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The Effect of Hydroxyurea on Adhesion Receptor Integrin-Associated Protein (CD47) Expression in Patients with Sickle Cell Disease

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ABSTRACT

Background: Patients with sickle cell disorders (SCD) are prone to episodes of micro vascular obstruction. Integrin-associated protein (CD47) is one of the important participating adhesion molecules in this process.

Aim of the Work: To study the role of red cells (RBCs) CD47 expression as a predictor for severity of sickle cell disease and its relation to Hb F% and hydroxyurea therapy.

Patients and Methods: The study included 48 sickle cell disease children, divided into three groups: G1; patients not on hydroxyurea (HU) therapy in steady state, G2; patients on HU in steady state, and G3; patients in painful crisis at the time of evaluation. Twenty normal children were included as a control group. For all individuals % expression of CD47 on RBCs was evaluated by flowcytometry.

Results: The mean RBCs CD47 expression was significantly higher in the patient group compared to the control group ($p<0.001$). The mean CD47 expression in G1 (87.25±8.46) was lower than in G3 (89.04±5.14) but higher than in G2 (85.74±18.37) yet the difference was not significant ($p>0.05$). CD47expression was significantly positively correlated with both total leucocytic count and absolute neutrophilic count with non significant negative correlation with HbF%.

Conclusion: The adhesion molecule CD47 expression could be a contributing factor to acute and chronic vaso-occlusion characteristic of SCD.

Key Words: *Sickle Cell Disease – Adhesion molecules – Integrin-associated protein CD47 – Hydroxyurea.*

INTRODUCTION

Sickle cell disease is an inherited hemolytic disorder caused by abnormal hemoglobin (Hb S) **[1]**, transmitted as incomplete autosomal dominant trait that results from a missense mutation in ß-globin chain determined gene **[2]**. It is characterized by the crescent-like or sickle shape that red blood cells acquire in response to decreased oxygen concentration or other physiologic stresses **[3]**.

One of the hallmarks of sickle cell disease is episodic occurrence of painful crises which are believed to originate from vascular occlusion and lead ultimately to organ failure **[4]**. Sickle red blood cells (RBC) were found to be more adhesive than normal **[5]**. This makes interactions between the sickle cells and endothelial cells central to the pathophysiology of the disease **[4]**.

Adhesion events are mediated by: (i) membrane-bound receptors at the circulating-cell surface; (ii) membrane-bound counter receptors at the vascular endothelial cell (VEC) surface; (iii) sub-endothelial matrix elements exposed after VEC injury, and finally by (iv) soluble proteins in the plasma forming bridges between the red cells and endothelial cells **[6]**.

Hydroxyurea is an important major advance in the treatment of sickle cell disease. Strong evidence supports the efficacy of hydroxyurea to decrease severe painful episodes, hospitalization, number of blood transfusions and the acute chest syndrome **[7,8]**.

Unfortunately the more understanding of the pathophysiology of sickle cell disease is not accompanied with advances in therapy for this disease **[9,10]**. However, hydroxyurea therapy is often associated with clinical improvement

before any measurable rise in fetal hemoglobin, suggesting that hydroxyurea could also act through other mechanisms, such as a decrease of VCAM-1 expression and release of endothelin-1 from human endothelial cells **[11]**. Hydroxyurea also decreases the adhesion of sickle red cells and reticulocytes to endothelial cells **[11]** and to the sub-endothelial matrix proteins, thrombospondin and laminin **[12]**. In vivo, hydroxyurea decreases the percentage of reticulocytes expressing α4ß1, CD36 **[13]**, soluble VCAM-1 **[14]** and plasma endothelin-1 levels **[15]**. Hydroxyurea could also exert its impact through a reduction in neutrophil activation **[16]**. Thus, adhesion receptors on both red blood cells and endothelial cells seem to be a target for hydroxyurea.

There are several cell surface receptors that appear to be involved in mediating the interaction between HbS erythrocytes and the endothelium. These include Lutheran blood group antigen (BCAM/Lu, CD239), CD147, intercellular adhesion molecule-4 (ICAM-4), CD36 on reticulocytes, very late activation antigen 4 (VLA-4) on reticulocytes, and sulfated glycolipids **[17]**. Membrane damage to the HbS erythrocyte also leads to the exposure of phosphatidylserine, which is normally restricted to the inner surface of the membrane lipid bilayer. This phosphatidylserine exposure is also thought to contribute to the adhesiveness of HbS erythrocytes to the endothelium **[18,19]**.

