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Correlation Between the Frequency of Painful Crises in Sickle Cell Anemia and the Biomarkers of Inflammation, Endothelial Dysfunction, Coagulation Activation and Bone Metabolism

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ABSTRACT

Objective: Sickle cell anemia is a widespread inherited hemolytic anemia, characterized by chronic hemolysis, infections, recurrent occlusion of the microcirculation and painful crises. In this cross-sectional study we investigated the correlation between biomarkers of inflammation, endothelial dysfunction, coagulation activation and bone metabolism, with the frequency of painful crisis in sickle cell anemia adult patients, to understand the potential role of theses biomarkers in the management of sickle cell anemia.

Methods: Sixty-three sickle cell anemia patients were enrolled in this study. They were divided into three groups based on the frequency of pain crises to mildly, moderately, and severely affected. Twenty-five healthy volunteers were enrolled as controls. Plasma levels of different biomarkers were tested, including tumor necrosis factor alpha, soluble intercellular adhesion molecule-1, D-dimer, sclerostin, and nitric oxide.

Results: Tumor necrosis factor alpha, soluble intercellular adhesion molecule-1, and D-Dimer plasma levels were significantly higher in sickle cell anemia patients when compared to the controls, while nitric oxide levels were notably lower in the patients group. Sickle cell anemia patients with severe pain showed higher tumor necrosis factor alpha, soluble intercellular adhesion molecule-1 and D-dimer levels than those mildly and moderately affected with painful crisis. Positive correlations were observed between all measured biomarkers and the frequency of the painful crisis, except for nitric oxide, which showed a negative correlation.

Conclusion: Tumor necrosis factor alpha, soluble intercellular adhesion molecule-1, nitric oxide, and D-dimer can be used to assess sickle cell anemia severity and prognosis, and thus may affect determining appropriate management strategies.

Keywords

Sickle cell anemia, Inflammation, Biomarkers, Adhesion molecules; Endothelial dysfunction.

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INTRODUCTION

Sickle cell anemia (SCA) is an autosomal inherited condition, characterized by chronic haemolysis, inflammation, and vascular occlusions. The latter results in recurrent vaso-occlusive crises (VOC), chronic organ damage and ultimately organ failure[1,2]. In fact, in SCA patients, inflammation plays a major role in the initiation of a painful VOC[3]. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine, involved in cell growth and differentiation, as well as the activation of endothelial cells and leukocytes[4]. Elevated levels of TNF-α is often associated with an inflammatory state in SCA patients[5], indicating its significance in VOC[6,7].

Moreover, the abnormal cellular adhesion to the endothelial layer of blood vessels is another initiating factor of VOC in SCA patients[8]. Intercellular adhesion molecule-1 (ICAM-1) is an endothelial protein expressed in response to the abnormal adhesion in SCA patients[9,10]. An increased level of plasma soluble ICAM-1 (sICAM-1) was reported before in SCA patients, reflecting the continuous endothelial activation occurring in a VOC[11]. A central aspect of SCA vasculopathy is the impairment of the endothelial regulation of vasomotor tone, thrombosis, and inflammation[12]. In SCA, hemolysis contributes to dysregulation of nitric oxide (NO), which is considered a potent vasodilator[13]. Depletion of NO bioavailability results in endothelial dysfunction and subsequently VOC[13,14].

In addition to the above, the hypercoagulable state of a SCA patient can contribute to VOC events in the microcirculation, leading to acute and chronic organ damage[15]. D-dimer is a small protein fragment found in blood after a blood clot, and is degraded by fibrinolysis[16]. An elevated level of D-dimer has been observed in patients with SCA, in both crisis and crisis-free states[17]. Lastly, bone involvement is one of the most common clinical manifestations of SCA, namely chronic, progressive processes such as osteoporosis, osteopenia and avascular necrosis. [18] Sclerostin is an important and known biomarker for several diseases associated with similar bone disorders[19].

This study aims to investigate the correlation between the different biomarkers of inflammation, endothelial dysfunction, coagulation activation, and bone metabolism, with frequency of painful VOC in adult SCA patients, and to assess the role of theses biomarkers in the prognosis and management of SCA.

