18
Balochi	3 (3.6%)	20 (23.8%)	17 (20.2%)	40 (47.6%)
Pathan	2 (2.4%)	5 (6%)	2 (2.4%)	9 (10.7%)
Sindhi	1 (1.2%)	4 (4.8%)	3 (3.6%)	8 (9.5%)
Punjabi	2 (2.4%)	2 (2.4%)	2 (2.4%)	6 (7.1%)
Memon	0	3 (3.6%)	0	3 (3.6%)
History of Consanguineous Marriage				
Yes	8 (9.5%)	22 (26.2%)	15 (17.9%)	45 (53.6%)
No	3 (3.6%)	22 (26.2%)	14 (16.7%)	39 (46.4%)
Family History of Thalassemia				
Significant	10 (11.9%)	25 (29.8%)	23 (27.4%)	58 (69%)
Non-Significant	1 (1.2%)	19 (22.6%)	6 (7.1%)	26 (31%)
Residence				
Rural Areas	4 (4.8%)	24 (28.6%)	19 (22.6%)	47 (56%)
Urban Areas	7 (8.3%)	20 (23.8%)	10 (11.9%)	37 (44%)

Table 3. Frequency of XmnI Polymorphism in Different Genotypes of Sickle ß-Thalassemia, (N=84).

Genotype	**XmnI Polymorphism**			
	+/+	+/-	-/-	**Total N=84**
	N=11 (13%)	**N=44 (52%)**	**N=29 (35%)**	
IVSI 5	4 (4.8%)	17 (20.2%)	16 (19%)	37 (44%)
Fr 8-9	1 (1.2%)	9 (10.7%)	3 (3.6%)	13 (15.5%)
Fr 41-42	2 (2.4%)	5 (6%)	2 (2.4%)	9 (10.7%)
Cd 30	1 (1.2%)	3 (3.6%)	4 (4.8%)	8 (9.5%)
Cd 15	0	2 (2.4%)	2 (2.4%)	4 (4.8%)
Cd 5	2 (2.4%)	1 (1.2%)	1 (1.2%)	4 (4.8%)
Del 619	0	3 (3.6%)	1 (1.2%)	4 (4.8%)
IVSI 1	1 (1.2%)	2 (2.4%)	0	3 (3.6%)
Fr 16	0	2 (2.4%)	0	2 (2.4%)

DISCUSSION

Besides other genetic factors which are known to modulate HbF levels one of them is γG-158 (C→T) polymorphism, located at the XmnI restriction site of the γG-globin gene, that affects the gene expression of fetal hemoglobin and its synthesis. This is further responsible for the observed clinical heterogeneity in SCD; therefore, acting as a target for genetic screening as well as therapy [6-8, 12, 13]. It has been demonstrated previously that the XmnI polymorphism influences output levels of fetal hemoglobin (HbF) in sickle cell disease and Sickle cell trait patients in Chattisgarh by Bhagat *et al.* [14]. A study carried out in the region on 100 sickle cell disease and 50 sickle cell trait patients revealed XmnI γG-158 (C→T) polymorphism variation increases the level of G globin chains expression that in turn raises the output of HbF. The study aimed at identifying the different genotypes of G XmnI polymorphism and its implications on HbF levels and concluded that patients with XmnI (+/+) genotype generate HbF above the mean compared to those lacking this polymorphism [14].

Comparable data trends are seen in Pandey *et al.* research [12] which included 60 homozygous sickle cell and 75 sickle β-thalassemia patients. Pandey's study revealed significant differences in hematological parameters between those with and without the Xmn1 carrier status and presence of XmnI polymorphism correlated with higher HbF levels and milder clinical manifestations. Anemia, splenomegaly, gallstones, painful crisis, frequency of blood transfusion were lesser in XMNI (+/+) and (+/-) groups in both HbSS and sickle/β thalassemia patients. In our study we did not find statistically significant differences on comparison of hematological indices of XmnI positive (+/+), heterozygous (+/-) and negative (-/-) in sickle ß-thalassemia group, but in HbSS, MCV and MCH are showing statistical sig-

