

RESEARCH ARTICLE

Altered levels of pro-inflammatory cytokines in sickle cell disease patients during vaso-occlusive crises and the steady state condition

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ABSTRACT. Objective: This study aimed to evaluate serum levels of pro-inflammatory cytokines and TGF- β in sickle cell disease (SCD) patients, and to compare the results during vaso-occlusive crisis (VOC) or steady state (StSt) conditions. **Methods:** 54 SCD patients (37HbSS and 17S β ⁺Thal) were enrolled in the study and evaluated in two groups as follows; group A consisted of 39 VOC patients and group B comprised 15 StSt patients. Nineteen healthy volunteers were included as controls. Circulating levels of IL-1, IL-6, IL-8, IL-17, TNF- α and TGF- β were measured using ELISA. **Results:** Patients in VOC showed higher mean levels of all cytokines than those found in steady-state patients, but this was only marginally significant for IL-8 levels ($P = 0.08$). Increased levels of TGF- β and IL-17 were found in StSt patients *versus* normal controls ($P = 0.004$ and $P < 0.0001$ respectively). A positive correlation was observed between IL-8 and IL-17 in both groups of patients ($P = 0.002$ and $P = 0.005$ respectively). Decreased levels of TNF- α , IL-1 β and IL-17 were found in hydroxyurea-treated patients. Additionally, significantly higher levels of IL-6 and IL-8 were observed in hydroxyurea-treated and untreated patients than in controls respectively ($P = 0.04$ and $P = 0.01$). **Conclusions:** Our findings indicate that pro-inflammatory cytokines, especially IL-8 and IL-17, could be used as related markers for assessing disease severity, and consequently therapeutic intervention.

Key words: pro-inflammatory, cytokines, sickle cell anemia

Sickle cell disease (SCD) is an inherited disorder of hemoglobin (Hb) structure and synthesis, which is characterized by chronic hemolysis, frequent infections and recurrent occlusions of the microcirculation. These complications cause painful crises and lead to chronic organ damage, disability and ultimately, premature death [1, 2]. Direct adherence of sickle red blood cells to the endothelium plays a major role in vaso-occlusion and this process is the main cause of morbidity and mortality in SCD patients [3, 4].

Vaso-occlusion is a complicated process governed by several contributing factors including, interaction between multiple adhesion molecules and receptors on erythrocytes, white blood cells, platelets and endothelial cells, in which sub-endothelial matrix components and plasma proteins forming bridges between blood cells, and vascular endothelium play critical roles [5-7].

Evidence is accumulating to support the hypothesis that unusual binding of pro-adhesive sickle cells to the vascular endothelium is a crucial event in pathophysiology of vaso-occlusion and other clinical manifestations of this

disease [8]. Additionally, it is believed that pro-adhesive mechanisms related to abnormal red cells, leukocytes or endothelial cells are also activated during steady-state, the period between a painful crisis, in which the patient feels well, and vaso-occlusion, which is an ongoing subclinical process in these stable SCD patients [8, 9].

The increased adhesiveness of sickle cells to endothelium during the steady-state of SCD leads to chronic activation of endothelium and damage, and consequently cytokine production (IL-1, IL-6, IL-8, TNF- α) by endothelial cells. These inflammatory cytokines enhance the adhesiveness of sickle cells to endothelium through different mechanisms such as induction of vascular cell adhesion molecule (VCAM) expression, fibronectin and chemokine production. In addition, $\alpha_4\beta_1$ integrin expression on sickle cells leads to subclinical vaso-occlusion during steady state periods [8-10]. In the case of inflammatory stress, cytokines further activate the vascular endothelium, increase the adhesion of sickle cells to endothelium, and ultimately lead to painful vaso-occlusive crisis [8].

Microvascular occlusion, either clinical or subclinical, infection and hemolysis are crucial factors that stimulate production of cytokines and acute phase proteins [10-12]. On the other hand, increased numbers of reticulocytes, as a result of chronic hemolytic anemia, are often present in the circulation of SCD patients. Reticulocytes express integrin $\alpha_4\beta_1$ (VLA-4), which binds to plasma and endothelial fibronectin, and to VCAM-1 on the surface of endothelial cells [3, 13, 14]. These interactions are enhanced by inflammatory cytokines such as TNF- α , IL-1 and IL-8, especially after the activation of endothelial cells [8, 9, 15, 16].

