Interleukin-6 Gene Polymorphisms Influencing in hematological indices from sickle cell Anemia Patients

Polimorfismos Genéticos da Interleucina-6 influenciando nos índices hematológicos de doentes com Anemia Falciforme

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ABSTRACT

The homozygous hemoglobin SS is characterized Sickle Cell Anemia (SCA), altering the original structure of erythrocytes to a sickle shape. The hemoglobinopathies encompass all genetic diseases of hemoglobin and the SCA is the one that presents the greatest clinical manifestations variability and also the most severe ones, causing chronic hemolysis, vaso-occlusive crises and severe anemia in patients. The present study aimed to investigate the role of rs2069832, rs2069835, rs2069840, rs2069845 and rs2069849 polymorphisms in the Interleukin-6 gene in the hematological values of SCA patients treated at Fundação HEMOAM, Manaus, AM. The inclusion of patients was carried out through outpatient care at HEMOAM. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) and molecular analyzes by TaqMan® probes on Applied Biosystems QuantStudio 6 Flex Real-Time PCR System. A total of 277 SCA patients were included in this study, having the female gender having a minimally higher frequency (55.3%). The mean age at diagnosis was approximately three years old, with brown race being the most predominant (77.6%). The rs2069832_AA and rs2069845_AA genotypes showed high values for red blood cell, hemoglobin and hematocrit indices, by

having an important role as a protective factor for hemolysis in these patients. While the rs2069835_CC genotype showed decreased values for the same hematimetric indices, demonstrating to be a potential risk factor for increased hemolysis. No significant correlation in hematimetric indices was observed for the rs2069840 and rs2069849 genotypes. There are few studies correlating the genetic variants of the IL-6 gene in SCA in the state of Amazonas, however, it is known that IL-6 is involved in cell proliferation and greater response to inflammatory cytokines, and may modulate the clinical response in these patients, such as chronic hemolysis, vaso-occlusion and infections. Our work demonstrated associations of risk and protective IL-6 genotypes for possible hemolysis in patients with sickle cell anemia. We understand that an investigation with a larger number of patients would be recommended to elucidate the roles of the studied polymorphisms in sickle cell anemia. In addition, elucidating the role of Il-6 in sickle cell anemia may lead to the development of new strategies and therapies to prevent the systemic effects of excessive cytokine production and, consequently, reduce the severity of crises in these patients, providing better prognosis, clinical follow-up and welfare.

Keywords: sickle cell anemia, molecular markers, interleukin 6, Manaus, Amazonas.

RESUMO

A hemoglobina homozigotosa SS é caracterizada Anemia Falciforme (SCA), alterando a estrutura original dos eritrócitos para uma forma de foice. As hemoglobinopatias abrangem todas as doenças genéticas da hemoglobina e a SCA é a que apresenta a maior variabilidade de manifestações clínicas e também as mais graves, causando hemólise crónica, crises vaso-oclusivas e anemia grave nos doentes. O presente estudo teve como objectivo investigar o papel dos polimorfismos rs2069832, rs2069835, rs2069840, rs2069845 e rs2069849 no gene Interleucina-6 nos valores hematológicos dos doentes com SCA tratados na Fundação HEMOAM, Manaus, AM. A inclusão de doentes foi efectuada através de cuidados ambulatórios na HEMOAM. O ADN genómico foi extraído utilizando o QIAamp DNA Mini Kit (Qiagen) e análises moleculares por sondas TaqMan® em Sistema de PCR Aplicado QuantStudio 6 Flex Real-Time. Foi incluído neste estudo um total de 277 pacientes com SCA, tendo o sexo feminino uma frequência minimamente mais elevada (55,3%). A idade média no diagnóstico era de aproximadamente três anos, sendo a raça castanha a mais predominante (77,6%). Os genótipos rs2069832_AAA e rs2069845_AAA mostraram valores elevados para os índices de eritrócitos, hemoglobina e hematócrito, tendo um papel importante como factor de protecção da hemólise nestes doentes. Enquanto o genótipo rs2069835_CC mostrou valores diminuídos para os mesmos índices hematimétricos, demonstrando ser um factor de risco potencial para o aumento da hemólise. Não foi observada correlação significativa nos índices hematimétricos para os genótipos rs2069840 e rs2069849. Existem poucos estudos que correlacionem as variantes genéticas do gene IL-6 na SCA no estado do Amazonas, no entanto, sabe-se que a IL-6 está envolvida na proliferação celular e maior resposta a citocinas inflamatórias, e pode modular a resposta clínica nestes doentes, tais como hemólise crónica, vaso-oclusão e infecções. O nosso trabalho demonstrou associações de genótipos de risco e protectores da IL-6 para possíveis hemólises em doentes com anemia falciforme. Compreendemos que uma investigação com um maior número de doentes seria recomendada para elucidar os papéis dos polimorfismos estudados na anemia falciforme. Além disso, a elucidação do papel da Il-6 na anemia falciforme pode levar ao desenvolvimento de novas estratégias e terapias para prevenir os efeitos sistémicos da produção excessiva de citocinas e, consequentemente, reduzir a