MATERIAL AND SUBJECTS

The participating subjects were recruited from patients attending the hematology clinic at King Abdulaziz University Hospital (KAUH), Jeddah, Saudi Arabia. The diagnosis of SCA was confirmed by alkaline electrophoresis of hemoglobin[20]. Ethical approval was granted from the Ethical and Technical Committee at KAUH, and written informed consents were obtained from all study participants.

During the study period of six months, a small number of patients fulfilled the study criteria, and agreed to participate in the study. Subsequently, a total of 63 homozygous SCA patients were included, and divided into three equal groups of 21 participants based on the frequency of painful VOC; 1. Mildly affected (patients who experienced ≤ 5 painful VOC/year), 2. Moderately affected (patients who reported 6-9 painful VOC/year), and 3. Severely affected (patients who had ≥ 10 painful VOC/year). We recruited 25 healthy controls from the same age group, and their HbAA status was confirmed, by performing sickling test in KAUH laboratories.

For all subjects, 10 ml of venous blood was collected into a vacutainer tube with EDTA anticoagulant. Blood samples were centrifuged at 5000 g for 15 minutes, and plasma samples were transferred into coded tubes, and then frozen at -80°C until assayed for the different biomarkers.

Levels of TNF-α, sICAM-1, D-dimer, and sclerostin biomarkers were estimated using ELISA kits. Enzyme-Linked Immunoassay (ELISA) Kit for TNF-α, was obtained from Life Technologies Corporation (USA), and ELISA Kit for sICAM-1, D-dimer, and Sclerostin were obtained from Thermo Fisher Scientific (USA). The principle of ELISA assay depended on a highly specific antibody-antigen interaction, in which an antibody specific for an antigen in the plasma sample was pre-coated onto a microplate. Plasma samples were added to the appropriate wells, and after washing away any unbound substances, a biotinylated monoclonal antibody specific for an antigen in the plasma sample was added. After removal of the excess second antibody, streptavidin-peroxidase enzyme was added to the wells to bind to the biotinylated antibody. All the unbound enzymes were then removed by washing. Detection was accomplished by assessing the conjugated enzyme activity *via* incubation with a substrate to produce a measureable colored product. The absorbance of the obtained product was read using a microplate reader (Spectro Reader State Fax-2100, Awareness Technology Inc., Palm City, FL USA) set at 450 nm.

For NO, blood levels were determined using a Chemiluminescence Assay Kit from the Research and Diagnostic Systems (USA). The principle of this method depends on spectrophotometric measurement of the stable decomposition products of NO, nitrate (NO_3^-) and nitrite (NO_2^-). This method requires that NO_3^- first be reduced to NO_2^- and then NO is determined by the Griess reaction. The absorbance of the formed chromophoric azo-derivative was read at 540 nm, using a microplate reader.

Statistical Analysis

Statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 20 (IBM Corp., Armonk, NY USA). Continuous variables were presented as mean values and standard deviations, and the categorical variables as counts and percentages. Chi-square test was used to compare gender distribution between groups. Independent *t*-test was used to compare means between groups. Differences between means of more than two groups were analyzed using One Way (ANOVA) for multiple comparisons. Correlations were tested using Pearson correlation test. A P-value (< 0.05) was considered statistically significant.

RESULTS

General Characteristic of the Study Participants

For the overall patients' group, the mean age of all included SCA patients was (25.83 ± 5.49) years, and males constituted (47.6%). On the other hand, the mean age of the control group was (25.84 ± 4.32) years, and males constituted (52.0 %). The general characteristic of the SCA patients and the healthy control groups are shown in (Table 1). Gender distribution and age were both comparable for SCA patients and controls (P = 0.915, and P = 0.990, respectively). Patients in the mildly, moderately and severely affected subgroup, reported a mean painful VOC/year of (2.9 ± 1.1, 7.6 ± 0.97, 24.5 ± 9.02), respectively.

Comparison of Biomarkers Concentrations between SCA Patients and the Healthy Controls.

