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EVALUATION OF ANTI-INFLAMMATORY RESPONSE IN SICKLE CELL ANEMIA PATIENTS: THE ROLE OF INTERLEUKIN-27 AND INTERLEUKIN-37

AVALIAÇÃO DA RESPOSTA ANTI-INFLAMATÓRIA EM PACIENTES COM ANEMIA FALCIFORME: O PAPEL DA INTERLEUCINA-27 E INTERLEUCINA-37

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Dissertação apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de mestre em Ciências Médicas, área de concentração em Patologia Clínica.

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DEDICATION

This work is dedicated to the glory of God in whom I live, I move and I have my being.

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To my mum, Mrs. Elizabeth O. Alagbe, my siblings- Mr. Samsom A. Alagbe and Mrs Abimbola O. Akadiri, I say thank you. I know that our fallen brother, Dayo Alagbe who died suddenly in January 2017, shall indeed be happy wherever he is. I am forever indebted to you, my best friend and wife, Dr. Olayemi Atinuke Alagbe and my angels, AyoOluwa Emmanuella, Oluwamayowa Angel and Oluwadamife Davida; for your patience, love, understanding, prayers, and perseverance. You are the best indeed. I love you. May God lengthen our days together in Jesus name.

RESUMO

Introdução e objetivo: A resposta inflamatória está envolvida em muitas das complicações observadas em pacientes com anemia falciforme (AF). O objetivo deste trabalho foi avaliar as concentrações séricas de duas citocinas anti-inflamatórias recentemente descritas, a IL-27 e IL-37, e de algumas citocinas inflamatórias em pacientes brasileiros com AF em tratamento com hidroxiureia (HU) e comparar com pacientes não tratados e indivíduos controle. Além disso, procuramos demostrar o efeito da IL-27, IL-37 e heme *in vitro* na secreção de IL-8 por neutrófilos e monócitos humanos.

Métodos: Foi realizado um estudo transversal com 82 pacientes com AF (35 sem tratamento com HU e 47 em tratamento com HU) com a doença estável e 49 indivíduos controle. Os dados clínicos foram obtidos a partir dos prontuários médicos e por meio de entrevista. As concentrações séricas de IL-27, IL-37, TGF-β, TNF-α, IL-1β, IL-6 e IL-8 foram determinadas por ELISA. Neutrófilos e monócitos foram isolados de indivíduos saudáveis e cultivados separadamente com ou sem citocinas (IL-27 e IL-37) e heme. As citocinas pró-inflamatórias no sobrenadante de cultura foram quantificadas por ELISA.

Resultados: As concentrações séricas de IL-27, IL-37, IL-1β, IL-6 e IL-8 em pacientes tratados com HU foram significativamente maiores em comparação a controles HbAA. Não foi observada diferença entre os níveis séricos de citocinas pró e anti-inflamatórias, exceto IL-8, em pacientes tratados com HU comparados a indivíduos controles. Correlação positiva foi detectada entre IL-27 e IL-37 em pacientes HbSS com e sem tratamento com HU. Em relação aos experimentos *in vitro*, verificamos que a produção de IL-8 foi significativamente inibida em

neutrófilos e monócitos pré-tratados com IL-27 e IL-37, apesar da adição de heme.

Conclusão: Os resultados mostraram que a IL-27 e IL-37 estão elevadas em pacientes com AF sem tratamento com HU. Estas citocinas podem exercer um papel regulatório nas vias pró inflamatórias, como sugerido pelos experimentos *in vitro*. A ação destas citocinas é provavelmente suficiente para prevenir danos celulares e teciduais mais tardios, mas não tem efeito na prevenção da inflamação. Assim, IL-27 e IL-37 poderiam ter um potencial terapêutico contribuindo para a diminuição das complicações associadas com a elevação do heme, como ocorre na AF e outras anemias hemolíticas.

Palavras chaves: Inflamação, anemia falciforme, IL-27, IL-37, hidroxiureia, heme.

ABSTRACT

Background and objective: Inflammation has been implicated in the pathogenesis of most complications seen in Sickle cell anemia (SCA) patients. This project aimed to evaluate serum levels of two newly discovered anti-inflammatory cytokines IL-27 and IL-37 and some pro-inflammatory cytokines among Brazilian SCA patients that are hydroxurea-naive and compared with hydroxyurea-treated patients and HbAA controls. Furthermore, to demonstrate the effect of IL-27, IL-37 and heme on *in vitro* secretion of IL-8 in human neutrophils and monocytes.

Methods: It is a cross-sectional study of 82 consenting SCA (35 hydroxyurea-naïve HbSS and 47 hydroxyurea-treated HbSS) patients in steady state and 49 HbAA consenting individuals. Their clinical details were obtained by interview and from patients' records. The serum levels of IL-27, IL-37, TGF-β, TNF-α, IL-1β, IL-6 and IL-8 were quantified by ELISA. Neutrophils and monocytes were isolated from healthy controls, cultured separately without or with cytokines (IL-27 and IL-37) and heme. The pro-inflammatory cytokines in the supernatant were detected by ELISA,

Results: Serum levels of IL-27, IL-37, IL-1β, IL-6 and IL-8 in hydroxyurea- naïve SCA patients were significantly elevated compared to HbAA controls There was no significant difference in the serum levels of both anti- and pro-inflammatory cytokines except IL-8 in the HU-treated SCA patients compared to controls. IL-27 and IL-37 were positively correlated in both HU-naive and hydroxyurea-treated HbSS patients. *In vitro* IL-8 production by IL-27 and IL-37 pre-treated neutrophils and monocytes was significantly inhibited despite addition of heme.