Integrin-associated protein (IAP), known as CD47 is an erythrocyte adhesion receptor expressed by both normal and HbS erythrocytes. For unclear reason, it was found that the HBS erythrocytes are more adherent to thrombospondin (TSP). It was suggested that in patients with SCD there are abundance of reticulocytes which express α4ß1 integrins, and CD47 is thought to function only in association with integrins **[20,21]**.

In this study we examined the status of CD47 on Red blood cells from children with SCD and the effects of hydroxyurea therapy on modulation of CD47 expression on erythrocytes. Our aim was to study the role of RBCs CD47 expression as a predictor for severity of sickle cell disease and its relation to Hb F% and hydroxyurea therapy.

PATIENTS AND METHODS

This study included 48 sickle cell disease children (The patient group) selected from inpatient and outpatient clinics of Menoufiya University Hospital and Abou El-Rish University Hospital. Egypt. Their ages ranged between 1 and 18 with a mean of 9±4.27 and a median of 8.5 years. They included 26 males and 22 females. This group was subdivided into 2 subgroups 1- Group A: Included 28 patients not on hydroxyurea (HU) treatment, their ages ranged from 1 to 18 with a median of 7.5 yrs. 2- Group B: Included 20 patients on HU treatment for at least one year with good compliance. The treatment duration period ranged from 1 to 12 yrs. Their ages ranged between 6 and 18 with a median of 10.5 yrs. To study the effect of CD47 expression, all patients (48) were divided into another three groups according to the disease status and HU therapy at the time of evaluation: Group 1 (G1) 10 patients in steady state (that was maintained for at least 6 months before the time of evaluation) not on HU, group 2 (G2) 12 patients in steady state (that was maintained for at least 6 months before the time of evaluation) on HU therapy and group 3 (G3) 26 patients with frequent painful attacks and in acute painful crisis at the time of evaluation. Twenty normal children and adolescents of matched age, gender, and socio-economic standard were enrolled as the control group. Their ages ranged from 4 to 18 with a mean of 9.67±4.53 and a median of 9 years. They were 10 males and 10 females. Any individual with pallor, abnormal CBC findings, history of blood transfusion, or family history of SCD or any hematological disorder were excluded from this group. The Ethics Committee approved the study and an informed consent was obtained from all the parents.

For all individuals the following was done:

1- Full history taking including the pain rate (in patient group) defined according to Bonds **[10]** as the average number of days of hospital stay due to painful episodes or days of extreme relevant illness at home from patient own calendar during the last year.

2- Clinical examination.

3- Laboratory investigations including complete blood picture, reticulocytic count, Liver enzymes (ALT and AST), renal function tests, Hb electrophoresis and serum ferritin level.

4 - CD47 expression on RBCs by flow cytometry (BECKTON, DICKINSON FACS caliber, BD immune cytometry systems, San Jose, CA). The expression of CD47 was evaluated by using FITC labeled mouse monoclonal antibodies against human CD47 (clone B6H12 BD Pharmingen). CD71 PE (clone: M- A712 BD Pharmingen) was used in some cases.

In short 10ul of each monoclonal antibodies were added to 100ul of PB diluted 1:200 in PBS. After 30-45 minutes incubation at room temperature, cells were washed twice and resuspended in 200-400ul PBS and analyzed on Flow Cytometer. Hundred ul of diluted PB served as auto-control **[22]**.

Flow cytometric analysis: Data were acquired on a FACS caliber flow cytometer (BD immune cytometry systems, San Jose, CA).

The instrument was checked weekly using QC windows beads (flowcytometry standard, San Juan, PR). Forward scatter and side scatter measurements were made using linear amplifiers, whereas fluorescence measurements were made with logarithmic amplifiers and flow cytometric parameters dot plots were generated by cell quest software **[23]**.

In some case we use mouse monoclonal antibodies against human CD71 PE for gating on CD47 positive cells (data not presented).