The mean plasma TNF-α, sICAM and D- Dimer levels were significantly higher for all patients' groups, when compared with controls (P < 0.001 for all). Conversely, NO levels were significantly lower for the patients versus the controls (P < 0.001). There was no significant difference in the plasma levels of sclerostin between patients and controls (Table 2).

Comparison of Biomarkers Concentrations across the Four Studied Groups

Data from Table 2 show significant differences in TNF-α levels in the mildly, moderately, and severely affected SCA patients (P = 0.041, P = 0.005, and P < 0.001, respectively), when each was compared with the control subjects. In fact, patients with severe pain showed a significant increase in TNF-α levels in comparison to the mildly and moderately affected patients (P < 0.001 and P < 0.001, respectively). Statistical difference was also observed between mildly and moderately affected patients (P = 0.020).

The plasma level of sICAM-1 followed the same trend as TNF- α, where a significant increase in both moderately and severely affected patients was detected when compared to controls (P = 0.002 and P < 0.001). Furthermore, severely affected patients showed a distinct increase in sICAM-1 levels when compared to patients with mild or moderate pain (P < 0.001). A significant difference was also observed between mildly and moderately affected patients (P = 0.009).

On the other hand, NO plasma levels were significantly lower in all three patients' subgroups, the mildly, moderately, and severely affected patients, in comparison to the controls (P = 0.024, P = 0.005 and P < 0.001, respectively), with a further distinct decrease in patients with severe VOC rates when compared to the mildly and moderately affected patients (P < 0.001 and P = 0.007). A significant difference was also observed between the latter two groups (P = 0.030).

ANOVA analysis showed a significant increase in the D-dimer levels in both moderately and severely affected SCA patients (P = 0.002 and P < 0.001) in comparison to the control group. A greater increase was noted in patients with severe pain, when compared to the mildly and moderately affected patients (P < 0.001 and P = 0.008). There was no significant difference in the plasma levels of sclerostin between the different subgroups of SCA patients and the control group.

Correlation Studies

A significant positive correlation was detected between TNF-α with sICAM-1 and D-dimer levels [(r = 0.327, P = 0.009), (r = 0.278, P = 0.027) respectively], and while sICAM-1 levels showed a similar significant positive correlation with the D-dimer concentrations (r = 0.258, P = 0.041), the NO levels negatively correlated with both TNF-α and sICAM levels [(r = -0.449, P < 0.001) and (r = -0.309, P = 0.014), respectively]. No correlations were found between sclerostin and the levels of TNF-α, sICAM-1, D- Dimer and NO. Furthermore, there was significant positive correlations between the frequency of

TABLE 1.
General characteristic of the study participants.

	Control (n=25)	Patients (n=63)	P-value
Age (Years)	25.84 ± 4.32	25.83 ± 5.49	0.990
Gender			
Male	13 (48.00%)	30 (47.62%)	0.915
Female	12 (52.00%)	33 (52.38%)	
Painful Crises/Year	No	11.69 ± 4.72	_

Data are expressed as mean ± Standard Deviation or number (%)

TABLE 2.
Comparison of biomarkers concentrations between sickle cell anemia patients and normal control subjects.

		ANOVA-One Way				Multiple Comparison - LSD			
Variables	**Groups**	**N**	**Mean**	**± SD**	P-Value	Control	Mild	Moderate	Severe
TNF-α	Control	25	14.45	2.59	<0.001	-	0.041*	0.005*	<0.001*
	Mild	21	17.91	3.45			-	0.020*	<0.001*
	Moderate	21	23.03	5.59				-	<0.001*
	Severe	21	29.98	7.04					-
sICAM-1	Control	25	162.23	49.27	<0.001	-	0.102	0.002*	<0.001*
	Mild	21	190.41	62.19			-	0.009*	<0.001*
	Moderate	21	243.41	79.68				-	<0.005*
	Severe	21	350.20	91.09					-
Nitric Oxide	Control	25	27.12	2.83	<0.001	-	0.024*	0.005*	<0.001*
	Mild	21	23.90	3.66			-	0.030*	<0.001*
	Moderate	21	21.01	3.31				-	0.007*
	Severe	21	16.94	3.29					-
D-Dimer	Control	25	369.39	43.92	<0.001	-	0.120	0.002*	<0.001*
	Mild	21	392.64	44.84			-	0.003*	<0.001*
	Moderate	21	485.34	75.85				-	0.008*
	Severe	21	616.12	94.59					-
Sclerostin	Control	25	281.61	46.09	0.691	-	0.685	0.553	0.235
	Mild	21	287.93	40.13			-	0.856	0.452
	Moderate	21	290.88	61.89				-	0.567
	Severe	21	300.19	60.02					-