Conclusions: Our findings show that IL-27 and IL-37 are elevated in HU-naïve patients. The study support that the use of HU did not significantly alter serum

levels of anti- and pro-inflammatory cytokines except IL-8 in SCA patients. IL-27 and IL-37 may play a regulatory role on the pro-inflammatory pathways, as suggested by the *in vitro* studies. This role is probably sufficient to prevent further cellular or tissue damage but not potent enough to prevent inflammation. Thus, IL-27 and IL-37 may be potential immuno-targets to ameliorate complications associated with elevated heme as seen in SCA and other hemolytic anemias.

Keywords: Inflammation, sickle cell anemia, IL-27, IL-37, hydroxyurea, heme.

LIST OF ABBREVIATIONS

- CBC : Complete blood count
- ELISA: Enzyme linked immunosorbent Assay
- Hb A: Adult hemoglobin A
- HbAS: Hemoglobin A+SSickle cell trait
- HbF: Fetal hemoglobin
- HbS: Sickle Hemoglobin
- HbSS: Hydroxyurea-naive homozygous hemoglobin SS
- HbSS: Homozygous Sickle cell anemiaHemoglobin
- HbSSHU: Hydroxyurea-treated homozygous hemoglobin SS
- HEMOPE: Hematology and Hemotherapy Foundation of PernambucoHematology and Hemotherapy center of Pernambuco, Recife.
- HPLC: High performance liquid chromatography
- HU: Hydroxyurea
- ICAM: Intercellular Adhesion Molecule
- IL: Interleukin
- K+ Cl- co-transporter: Potassium / Chloride co-transporter
- LDH: Lactate dehydrogenase
- MCH: Mean cell hemoglobin
- MCHC: Mean cell hemoglobin concentration
- MCP: Monocyte Chemoattractant Protein
- MCV: Mean cell hemoglobin
- MIP: Macrophage Inflammatory Protein
- NF-Kb: Nuclear Factor-kappa beta
- NO: Nitric oxide

- RBC: Red blood cells
- RNS : Reactive Nitrogen species
- ROS : Reactive Oxygen species
- SCA: Sickle Cell Anemia
- SCD: Sickle Cell Disease
- STAT3: Signal Transduction and Transcription 3
- TGF-Transforming growth factor
- TNFR: TumourTumor Necrosis Factor Receptor
- TNF-α: TumourTumor Necrosis Factor-alpha
- VCAM: Vascular-Cellular Adhesion Molecule
- VOC: Vaso-Occlusive Crisis
- WBC: White blood cell.

CONTENTS

1. INTRODUCTION

1.1. Definition of Sickle Cell Anemia

Sickle cell disease (SCD) is a major public health problem in the world. Sickle cell disease (SCD) represents a group of inherited red blood cell disorder characterized by the presence of HbS gene in combination with other abnormal hemoglobin gene in homozygote, heterozygote or compound heterozygote states. The term sickle cell anemia (SCA) refers to the homozygous inheritance of HbS gene; and represents the most severe and most common form of SCD. It is usually mild with hemoglobin SC (HbSC) but a spectrum of clinical course is observed when there is co-inheritance with β-thalassemias. The severity is known to be mild with HbSβ^{+} and similar in HbSS and HbSβ^{0} (1). The resultant sickle hemoglobin (HbS) has its negatively charged hydrophilic glutamic amino acid replaced by hydrophobic valine at the 6th position of the hemoglobin beta globulin chain (1, 2). The inheritance of the mutant hemoglobin with normal hemoglobin results in the sickle cell trait (1-3).

The mutant HbS gene originated in sub-Saharan Africa, but its distribution is now global. Piel *et al* (2013) using detailed geo-referenced data estimated that 5 476 000 HbAS neonates and 312 000 SCA neonates were born in 2010. The prevalence of the deleterious gene ranges from 1.1% to 9.8% for HbAS and from 0.8 to 60 per 100,000 live births SCD in different regions of Brazil (1, 2). Data from the national neonatal screening of SCD in Brazil, 2010, the prevalence of SCD was 1 per 650, 1 per 1,400 and 1 per 4,000 live births in Bahia, Pernanbuco and Sao Paulo respectively (4).

The quality of life and longevity of patients with sickle cell anemia (SCA) is determined by various complications each patient experiences. Based on the

predominant pathophysiology, the complications of SCA are classified into two sub-phenotypes, the first group is characterized by hemolytic endothelial dysfunction and the second by vaso-occlusion (5). The complications are influenced by percentage of fetal hemoglobin (HbF), which varies with β^s globin haplotypes, presence of alpha thalassemias and other genetic modifiers (3).