Growing bodies of literature suggest that white blood cells (WBC), especially neutrophils, may be involved in the initiation and propagation of vaso-occlusive crisis in SCD. It has been demonstrated that an increased steady-state leukocyte count is a risk factor for acute chest syndrome, stroke and mortality. Also, it is postulated that vascular endothelium activation promotes leukocyte recruitment, activation and adhesion, culminating in adhesive interactions between circulating erythrocyte and adherent leukocytes [9, 17]. More importantly, increased levels of proinflammatory cytokines, particularly TNF- α and IL-8, which are potent mediators of neutrophil activation, enhance abnormal adhesion of activated neutrophils to endothelial cells, and to plasma fibronectin, leading possibly to vaso-occlusion and sickle cell crisis [8, 10, 17]. IL-8, a chemokine produced by endothelial cells in areas of endothelial injury, activates $\alpha_4\beta_1$ integrin on sickle reticulocytes and endothelial surface-associated fibronectin, thereby increasing the adherence of sickle red blood cells to endothelium [13]. Additionally, IL-8 acts as a chemotactic factor for neutrophils and contributes to the initiation of vascular occlusion and painful crisis in SCD patients. From this point of view, IL-8 production is regulated by TNF- α and IL-1, synthesis of the plasma form of fibronectin being regulated by IL-1 and IL-6 [17-20]. Likewise, VCAM-1 expression on the surface of endothelial cells is increased by tumor necrosis factor (TNF)- α , platelet activating factor (PAF) and interleukin-1 [15].

Another pro-inflammatory mediator that possibly aggravates this condition is IL-17, a cytokine produced by Th17, which initiates a neutrophil-dominant inflammatory response. Binding through IL-17R induces target cells to produce proinflammatory factors such as IL-1, IL-6, IL-8, TNF- α and matrix metalloproteinase [21, 22]. Hence, cytokines seem to be one of the main contributing factors in the pathogenesis of the vaso-occlusion phenomenon in SCD [8, 10].

In spite of encouraging data on the physiopathology of SCD, progress in its treatment remains unimpressive [23-27]. Hydroxyurea (Hu), the only drug with any proven clinical efficacy, can affect cell adhesion through several possible mechanisms such as decreased HbSS erythrocyte adhesion, down-regulation of VCAM-1 and endothelin-1 expression, which presumably contribute to the reduction of vaso-occlusive episodes [17, 27].

In this study, we evaluated serum levels of IL-1, IL-6, IL-8, IL-17, TNF- α and TGF- β in SCD patients and a group of healthy volunteer controls, and compared the results considering vaso-occlusive crisis and the steady-state condition, in patients receiving treatment with hydroxyurea, or not.

PATIENTS AND METHODS

A total of 54 SCD patients (37 HbSS homozygous type and 17 S β^+ Thal heterozygous type), attending the Thalassemia and Hemoglobinopathy Research Center in Shafa University Hospital (southern Iran) were enrolled in this cross-sectional study, which was approved by the institutional ethics committee. Informed consents were obtained from all patients or their guardians for participation in the study. Documentation of homozygous sickle cell (HbSS) and S β^+ Thal heterozygous patients had been determined by hemoglobin electrophoresis at pH 9.2 on cellulose acetate strips, tests for solubility and sickling, and quantification of Hb A₂ and Hb F. Diagnosis of HbSS homozygous or S β^+ heterozygous type in these patients had been confirmed by genetic analysis in the molecular laboratory of this center. The exclusion criterion was previous transfusion within three months before sampling. Patients were evaluated in two groups as follows; group A consisted of 39 patients experiencing vaso-occlusive crisis who were admitted to hospital after visiting the clinic (eight with abdominal crisis and 31 with musculoskeletal crisis), and group B consisting of 15 non-symptomatic, sickle cell patients (no acute illness or crisis), who had been referred to the outpatient clinic for routine check-ups. However, vaso-occlusive crisis was defined as an episode of acute pain in the abdomen, bone, joint, or multiple sites of pain, necessitating hospital admission and analgesic administration. Clinical infection was assessed by hematology specialist by taking a history for possible exposure to infectious disease and recording clinical signs and symptoms of fever, rashes, jaundice, or swelling, as well as organ-specific signs and symptoms, such as coughing, diarrhea, and dysuria.