gravidade das crises nestes doentes, proporcionando um melhor prognóstico, acompanhamento clínico e bem-estar.

Palavras-chave: anemia falciforme, marcadores moleculares, interleukin 6, Manaus, Amazonas.

1 INTRODUCTION

Hemoglobinopathies comprise genetic disorders by alteration of the hemoglobin molecule, classified as structural or synthesis.[1] While sickle cell anemia (SCA) presents hemoglobin S (HbS) in the homozygous form (HbSS), the term sickle cell disease (SCD) defines the hemoglobinopathies in which HbS, in heterozygosis, is associated with another variant hemoglobin, the most frequent being HbSC, HbSD and Sβ-thalassemia.[2]

The characterization of SCA is the A→T change in the sixth codon of the β-globin gene (located on chromosome 11), which leads to the replacement of the amino acid glutamic acid by valine in the at 6th codon of β-globin gene, modifying its molecular structure.[3.4]

While hemolytic anemia and vaso-occlusive events are found to varying degrees in all disease genotypes, some genotypes are more clinically severe than others.[5-7] This is largely due to variation in HbS concentration and the propensity for polymer formation, which is highly dependent on HbS concentration.[8-11]

HbS polymerization normally triggers two main pathophysiological processes: vaso-occlusion, with ischemia and reperfusion, and intense hemolytic anemia, which is the main characteristic of this disease, which stands out for its laboratory variability. Successive intracellular HbS polymerization alters the red blood cell membrane, especially resulting in increased adhesion to the endothelium, shortening of its survival in the circulation, damage to the microvasculature, inflammation and activation of coagulation, with perpetuation of hemolysis.[12,13]

Interleukin-6 is a multifunctional cytokine exhibiting pro and anti-inflammatory properties and with a strategic role in the body's defense. In addition to stimulating the proliferation and activation of plasma B and T cells, it has biological actions including a role in hematopoiesis. Even in a stable state, that is, without clinical manifestation, several studies have demonstrated elevated levels of many cytokines in patients with SCA.[14]

In SCA patients, pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) cause chronic endothelial activation and adhesion mainly

of sickled erythrocytes, directly related to constant tissue ischemia site and observed necrosis.[15] In addition to increased leukocyte aggregation in response to inflammatory cytokines.[16,17]

Several genetic studies have demonstrated its enormous importance in the modulation of the SCA phenotype, especially related to inflammation, vaso-occlusive crisis, oxidative stress and vascular homeostasis, with inflammatory interleukins being highlighted in the clinical severity.[18,19] VICARI et al., (2015) demonstrated that polymorphisms (SNPs) in the IL-1β and IL-6 genes are associated with complications of SCA and may act as genetic predictors of the clinical heterogeneity of SCA.[20]

Genetic variants of the IL-6 gene (rs2069840, rs2069845, rs2069849 and rs2069832) have already been associated with several diseases, including different types of cancer such as: cervix,[21] lung adenocarcinoma,[22,23] prostate,[24] breast cancer,[25] development of obesity[26] and implications in neuroinflammatory diseases[27] and leprosy.[28,29]

Currently, there are few studies related to the frequency and clinical correlation of these polymorphisms in patients with Sickle Cell Anemia patients at Fundação HEMOAM. The present work investigated the role of polymorphisms rs2069840, rs2069845, rs2069849, rs2069832 and rs2069835 in the Interleukin-6 gene in hematological and biochemical data of patients with Sickle Cell Anemia assisted at Fundação HEMOAM, Manaus, AM.

