



THE IMPACT OF THE COINHERITANCE OF ALPHA THALASSEMIA AND HEMOGLOBIN F CONCENTRATION ON SICKLE CELL DISEASE

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ARTICLE INFO

Received:

29 May 2023

Received in revised form:

29 September 2023

Accepted:

05 Oct 2023

Available online:

28 Oct 2023

Keywords: HbF, Alpha thalassemia, Sickle cell disease, Phenotype, Iraq

ABSTRACT

Alpha thalassemia and hemoglobin F concentration are major modulators of sickle cell disease in many populations. To address the impact of these two modulators on phenotype in sickle cell disease patients from Northern Iraq this study was initiated. Seventy-four Iraqi patients with sickle cell anemia or sickle/ β^0 thalassemia in steady state were enrolled. They had a median age of 16 years and included 56.8% males. Patients were clinically evaluated and had their blood and reticulocyte counts, hemoglobin F, serum lactic dehydrogenase, and bilirubin assayed. Furthermore, they were screened for α -thalassemia mutations by multiplex PCR and reverse hybridization. Hemoglobin F was positively correlated with hemoglobin, negatively correlated with reticulocyte count, hemoglobin A2, and blood transfusion frequency ($P=0.033$, 0.041 , 0.037 , and 0.02 respectively), but it was not correlated to other clinical manifestations. Alpha thalassemia was detected in nine patients (including eight with $-\alpha^{3.7}/\alpha\alpha$ and one with $-\alpha^{4.2}/\alpha\alpha$), but it did not show any significant hematological or clinical associations. The current study revealed that among SCD patients from Northern Iraq, hemoglobin F and not α -thalassemia had a modulating effect on phenotype, which is in contrast to the situation among SCD patients from Africa, the Arabian Peninsula, and Southern Iraq, where α -thalassemia plays an important modulatory role, sometimes even exceeding HbF.

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To Cite This Article: Saeed HM, Al-Allawi N, Eissa AA. The Impact of the Coinheritance of Alpha Thalassemia and Hemoglobin F Concentration on Sickle Cell Disease. *Pharmacophore*. 2023;14(5):76-83. <https://doi.org/10.51847/M2WwRD0iCw>

Introduction

Sickle cell disease (SCD) is an autosomal recessive structural hemoglobin disorder, due to a missense mutation involving codon 6 of the β -globin gene, leading to the formation of hemoglobin S ($\alpha_2\beta^S_2$), which is characterized by its tendency to polymerize upon deoxygenation [1]. The polymerization of HbS leads to shape change of their containing red cells (sickling), as well as shortened survival (hemolysis) and decreased deformability. The latter reduces the ability of the red cells to negotiate smaller blood vessels with consequent vaso-occlusion, and multi-organ damage [2]. It is prevalent in Africans and those of African origin, as well as in India, the Arabian Peninsula, and the Mediterranean Basin [1]. In Iraq, it has a characteristic distribution where it is seen in polymorphic frequencies in the Southern province of Basrah and the Northern province of Duhok, while it is sporadic in other parts of the country [3]. Sickle cell disease is associated with a variety of genotypes, the most common of which is homozygosity to the sickle cell gene (sickle cell anemia), other genotypes include compound heterozygosity to sickle cell and β -thalassemia, and sickle cell with other β -structural hemoglobinopathies, like Hb SC and SD [4]. The clinical course of sickle cell disease is quite heterogeneous, and its prediction is challenging. Much research has gone into identifying modifiers that may help in predicting the severity of this disease, and in defining genetic variations that may provide targets for future therapeutic interventions. Two major modulators of SCD have been identified, namely: the ability to augment Hb F production and coinheritance α -thalassemia [5]. However, the contributions of these two modulators vary in different populations and it is the aim of the current study to look at the contributions of these modulators and assess their impact on hematological and clinical variables in patients with SCD in Northern Iraq.

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Materials and Methods

Patients

A total of 74 sickle cell disease patients registered at the inherited blood disorders center in Duhok-Iraq were enrolled. The enrollment was restricted to confirmed cases of sickle cell anemia (SCA), and sickle/ β^0 thalassemia, while cases of sickle/ β^+ thalassemia were excluded. The inclusion criteria also included age ≥ 2 years, confirmed diagnosis of SCD, and a steady state at the time of enrollment (as defined by no history of any sickle cell crises for at least 4 weeks). Moreover, patients who were on hydroxyurea were excluded. The study was approved by the ethics committee at the directorate of Health-Duhok, Iraq, and information was obtained from all patients or their guardians.