1.2. Pathophysiology of sickle cell anemia:

It is still not well elucidated why SCA, a disease with a single missense mutation in a gene whose expression is restricted to the erythroid lineage, is characterized by a wide spectrum of clinical manifestations. The sickle gene mutation in SCA is a single base change from adenine to thymine (GAG to GTG) on the 17th nucleotide of the β globin gene. This mutation results in the replacement of the hydrophilic glutamate by hydrophobic valine at the sixth amino acid residue of the β-globin polypeptide chain (6, 7). The human hemoglobin is a tetramer of two pairs of identical peptide chains (α-like and β-like) coded by distinct genes. The genes of the β-globin gene cluster (ε, Gγ, Aγ, δ and β) are present on chromosome 11 in the same order in which they are expressed during ontogenic development. The β- locus control region (β–LCR) is a major regulatory element located far upstream of the genes of the cluster that is necessary for the high level of expression of those genes. The genes of the α-globin gene cluster (ζ, α1 and α2) are present on chromosome 16, also in the same order in which they are expressed during ontogenic development. During fetal life, HbF ($\alpha_2\gamma_2$) is the predominant type of hemoglobin. The hemoglobin switching refers to the developmental process that leads to the silencing of gamma-globin gene

expression and the reciprocal activation of adult β-globin gene expression. This results in the replacement of HbF by HbA (α2β2) as the predominant type of hemoglobin in adult life with about 2-5% of HbA2. This is the thrust for the use of hydroxyurea (HU) in the management of SCA thereby increasing the level of HbF and ameliorating the clinical severity of SCA (8, 9).

Patients with SCA begin to manifest symptoms about 6 months of life, during which time there is a completion of switch from the HbF to the abnormal adult hemoglobin. (5, 7) The fetal hemoglobin is related to the beta globin haplotype and correlates to the clinical course of SCA. The beta hemoglobin haplotypes in SCA include Senegal, Benin, Bantu, Cameroun and the Arab-Indian (10-12). The Senegal and Arab haplotypes are associated with the highest HbF levels, milder clinical manifestations of SCA and with lower occurrence of organ damage. The Benin and Cameroun haplotypes are associated with intermediate levels of HbF and clinical severity. However, the Bantu haplotype is associated with the lowest levels of HbF and consequently with the worse clinical course. However, some studies have shown that the risk of acute chest syndrome, pain crisis and infections may be similar in individuals with the Benin and Bantu haplotypes (3, 12).

The mechanisms involved in the pathophysiology of sickle cell anemia are complex and multifactorial. The abnormal globin chain polymerizes resulting in abnormal sickle hemoglobin molecule particularly during deoxygenation. In deoxygenated state, the sickle mutation leads to instability of the hemoglobin molecule resulting in hemoglobin polymerization (gelation), tactoid formation, RBC rigidity and subsequent red blood cell (RBC) membrane damage (Figure 1). This results in cellular dehydration, hemolysis, nitric oxide (NO) depletion,

secretion and activation of inflammatory markers and increased propensity for thrombo-embolic phenomenon (7, 13).

The gelation theory emphasizes that during deoxygenation, the delay time of gelation is inversely proportional to about 30th power of the deoxygenated Hb concentration within the RBC. Therefore, if delay time of gelation is shortened, polymerization and vascular effect would be avoided and any factor that may cause a prolonged delay time would predispose to gelation and vaso-occlusion (5, 13-16). Alteration of pH (acidosis), oxygen tension (low), temperatures (extreme), erythrocyte 2, 3 diphosphoglycerate (DPG) levels (low) among others. are known to promote HbS polymerization (17).