2 MATERIALS AND METHODS

2.1 STUDY DESIGN

The study was a cross-sectional observational model in SCA patients diagnosed with SCA, of both genders, regardless of age, race, ancestry, pre-existing diseases, drug prophylaxis and blood transfusions, treated at the Hospital Foundation for Hematology and Hemotherapy of Amazonas (HEMOAM), Manaus, Amazonas, Brazil. The SCA diagnosis was confirmed by the High Performance Liquid Chromatography (HPLC) technique and the real-time PCR (qPCR) technique, after agreeing to participate in the study, by signing the Free and Informed Consent Form (TCLE) or the Term of Assent.

Patients were included in the study during outpatient care provided periodically at HEMOAM, according to the National Policy Guidelines for Comprehensive Care for People with Sickle Cell Disease implemented by the Ministry of Health. After the inclusion of the patient, observing the eligibility criteria, an individual clinical form was

generated, consisting of personal information (identification, age, gender, family history) and medical information (hemoglobin genotype, hospitalizations, transfusions, clinical manifestations, surgeries) according to information collected during interviews with patients and through medical records.

2.2 DNA EXTRACTION AND LABORATORY ANALYSIS

For genomic DNA extraction and hematological determinations, 3 ml of venous blood in anticoagulant (Ethylenediaminetetraacetic acid disodium salt dihydrate - EDTA) and 5 ml of venous blood without additives were collected to obtain serum for biochemical analysis. All analyzes and results were entered into a database for statistical analyses. The hematimetric and biochemical data analyzed are described in Table 1.

Chart 1. Hematological and biochemical parameters analyzed in the study

Hematological Data	**Biochemical Data**
RBC (10^6/mm³)	Urea (mg/dL)
Hemoglobin (g/dl)	Creatinine (mg/dL)
Hematocrit (%)	DB (mg/dL)
MCV (fL)	IB (mg/dL)
MCH (pg)	TB (mg/dl)
MCHC (g/dl)	Glucose (mg/dL)
RDW (%)	Iron Serum (mcg/dL)
WBC (x10^9/L)	Ferritin (ng/mL)
Platelets (x10^9/L)	Transferrin (mg/dL)
MPV (fL)	TIBC (µg/dL)

Hematological: RBC: Red Blood Cell; MCV: Mean Cellular Volume; MCH; Mean Cellular Hemoglobin: MCHC: Mean Cellular Hemoglobin; RDW: Red cell distribution width; WBC: white blood count; VPM: Mean Platelet Volume.
Biochemical: DB: Bilirubin Direct; ID: Bilirubin Indirect; TB: Total Bilirubin; TIBC: Total iron binding capacity

All blood samples were collected at the Laboratory of Hematology at HEMOAM and sent to the Laboratory of Specialized Analysis in Hematology and Molecular Biology (LAEBM), located at the Faculty of Pharmaceutical Sciences (FCF) at UFAM, to perform DNA extractions, analysis molecular, hematological and biochemical.

For molecular analyses, genomic DNA was isolated from leukocytes from 200µL of blood, using QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's protocol. The DNA was stored at -20oC until the time of analysis, in compliance with Resolution 441/2011-CNS during the period necessary for molecular analyses.

Polymorphisms were determined by qPCR using by TaqMan® probes on Applied Biosystems QuantStudio 6 Flex Real-Time PCR System. The amplification reaction was performed to a final volume of 10uL/reaction, containing 5µL of 2x TaqMan Universal

Master Mix, 0.2µL of 20x SNP Genotyping Assay, 2.8µL of sterile water and 2,0 µL of DNA sample.