Methods

All patients had a detailed history and examination, as well as a thorough review of their records. In addition to demographic data, clinical events including sickle cell crises, frequency of blood transfusion sessions, number of pain episodes, frequency of hospitalization for vaso-occlusive crises, as well as hospitalization for any sickle cell-related cause over the last year were scrutinized.

Investigations performed at the time of enrollment include full blood counts (Swelab, BouleMedical AB, Spånga, Sweden), reticulocyte counts, high-performance liquid chromatography (HPLC) for HbF and A2 [(D10, BioRad Laboratories, Hercules, CA, USA), and biochemical tests including S. bilirubin, ferritin as well as serum lactic dehydrogenase (LDH), which were assayed using a biochemistry autoanalyzer (Cobas c501, Roche Diagnostics, HITACHI, Tokyo, Japan).

DNA was extracted using a modified salted-out extraction method that yield a high quantity with high purity DNA [6]. Alpha thalassemia was screened for using multiplex polymerase chain reaction (PCR) and reverse hybridization to allele-specific oligonucleotide probes (ViennaLab diagnostics GmbH, Vienna, Austria) to simultaneously detect 21 α -globin mutations. The latter included the following deletions: $-\alpha^{3.7}$, $-\alpha^{4.2}$, $-\alpha^{\text{MED}}$, $-\alpha^{\text{SEA}}$, $-\alpha^{\text{THAI}}$, $-\alpha^{\text{FIL}}$, and $-(\alpha)^{20.5}$, as well as the $\alpha\alpha^{\text{anti-3.7}}$ gene triplication. It also screened for non-deletional α -thalassemias including two point mutations on the $\alpha 1$ gene [codon 14 (G>A); and Hb Adana (codon 59 (G>A)], and another 11 point mutations on the $\alpha 2$ gene including initiation codon ATG>ACG; codon 19 (-G);, IVS-I, -5 nucleotides (nts) (-TGAGG); Hb Adana [codon 59 (G>A)]; Hb Quong Sze [codon 125 (T>C)]; Hb Constant Spring [codon 142 (T>C)]; Hb Icaria [codon 142 (T>A)]; Hb Paksé [codon 142 (A>T)]; Hb Koya Dora [codon 142 (A>C)]; polyadenylation signal site (poly A1) (AATAAA>AATAAG)[Saudi Type]; and poly A2 (AATAAA>AATGAA) [Turkish type].

Statistical Methods

SPSS software was used to perform statistical analysis (Version 22, SPSS Corporation, Chicago, IL, USA). For the description of continuous parameters, the median and interquartile range (IQR) were used. Mann-Whitney test, Kurskel Wallis tests and Spearman correlation was used as appropriate. A two-tailed *P* value was considered significant if it was less than 0.05.

Results and Discussion

The median age of the enrolled patients was 16.0 years (range 2 - 47 years) and consisted of 42 males and 32 females. They included 59 cases of sickle cell anemia (SCA) and 15 of sickle/ β^0 thalassemia. The main clinical and laboratory characteristics of the enrolled patients are outlined in **Table 1**.

Table 1. Main Laboratory and clinical characteristics of 74 enrolled patients with Sickle cell disease.

Parameter	Median	IQR
Age (years)	16.0	10 – 23
Sex (Male: Female)		42: 32
Hb (g/L)	88	79-98
Reticulocyte (%)	12.5	8 -15
MCV (fL)	84.2	75.8 - 91.7
MCH (pg)	28.4	25.1- 32.0
WBC ($\times 10^9/L$)	12.9	9.6-16.9
Platelets ($\times 10^9/L$)	440.0	251-616
Bilirubin ($\mu\text{mol/L}$)	58.1	41.9-94.6
HbF (%)	11.4	6.7-20.1
HbA2 (%)	3.15	2.78-3.85
LDH (IU/L)	555.0	304-934
Ferritin (ng/mL)	155.1	92-382
Overall hospitalization/year	1.0	0-3

Hospitalization for VOC/year	1.0	0-2
Pain episodes /year	2.0	0-6
Blood transfusion/year	0	0-1.25
	Number (%)	
History of Acute Chest Syndrome	13 (17.6)	
History of Splenectomy	13 (17.6)	
Avascular necrosis of femoral head	7 (9.5)	
History of Aplastic Crisis	4 (5.4)	
History of Splenic Sequestration	4 (5.4)	
History of leg ulcers	1 (1.4)	

*IQR: interquartile range.