Data are expressed as mean ± Standard Deviation.
** Significant value (p<0.05).*
TNF-α: Tumor necrosis factor alpha; sICAM-1: Soluble Intercellular Adhesion Molecule-1

painful VOC and the plasma levels of TNF-α (r = 0.605, $P < 0.001$), sICAM-1 (r = 0.540, $P < 0.001$), and D-dimer (r = 0.530, $P < 0.001$). A significant negative correlation between VOC frequency and NO plasma levels (r = -0.529, $P < 0.001$) was also found (Fig. 1, 2, 3, 4). No correlation between sclerostin levels and frequency of painful crises in SCA patients was detected.

DISCUSSION

Data from this study revealed an up regulation of inflammatory, endothelial dysfunction and coagulation activation biomarkers in SCA patients when compared to the controls. Significantly elevated levels of TNF-α was observed in SCA patients when compared with the normal control subjects, and across the patients' subgroups, a significant increase of TNF-α was observed in the severely affected patients. This was again evidenced by the strong correlation between increased TNF-α secretion and the frequency of VOC in these patients. Our findings were in accordance with another study that showed elevated levels of TNF-α in SCA patients.[6] Studies reported a significant increase in TNF-α levels in SCA patients at steady state as well as in painful crises, when compared to healthy controls[6, 21]. Furthermore, Keikhaei *et al*[22]. (2013) reported a significant increase in TNF-α levels in crises state namely when compared to the steady state[22].

This could be due to subclinical microvascular occlusions, initiated by the increased adhesiveness of sickle red blood cells (RBCs) to the vascular endothelium, leading to chronic activation of endothelium, and consequently stimulation of cytokines' production such as TNF-α[21]. Moreover, it could be associated with the numerous infections, which are important risk factors in the pathogenesis of VOC in patients with SCA[23]. The

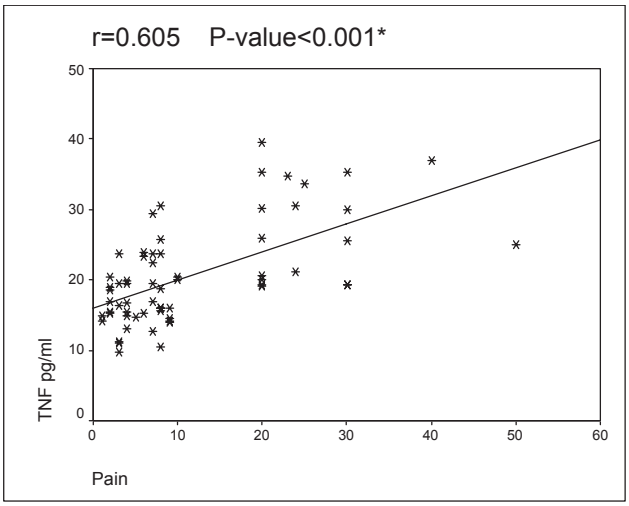

FIGURE 1.
Linear positive correlation between pain frequency and plasma level of tumor necrosis factor alpha.