Nineteen age-, sex- and race-matched, healthy, normal subjects were included in the study as the control group. Ten milliliters of venous blood was drawn from healthy volunteers and sickle cell patients. Of this amount, 5 ml was transferred to a vacutainer tube with EDTA anticoagulant for determination of the basic hematological indices. Five milliliters was dispensed into a plain, sterile tube for serum separation. Thereafter, serum samples were kept frozen at -70°C until cytokine assay. Peripheral blood cell counts were determined using an automated cell counter, Celltec α (Nihon Kohden, Tokyo, Japan).

Serum cytokine assays

Serum levels of IL-1 β , TNF- α , IL-6, IL-8, IL-17 and TGF- β were measured using commercial enzyme-linked immunosorbent assay kits (Bender MedSystems, Vienna, Austria), in accordance with the manufacturer's instructions. All serum samples were assayed in duplicate.

Statistical analysis

Data were analyzed using the SPSS, version 15, for Windows. Statistical significance was calculated using Student's unpaired *t* test, the Mann-Witney U test, Chi-square and Fisher's exact test. Spearman's correlation coefficient was used to determine the correlation between some quantitative variables. Statistical test results with a *P* value ≤ 0.05 were considered to be significant.

RESULTS

Demographics of the study subjects, as well as their hematological parameters and some clinical features of the patients are summarized in *table 1*. Fifty-four patients with a diagnosis of sickle cell disease (SCD) comprising 25 males (46.3%) and 29 females (53.7%), were evaluated in this study. Of the SCD patients, 39 (72.2%) were in vaso-occlusive crisis, and 15 (27.8%) were in a steady-state condition and represented our stable control patients. There was a significant difference in total white blood cell (WBC) counts and neutrophil percentages between both groups of patients ($P = 0.005$ and $P = 0.0005$ respectively), and also between patients and healthy controls ($P < 0.0001$) (*table 1*). Similarly, there was a statistically significant difference in total red blood cell (RBC) counts, hemoglobin concentrations and hematocrit percentages between both groups of patients and controls (*table 1*). Fifteen patients (12 in vaso-occlusive crisis and 3 in the

steady-state condition group) had been receiving treatment with hydroxyurea at least for six months (1000-1500 mg/day).

The mean serum levels of pro-inflammatory cytokines IL-1 β , TNF- α , IL-6, IL-8, IL-17 and TGF- β as a regulatory factor, were compared between all patients and healthy control volunteers, and also between the two groups of patients *versus* controls. Significantly higher, levels of all cytokines mentioned, except for IL-1 β , were observed in SCD patients compared to the control group (*table 2*).

Patients in vaso-occlusive crisis showed higher mean levels of TNF- α , IL-6, IL-8, TGF- β , IL-1 β and IL-17 than those in the steady-state condition. However, this difference was only marginally significant for IL-8 between both groups of patients (175.7 ± 44.6 *versus* 61.39 ± 5.02 , $P = 0.08$, *table 3*). In addition, patients in vaso-occlusive crisis had significantly higher levels of all cytokines except for IL-1 β in comparison with control subjects (*table 3*). Comparison of the mean cytokine concentrations between steady-state

Table 1

Characteristics of all sickle cell disease patients (N = 54) grouped by steady-state (StSt) *versus* vaso-occlusion crisis (VOC) conditions and healthy controls.

Variables	VOC Patients (group A, n = 39)	StSt Patients (group B, n = 15)	Normal controls (group C, n = 19)	P values	
				A vs. C	B vs. C
Age (Mean \pm SD)	23.4 \pm 8.4	23.25 \pm 11.7 $P = 0.96^a$	23.37 \pm 4.83	0.98	0.96
Male/Female	18 / 21	7 / 8 $P = 0.78^b$	9 / 10	0.84	0.76
SS / S β	26 / 13	11 / 4 $P = 0.75^b$	—		
Hu / No-Hu	12 / 27	3 / 12 $P = 0.51^b$	—		
WBC ($\times 10^6$ /ml)	13.98 \pm 6.4	8.3 \pm 2.6 $P = 0.005^a$	7.47 \pm 0.75	<0.0001	0.20
RBC ($\times 10^9$ /ml)	3.58 \pm 0.83	3.51 \pm 0.75 $P = 0.80^a$	4.79 \pm 0.62	<0.0001	<0.0001
Neutrophil (%)	65.39 \pm 6.5	56.68 \pm 7.33 $P = 0.0005^a$	52.79 \pm 5.2	<0.0001	0.09
Hemoglobin (gr/dl)	9.12 \pm 1.82	8.76 \pm 1.66 $P = 0.56^a$	14.34 \pm 1.16	<0.0001	<0.0001
Hematocrit (%)	28.98 \pm 5.36	28.28 \pm 4.81 $P = 0.69^a$	43.08 \pm 3.5	<0.0001	<0.0001