Hemoglobin F (%) was investigated for its correlation with various laboratory and clinical variables and the results are outlined in **Table 2**. The main significant correlations were positive correlation with hemoglobin concentration (**Figure 1**), and RBC counts ($P=0.033$, and 0.009 respectively), and negative ones with reticulocyte count (**Figure 2**), Hb A2 (%), blood transfusion frequency, and platelet count ($P=0.041$, 0.037 , 0.020 , and 0.030 respectively). Furthermore, HbF was not significantly correlated with an annual number of pain episodes or hospitalizations, and it was not significantly different between those with or without a history of various sickle cell crises.

Table 2. Non-parametric correlations and associations between Hb F (%) and various hematological and clinical variables in 74 patients with Sickle cell disease.

Parameter	HbF	
	Spearman Coefficient	P Value
Age	-0.131	0.265
Hb	0.248	0.033*
RBC count	0.303	0.009*
MCV	-0.108	0.360
MCH	-0.177	0.131
Reticulocyte count	-0.238	0.041*
WBC count	-0.109	0.354
Platelet count	-0.253	0.030*
Hb A2	-0.242	0.037*
LDH	0.016	0.894
S. Ferritin	-0.003	0.980
S. Bilirubin	-0.142	0.227
Frequency of blood Transfusions/year	-0.270	0.020*
Overall Hospitalization/year	0.051	0.615
Hospitalization for VOC/year	0.124	0.294
Pain episodes/year	0.125	0.289
	Mann Whitney Test (P value)	
Sex	0.639	
Acute chest Syndrome	0.972	
Splenectomy	0.842	
Avascular necrosis of the Femoral Head	0.305	
Splenic Sequestration	0.311	
Aplastic crisis	0.990	

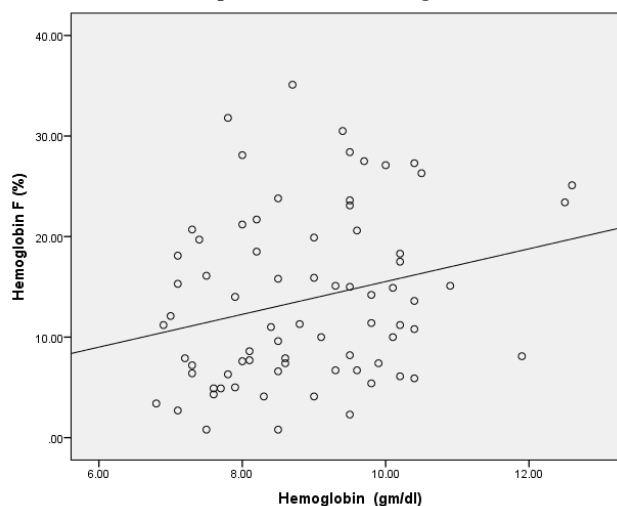


Figure 1. A scatterplot showing the positive correlation between HbF and Hemoglobin concentration (Spearman coefficient 0.248; $P=0.033$)

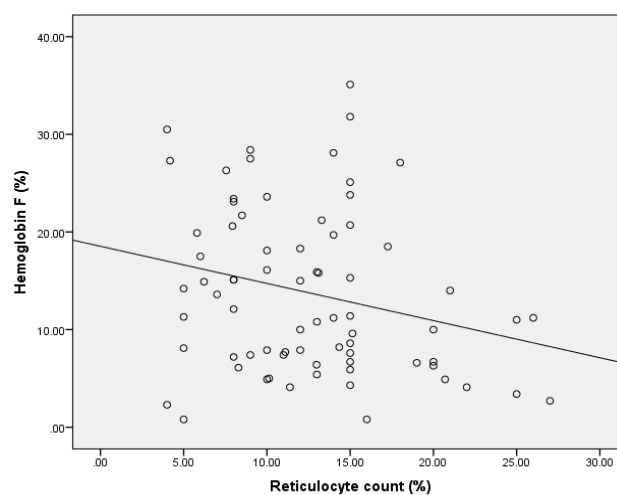


Figure 2. A scatterplot showing the negative correlation between HbF and the reticulocyte count (Spearman coefficient -0.238, $P=0.041$)