Statistical analysis:

Data were collected, tabulated, and analyzed by using SPSS (11) statistical package for windows XP. Quantitative data were analyzed by student test for comparison of the means of two normally distributed variables and Mann-Whitney (U) test for comparison of the means of two non normally distributed variables. Kruskal Wallis test is a test of significance used for comparison between three groups not normally distributed having quantitative variables. ANOVA (f) is a test of significance used for comparison between three groups having quantitative variables normally distributed. Pearson correlation coefficient (*r*) is used to test association between two normally distributed quantitative continuous variables. Qualitative data were analyzed by X^2 test. The level of significance was set as 95% confidence interval so *p*-value was <0.05 **[24]**.

RESULTS

Clinical and laboratory characteristics of patients with sickle cell disease.

Our data (Table 1) showed that patients on HU therapy are significantly taller than those not on HU therapies. The frequency of pain episodes was significantly lower in patients on hydroxyurea therapy compared with those not on HU therapies with no significant difference between the two groups regarding the transfusion index yet it was lower in the HU group. The HU group (GB) had significantly lower reticulocytic count%, total leucocytic count and absolute neutrophilic count compared to that not on HU (GA). No significant difference was found between the two groups regarding Hb level (yet higher in patients on HU therapy), platelets count, Hb F% (at the time of the study), serum ferritin, liver or renal function tests (Table 1).

Table (1): Comparison between Sickle Cell Disease patients not on HU therapy and those on HU therapy.

Parameters	Patients not on hydroxyurea (GA) No=28	Patients on hydroxyurea (GB) No=20	*p* value
Height (cm)	120.8±20.85*	134.75±19.40	<0.05
Pain episodes (days/year)	3.89±2.28 89.64±16.88	2.3±0.88 84.0±16.35	<0.05 >0.05
Transfusion index (ml/kg/yr)			
Hb (g/dl)	7.43±1.09	7.98±1.59	>0.05
Reticulocytic (%)	5.78±3.67	1.78±0.86	<0.001
WBC($x10^9$/L)	12.79±4.64	10.91±6.44	<0.05
ANC ($x10^9$/L)	6.0±2.94	3.98±1.75	<0.001
Platelets count ($x10^9$/L)	285.15±111.61	312.07±138.95	>0.05
Hb F%	20.16±6.27	23.0±2.77	>0.05
Ferritin (ng/ml)	484.6±330.77	414.35±210.37	>0.05
AST (IU/L)	30.86±11.21	34.15±14.87	>0.05
ALT(IU/L)	28.11±8.41	27.8±10.83	>0.05
BUN (mg/dl)	12.43±3.54	12.08±4.09	>0.05
Creatinine (mg/dl)	0.67±0.72	0.54±0.19	>0.05

* Mean±SD

The frequency of pain episodes was significantly lower in patients in steady state on HU therapy (G2) compared to patients in steady state not on HU therapy (G1). The transfusion index was significantly lower in G2 compared to G1 and to patients in acute painful crisis

(G3), while it was significantly higher in G3 compared to G1. Reticulocytic count% was significantly lower in G2 compared to G1 and G3. There was no significant difference between the three groups regarding Hb level, total leucocytic count, ANC, platelets count, or HbF% (Table 2). Comparing patients on HU therapy before and after at least one year of compliant therapy; the frequency of pain episodes, transfusion index, reticulocytic count% and the absolute neutrophilic count had significantly decreased after HU therapy with insignificant increase in Hb F% (Table 3).

CD47 expression on RBCs is summarized in (Table 4). There was significantly higher expression in the patient group compared to the control group. No significant difference between GA and GB was encountered, yet CD47 expression was lower in (GB). CD47% expression was found to be highest in G3 (patients in acute painful crisis) followed by that of G1 (patients without HU in steady state) and was least in G2 (patients on HU in steady state) but the difference between the three groups did not reach a significant level (Table 4). The frequency of pain episodes showed a significant negative correlation with Hb F% (r=–0.49, p=<0.05) and a significant positive correlation with Hb level (r=0.52, p=<0.05) There was a significant positive correlation between CD47% and both WBCs count and ANC (Table 5, Fig. 3).

Table (2): Comparison between different Sickle Cell Disease groups.