Quantitative data are presented as mean \pm SD. SS: homozygous Hb SS, S β : heterozygous HbS and β thalassemia, Hu: hydroxyurea treatment, No-Hu: not receiving hydroxyurea.

^a: two-tailed P values by unpaired t-test, b: two-tailed P values by Chi-square or Fisher's exact test.

Table 2

Comparison of serum cytokine levels between SCD patients and normal controls.

Cytokines (pg/ml)	Patients (N = 54)	Controls (N = 19)	P Values ^a
IL-1 β	3.24 \pm 0.16	3.33 \pm 0.19	0.33
TNF- α	16.02 \pm 4.58	12.17 \pm 0.55	0.02
TGF- β	11.15 \pm 1.86	1.04 \pm 0.30	<0.0001
IL-6	12.13 \pm 1.98	5.03 \pm 0.69	0.06
IL-8	144.0 \pm 32.88	55.74 \pm 6.15	0.02
IL-17	7.70 \pm 0.32	4.67 \pm 0.21	<0.0001

All data are presented as mean \pm SE. a: two-tailed P values by Mann-Witney U test.

Table 3Serum levels of cytokines in all patients grouped by vaso-occlusive crisis (VOC) *versus* steady-state (StSt) conditions and in normal controls.

Cytokines (pg/ml)	VOC patients (group a, n = 39)	StSt patients (group b, n = 15)	Controls (group c, n = 19)	P Values ^a		
				a vs. b	a vs. c	b vs. c
IL-1β	3.26 \pm 0.20	3.19 \pm 0.22	3.33 \pm 0.19	0.95	0.37	0.44
TNF-α	17.8 \pm 6.33	12.03 \pm 0.85	12.17 \pm 0.55	0.13	0.009	0.42
TGF-β	11.18 \pm 2.23	11.07 \pm 3.74	1.04 \pm 0.30	0.89	<0.0001	0.004
IL-6	14.18 \pm 2.26	6.78 \pm 1.73	5.03 \pm 0.69	0.15	0.04	0.45
IL-8	175.7 \pm 44.6	61.39 \pm 5.02	55.74 \pm 6.15	0.08	0.01	0.29
IL-17	8.25 \pm 0.58	7.48 \pm 0.38	4.67 \pm 0.21	0.59	<0.0001	<0.0001

All data are presented as mean \pm SE. a: two tailed P values by Mann-Witney U test.

patients and controls revealed a significant difference for TGF- β and IL-17 levels ($P = 0.004$ and $P < 0.0001$ respectively, *table 3*).

Spearman correlation analysis revealed a significant, positive correlation between IL-8 and IL-17 in vaso-occlusive crisis and steady-state patients ($P = 0.002$ and $P = 0.005$ respectively, *figure 1*), and an inverse correlation between neutrophil percentages and IL-8 concentrations in the steady-state group ($P = 0.07$, *figure 1*).

With regard to receiving hydroxyurea therapy, decreased levels of TNF- α , IL-1 β and IL-17, as well as increased levels of TGF- β , IL-6 and IL-8 were observed in hydroxyurea-treated patients compared to those not receiving hydroxyurea (*table 4*). In comparison with healthy controls, mean levels of TGF- β and IL-17 were significantly higher in both groups of patients (*table 4*). Also, significantly higher levels of IL-6 and IL-8 were observed in hydroxyurea-treated and untreated patients compared to normal controls respectively ($P = 0.04$ and $P = 0.01$, *table 4*).

