Hematology/Oncology and Stem Cell Therapy

Volume 16 | Issue 3 Article 8

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Recommended Citation

Elbostany, Eman A.; Elghoroury, Eman A.; Thabet, Eman H.; Rashad, Alaa A.; Rasheed, Enas A.; El-Saeed, Gamila S.M.; Abdelhalim, Dalia A.; Abdelfattah, Safa N.; Salama, Iman I.; and Salama, Niveen (2023) "Oxidative stress and hepcidin expression in pediatric sickle cell anemia with iron overload," *Hematology/Oncology and Stem Cell Therapy*: Vol. 16 : Iss. 3 , Article 8.
Available at: https://doi.org/10.1016/j.hemonc.2021.11.003

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ORIGINAL ARTICLE

Oxidative stress and hepcidin expression in pediatric sickle cell anemia with iron overload

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Abstract

Background: **Blood transfusion (BT) is essential in treating sickle cell disease (SCD); however, it leads to iron overload (IO) and oxidative stress. We studied the relationship between oxidative stress, iron status parameters, hepcidin mRNA gene expression, and IO in SCD patients.**

Methods: **We classified all SCD patients ($n = 90$) into two groups: Group I, 45 children (s.ferritin ≥ 938 ng/mL) and Group II, 45 children (s.ferritin < 938 ng/mL). A total of 55 children, age and sex matched, participated as a control group. Malondialdehyde (MDA), nitrite, s.iron, s.total iron-binding capacity (sTIBC), transferrin saturation %, s.ferritin, s.hepcidin, and hepcidin mRNA gene expression were assessed.**

Results: **Among SCD BT-dependent patients (>3 times/year), 63% were from Group I and 37% from Group II, $p < .01$. The two patient groups had significantly lower s.hepcidin and hepcidin gene expression than controls ($p < .001$). TIBC, s.iron, s.ferritin, transferrin saturation %, ferritin/hepcidin ratio, and MDA levels were higher among SCD patients than controls ($p < .001$). Group I had higher mean level of ferritin/hepcidin ratio and MDA than Group II ($p < .01$). The higher level of MDA and increased frequency of BT were the significant predicting risk factors for IO ($p < .05$). A receiver-operating characteristic curve indicates that MDA is the outstanding significant biomarker for high level of s.ferritin with subsequent IO progression.**

Conclusion: **MDA may serve as a biomarker of oxidative stress and IO in SCD patients. This result paid attention for urgent initiation of antioxidant and chelation therapy on detecting increased MDA level.**

Keywords: Antioxidants, Hepcidin, Iron overload (IO), Malondialdehyde (MDA), Sickle cell anemia, s.ferritin

1. Introduction

Sickle cell disease (SCD) is the most common genetic hematologic disorder [1]. It is estimated that 75–85% of children born with SCD are from Africa. In Egypt, the prevalence of SCD is 0.3%. HbS carrier rates vary from 9% to 22% in some regions. In 1951, Abbasy [47] reported the first case of SCD in Egypt [2].

Blood transfusion (BT) plays a major role in the management of SCD patients but causes marked iron overload (IO) [3], about 200–250 mg of iron per unit of transfused blood [4]. Normal content of the body's iron is about 4 g; individuals with chronic BT can store 5–10 g per year [5]. Iron becomes toxic with the progression of IO because of its tendency to catalyze the processing of reactive oxygen species, consequently leading to oxidative stress and enhancing cellular damage [6].

Biomarkers of oxidative damage are increased in SCD [7]. Malondialdehyde (MDA), a product of lipid peroxidation, is an important marker in the

Received 7 June 2021; revised 10 October 2021; accepted 14 November 2021.
Available online 4 April 2023

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https://doi.org/10.1016/j.hemonc.2021.11.003

evaluation of oxidative stress; it was found to be increased in SCD patients compared with healthy individuals [8].

During hemolysis, hemoglobin dimers and arginase are released into the plasma; these consume nitric oxide (NO) generating inactive nitrates and L-arginine—the substrate for NO production—causing a reduction in the bioavailability of NO and contributing to the vaso-occlusive process. NO is a potent vasodilator and its reduction is associated with endothelial damage [9]. In turn, low levels of NO may result in hemodynamic instability and reduction of antioxidant capacity [10].