Parameter	Patients (G1) No=10	Patients (G2) No=12	Patients (G3) No=26	*p*1	*p*2	*p*3
Pain rate (days/year)	4.3±2.11	2.25±0.62	3.34±2.13	<0.05	>0.05	>0.05
Transfusion index (ml/kg/yr)	100±22.71	71±28.06	132.5±17.12	<0.05	<0.05	<0.05
Hb (g/dl)	7.84±0.84	7.5±0.92	7.83±1.76	>0.05	>0.05	>0.05
Reticulocytic (%)	4.5±3.66	1.98±0.87	5.65±3.91	<0.05	>0.05	<0.05
WBC ($x10^9$/L)	12.39±7.71	11.61±5.08	12.57±3.34	>0.05	>0.05	>0.05
ANC ($x10^9$/L)	5.49±3.14	4.13±1.77	5.52±2.11	>0.05	>0.05	>0.05
Platelets count ($x10^9$/L)	282.5±121.3	258.83±103.1	327.3±137.7	>0.05	>0.05	>0.05
HbF %	19±7.34	21.15±7.07	16.5±4.2	>0.05	>0.05	>0.05

*p*1: Between G1 & G2. G1: Patients not on hydroxyurea (HU) therapy in steady state.
*p*2: Between G1 & G3. G2: Patients on HU in steady state.
*p*3: Between G2 &G3. G3: Patients in painful crisis.

Table (3): Comparison between Sickle Cell Disease patients on hydroxyurea before and after at least one year of treatment.

Parameter	Before therapy (No=20)	After therapy (No=20)	*p* value
Pain rate (days/year)	3.65±1.13*	2.3±0.88	<0.001
Transfusion index (ml/kg/yr)	123.5±24.97	84.0±16.35	<0.001
Hb (g/dl)	7.19±0.87	7.98±1.59	>0.05
Reticulocytic (%)	6.89±4.17	1.78±0.86	<0.001
WBCs ($x10^9$/L)	11.37±6.42	10.91±6.44	>0.05
ANC ($x10^9$/L)	6.13±3.63	3.98±1.75	<0.05
Platelets ($x10^9$/L)	301.55±111.5	312.07±138.95	>0.05
Hb F%	20.14±7.35	23±2.77	>0.05
AST (IU/L)	30.4±12.03	34.15±14.87	>0.05
ALT (IU/L)	33.65±10.99	27.8±10.83	>0.05
BUN (mg/dl)	11.25±3.59	12.08±4.09	>0.05
Creatinine (mg/dl)	0.49±0.16	0.54±0.19	>0.05

* Mean±SD

Table (4): Comparison between different Sickle Cell Disease patients groups regarding expression of CD47% on red cells.

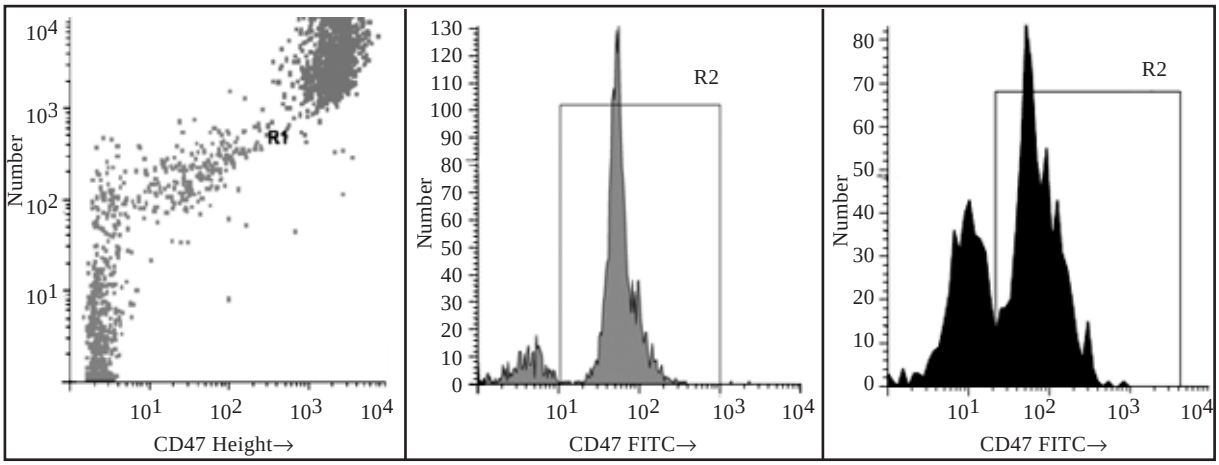
Group	CD47%	*p*-value
Patients	86.8±14.4*	
Controls	59.1±14.18	<0.001
Patients not on HU (GA)	89.64±6.91	
Patients on HU (GB)	82.98±20.25	>0.05
Patients in Steady state without HU (G1)	87.25±8.46	
Patients in Steady state with HU (G2)	85.74±18.37	>0.05
Patients with Painful crisis (G3)	89.04±5.14	

* Mean±SD.
HU:Hydroxyurea.