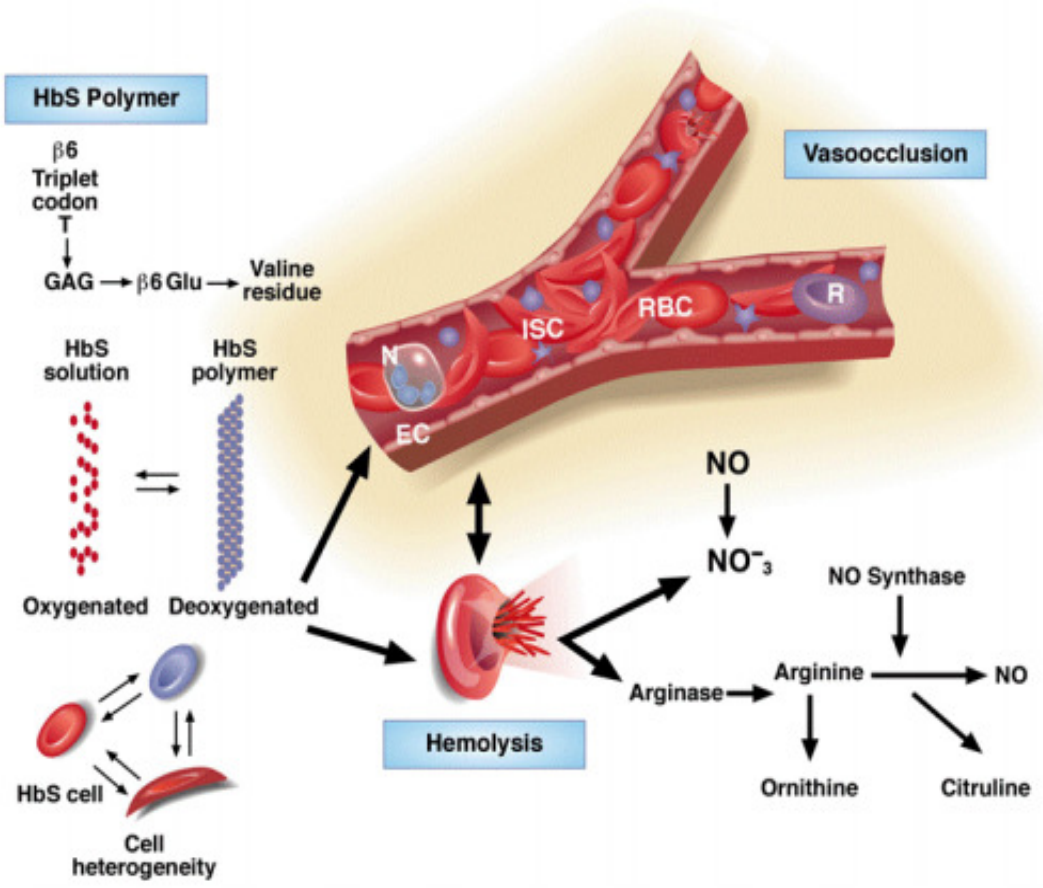

Figure 1: The pathophysiology of sickle cell anemia. The HbS mutation, *HBB* glu6val, leads to β-globin chains that, when incorporated into hemoglobin tetramers with normal α-globin chains, produce a sickle hemoglobin, HbS, which can undergo reversible polymerization when deoxygenated. The sickle polymer injures the erythrocyte and eventually produces irreversible membrane damage. These cells have a shortened life span (hemolysis), some of which occurs intravascularly consuming nitric oxide (NO). Arginase released from Sickle erythrocytes favors the biosynthesis of ornithine hence NO becomes depleted and worsens vaso-occlusion. (From Steinberg 2008)(18).

1.3. Inflammation in sickle cell anemia:

Sickle cell anemia has been long recognized as a chronic inflammatory disease. Evidences have shown that though the mutation in SCA affect the erythrocytes, other cells - leukocytes, endothelial cells and platelets play important roles in the pathophysiology of this disease, Figure 2 (19). Neutrophils are the most abundant immune cells in the circulation and have a critical role in the innate immunity. High neutrophil count has been positively correlated with severe clinical manifestations, painful crises, early death, silent infarcts, hemorrhagic strokes, and acute chest syndrome (ACS) in SCA patients (20, 21, 22). Reduction in neutrophil count is beneficial for SCA patients as seen in those on HU, who have marked reduction in the frequency of bone pain episodes and ACS (23). Effect of HU include increase HbF expression, increase NO synthesis, reduction of vascular cellular adhesion molecule-1 (VCAM-1) levels in plasma and reduction of RBCs to adhere to endothelial cells. Besides neutrophilia in SCA patients, the neutrophils are activated and HU has been reported to suppress the activation and recruitment of these cells (24, 25).

Platelets have inflammatory property in addition to being pivotal to coagulation process. Platelet activation is enhanced in SCA patients under steady state conditions and even further during vaso-occlusion crisis (VOC) (26, 27). The activated platelets promote adhesion of sickle RBCs to endothelial cells by secreting thrombospondin, hence may contribute to thrombosis and pulmonary hypertension in SCA (28). Platelets, that are increased as well as been activated in SCA, form aggregates with neutrophils and monocytes. These aggregates are elevated in SCA patients compared to controls and their inhibition

of formation of such aggregates could be protective against chronic inflammation and certain complications (29).

Similarly, monocytes are increased, activated and promote inflammation in SCA patients. Monocytes from SCA patients activate endothelial cells through the nuclear factor kappa B (NF-kB) pathway and enhance expression of adhesion molecules including ICAM-1, VCAM-1, E-selectin, thus promoting adhesion of mononuclear leukocytes to endothelial cells.(30, 31). Leukocytes adhesion is enhanced by pro-inflammatory cytokines produced by monocytes. Evidence exist that blocking the tumor necrosis factor (TNF)-α and IL-1β from monocytes by neutralizing antibodies abrogated the increased expression of E-selectin in endothelial cells. (30, 31).