nificance. Association of XMNI polymorphism with elevated HbF was also demonstrated in a study done on homozygous sickle patients from Sudan [2]. Another study done on Sudanese homozygous sickle and sickle cell trait revealed higher HbF levels with presence of XMNI polymorphism [1]. Another study from Kermanshah province of Iran [15] on 197 β-thalassemia major patients provided valuable insights, where presence of XmnI polymorphism frequency was determined as 0.39 and a positive association was found between XmnI polymorphism (+/+) with level of HbF and also alleviation of clinical features such as splenomegaly and bone marrow expansion thus supporting overall milder phenotype of disease. Similarly, Hossain *et al.* study [16] highlighted the role of genetic determinants in HbF expression, dominated by three loci: HBG2, BCL11A, and the HBS1L-MYB intergenic region. According to this review of published literature, XmnI polymorphism is heterogeneously distributed in various population across world and in patients of homozygous β thalassemia and thalassemia intermedia HbE/β, this polymorphism greatly influences HbF levels and has been associated with positive response to hydroxyurea.

Ali *et al.* evaluated the Xmn1 polymorphism in various patient groups, noting its frequency and association with different β-thalassemia genotypes [17]. Their findings emphasized XmnI polymorphism is one of the modifiers that can increase HbF production, and it has demonstrated a diverse distribution in different regions of the world including Pakistan as evidenced in the disparity of prevalence rates among various ethnic populations within the country. This study was conducted in northern Pakistan where population consists of more Punjabis and Pathans and homozygous β thalassemia /compound heterozygous and heterozygous β thalassemia were studied and XmnI polymorphism was found at a frequency of 13%. Common β globin mutations and their association with XmnI polymorphism was also studied in the study. Fr8-9/Fr8-9 was found as most common β thalassemia genotype with none of the patient having XmnI polymorphism. Second most common genotype was IVSI-5/IVSI-5 and XmnI polymorphism was found in 15% of cases. In our study group of sickle/β thalassemia, most common mutation was IVSI- followed by Fr8-9 and Fr41-42. Our study population had considerable ethnic diversity with majority of patients were Baloch (49.3%), followed by Urdu speaking (23.3%). Overall XmnI frequency in our study population was 78% which included +/+ and +/- genotypes, with higher numbers of positive patients reported in sickle homozygous patients. This is comparable to Ansari *et al.* study, conducted on thalassemia major patients in southern Pakistan where frequency of XmnI polymorphism was found to be 45% [18].

With a variable global prevalence, it is one of the most widespread genetic diseases. Currently Hydroxyurea (HU) is the only FDA approved treatment for HbF induction in patients with SCD. HU has shown variable response among individuals that in turn could be due to genetic factors as potential influencers of therapeutic response [19-25]. Therefore, further research is essential to assess how these genetic variations affect treatment outcomes in SCD.

LIMITATIONS

This research presents several limitations. First, the limited sample size might not adequately represent the diverse sickle cell patient population in Pakistan, affecting the applicability of the results more broadly. Furthermore, genotype/phenotype correlation was not done, also impact of other genetic factors and unidentified genetic variations, also play a role in the different manifestations of sickle cell disease observed and should be considered as possible confounders [5].

CONCLUSION

It is a preliminary study done on Pakistani patients with sickle cell diseases. This study concludes that presence of XmnI polymorphism positively influences HbF levels in sickle cell disease patients in Pakistan, emphasizing the need for further comprehensive studies studying genotype/phenotype correlation. Ultimately, such investigations could pave the way for a more individualized approach to therapy in SCD management.

AUTHORS' CONTRIBUTION

Almas Khan: Study design, Writing draft.

Saima Siddiqui: Conceptualization.

Haya ul Batool Abbasi: Methodology, Data analysis and interpretation.

Danish Zahid: Final approval, Final proof to be published.

Arpana Nihal: Critical review and revision of the manuscript.

Sumaira Sharif: Study design.

FUNDING DISCLOSURE

Declared none.

CONFLICT OF INTEREST

Declared none.

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