Table (5): Correlation between Hb F%, the pain rate and CD47% and different parameters in Sickle Cell Disease patients.

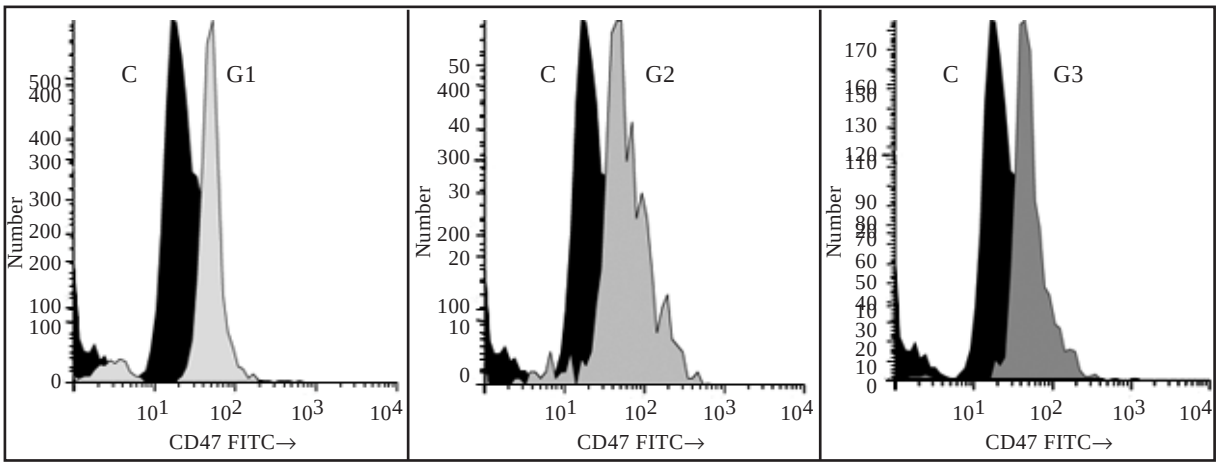
Parameter	Hb F%		Pain rate		CD47	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Pain rate (days/year)	–0.49	<0.05			0.20	>0.05
Duration of HU in yrs	0.24	>0.05			0.29	>0.05
Transfusion index (ml/kg/yr)	0.23	>0.05			0.32	>0.05
Hb (g/dl)	–0.12	>0.05	<0.05	0.52	0.09	>0.05
WBCs ($x10^9$/L)	–0.14	>0.05	>0.05	0.21	0.39	<0.05
ANC ($x10^9$/L)	–0.09	>0.05			0.39	<0.05
Reticulocytic count %	0.32	>0.05			–0.22	>0.05
Platelets ($x10^9$/L)	–0.33	>0.05			–0.04	>0.05
HbF%					–0.46	>0.05

HU: Hydroxyurea.



Gating on RBCs | CD47 expression (on patients group) | CD47 expression (on control group)

Fig. (1): CD47 expression on normal control and Sickle Cell Disease patients.



CD47 expression (histogram)

Fig. (2): Comparing of CD47 expression on different groups.

G1: Patients not on hydroxyurea (HU) therapy in steady state.
G2: Patients on HU in steady state.
G3: Patients in painful crisis.

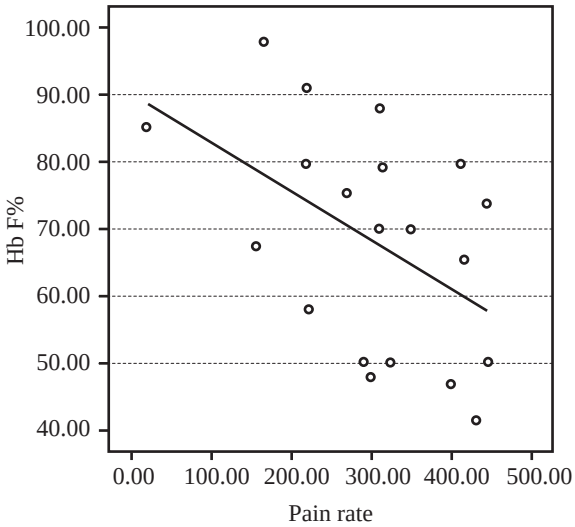


Fig. (3A): Correlation between Hb F% and the pain rate.