2.3 STATISTICAL ANALYSIS

Laboratory and molecular data were analyzed in a database generated using Graphpad Prism 5.0 software (Graphpad Software, San Diego, CA-USA) and SPSS version 19, according to the type of variable. The analysis of qualitative or categorical variables of three or more groups was performed using the non-parametric Chi-square test ($\chi 2$), duly corrected using the Mantel-Haenszel and Yates tests. The 95% confidence intervals and the prevalence ratio were calculated for these variables. Values of $p<0.05$ were considered significant for the performed analyses.

2.4 ETHICAL CONSIDERATIONS

This project was approved by the Research Ethics Committee (CEP) of the Hospital Foundation for Hematology and Hemotherapy of the State of Amazonas (CAAE n° 87700518.7.0000.0009). All participants were informed about the objectives of carrying out this study, necessary procedures, expected duration, relevance and, above all, about the possibility of ceasing to participate at any time without prejudice to medical care or any other type of penalty.

3 RESULTS AND DISCUSSION

The study presented a total of 277 patients with sickle cell anemia, with a mean age at diagnosis of approximately three years of age. The female gender had a minimally higher frequency (55.3%) and the brown race in 77.6% was predominant.

We observed that the rs2069832_AA and rs2069845_AA genotypes showed significantly higher values of hematimetric indices, which leads us to hypothesize as a protective factor in the hemolysis in these patients, while the genotype rs2069835_CC significantly decreased hematimetric indices, where we suppose as a risk factor in hemolysis (Figure 1). Genotypes in rs2069840 and rs2069849 did not show significant differences in hematimetric indices (Figure 2). No significant association was demonstrated in the biochemical data analyze (data no showed).

Since IL-6 is one of the most important mediators of the inflammatory response, mainly related to the elevation of pro-inflammatory cytokines and understanding that hematimetric indices are important values in the laboratory and clinical heterogeneity in

these patients, our results suggest that the rs2069832_AA, rs2069845_AA and rs2069835_CC genotypes can modulate clinical comorbidities in patients with sickle cell anemia, such as vasoocclusion crisis, severe hemolysis, anemia, transfusions and infections.

Several studies demonstrate the presence of high levels of Il-6 in patients with sickle cell anemia, however, with no significant association between clinical features and hematological and biochemical values.[30-33] RINCÓN-LÓPEZ et al. (2021)[34] demonstrated high levels of Il-6 as a marker of severe bacterial infection in children with sickle cell disease in a case-control study. SARRAY et al. (2015)[35] reported a significant association between high levels of IL-6 and duration of occlusive episodes. In a recent study, high levels of pro-inflammatory markers, including IL-6, were associated with a higher frequency of occlusive seizures, acute chest syndrome, malleolar ulcers, osteonecrosis, stroke and priapism, emphasizing the role of inflammation in the pathophysiology of the disease. sickle cell disease, indicating that IL-6 levels may be a useful predictor of poor patient outcomes.[36]

Our study did not demonstrate significant correlations between the genotypes studied and the phenotypes of the patients. However, the rs2069835_CC genotype predisposed patients to a higher number of transfusions and seizures, even if not significantly compared to the other genotypes (data not shown)

Figure 1. Associations between the polymorphisms rs2069845, rs2069832 and rs2069835 and hematimetric indices of patients with Sickle Cell Anemia treated at Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas.