Although most studies have generated the hypothesis that hemolytic anemias including SCD are associated with decreased NO bioavailability [11], the conclusive evidence that NO bioavailability is decreased in SCD is lacking, and the hypothesis that NO deficit contributes to SCD pathobiology has been challenged [12].

Hepcidin—a polypeptide hormone with a 25-amino acid sequence, from the transcription of the gene HAMP (hepcidin antimicrobial peptide)—performs an important role in iron homeostasis by binding to ferroportin, a protein which exports iron from different cell types, especially enterocytes and macrophages of the reticuloendothelial system [13]. The enterocytes and macrophages internalize the hepcidin–ferroportin complex with degradation of ferroportin, thus blocking iron output and consequently minimizing the absorption of intestinal iron and its bioavailability [14]. The ineffective intensive erythropoiesis and congenital anemia of the SCD potentially cause reduction of hepcidin level, leading to increased intestinal iron absorption. In contrast, the characteristic inflammatory feature as well as the increased serum iron (s.iron) concentration can cause induction of the transcription of this hormone, reducing iron absorption [15].

Ferritin is a high-molecular-weight protein that contains approximately 20% iron. It occurs normally in almost all tissues of the body, especially in hepatocytes and reticuloendothelial cells, where it serves as an iron reserve [16].

Hepcidin and serum ferritin (s.ferritin) respond similarly to inflammation and changes in iron stores. However, hepcidin responses take place within a few hours, whereas changes in ferritin concentration are much slower [17] as the factors that accelerate and inhibit hepcidin formation may be simultaneously present in SCD patients [18].

Transferrin is the plasma iron transport protein that binds iron strongly at physiologic pH. Transferrin is normally only 25–30% saturated with iron [19]. Although excess iron elevates both ferritin levels and transferrin saturation (TSAT) in SCD patients, there is notorious discrepancy between these parameters [20].

Hepcidin in SCD has not yet been sufficiently studied. The few available studies present inconclusive data regarding hepcidin plasma level, mostly without differentiation between SCD patients with or without IO, and also do not compare these levels with healthy control groups [6]. The current study aim was to assess oxidative stress, antioxidant, some of iron status parameters, and hepcidin mRNA gene expression among children with SCD in relation to IO.

2. Patients and methods

The study was approved by the Ethical Committee of the National Research Centre (approval No. 16128), Egypt, and a written informed consent was taken from patients and controls in accordance with the Declaration of Helsinki.

Ninety children with SCD, aged 6–18 years, were recruited from Pediatric Hematology Clinic at National Research Centre, and Abo El-Ryish Children hospital, Cairo University. The patients were at quiescent stage (excluded infection or any crisis) and classified into two groups according to the level of s.ferritin 938 ng/mL (which is established according to median value of the studied patients): Group I, 45 children (s.ferritin $\geq$ 938 ng/mL) and Group II, 45 children (s.ferritin $<$ 938 ng/mL). A control group of healthy 55 children with age and sex matched also participated. All patients and controls were subjected to the following:

1. Clinical assessment. Full medical history was taken, considering blood transfusion (BT) (type, frequency, amount/year, and chelation and hydroxyl urea (hydra) medication) and complications (attacks of hemolytic crisis, thromboembolic manifestations, or chest problems). Clinical examination was done stressing on the presence of hepatosplenomegaly, pallor, jaundice, bone aches.
2. Laboratory investigations. Venous blood samples (5 mL) were withdrawn for laboratory assessment. The s.iron was measured using automated analyzer Olympus AU-400 Manufactured by Diagnostic Systems Group of Olympus America. Enzyme-linked immunosorbent assay according to manufacturer's protocol was performed for assessing serum hepcidin and ferritin. The percentage of TSAT with iron was calculated. Normal values are 20–50%. Estimation of oxidative stress (MDA and nitrite)

was measured colorimetrically according to methods described by Ruiz-Larrea et al. [21] and Moshage et al. [22], respectively. NO measured as nitrite was determined by using Griess reagent [23].