Alpha thalassemia was identified in nine patients, and they included eight with the $(-\alpha^{3.7}/\alpha\alpha)$ and one with $(-\alpha^{4.2}/\alpha\alpha)$. No cases with double α - gene deletions or with non-deletional α -thalassemia were detected. None of the laboratory or clinical parameters were significantly different in those with or without α -thalassemia (**Table 3**). Although it is worth noting that none of the nine carriers of α -thalassemia, had a history of avascular necrosis of the femoral head, splenic sequestration, aplastic crisis, or leg ulcers, and only one had a history of acute chest syndrome (all $P>0.05$). When patients were categorized as SCA, sickle/ β^0 -thalassemia and HbSS/ α -thalassemia (**Table 4**), it was found that Hb F was significantly higher in sickle/ β^0 -thalassemia followed by SCA, and then HbSS/ α -thalassemia ($P=0.032$); Hb A2 was highest in sickle/ β^0 -thalassemia then HbSS/ α -thalassemia and least in SCA ($P=0.001$), while MCV and MCH were both significantly lowest in sickle/ β^0 -thalassemia, followed by HbSS/ α -thalassemia and SCA ($P<0.001$ in both). Furthermore, blood transfusion frequency was highest in SCA followed by HbSS/ α -thalassemia and Sickle/ β^0 -thalassemia ($P=0.039$).

Table 3. Comparison between various continuous variables in sickle cell disease with or without α -thalassemia using Mann Whitney U test.

Parameter	Median (IQR)		P value
	SCD without α -thal	SCD with α -thal	
Age (years)	16 (10-23)	15 (10.5-25.5)	0.993
Hb (g/L)	88 (79.5-98)	86 (76-100)	0.766
RBC ($\times 10^{12}/L$)	3.04 (2.5-3.7)	3.05 (2.9-3.3)	0.960
Reticulocyte count (%)	12 (8.0-15.0)	13.0 (10.1-15.0)	0.476

MCV (fL)	84.3 (75.6-94)	79.2 (74.9-88.2)	0.350
MCH (pg)	28.4 (24.9-33.1)	26.4 (25.5-29.9)	0.376
HbF (%)	13.6 (7.3-20.95)	7.4 (5.213.1)	0.077
HbA2 (%)	3.1 (2.65-4.0)	3.5 (3-3.75)	0.466
LDH (IU/L)	540 (297-917)	570 (422-1204)	0.602
Ferritin (ng/ml)	152 (83-363)	162 (103-538)	0.710
Overall hospitalization annual	1 (0-3)	0 (0-2)	0.311
Hospitalization for VOC (Annual)	1 (0-2)	0 (0-1)	0.182
Pain episodes annual	2 (0-6)	0 (0-2)	0.092
Blood transfusion annual	0 (0-1.5)	0 (0-1.5)	0.837
Bilirubin (umol/L)	56.4 (39.3-95.1)	59.9 (47-93.2)	0.882
WBC (x10 ⁹ /L)	12.6 (9.1-17.0)	14.1 (11.8-17.2)	0.350
Platelets (x10 ⁹ /L)	438 (248-618)	489 (269-581)	0.862

*VOC: Vaso-occlusive crisis.

Table 4. Comparison between various continuous variables in sickle cell anemia (SCA), sickle/ β^0 -thalassemia, and Hb SS/ α -thalassemia using the Kruskal Wallis test.

Parameter	Median (IQR)			P value
	SS/ α -thal	Sickle/ β^0 thal	SCA	
Age (years)	15 (10.5-25.5)	16 (10-25)	16.5 (9.8-23)	0.896
Hb (g/L)	86 (76-100)	95 (80-99)	86 (77-97)	0.445
RBC (x10 ¹² /L)	3.05 (2.9-3.3)	4.0 (3.7-4.3)	2.86(2.40-3.45)	< 0.001
Reticulocyte count (%)	13.0 (10.1-15.0)	14.0 (9-15)	11.7 (8-15)	0.587
MCV (fL)	79.2 (74.9-88.2)	71.8 (68.7-73.4)	86.4 (83-95.9)	< 0.001
MCH (pg)	26.4 (25.5-29.9)	23.5 (22.7-24.2)	30.0 (27.4-33.6)	< 0.001
HbF (%)	7.4 (5.213.1)	21.2 (8.2-28.1)	12.9 (6.7-18.8)	0.032
HbA2 (%)	3.5 (3-3.75)	4.8 (3.1-5.4)	3.0 (2.5-3.4)	0.001
LDH (IU/L)	570 (422-1204)	700 (446-1107)	521 (278-880)	0.140
Ferritin (ng/ml)	162 (103-538)	181 (102-350)	146 (71-382)	0.661
Overall hospitalization annual	0 (0-2)	1 (0-2)	1 (0-4.25)	0.369
Hospitalization for VOC (Annual)	0 (0-1)	0 (0-2)	1 (0-2.25)	0.283
Pain episodes annual	0 (0-2)	4 (2-7)	2 (0-6)	0.115
Blood transfusion annual	0 (0-1.5)	0 (0-0)	0 (0-2.25)	0.039
Bilirubin (umol/L)	59.9 (47-93.2)	44.5 (34.2-56.4)	63.3 (46.2-99.2)	0.043
WBC (x10 ⁹ /L)	14.1 (11.8-17.2)	12.6 (9.1-17.0)	12.7 (9.1-16.9)	0.645
Platelets (x10 ⁹ /L)	489 (269-581)	438 (181-580)	438 (251-638)	0.895