Comparison of the mean concentration of cytokines between SCD (HbSS) and sickle-thalassemia ($S\beta^+$) patients did not show any statistically significant differences. However, increased levels of TGF- β and IL-8 were observed in $S\beta^+$ patients *versus* SCD patients ($P = 0.68$ and $P = 0.75$ respectively, *table 5*). It is interesting to note that we found significantly increased levels of TNF- α , TGF- β and IL-17 in both group of patients compared to the controls (*table 5*). Likewise, control subjects had significantly decreased levels of IL-8 and IL-6 compared with SS patients and $S\beta^+$ patients respectively ($P = 0.01$ and $P = 0.05$, *table 5*).

DISCUSSION

Sickle cell anemia has been defined as a chronic inflammatory state with significant immunological components including; elevated leukocyte counts, abnormal activation of granulocytes, monocytes and endothelial cells, and increased levels of multiple inflammatory mediators [8-10]. One of the main reasons for painful crisis and consequent chronic organ failure in SCD patients is recurrent occlusion of the microcirculation. In spite of general agreement denoting the increased binding of sickle red blood cells to vascular endothelium as a crucial factor in vaso-occlusive crisis, the pathogenic mechanisms involved in this phenomenon have remained controversial [1, 2, 4].

Several studies have demonstrated that vaso-occlusive crisis is a multi-factorial process involving hematological, immunological and thrombotic disturbances [14, 28]. In this context, endothelial abnormalities, enhanced adherence of sickle red cells to vascular endothelium, plasma proteins and cytokines, and leukocyte activation, especially neutrophils, play important roles in the vaso-occlusion and in the pathophysiology of SCD [29].

Interaction between HbSS erythrocytes and vascular endothelium is enhanced by further activation of endothelial cells through inflammatory cytokines released from activated endothelial cells (IL-1, IL-6, IL-8 and TNF- α), activated platelets (IL-1 and TNF- α) and from monocytes-macrophages (IL-1, IL-6 and TNF- α) [8, 10]. Therefore, a study of the effects of cytokines on vascular endothelium and adhesion molecule expression, and their presumptive role in clinical outcomes in SCD patients is an interesting and relevant area of research for this disease [14, 28].

Evaluation of serum cytokines in all SCD patients in the present study, either in a steady-state condition or in vaso-occlusive crisis revealed an increased level for all inflammatory cytokines apart from IL-1 β , when compared to normal controls (*table 3*). Likewise, significant differences were found between vaso-occlusive crisis patients and controls for all factors except IL-1 β , and between steady-state patients and control for TGF- β and IL-17 (*table 3*). In a similar study by Taylor *et al.* [30], no significant, detectable levels of IL-1 were found in the serum of patients in the steady-state condition or normal controls.

Increased levels of IL-6 were observed in both groups of patients *versus* controls although this was statistically significant only between vaso-occlusive crisis patients and healthy controls ($P = 0.04$, *table 3*), whereas two previous studies showed this difference between steady-state patients and normal controls [31, 32]. More importantly, patients in vaso-occlusive crisis showed increased concentrations of all factors compared with steady-state patients, although only the differences in IL-8 levels were marginally significant between the two groups ($P = 0.08$, *table 3*).

High levels of IL-8, a chemotactic factor for neutrophils, during painful crisis in SCD have been reported independently of the crisis-inducing factors [20]. As expected, we observed higher levels of IL-8 in vaso-occlusive crisis patients compared to steady-state patients and healthy controls ($P = 0.08$, $P = 0.01$ respectively). This finding is supported by Goncalves *et al.* [16] who showed a significantly elevated concentration of IL-8 in crisis *versus*