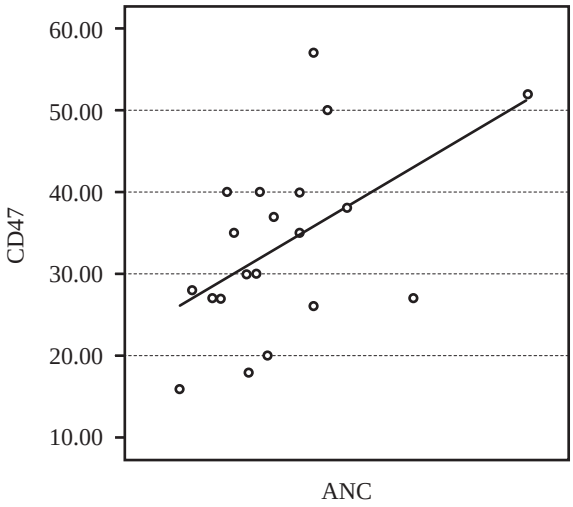


Fig. (3B): Correlation between CD47% and the ANC.

Fig. (3): Correlation studies in 20 sickle cell disease patients.

DISCUSSION

Hydroxyurea is the most prescribed therapy for sickle cell disease **[25]**. The clinical improvement after hydroxyurea therapy is out of question; there is reduction in painful vaso-occlusive crisis and adhesion of sickle cells to vascular endothelium that is a critical factor in pathogenesis of this event **[4]**.

Comparing the group of patients not on HU therapy (GA) and the patients on HU therapy (GB), the pain rate was significantly lower in the HU group than the other group with no significant difference between the two groups regarding the transfusion index (Table 1) indicating that the beneficial clinical effect of HU therapy was reflected on the decline of the pain rate before the effect on RBCs transfusion requirement. It is speculated that reduction in leukocytic count may be an important component of the beneficial effects of hydroxyurea therapy **[9,15,25]**.

The results of this study revealed that the reticulocytic count%, WBCs and ANC were significantly lower in (GB) compared to (GA) with insignificant difference between the two groups regarding the Hb level yet it was higher in (GB) (Table 1). This data is consistent with that obtained by Borba et al. **[16]**.

Hb F% did not significantly differ between the two groups yet was higher in the HU group (Table 1) indicating that HU treated patients had improved clinically and hematologically even before the peak level of Hb F was reached. This is consistent with results reported by Bachir et al. **[26]** who found no correlation between Hb F% and HU serum level but is not in agreement with results of other studies done by Rodgers **[27]** Maier-Redelsperger et al. **[28]** and Koren et al. **[29]**.

The multi-center phase I/II pediatric hydroxyurea trial (HUG-KIDS) confirmed a wide variability in the Hb F% response; a few children who reached the maximal tolerated dose (MTD) had Hb F% levels that were persistently below 10% whereas several others had levels that exceeded 25% **[30]**. So, apparently, the beneficial effect of HU may be exerted through reduction of the total leucocytic count, ANC and reticulocytic count even before the increase of Hb F%.

The pain rate and the transfusion index were significantly lower in patients in steady state on HU therapy (G2) compared to patients in steady state not on HU therapy (G1). The transfusion index was significantly higher in G3 (patients in acute painful crisis) compared to both G1 and G2. There was no significant difference between the three groups regarding the Hb level or Hb F%.

For the group of patients who received HU therapy (for at least one year), there was a significant decrease in the pain rate and transfusion index after HU therapy and this is going with several studies done by Charache et al.

[31], Al-Jam'a and Al-Dabbous **[32]** and Anderson **[33]**.

The Hb level insignificantly increased after HU therapy and this is in contrast to Wang et al. **[30]** who reported a significant elevation of Hb level after HU therapy. There was a significant decrease in reticulocytic count and absolute neutrophilic count (ANC) after HU therapy and this is in agreement with Bagdasaryan et al. **[34]** and Debaun & Field **[35]**. In contrast, Koren et al. **[29]** found no significant effect of HU on reticulocytic count.