3. RNA extraction and cDNA synthesis. Total RNA was extracted from peripheral mononuclear cells using a QIAamp RNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions.
   Reverse transcription reactions were performed using random primers with a high-capacity complementary DNA (cDNA) archive kit (Applied Biosystem, Foster City, CA, USA).
4. Real-time quantitative PCR. Real-time PCR was performed with Stratagene Mx3000P (Agilent Technologies, USA). The PCR reaction was carried out in a final volume of 25 μL containing 2 μL cDNA, 12.5 μL 2 × SYBR Green master mix (Applied Biosystems), 0.5 μL of 25 nM sense and antisense primers, and deionized water up to 25 μL. The PCR conditions consisted of 40 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. The sequences of the primers were as follows: GAPDH: sense primer 5′-CCACCCAGAAGACTGTGGAT-3′, antisense primer 5′-TTCAGCTCAGGGATGACCTT-3′; and hepcidin: sense primer 5′-CACAACAGACGGGACAACTT-3′, antisense primer 5′-CGCAGCAGAAAATGCAGATG-3′. We used the comparative (ΔΔCT) method where we compared the CT values of the target gene (hepcidin) with the reference gene (GAPDH) [24].

2.1. Statistical analysis

Data entry and analysis were performed using SPSS 18.0 software for Windows (SPSS Inc., Chicago, IL, USA). The quantitative data are expressed as mean ± standard deviation, and the qualitative variables are expressed as percentages. Chi-square test was used to compare between two qualitative variables, t test was used to compare between two means, and analysis of variance test was used for comparison of more than two groups. When the results were significant, differences between groups were identified using the Bonferroni post hoc test. When data were not normally distributed, Mann–Whitney U test was used for comparing between two groups and Kruskal–Wallis test for comparing between three groups. Multivariate logistic analysis was performed to predict risk factors significantly associated with high level of s.ferritin. A p value < 0.05 was considered statistically significant, and $p < .01$ was considered statistically highly significant. For evaluating the diagnostic performance of each biomarker for IO, receiver-operating characteristic (ROC) analysis was performed for obtaining the area under the curve (AUC) and the corresponding 95% confidence interval (CI). The maximum diagnostic discrimination cut-off point was calculated corresponding to the highest Youden index.

3. Results

A total of 145 children (90 with SCD and 55 healthy controls) participated in the study. There was no significant difference regarding age and sex between the three studied groups. The demographic and medical history data of SCD patients are shown in Table 1. The percentage of SCD patients receiving BT > 3 times/year or receiving chelation therapy was significantly higher among patients with s.ferritin ≥ 938 ng/mL than those with s.ferritin < 938 ng/mL ($p < .01$).

Table 2 shows comparison between hepcidin, hepcidin gene expression, iron status, and oxidant and antioxidants among two groups of SCD patients and controls. The two groups of SCD patients had significantly lower s.hepcidin and hepcidin gene expression than controls ($p < .001$). The mean hemoglobin, s.iron, total iron-binding capacity (TIBC), s.ferritin, TSAT %, ferritin/hepcidin ratio, and MDA were increased significantly among SCD patients compared with controls ($p < .001$). Group I SCD patients (s.ferritin ≥ 938 ng/mL) had significantly higher mean level of s.ferritin, ferritin/hepcidin ratio, and MDA than Group II (s.ferritin < 938 ng/

Table 1. Demographic and Medical History Among SCD Patients According to Our Result Cut-Off Point of Serum Ferritin = 938 ng/mL.