*VOC: Vaso-occlusive crisis

Correlations between various red cell indices, laboratory, and clinical parameters were also investigated and some of the significant ones are outlined in **Table 5**. Some of the important correlations include: hemoglobin's negative correlation with both reticulocytes count and LDH ($P=0.001$ and 0.022 respectively); LDH's positive correlation with the reticulocyte count, frequency of pain episodes and transfusions ($P=0.007$, 0.030 and 0.006 respectively); MCV and MCH positive correlations with transfusion frequency ($P=0.034$ and 0.020 respectively). Other correlations are listed in **Table 5**.

Table 5. Some Significant bivariate correlations between various hematological and clinical parameters in 74 SCD disease patients enrolled.

Parameters	Spearman coefficient	P value
Hemoglobin and Age	+ 0.431	<0.001
Hemoglobin and RBC count	+ 0.666	<0.001
Hemoglobin and Reticulocyte count	- 0.389	0.001

Hemoglobin and Hb F (%)	+0.284	0.033
Hemoglobin and LDH	-0.266	0.022
Hemoglobin and Platelets	+0.318	0.006
Reticulocyte count and RBC count	-0.318	0.006
Reticulocyte count and Hb F(%)	-0.238	0.041
Reticulocyte count and LDH	0.309	0.007
Hb F and RBC count	0.303	0.009
Hb F and Transfusion frequency/year	-0.270	0.020
Hb F (%) and Hb A2 (%)	-0.242	0.037
Hb F(%) and Platelets	-0.253	0.030
LDH and frequency of pain episodes/year	+0.253	0.030
LDH and frequency of transfusions/year	+0.318	0.006
Frequency of Hospitalization and pain episodes /year	+0.688	<0.001
Frequency of Hospitalization and transfusions/year	+0.272	0.019
Leucocytes and frequency of hospitalization/year	+0.219	0.061
Leucocytes and platelet counts	+0.374	0.001

When the history of the occurrence of sickle cell crisis was assessed in relevance to other laboratory or clinical parameters, it was found that acute chest syndrome was associated with higher frequencies of pain episodes and admissions for this episode, though only the former was significant ($P=0.037$ and 0.059 respectively). Avascular necrosis of the femoral head was not associated significantly with any of the parameters tested, though patients with this complication were slightly older, but this did not reach significance ($P=0.102$). On the other hand, a history of splenectomy was associated with a significantly older age, and higher hemoglobin and platelet counts ($P=0.020$, 0.048 , and 0.043 respectively).

Sickle cell disease has a unique distribution in Iraq, with two epicenters, one at the extreme North [7] and the other at the extreme South, with the former being associated mainly with Benin and the latter with the Arab Indian haplotype [8, 9]. The Benin haplotype is associated with moderate disease, while the Arab Indian haplotype has been associated with higher HbF and less severe disease [10]. Earlier reports from Northern Iraq revealed that SCD is indeed of moderate severity with less SCD crisis than that reported among Africans. Consistent with the latter observations, the current study documented a median HbF of 11.4% and hemoglobin of 88 g/L which were both lower than reported in those with the Arab Indian haplotype from the Arabian Peninsula and Southern Iraq, but comparable to those reported in Southwest Saudi Arabia where the Benin haplotype is similarly the predominant haplotype [11].