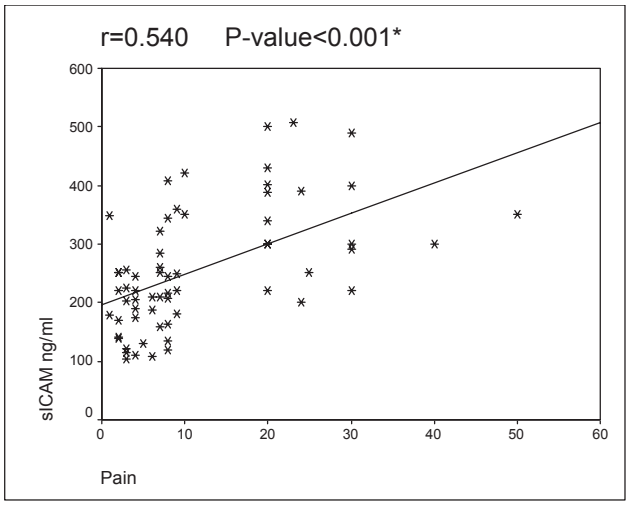

FIGURE 2.
Linear positive correlation between pain frequency and plasma level of soluble intercellular adhesion molecule-1.

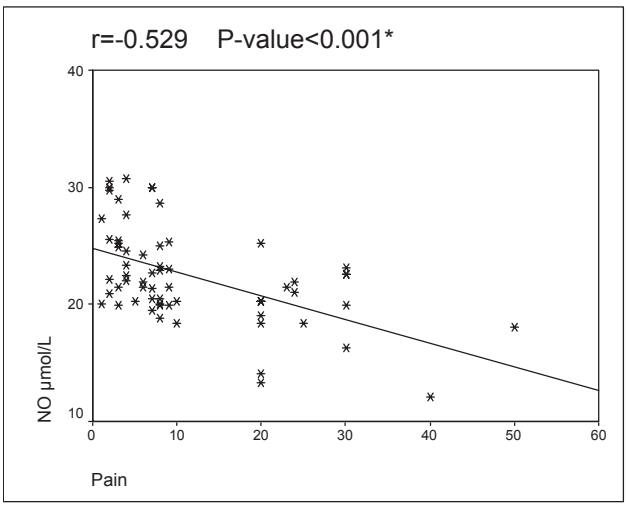

FIGURE 3.
Linear negative correlation between pain frequency and plasma level of nitric oxide.

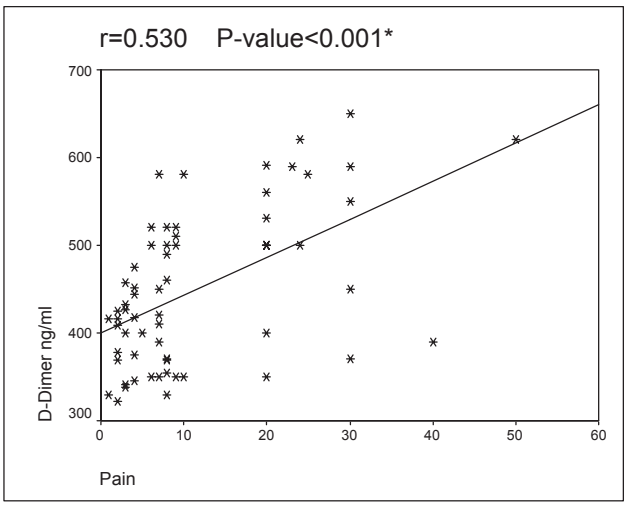

FIGURE 4.
Linear positive correlation between pain frequency and plasma level of D-Dimer.

observed increase in TNF- α levels in severely affected patients confers an increased propensity to chronic inflammation and its complication, such as the promotion of prothrombotic state as seen from the elevated levels of D-dimer in the present study, as well as endothelial dysfunction.

Soluble ICAM-1 represents a circulating form of ICAM-1 that is usually expressed to a lesser degree on the luminal surface of the vascular endothelial cells but is up regulated on account of the inflammatory stimulus from TNF-α. Subsequent release of soluble adhesion molecules into blood plasma may serve as markers of endothelial dysfunction[24]. This study revealed that the plasma levels of sICAM-1 were significantly increased in SCA patients when compared to the controls. This finding confirmed previous reports of increased sICAM-1 levels in SCA patients in comparison to normal individuals, and its positive correlation with the frequency of painful crises[24,25]. Furthermore, it is worthy to note that significant association was observed between sICAM-1 and the patients with severe VOC attacks. Therefore, the observed increases in sICAM-1 levels in patients probably reflect an increased expression of endothelial cell adhesion molecules, as well as an increased capacity for the adhesion of Sickle RBCs and leukocytes to the endothelium, reflecting an endothelial function impairment. Hence, sICAM-1 can reflect disease severity and frequency of painful crises. We may even speculate that targeted therapies that inhibit abnormal adhesions can provide a control state, and ultimately reduce the frequency and severity of pain[26].