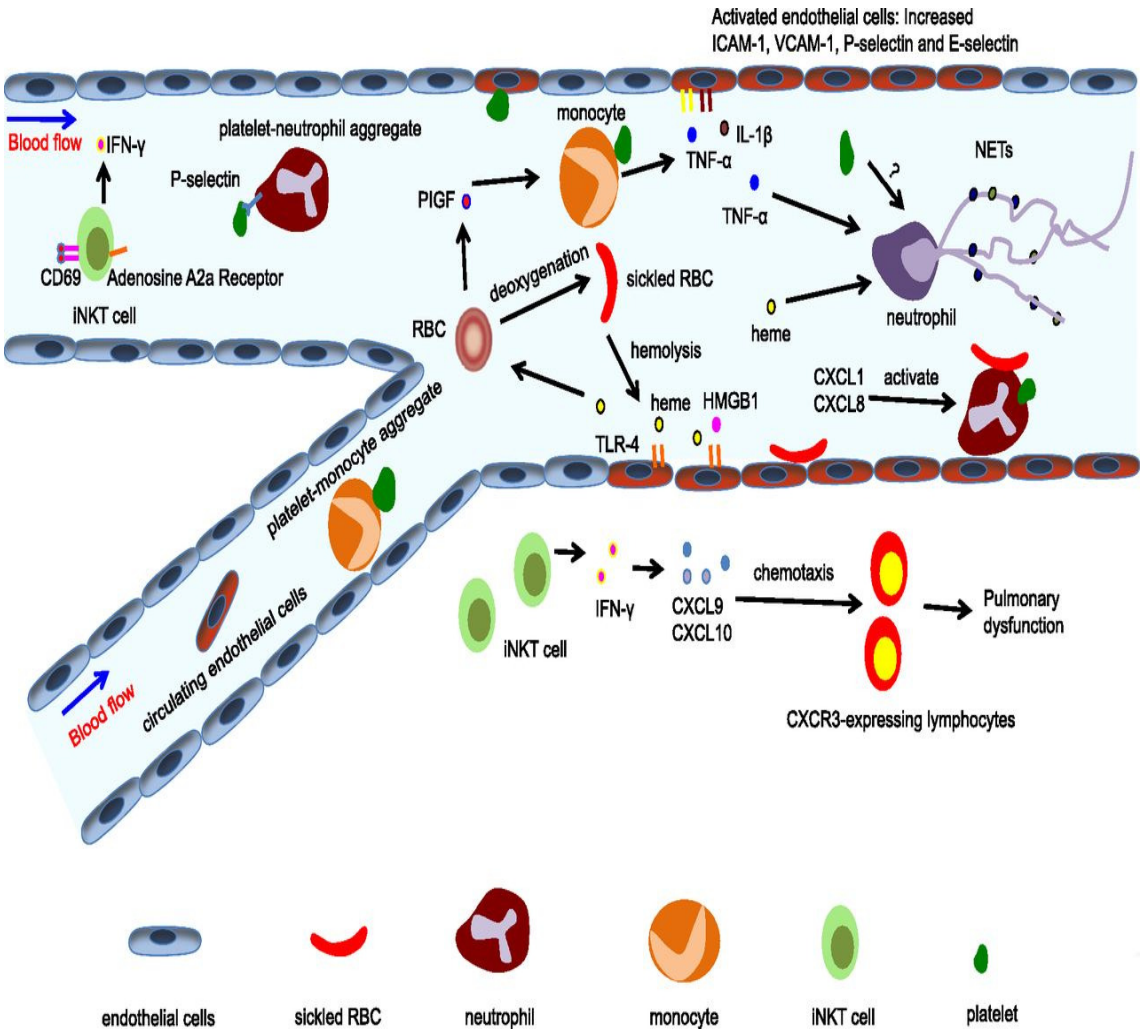

Figure 2: Inflammation in Sickle cell disease. SCD has been recognized as a chronic inflammatory disease. Multiple cell types and molecules are involved in its inflammatory pathways. Hemolysis of RBCs leads to the release of heme into the circulation, which can activate endothelial cells through the TLR4 pathway and induce PlGF release from RBCs. PlGF activates monocytes, leading to the production of pro-inflammatory cytokines TNF-α and IL-1β. The adhesion of platelets or sickle RBCs can also activate endothelial cells, producing increased expression of adhesion molecules including ICAM-1, VCAM-1, P-selectin, and E-selectin, and thus promoting the recruitment of neutrophils. iNKT cells exhibit an activated phenotype and contribute to pulmonary dysfunction in SCD by producing IFN-γ and inducing CXCR3 chemokines. Platelets can form aggregates with circulating leukocytes, including neutrophils and monocytes. Activated neutrophils roll and adhere to the endothelium, and initiate VOC by capturing sickle RBCs. Heme can also induce NET formation, which promotes acute pulmonary injury in SCD. Another damage-associated molecular pattern released from activated immune cells and necrotic cells, HMGB1, is a key player in promoting inflammation in SCD through activation of TLR4 signalling pathway. HMGB1, high mobility group box 1; iNKT cell, invariant natural killer T cell. (From Dachuan Zhang *et al*. 2016)(19).

1.4. Cytokines in sickle cell anemia

Pro-inflammatory cytokines play critical role in the pathophysiology of SCA. SCA patients exhibit higher levels of TNF-α, IL-1β, IL-6, IL-3, IL-10, IL-17. Following hemolysis, free hemoglobin, heme and other products are released. Placental growth factor (PlGF), from the red cells activate monocytes to secrete IL-8 and other cytokines. These cytokines activate endothelial cells and leukocytes via the NF-kB pathways resulting in increased expression of endothelial adhesion molecules such as ICAM-1, VCAM-1, E-selectin. This leads to leukocyte adhesion and triggers vaso-occlusion (32) Interleukin-8 (IL-8) is a pro-inflammatory member of the CXC chemokine family, involved in both endothelial cell proliferation and angiogenesis (33). Several types of cells, such as neutrophils, endothelial cells, macrophages and fibroblasts, produce IL-8 (34). IL-8 plays a role in the re-arrangement of the cytoskeleton, changes in intracellular Ca++ levels, activation of integrins and promotion of protein-granule exocytosis and the respiratory burst (35). IL-8 require CXCR1 and CXCR2 as receptors, which are expressed mainly by neutrophils, to enhance neutrophil recruitment and function (33).