There was insignificant increase in HbF% after HU therapy. Again these results support that patients on HU therapy had improved clinically (reflected on decline in the pain rate and the transfusion index) even before the peak of Hb F% has been reached and reticuolocytic count improved before improvement of the Hb level. In contrast to what was reported by Al-Jam'a & Al-Dabbous **[32]**, the result of this study revealed that the total leucocytic count had insignificant decrease after HU therapy.

It has been shown that CD47 on sickle red blood cells activates G-protein-dependent signaling, which promotes cell adhesion to immobilized thrombospondin through a receptor called $\alpha 4\beta 1$ **[20]**.

Studying the role of CD47 as an adhesion molecule in the present study, the results revealed that the mean CD47 expression on the RBCs was significantly higher in patient group compared to the control group suggesting that this adhesion molecule could have a role in the pathology of SCD. A few studies have investigated the expression of CD47 in SCD. In contrast to the results of this work, Brittain et al. **[36]**, had reported that CD47 levels are similar or identical on SS and AA RBCs. However, they stated that the structure of CD47 on SS RBCs is different from the structure of CD47 expressed on normal AA RBCs.

The role of adhesion molecules as mediators of HU effectiveness was studied by some researchers **[37]**; their results supported the hypothesis that HU reduces the adhesive properties of sickle cells and suggested that this decrease may be mediated, at least in part, by a decrease in the gene and consequently, surface protein expression of adhesion molecules such as VLA-4 and CD36.

In the present study, CD47 expression was lower in the group on HU therapy (GB) compared to those not on HU therapy (GA), however, the difference did not reach a significant level. This may suggest that HU produces clinical improvement through pathways not involving CD47 as the pain rate was significantly lower in patients on HU therapy. Also when comparing CD47 expression in patients in steady state not on HU (G1), patients in steady state on HU (G2) and patients in acute painful crises (G3); the mean of CD47 expression in G1 was lower than that in G3 but higher than that in G2, yet the difference was not significant. The last results may indicate that CD47 expression is lower in steady state in general than during the painful crisis. The results also indicate that HU therapy effect is not exerted through decreased CD47 expression.

Fetal Hb concentration is the most important disease modifier in SCD as it is protective to the HbS-containing RBCs. Increased Hb F% is associated with decreased mortality in children and adults with SCD **[38]** and it protects against painful episodes, acute chest syndrome (ACS) and leg ulcers **[39]**.

In this study significant negative correlation between Hb F% and the pain rate was found. This is in agreement with numerous studies **[27,33,40]**. Thus, it is expected that patients with higher Hb F% level would have milder disease course and agents that increase Hb F% level would provide significant amelioration of disease severity.

Significant positive correlation between the pain rate and Hb level was found and this is consistent with Platt et al. **[41]** who stated that increased Hb concentration is a predictor of pain. Positive correlation between the pain rate and the WBCs count was found, yet insignificant. In this regard Redding-Lallinger & Knoll **[42]** reported that steady state WBCs count >20.000 is a risk factor for SCD complications including recurrent painful crises.

Studying the correlation between CD47 expression and some parameters significant positive correlations between CD47% and both WBCs count and absolute neutrophilic count were found supporting its role in vaso-occlusion as it is matched with other risk factor for vaso-occlusion (WBCs and ANC). This agrees with

Lindberg et al. **[43]** who stated that CD47 activates neutrophils and with Ticchioni et al. **[44]**, who reported that CD47 activates mature T cells. The results of this work had revealed non significant positive correlation between CD47 expression and both the pain rate and the duration of HU therapy that could be a form of drug tolerance over time.

Chung et al. **[45]** had reported that CD47 activates platelets and increases platelet adhesion via a Gi-linked signal transduction pathway. In this study negative correlation was found between CD47% and both reticulocytic and platelet counts yet did not reach a significant level.

From the results of this study, it can be concluded that Hydroxyurea is an effective treatment for amelioration of the disease severity of sickle cell disease through its effect on the pain rate and transfusion index. This beneficial effect of hydroxyurea is probably achieved through reduction of total leucocytic and the absolute neutrophilic counts.

In conclusion, the adhesion molecule CD47 expression is increased in SCD patients compared to the normal control and it is positively correlated with WBC and absolute neutrophilic count which could be a factor contributing to acute and chronic vaso-occlusion characteristic of SCD. As CD47 has a potential role in the pathophysiology of vaso-occlusion in SCD, consequently, it should be a target of further research to establish this role as well as to search for useful therapeutic agents capable of antagonizing it and alleviating disease severity.

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