Nitric oxide acts as a potent vasodilator of smooth muscle cells[27], inhibiting platelet activation, down-regulating the expression of endothelial adhesion molecules and reducing reactive oxygen species levels[13]. The results from this study showed that plasma levels of NO are markedly decreased in SCA patients when compared to healthy controls, with more decrease in the severely affected patients. This may indicate endothelial dysfunction and hemolysis in these patients. The deficiency of NO bioavailability is mainly due to intravascular hemolysis, which leads to a high consumption of NO by the cell-free hemoglobin released from lysed RBCs. This latter deficiency is associated with endothelial dysfunction, and results in pathological activation of endothelial cells and vascular instability.[28] Our findings were in agreement with previous studies which reported a decrease in NO bioavailability in both patients, and mice with SCA[24,29]. Since NO suppresses the expression of ICAM-1, VCAM-1 and E-selectin, the elevated levels of their soluble forms were suggested to indicate impaired NO availability [30]. This hypothesis is supported by another study finding, where we detected significant negative correlation between the levels of sICAM-1 and NO in SCA patients. On the other hand, an overproduction of reactive oxygen species seen in SCA such as superoxide, encourages intravascular oxidant stress and in turn may perturb NO homeostasis, contributing to vascular dysfunction and increasing the frequency of painful crises[31].

The present study revealed a significant increase in the level of coagulation activation marker D-dimer, in patients with SCA when compared to normal control subjects. This confirms the activation of coagulation and fibrinolytic systems SCA patients. Our findings were consistent with previous studies, suggesting an increased risk of thrombosis in SCA patients[4,32]. In fact, one study reported a significant increase in D-dimer levels in SCA patients during a VOC when compared to the steady state, accompanied with an increase in thrombin generation [33]. A possible explanation of this increase in the D-dimer levels is that in SCA biochemical peculiarity of polymerization of sickled haemoglobin under deoxygenation leads to generation of distorted red cells, and these damaged cells show a loss of phospholipid asymmetry with externalization of procoagulant phosphatidyl serine (PS). Phosphatidyl serine on red cell membrane is believed to play a role in facilitating the assembly of coagulation factors on the erythrocytic membrane, which in turn contributes to thrombin generation and coagulation activation[34]. Therefore, the generation of thrombin is coupled with an increase in fibrinolytic activity, leading to elevation of D-dimer levels in both the acute VOC crises as well as in the steady state.

In SCA, bone pains occur acutely during vaso-occlusion and osteomyelitis, and more chronically as a vascular necrosis of the hips and shoulders[35]. The wingless-type/beta catenin (Wnt/β-catenin) signaling pathway plays an important role in both bone architecture remodeling and healing[36]. The activation of this pathway raises the number of osteoprogenitor cells, and at the same time decreases osteoblast apoptosis, resulting in an anabolic effect on bone tissue. Sclerostin is a recently identified osteocyte-derived bone morphogenetic protein, that is reported to have a regulatory effect upon Wnt/β-catenin and modulates bone mass, through inhibition of Wnt signaling, and the repressing of osteoblasts proliferation[37]. This protein is proposed to serve as a potential biomarker for diseases associated with bone disorders[38], such as osteoporosis-related fracture risk[19]. Numerous studies demonstrated that increased sclerostin levels were found to be associated with low bone mineral density,[19,39] a common feature in SCA[35].

To our knowledge, there are no reports in the literature outlining the changes of sclerostin in SCA in relation to severity and the frequency of pain episodes. Our present study revealed an intriguing finding, as no significant differences were detected in sclerostin concentrations between patients and controls. Moreover, we observed no differences between the patients' subgroups with the different pain severity, indicating that sclerostin levels do not change in relation to disease severity. Therefore, further studies should be conducted to investigate other bone metabolism biomarkers that could be used in assessing SCA severity.