	SCD patients		p
	s.ferritin ≥ 938 ($n = 44$)	s.ferritin < 938 ($n = 45$)	
Age (yr)	17.7 ± 4.42	16.2 ± 4.11	0.120
Sex			
Male	26 (50.0)	26 (50.0)	1.0
Female	19 (50.0)	19 (50.0)	
Disease duration (yr)	13.1 ± 3.0	12.6 ± 3.4	0.483
Hemoglobin (g/dL)	7.8 ± 1.2	8.4 ± 1.1	0.016
Complication			
+ve ($n = 54$)	29 (53.7)	25 (46.3)	0.389
−ve ($n = 36$)	16 (44.4)	20 (55.6)	
Chelation therapy			
+ve ($n = 45$)	29 (64.4)	16 (35.6)	0.006
−ve ($n = 45$)	16 (35.6)	29 (64.4)	
Blood transfusion/year			
>3 ($n = 54$)	34 (63.0)	20 (37.0)	0.003
≤3 ($n = 36$)	11 (30.6)	25 (69.4)	

Note. Data are presented as mean ± SD or n (%). SCD = sickle cell disease; s.ferritin = serum ferritin.

Table 2. Iron Status and Oxidant and Antioxidants Among Two Groups of SCD Patients and Controls.

	SCD patients		Controls ($n = 44$)	p
	s.ferritin ≥ 938 ng/mL ($n = 44$)	s.ferritin < 938 ng/mL ($n = 45$)		
Hepcidin (ng/mL)	16.58 ± 54.66**	77.8 ± 87.08***	94.52 ± 13.55	<0.001
Hepcidin mRNA expression (ΔΔCT)	0.125 ± 0.202**	0.129 ± 0.191***	1.03 ± 0.113	<0.001
Iron (ug/dl)	387.09 ± 82.34**	374.46 ± 67.62***	91.63 ± 15.35	<0.001
Ferritin (ng/mL)	1686.84 ± 749.06*,**	562.60 ± 230.47***	82.47 ± 30.86	<0.001
TIBC (ug/dl)	331.64 ± 49.72**	326.86 ± 50.71***	306.68 ± 15.43	<0.001
TSAT %	118.4 + 27.50	117.4 + 30.80	29.86 + 4.60	<0.001
Nitrite (umol/L)	29.28 ± 6.06*	30.02 ± 6.26***	27.06 ± 4.56	<0.01
MDA (nmol/mL)	19.90 ± 32.56*,**	8.23 ± 6.83***	2.83 ± 1.03	<0.001

Note. Data are presented as mean ± SD. MDA = malondialdehyde; SCD = sickle cell disease; s.ferritin = serum ferritin; TIBC = total iron-binding capacity; TSAT = transferrin saturation.
* Significant p value between two groups of SCD patients.
** Significant p value between SCD patients with s.ferritin ≥ 938 ng/mL and controls.
*** Significant p value between SCD patients with s.ferritin < 938 ng/mL and controls.

mL), $p < .01$. Serum nitrite (s.nitrite) was slightly significantly higher among Group II than among controls ($p < .05$), and there was no significant difference between the two groups of SCD or between SCD Group I and controls ($p > .05$).

There was no significant difference of all studied parameters (hepcidin, real hepcidin, s.iron, TIBC, TSAT, nitrite, and MDA) between SCD children who received BT ≥ 3 times/year and who received < 3 times/year.

Correlation analysis among SCD patients revealed that s.ferritin was found to be significantly positive correlated with MDA ($p < .01$) and frequency of BT per year ($p < .01$). Nitrite level was significantly positive correlated with hepcidin level ($p < .05$) and TIBC ($p < .05$) (Table 3).

Multiple regression analysis revealed that higher level of MDA and increased frequencies of BT were the significant predicting risk factors for IO (Fig. 1, table insert).

4. Discussion

SCD is one of the most common genetic hematologic disorders. BT plays a major role in the management of SCD patients but leads to significant IO results in considerable morbidity and mortality.

Table 3. Predictors for Risk of Iron Overload Among SCD Patients Using Logistic Regression.[a]

	B	SE	Wald	p	AOR (95% CI)
Hepcidin (ng/mL)	0.016	0.005	8.965	0.003	1.02 (1.0–1.03)
Nitrite (umol/L)	−0.113	0.051	4.899	0.027	0.89 (0.81–0.99)
MDA (nmol/mL)	0.129	0.040	10.486	0.001	1.14 (1.05–1.23)
Ferritin/Hepcidin ratio	0.031	0.009	10.635	0.001	1.03 (1.01–1.05)
Constant	−0.545	1.356	0.162		

Note. AOR = adjusted odds ratio; CI = confidence interval; MDA = malondialdehyde; SCD = sickle cell disease; SE = standard error.
[a] Variable(s) entered on step 1: hepcidin, Fe, HYDRA, TIBC, nitrite, MDA, Hepcidin mRNA expression. Blood transfusion > 3, Ferritin-Hepcidin ratio, Chelation therapy.