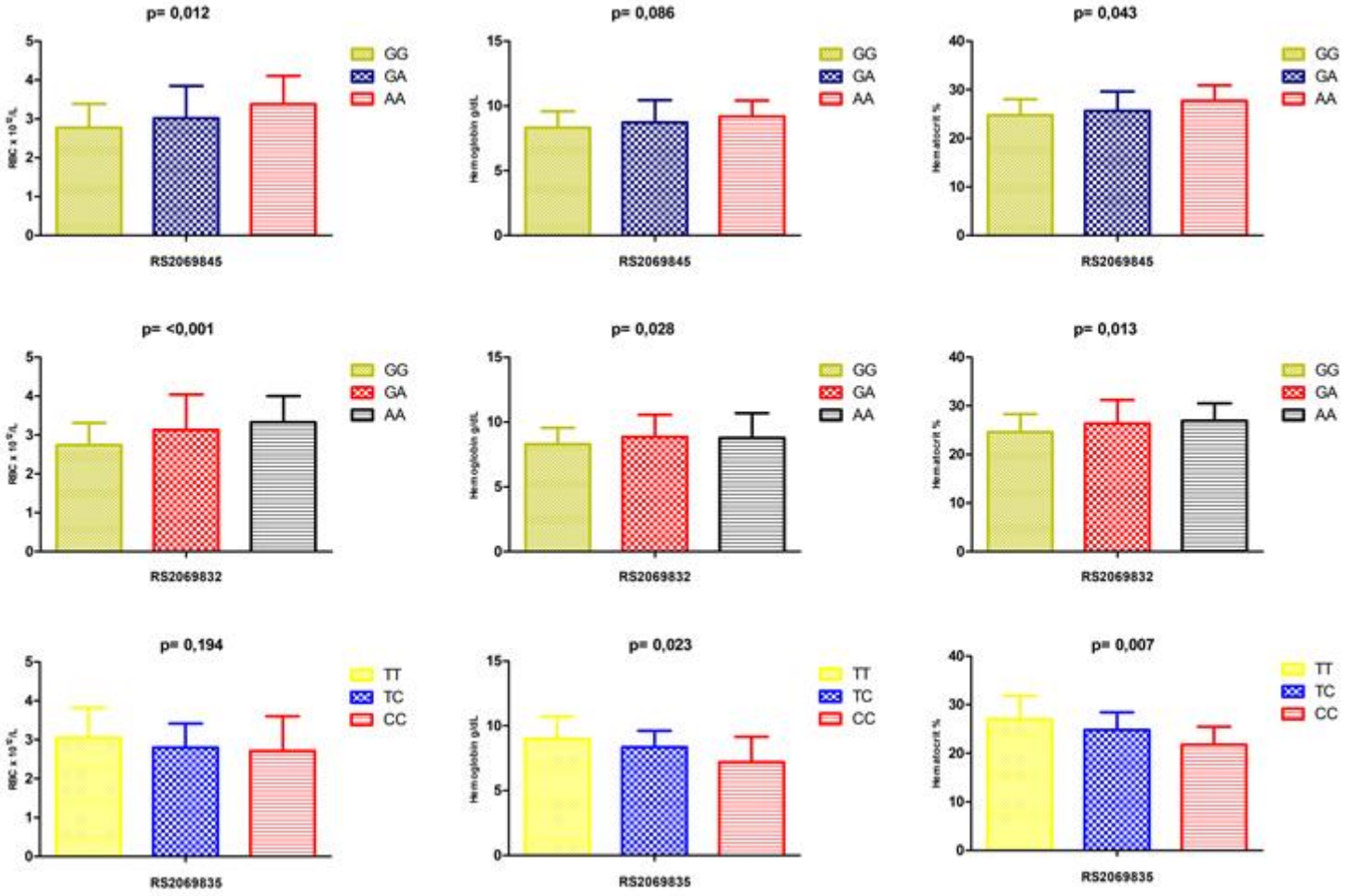
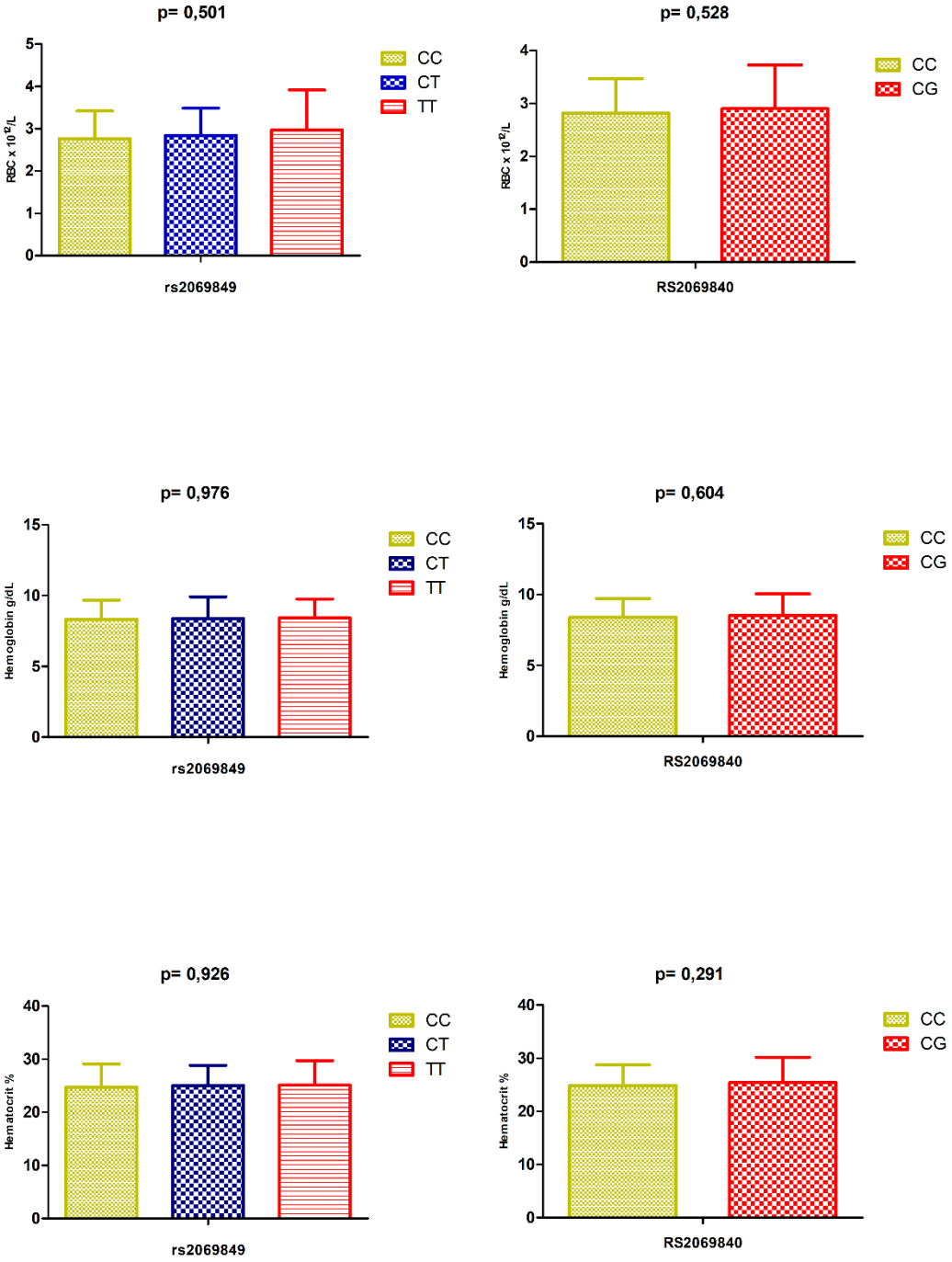


Figure 2. Associations between the rs2069840 and rs2069849 polymorphisms and hematimetric indices of patients with Sickle Cell Anemia treated at Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas.



4 CONCLUSION

Recent studies involving pro-inflammatory cytokines and their role in Sickle Cell Anemia allowed the development of new clinical approaches for the disease. Our work demonstrated an association of risk and protection for possible severe hemolysis in patients with sickle cell anemia treated at Fundação HEMOAM, Amazonas, Brazil.

We understand that an investigation with a larger number of patients, as well as in the cohort study model, would be recommended to elucidate the roles of the studied polymorphisms in sickle cell anemia. Added to this, elucidating the role of Il-6 in sickle cell anemia may lead to the development of new strategies and therapies to prevent the systemic effects of excessive cytokine production and consequently reduce the severity of crisis in these patients, providing better prognosis, clinical follow-up and well-being.

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