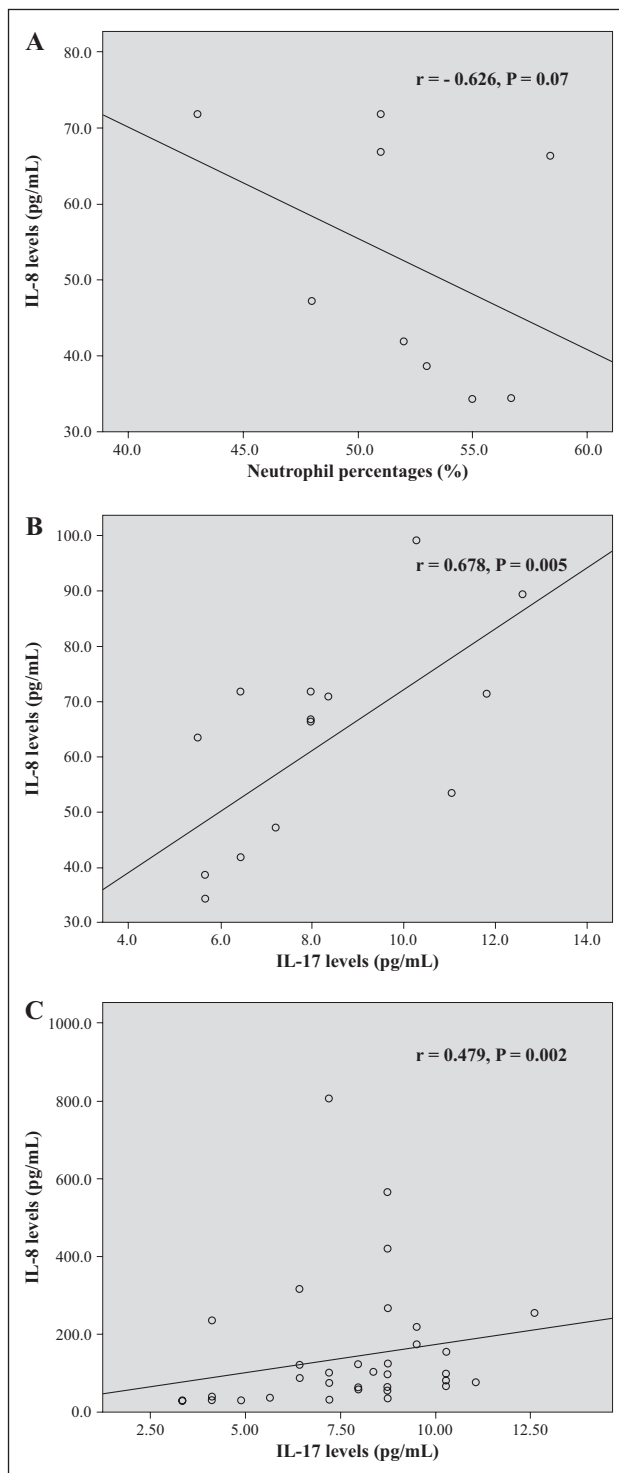


Figure 1

Spearman correlation analysis for IL-8 and IL-17 levels (pg/ml) and neutrophil percentages. Negative correlation between neutrophil (%) and IL-8 concentration (pg/ml) in steady-state patients (A) and significantly positive correlation between IL-8 and IL-17 levels in steady-state (B) and VOC patients (C).

non-crisis sickle cell patients. However, another study by Michaels *et al.* [34] demonstrated no differences between crisis and non-crisis patients.

Various investigations have shown that neutrophils are activated in sickle cell patients, particularly during a vaso-occlusive crisis, and that this event is mainly mediated by the potent neutrophil activator IL-8 [19, 35] and to a certain extent by IL-17 as a pro-inflammatory cytokine [14, 21, 22]. As expected, significantly increased cir-

culating levels of both cytokines were observed in our patients *versus* controls and remarkably in vaso-occlusive crisis patients compared to those in the steady-state condition. IL-17 is an essential, proinflammatory, T cell-derived cytokine with several biological actions such as induction of IL-1 and TNF- α production, and recruitment of neutrophils during inflammatory responses [14].

Generally, differentiation of naïve T cells to Th17, the main source of IL-17 production, is induced in the presence of TGF- β plus IL-6, and this process is also enhanced by IL-21 and IL-1 β [21]. Our findings demonstrate increased serum levels of TGF- β , IL-6 and IL-17 in vaso-occlusive crisis patients *versus* normal controls, which is in agreement with previous studies [9, 18, 36].

TGF- β is a member of the cytokine family whose main effects are on differentiation and inhibition of cell growth. It is believed that the balance between TGF- β as a negative regulator and SCF, IL-3, and GM-CSF as positive regulators plays a major role in the control of hematopoiesis in SS disease [36]. It has been shown that high levels of TGF- β in SS patients with high levels of Hb F are associated with lower levels of SCF. This suggests that TGF- β may inhibit SCF production and/or repress hemopoietic progenitor expression of c-kit [36]. As we did not quantify the concentrations of the positive regulators mentioned, our data should be interpreted cautiously in relation to other studies.