Hemoglobin F ($\alpha_2\gamma_2$) has been coined as the most potent modulator of the sickle cell disease phenotype. Increased HbF retards the polymerization of deoxy HbS (which is key in the pathophysiology of SCD) since the former is unable to enter the HbS polymer; Moreover, it reduces the mean red cell HbS concentration [12]. In the current study, it was found that HbF (%) is positively correlated with hemoglobin concentration and negatively with reticulocyte count and transfusion frequency, and this is consistent with the protective role of HbF and its ability to reduce hemolysis. Similar findings were observed in Jamaican and American patients with sickle cell anemia [13-15]. The negative correlation between Hb F and Hb A2 noted in the current study is also shared by previous studies [13, 15, 16], and it may be due to cell selection and preferential survival of cells containing high F and low A2 than other populations of cells [13]. In the current study, and except for the negative correlation with transfusion frequency, we did not find a significant correlation between Hb F and other clinical manifestations of SCD, particularly the frequency of pain episodes or other sickle cell crises and this is not unique and has been also documented by previous investigators in Jamaicans [15], and in Saudi Arabian sickle cell anemia patients [11, 17]. However, these observations are contrary to other studies from several SCD populations, including African, American, Indian, and French West Indies patients which demonstrated that HbF reduces the frequency of vaso-occlusive episodes [12, 18-22]. The absence of an association of HbF with vaso-occlusive events in the current study may be related to the sample size and short time frame for assessment of these episodes (past year).

Alpha thalassemia leads to a reduction in the α -chain production with a resultant lowering in intracellular HbS concentration, thus reducing sickling and consequent hemolysis [23]. The frequency of α -thalassemia among the studied sample was 12.2% which is close to the frequency reported in an earlier study on SCA in Northern Iraq at 10% [8]. It is lower than the frequency of α -thalassemia among SCD patients in Southern Iraq, where it was reported in 18.4% [24], and much lower than that reported in Arabian Peninsula countries where the frequencies of α -thalassemia among SCD patients range from 77.6% in Oman to 23% in Bahrain [11, 25-27]. The frequency of α -thalassemia reported among Africans and populations of African origin with SCD ranged from 30 to 45% [18, 28-30], while its frequency among Indian SCD patients is 30% [21]. Coinheritance of α -thalassemia has been associated with higher hemoglobin, lower reticulocyte counts, lower MCV, and MCH; Furthermore, it may confer protection against acute chest syndrome, stroke, leg ulcers, kidney disease, and splenic sequestration, while it may lead to a paradoxical increase of painful vaso-occlusive events [12, 18, 20, 22, 31]. However, the latter association has been

disputed by some reports which showed either no effect or decreased frequency of pain episodes in association with α -thalassemia [19, 24, 29, 32]. In the current study, there were no significant differences in hemoglobin or reticulocyte counts between those with or without α -thalassemia. Moreover, although the frequency of pain episodes was less in those with HbSS/ α -thalassemia, yet neither this nor any of the other clinical events were significantly associated with coinheritance of α -thalassemia. This observation may be because all nine of our α -thalassemia carriers, had a single α -gene deleted, unlike earlier studies on other populations, where α -thalassemia with double gene deletions was also a frequent occurrence [21, 24, 26]. It is believed that an ameliorating effect of α -thalassemia on SCD is observed mainly in those with double gene deletions, while such an effect is limited or minimal in those with single α -gene deletions [5, 28]. It is, however, interesting to note that HbF was lower in association with HbSS/ α -thalassemia when compared to the other two genotypes (**Table 4**). Such an association has been also reported by Dover *et al.* [33], though not by others [28, 29]. The former researchers attributed the lower HbF to the relatively prolonged survival of non-F cells in HbSS/ α -thalassemia compared to non-F cells in SCA (without α -thalassemia), and not due to reduction in F cell production or percentage of HbF per F cell [33].

The limitations of the current study are that it is cross-sectional, restricted to patients in a steady state, and not on hydroxyurea. This may have limited the patients eligible for inclusion.

Conclusion

In conclusion, the current study revealed that among SCD patients from Northern Iraq hemoglobin F and not α -thalassemia had a modulating effect on the phenotype, which is in contrast to the situation in most studies on SCD patients from Africa, the Arabian Peninsula, and Southern Iraq, where α -thalassemia plays an important modulatory role, sometimes even exceeding HbF.

Acknowledgments: None

Conflict of interest: None

Financial support: None

Ethics statement: None

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