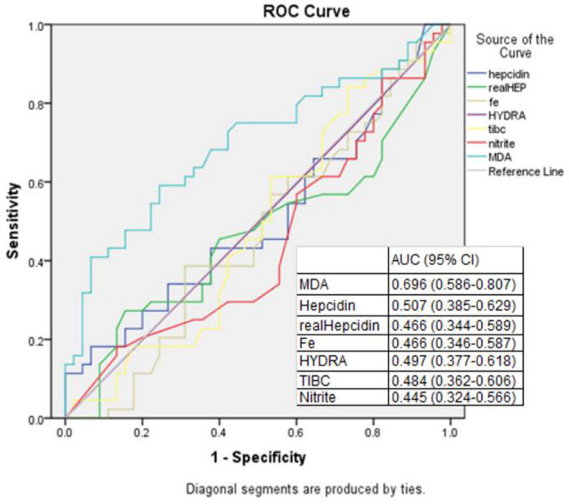

Fig. 1. ROC curves obtained to assess the discriminatory value of different biomarkers for prediction of high level of s.ferritin (≥938 ng/mL). The ROC curve shows that MDA was the only significant biomarker, with AUC: 0.70 (95% CI: 0.59–0.81). The cut-off point for MDA was 10.6 with sensitivity 0.60 (95% CI: 0.44–0.74) and specificity 0.76 (95% CI: 0.60–0.87). Note. AUC, area under the curve; MDA, malondialdehyde; ROC, receiver-operating characteristic.

The current study assessed the association between IO among patients with SCD and hepcidin, hepcidin gene expression, oxidative stress, and iron parameters status. The study demonstrated that SCD patients with s.ferritin ≥ 938 ng/mL had lower mean s.hepcidin concentrations (61.6 ng/mL) than those with s.ferritin < 938 ng/mL (77.8 ng/mL; $p > .05$) and controls (94.5 ng/mL; $p < .001$). These results were in agreement with those by Fertrin et al. [20]. However, other studies detected equal hepcidin level among SCD patients and controls

[25–28] or even higher levels among controls [17,29]. Similar to the current study, Rashidy, et al. [17] reported that there was no significant correlation between ferritin and hepcidin levels among SCD patients. Such variability among several studies was probably due to the heterogeneity of the studied groups or association of other chronic diseases which affect the level of hepcidin. These variations can be explained by lacking data distinguish the participants whether BT dependent or not also do not determine IO by ferritin concentration. However, ineffective erythropoiesis with low or inappropriately normal hepcidin levels, and consequent IO, are known features of the "iron-loading anemias" [30].

Iron profile was made up in current study by measuring the s.iron, s.ferritin, TSAT, and TIBC. The TIBC measures the availability of iron-binding sites, whereas the s.ferritin is known to reflect mainly iron stores. The TSAT is a measure of amount of iron bound to the protein transferrin and reflects iron transport. As the body iron stores are depleted, the TSAT rises. In the present study, the mean levels of TIBC, s.ferritin, and TSAT % were higher among SCD patients than among controls ($p < .001$). The finding of higher body iron stores and higher mean TSAT among the studied SCD patients potentially resulted from the increased red cell turnover and from BTs. By contrast, Akodu et al. [16] observed higher mean TSAT % among controls than among SCD patients. Fertrin et al. [20] reported notorious discrepancy of s.ferritin concentration and TSAT in SCD.