This study represents an advanced understanding of SCA pathogenesis, which may contribute to better

targeted management in SCA. Principally, and based on the presented biomarkers' profile, the clinical utilization of this work may enable better understanding and innovative use of monoclonal and targeted therapy in treatment of SCA, as well as risk assessment and prognostication. Some limitations to these findings are noteworthy, namely the small sample size for all groups, associated with difficulties in selection and recruitment. Thus, this study is considered a pilot study, with no firm conclusions, and further research is needed to enable generalizable inferences.

CONCLUSION

A positive correlation was detected between elevated plasma levels of TNF-α, sICAM-1 and D-dimer, and a higher incidence of painful VOC in SCA patients. Conversely, decreased NO concentrations were associated with an increased risk of painful crises. Therefore, TNF-α, sICAM-1, NO, and D-dimer could be considered valuable biomarkers in determining prognosis and the management of patients with SCA.

Conflict of Interest

The authors have no conflict of interest.

Disclosure

None of the authors received any type of commercial support either in forms of compensation or financial for this study. They have no financial interest in any of the products or devices, or drugs mentioned in this article.

Ethical Approval

Obtained.

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الارتباط بين تواتر الأزمات المؤلمة في مرض فقر الدم المنجلي والمؤشرات الحيوية للالتهاب، الخلل الوظيفي في الخلايا البطانيه، تنشيط التخثر، واستقلاب العظام

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المستخلص.

الأهداف: الأنيميا المنجليه هو فقر دم انحلالي وراثي واسع الانتشار، يتميز هذا المرض بانحلال الدم المزمن، الالتهابات، العدوى، الانسداد المتكرر لدوران الدم في الأوعية الدقيقة، والأزمات المؤلمة، تهدف هذه الدراسة إلى التعرف على الارتباط بين المؤشرات الحيوية للإلتهاب، الاختلال الوظيفي للخلايا البطانية، تنشيط التخثر، واستقلاب العظام مع تواتر الأزمات المؤلمة لدى مرضى الأنيميا المنجلية عند البالغين.

طريقة العمل: تم تسجيل ثلاثة وستين مريضاً مصاباً بالأنيميا المنجلية في هذه الدراسة، وتم تقسيمهم إلى ثلاث مجموعات (٢١ مريضاً في كل مجموعة) بناءا على مدى تكرار الأزمات المؤلمة (من شديد إلى متوسط وخفيف) وتم تسجيل خمسة وعشرين متطوعاً صحيحا خاليا من الأمراض، واستخدمت طريقة التحليل المناعي المرتبط بالإنزيم لتعيين مستويات كلاً من: عامل نخر الورم ألفا، جزيء الالتصاق الذائب بين الخلايا، دي دايمر، والسكليروستين، وتم تعيين مستوى أكسيد النيتريك باستخدام تقنية اللمعان الكيميائي.

النتائج: كانت معدلات البلازما الخاصة بعامل نخر الورم ألفا، جزيء الالتصاق الذائب بين الخلايا، ودي دايمرمرتفعة اعتداديا بينما كانت معدلات أكسيد النيتريك منخفضة لدى المرضى مقارنة بالمجموعة الضابطة السليمة، وقد لوحظ ارتفاع إعتدادي في معدلات كل من المؤشرات الحيوية السابق ذكرها في المرضى الذين يعانون من التكرار الحاد للأزمات المؤلمة، مقارنة بالمرضى الذين يعانون من تكرار خفيف أومتوسط، عدا أكسيد النيتريك الذي أظهر انخفاضا اعتداديا وقد ظهرت العلاقة الإيجابية المعنوية بين معدلات كل من عامل نخر الورم ألفا، جزيء الالتصاق الذائب، ودي دايمر وتكرار الألم في جميع مجموعات المرضى.

الاستنتاج: تشير هذه الدراسه لإمكانية استخدام كلاً من عامل نخر الورم ألفا، جزيء الالتصاق الذائب بين الخلايا، دي دايمر وحمض النيتريك كمؤشرات حيويه لقياس شدة المرض ومدى خطورته، وبالتالي تخطيط العلاج.