Observation of a reduced neutrophil count in our steady-state patients *versus* those in vaso-occlusive crisis is in line with previous data highlighting the correlation between low neutrophil counts and improved clinical manifestation [37]. As anticipated, we found an inverse correlation between IL-8 levels and neutrophil percentages ($P = 0.07$, figure 1A) among the steady-state patients. Interestingly, a significantly positive correlation was observed between IL-8 and IL-17 in both groups of patients (figures 1B-C) that could be indicative of an ongoing inflammatory process in stable patients as well as vaso-occlusive crisis patients. Consistently, previous studies have demonstrated neutrophil activation, especially during vaso-occlusive crisis, and increased serum levels of IL-8 in SCD patients [19, 35].

With regard to the positive correlation between IL-8 and IL-17 concentrations in both groups of patients, and the suggested roles of these contributors to the pathogenesis of vaso-occlusive crisis, IL-8 and IL-17 could be useful markers not only in vaso-occlusive crisis patients, but also in steady-state patients [14, 20, 33, 34]. Thus, it might be worth observing the non-significant differences for IL-8 and IL-17 levels between both groups of patients, despite the higher levels of IL-8 and IL-17 in vaso-occlusive crisis patients (table 3). However, the increased levels of IL-8 in vaso-occlusive crisis patients *versus* normal controls and steady-state patients are more prominent than changes in IL-17 content. Another explanation for these non-statistically significant differences might be the low number of samples in our study.

In agreement with Pathare *et al.* [9], we found higher concentrations of TNF- α and IL-6 in vaso-occlusive crisis patients *versus* steady-state patients, although this was not statistically significant. Increased levels of IL-8 were observed in our vaso-occlusive crisis patients compared to steady-state patients, whereas the study mentioned above

Table 4
Comparison of cytokine levels in all patients grouped by hydroxyurea (Hu) treatment and in healthy controls.

Cytokines (pg/ml)	Patients on Hu (group a, n = 39)	Patients not on Hu (group b, n = 15)	Controls (group c, n = 19)	P Values ^a		
				a vs. B	a vs. c	b vs. c
IL-1 β	3.03 \pm 0.27	3.33 \pm 0.19	3.33 \pm 0.19	0.61	0.51	0.34
TNF- α	13.05 \pm 2.26	17.41 \pm 6.29	12.17 \pm 0.55	0.60	0.14	0.02
TGF- β	14.81 \pm 4.46	9.74 \pm 1.92	1.04 \pm 0.30	0.18	<0.0001	0.0004
IL-6	14.46 \pm 4.37	11.23 \pm 2.19	5.03 \pm 0.69	0.43	0.04	0.15
IL-8	144.6 \pm 53.02	143.7 \pm 41.11	55.74 \pm 6.15	0.38	0.28	0.01
IL-17	7.30 \pm 0.65	7.85 \pm 0.37	4.67 \pm 0.21	0.34	0.001	<0.0001

All data are presented as mean \pm SE. a: two tailed P values by Mann-Witney U test.

Table 5
Comparison of cytokine levels between HbSS sickle cell anemia (SCA) and sickle β -thalassemia (S β Thal) patients and healthy controls.

Cytokines (pg/ml)	HbSS-SCA patients (group a, n = 37)	S β Thal patients (group b, n = 17)	Controls (group c, n = 19)	P Values ^a		
				a vs. B	a vs. c	b vs. c
IL-1 β	3.38 \pm 0.21	2.95 \pm 0.20	3.33 \pm 0.19	0.29	0.52	0.20
TNF- α	17.83 \pm 6.63	12.65 \pm 2.01	12.17 \pm 0.55	0.96	0.03	0.05
TGF- β	10.92 \pm 2.33	11.65 \pm 3.12	1.04 \pm 0.30	0.68	<0.0001	0.001
IL-6	12.3 \pm 2.65	11.74 \pm 2.63	5.03 \pm 0.69	0.68	0.14	0.05
IL-8	142.4 \pm 45.0	147.3 \pm 38.21	55.74 \pm 6.15	0.75	0.01	0.11
IL-17	7.76 \pm 0.38	7.56 \pm 0.60	4.67 \pm 0.21	0.72	<0.0001	0.0003

All data are presented as mean \pm SE. a: two tailed P values by Mann-Witney U test.

reported increased levels of IL-6 in crisis patients. In addition, our results demonstrated significantly increased levels of all cytokines, apart from IL-1 β , in vaso-occlusive crisis patients compared to normal controls, although Pathare *et al.* showed this difference only for IL-8 and IL-6. The steady-state patients in our study showed increased levels of TGF- β and IL-17 *versus* controls, which is also discordant with Pathare's study that reported increased concentrations of IL-6, IL-1 β and IFN- γ in steady-state patients.