Conflicting results have been obtained by several studies investigating s.ferritin in SCD patients compared with controls. Akinbami et al. [31] found that s.ferritin level was normal in 90% of studied SCD patients, Russo-Mancuso et al. [32] reported it to be normal or moderately elevated, and Garba et al. [33] found it to be at lower level. Contributing factors may explain the variability of s.ferritin measurements in SCD patients including increased mobilization and utilization of ferritin iron from the stores to erythropoietic precursors in the bone marrow to meet increased demand for new red blood cells production in SCD patients as a result of chronic hemolytic/hyper hemolytic process or unselected BT-dependent SCD patients [16,31].

The role of oxidant damage to red cells and role of antioxidant in sickle cell anemia has been of interest in recent years. The present study showed that MDA level was significantly elevated level among SCD patients with s.ferritin $\geq$ 938 ng/mL compared with those with s.ferritin < 938 ng/mL and among controls ($p < .01$). Similarly, many studies reported higher level of oxidant among SCD patients than controls [34,35]. On contrary, MDA in SCD was not found significantly different from controls [36]. The higher activities of MDA reflect the extent of oxidative damage in red cells. In addition, a positive correlation was detected between MDA level and s.ferritin and frequency of BT among studied sickle cell cases. Similarly, Dos Santos et al. [37] obtained a positive correlation between ferritin and MDA, which supported that iron store was a major risk factor for increased oxidative stress in SCD.

Nitrite level was assessed as an index of antioxidant NO among the studied participants. A significant slightly higher mean level of s.nitrite was found among SCD patients with s.ferritin < 938 ng/mL (30.0 umol/L) compared with controls (27.1 umol/L), $p < .05$. Antwi-Boasiako and Campbell [38] reported that among SCD patients with vaso-occlusive crisis, the mean NO levels were significantly higher in the immediate post crisis period. In contrast, Emokpae et al [39], Arinola et al. [40], Foluke et al. [41], and Hasanato [42] reported that SCD patients have lower levels of NO and total antioxidant capacity than normal healthy controls.

In the current study, the percentages of SCD patients receiving BT > 3 times/year or receiving chelation therapy were significantly higher among patients with s.ferritin $\geq$ 938 ng/mL (63.0% and 64.4%, respectively) compared with those having a lower ferritin level (37.0% and 35.6%, respectively), $p < .01$. These findings suggested that increased frequency of BTs among patients with SCD enhance the IO risk, contributing to oxidative stress.

Similar results were published by Harmatz et al. [43] and Hafsia et al. [44] who reported correlation between s.iron and s.ferritin levels and increased frequency of BT. Ikusemoro et al. [45] also reported high level of s.ferritin among SCD patients receiving repeated red cell transfusion ($\geq$3 units/year) compared with those with rare red cell transfusion history. Conversely, Sahasrabhojaney and Solanki [46] found that IO state was detected only among SCD patients who received multiple BTs.

Multivariate regression analysis was performed and included hepcidin, hepcidin gene expression, ferritin/hepcidin ratio, TIBC, and frequency of BT/year. It revealed that a higher level of MDA and increased frequencies of BT were the significant predicting risk factors for IO. The ROC curve analysis indicated that MDA was the only significant biomarker for high level of s.ferritin with subsequent IO progression. This result paid attention for urgent initiation of chelation therapy on detecting increased MDA level, which reflected enhanced oxidative stress from IO independent of s.ferritin

level as it was considered as a slowly growing marker of IO.

The current study concluded that MDA can be considered as a sensitive predictor of oxidative stress due to IO in SCD patients, and it is correlated with iron indices of IO. A routine early screening of MDA and s.ferritin levels in SCD patients is recommended as future strategy by early intervention with antioxidant and chelation therapy to ameliorate morbidity of IO. Further research to determine the lower cut-off level of s.ferritin is warranted for early prediction of IO complication.

Authors' contributions

Elbostany Eman, Elghoroury Eman, and ET designed the study. Elbostany Eman, Elghoroury Eman, NS and AR interpreted the data and wrote the manuscript. Elbostany Eman and IS collected the data and performed statistical analysis. Elbostany Eman, ET, E.A Rasheed, DA, and GS performed laboratory assessment of patients. IS and Elbostany Eman provided valuable input into the study design, interpretation, and reviewed the manuscript. All contributing authors reviewed and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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