Tumor necrosis factor-α (TNF-α) is another pro-inflammatory cytokine produced mainly by monocytes/macrophages and other cells, such as T-cells, smooth muscle cells, adipocytes and fibroblasts. The main function of TNF-α is to stimulate tumor necrosis and regression *in vivo* (34). Its actions are mediated by tumor necrosis factor receptors (TNFR 55 and TNFR 75), which are present on the membrane of several types of cells, excluding RBCs (36). Synergistically, IL-8 and TNF-α induce increased adhesion of RBC and leukocytes to the vascular endothelium, and this adhesion can cause vaso-occlusion and local hypoxia (37).

Local hypoxia will further worsen vaso-occlusion leading to vicious cycle. Increasing evidences have shown that IL-8 and other cytokines are elevated in SCA patients during VOC as well as in steady state (37-42). Contrarily, other studies have reported that IL-8 levels was not different in SCA patients during VOC or steady state (43, 44). Result with use of HU is not clear-cut. Some researchers reported increased serum levels of circulating IL-8 in HU-treated patients (38). Others reported conflicting results, demonstrating that patients undergoing HU therapy displayed significantly lower plasma levels of IL-8 (41). Other studies showed that HU did not significantly alter plasma TNF-α concentrations (41, 44) These suggest that increased levels of circulating IL-8 and TNF-α are associated with increased hemolysis, vascular occlusion and inflammation. Hydroxyurea is a potent inducer of HbF and the only FDA-approved therapy for SCA. Hydroxyurea has multiple mechanisms by which it produces its clinical effects. The recommended escalation of HU is associated with reduction of neutrophils and thrombocytosis in SCA thereby reducing inflammation. Reports show that short-term administration of HU ameliorates VOC in a SCD murine model via NO-mediated pathways.

One study observed higher plasma concentrations of IL-1β in Saudi Arabian SCA patients during both steady state and painful crises than in control subjects. However, higher levels were observed during the steady state than during painful crises (45). Similarly, plasma IL-1β levels were reported to be good predictor of stroke outcome and that high plasma IL-1β levels were associated with protection against stroke development in juvenile SCA patients with altered transcranial Doppler studies (46). Another study among SCA patients, found a positive correlation between elevated serum IL-18 levels and classic danger

signals, including uric acid and lactic dehydrogenase (LDH) (47). LDH and other markers of hemolysis were reported to be linked to oxidative stress. The release of reactive oxygen species (ROS) enhances VOC by classical endothelial activation and ROS-mediated activation of inflammasome via membrane receptors. Inflammasome, a multiprotein complex involved in the activation of other plasma proteins such as caspase-1, is formed at the junction of many inflammatory molecules in response to danger signals (48).

The anti-inflammatory cytokines that are known are fewer. Interleukin-10 is an anti-inflammatory cytokine, secreted by activated CD8+ T cells as well as activated T-helper (TH0, TH1 and TH2) cells, B-lymphocytes, mast cells and lipopolysaccharide-activated monocytes (49). IL-10 mainly inhibit the synthesis of pro-inflammatory cytokines such as TNF-α, GM-CSF, IL-1, IL-6, IL-8 and IL-12. IL-10 also inhibits the proliferation of TH1 cells, decreasing cytolytic function and the secretion of TH1 cytokines and facilitating the development of a TH2 response (32). The role of anti-inflammatory cytokine in SCA patients is conflicting. While some researcher found elevated IL-10 levels among HU-treated Brazilian SCA patient, Cerqueira et al. did not find a significant difference in the IL-10 level between the SCA and HbAA controls. However, these patients were HU-naive (41, 47). Another anti-inflammatory cytokine, IL-4, was observed to be elevated during VOC and during the steady-state (50, 51). In 2000, an investigation of the TH2 cytokine levels in SCA patients revealed that plasma IL-4 levels were significantly higher among steady-state HbSS patients than HbAA individuals. In addition, the ratio of plasma IL-2 to IL-4 and IFN-γ to IL-4 were significantly lower in HbSS patients than in the other groups (51).