Increased levels of proinflammatory cytokines such as TNF- α , IL-6, IL-8 and IL-17 in vaso-occlusive crisis patients compared to the controls and steady-state patients could be indicative of a chronic inflammatory response and further production of these cytokines from activated endothelial cells, platelets and accumulated monocytes/macrophages in the vaso-occlusion area [8-10]. These inflammatory mediators enhance red blood cell adhesiveness to endothelium and form a vicious cycle leading to more dense aggregations of sickle erythrocytes, platelets and neutrophils, and eventually to clinical vaso-occlusion [10].

With respect to the cytokine profile alterations during anti-infection responses, this confounding factor should be considered for interpretation of the results. In this case, we had only five patients (in the vaso-occlusive crisis group) with mild bacterial or viral infection, which is unlikely to have contributed to the cytokine alterations in all vaso-occlusive crisis patients.

Lanaro *et al.* reported a significantly decreased plasma level of TNF- α and elevated levels of IL-10 in patients

on hydroxyurea therapy [38]. This study also showed significant alterations in gene expression levels for TNF- α and IL-8 between patients receiving and not hydroxyurea therapy. In line with the above study, we observed slightly, but not significantly, lower concentrations for TNF- α and IL-1 and IL-17, and a higher content of TGF- β , IL-6 and IL-8 in hydroxyurea -receiving patients compared to those not receiving hydroxyurea treatment. However, the beneficial effects of hydroxyurea in SCD is mediated partially by increased Hb F concentrations, which, in turn, inhibits polymerization of HbS in red cells and is associated with a less severe form of the disease [36, 39]. Accordingly, demonstration of higher levels of TGF- β in hydroxyurea-treated patients in the present study could be supported somewhat by Croizat *et al.* who reported a direct correlation between HbF concentration and TGF- β levels in relation to erythropoietic stress in SCD patients [36].

Conversely, Tavakoli *et al.* [39] reported the beneficial effects of hydroxyurea-induced increases in TNF- α levels in SCD patients. Although the previous findings support a role for hydroxyurea in the reduction of vaso-occlusive episodes, further studies are needed to clarify the exact mechanism of action of hydroxyurea in relation to adhesion molecules and other inflammatory mediators such as cytokines in SCD patients [17, 27, 40]. Moreover, as many of SCD patients in crisis are on hydroxyurea treatment, any comparison with non-hydroxyurea patients regarding inflammatory mediators should be viewed cautiously.

Comparison of cytokine levels between HbSS and S β -Thal patients depicted increased but nonsignificant

concentrations of TGF- β and IL-8 in the S β -Thal group and significantly increased levels of TGF- β , TNF- α , IL-6 and IL-17 in this group of patients *versus* controls (table 5). It has been reported that both diseases manifest increased levels of inflammatory mediators, as well as activation of platelets, monocytes, endothelial cells and granulocytes that are the main sources of cytokine production [11].

In conclusion, our results support the view that the chronic inflammatory response is an ongoing process, not only during crisis, but also during steady-state conditions in SCD patients. Evaluation of proinflammatory cytokines, especially IL-8 and IL-17 as important, contributing factors to this process could show them to be useful markers for assessing disease severity and, consequently, therapeutic intervention. However, our findings presented here should be interpreted cautiously as there are a wide variety of major contributors to the pathogenesis of sickle cell disease such as adhesion molecules, other plasma proteins, leukocyte activation status, endothelial injury, and other unknown factors that needed to be investigated further for a better understanding of the pathophysiology of this disease.

AUTHORS' CONTRIBUTION

GS, ARM and BK conceived the project, designed and performed experiments, analyzed and interpreted data and wrote the manuscript. RN, MA, SG and FS performed experiments.

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