From the aforementioned, it is obvious that inflammatory markers such as pro-inflammatory cytokines, chemokines and adhesion molecules are being extensively studied in SCA; however, only a few studies exists on the anti-inflammatory aspect of the immune response. This stimulated the conception of these recently characterized anti-inflammatory cytokines (IL-27 and IL-37) in SCA patients. Though no existing report of evaluation of these cytokines in patients with SCA, recent works have described their important participation in modulation of chronic inflammatory diseases (52-54). The duo of IL-27 of the IL-12 family and IL-37 of the IL-1 family member have received special attention due to their potent anti-inflammatory and potential therapeutic use in patients with chronic inflammatory disorder such as atherosclerosis, colitis, and rheumatoid arthritis (52, 53, 55-57). Dendritic cells and macrophages produce interleukin-27 (IL-27) after stimulation via Toll-like receptors (TLR2, TLR4 and TLR9). IL-27 is composed of two subunits, Epstein Bar induced gene EBI3 and p28, which bind to a specific receptor made up of the WSX-1 subunits (known as IL-27R) and gp130 (58, 59). In mice, the activation of naive CD4 + T cells in the presence of IL-27 or transforming growth factor-β (TGF-β) results in their differentiation into IL-10 producing Tr1 cells, a potent suppressor of inflammation. In addition, IL-27 is capable of inhibiting the differentiation of naive CD4 + T cells to Th17 and / or Treg Foxp3 + (60). Some researchers have shown the potential inhibitory role of IL-27 in inflammatory diseases such as autoimmune encephalomyelitis, rheumatoid arthritis and uveitis (61-63). In ApoE-/- mice, an experimental atherosclerosis model, it was shown that the adoptive transfer of Tr1 IL-10 promotes the reduction of lesion formation. In addition, the authors verified the co-localization of EBI3 and p28, subunits of IL-27, in human atherosclerotic

lesions (52). In mice induced zymosan peritonitis, the early administration of IL-27 was able to control mobilization and recruitment of neutrophils into the peritoneal cavity as well as decreased chemokines such as Monocyte Chemoattractant Protein-1 (MIP-1) and Macrophage Inflammatory Protein-1α (MIP-1α) (64). Thus, buttressing their anti-inflammatory role. Li et al. recently demonstrated the constitutive expression of IL-27 receptor complex on human neutrophils and that IL-27 mainly down modulate human neutrophil function (65). Similarly, other researchers demonstrated that IL-37 suppresses TNF-α-induced neutrophil activation and reduces pro-inflammatory cytokines and chemokine production from Kupffer cells and hepatocytes, hence this cytokine was reported to be protective for hepatitis (66).

The biological role of IL-37 (IL-1F7) is still unclear, but studies in murine models have shown their marked ability to inhibit inflammatory response (67, 68). IL-37 transgenic mice are less susceptible to LPS-induced shock and to dextran sulfate-induced colitis (69). Unlike IL-27, the effect of IL-37 is likely independent of IL-10, since receptor blockade of this cytokine receptor (IL-10R) does not reverse the protection induced by IL-37 (69). The expression of IL-37 in macrophages and epithelial cells decreases the secretion of pro-inflammatory cytokines, whereas the silencing of the IL-37 gene in human cells led to a greater expression of these cytokines. Transient expression of IL-37 in the liver of mice protects against hepatitis induced by concanavalin-A (67). The anti-inflammatory properties of IL-37 were also demonstrated *in vitro* when RAW macrophages incubated with IL-37 produced reduced concentrations of pro-inflammatory cytokines such as IL-1α, IL-6, TNF-α, and CXCL2 after activation by LPS. A similar reduction in cytokine production was observed in THP-1 macrophages

and in epithelial cells stimulated with LPS and IL-1 (67). Similarly, Sharma and colleagues (2008) showed that the silencing of endogenous IL-37 in endothelial and muscular cell lines induced a significant increase of 13 pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6, TNFα and GM-CSF (68). Participation of IL-37 in the anti-inflammatory response was also evidenced in a study with colon extract cultures in which the presence of this cytokine was shown to reduce the production of TNF-α and IL-1β *in vitro* and *in vivo*, in addition to inducing a six-fold increase in IL-10, an anti-inflammatory cytokine (69). Based on the potential anti-inflammatory role of IL-27 and IL-37 in chronic inflammatory disorders and lack of studies evaluating their role in SCA, this study aimed to evaluate the serum levels of IL-27, IL-37 and other cytokines in Brazilian hydroxurea-naïve SCA patients compared with hydroxyurea-treated patients and HbAA controls. In addition, to demonstrate the anti-inflammatory role of these cytokines in monocyte and neutrophil cultures stimulated by heme.

2. OBJECTIVES

2.1. General objective

To evaluate the inflammatory response in sickle cell anemia patients and to evaluate the inhibitory role of IL-27 and IL-37 on inflammation in heme-stimulated cells *in vitro*.

2.2. Specific objectives

- To determine the serum concentration of pro- and anti-inflammatory cytokines in hydroxyurea-naïve and hydroxyurea-treated sickle cell anemia patients; and HbAA controls.
- To evaluate the effect of IL-27 and IL-37 on cytokine production by heme-stimulated cells *in vitro*.

3. MANUSCRIPT

Interleukin-27 and interleukin-37 are elevated in Sickle Cell Anemia patients and inhibit *in vitro* secretion of interleukin-8 in neutrophils and monocytes

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Interleukin-27 and interleukin-37 are elevated in Sickle Cell Anemia patients and inhibit *in vitro* secretion of interleukin-8 in neutrophils and monocytes

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ABSTRACT

Background and objective: Inflammation has been implicated in the pathogenesis of most complications seen in Sickle Cell Anemia (SCA) patients. We aimed to evaluate serum levels of two newly discovered anti-inflammatory cytokines (IL-27 and IL-37) and some pro-inflammatory cytokines among Brazilian SCA patients that are hydroxurea (HU)-naive and compared with hydroxyurea-treated patients and healthy controls (HbAA). Furthermore, we demonstrated the effect of IL-27, IL-37 and heme on *in vitro* secretion of IL-8 in human neutrophils and monocytes.

Methods: It is a cross-sectional study of 82 consenting SCA (35 HU-naive HbSS and 47 HU-treated HbSS) patients in steady state and 49 HbAA consenting individuals. Their clinical details were obtained by interview and from patients' records. The serum levels of IL-27, IL-37, TGF-β, TNF-α, IL-1β, IL-6 and IL-8 were quantified by ELISA. Neutrophils and monocytes were isolated from healthy controls, cultured separately without or with cytokines (IL-27 and IL-37) and heme. Using ELISA, the concentration of IL-8 in the supernatant was detected.

Results: Serum levels of IL-27, IL-37, IL-1β, IL-6 and IL-8 in HU-naive SCA patients were significantly elevated compared to HbAA controls. There was no significant difference in the serum levels of the anti- and pro-inflammatory cytokines in HU-treated SCA patients compared to controls except IL-8 level that was higher in both HU-naïve and HU-treated SCA patients than the controls. IL-27 and IL-37 were positively correlated in both HU-naive and hydroxyurea-treated HbSS patients. *In vitro* IL-8 production by IL-27 and IL-37 pre-treated neutrophils and monocytes was significantly inhibited despite addition of heme.

Conclusions: Our findings show that IL-27 and IL-37 are elevated in HU-naïve patients. The study support that the use of HU did not significantly alter serum levels of anti- and pro-inflammatory cytokines except IL-8 in SCA patients. IL-27 and IL-37 may play a

regulatory role on the pro-inflammatory pathways, as suggested by the *in vitro* studies. This role is probably sufficient to prevent further cellular or tissue damage but not potent enough to prevent inflammation. Thus, IL-27 and IL-37 may be potential immuno-targets to ameliorate the complications associated with elevated heme as seen in SCA and other hemolytic anemias.

Keywords: Inflammation, sickle cell anemia, IL-27, IL-37, hydroxyurea, heme.

1. Introduction

Sickle Cell Anemia (SCA) is an inherited disease of global health burden characterized by chronic hemolysis and chronic end organ damage (1, 2). Based on the predominant pathophysiology, the complications in SCA patients are classified into hemolytic endothelial dysfunction and vaso-occlusion sub-phenotypes (3). Chronic inflammatory state in SCA patients is associated with high expression of markers of inflammation such as adhesion molecules and cytokines because of complex interactions among endothelial cells, erythrocytes, leukocytes and platelets (4). Following hemolysis, hemoglobin, heme and other products are released. Placental growth factor (PlGF) from the red cells activate monocytes to secrete IL-8 and other cytokines. These cytokines activate endothelial cells and leukocytes via the NF-kB pathways resulting in increased expression of endothelial adhesion molecules such as ICAM-1, VCAM-1, E-selectin (5). This leads to leukocyte adhesion and subsequently triggers vaso-occlusion (6). Several types of cells, such as neutrophils, endothelial cells, macrophages and fibroblasts produce IL-8, a CXC chemokine family member, involved in neutrophil recruitment/function, endothelial cell proliferation and angiogenesis (7-9). Tumor necrosis factor-α (TNF-α), produced mainly by monocytes/macrophages and other cells, such as T-cells, smooth muscle cells, adipocytes and fibroblasts, stimulates necrosis in tumors (8, 10). IL-8 and TNF-α induce increased adhesion of erythrocytes and leukocytes to the vascular endothelium, and this adhesion can cause vaso-occlusive crisis (VOC) and local hypoxia (11).

Several studies have shown that SCA patients display higher levels of IL-8 as well as other cytokines during VOC and steady state compared to controls but a few did not find a statistical difference (11-18). Results with use of hydroxyurea are not clear-cut. Some researchers reported increased levels of circulating IL-8, some reported significantly lower levels of IL-8 while others reported unaltered TNF-α level in hydoxyurea (HU)-treated patients (12, 15, 18). Similarly, a higher plasma concentration

of IL-1β observed during both steady state and painful crises than in control subjects is demonstrated to be a good predictor of stroke outcome in SCA patients (19, 20). This increase in pro-inflammatory cytokines leads to imbalance between the pro- and anti-inflammation with the pro-inflammatory been favored. Thus, endothelial activation is worsened and the vicious cycle continuous. The pattern of anti-inflammatory cytokines in SCA are similar to the pro-inflammatory as studies show that IL-10 and IL-4 may be higher, lower or unchanged in SCA compared to controls (15, 21-23).

Only a few studies evaluated the role of anti-inflammatory cytokines in SCA and there are no studies on two recently characterized anti-inflammatory cytokines, IL-27 and IL-37. However, recent works have described the importance of IL-27 and IL-37 in modulation of chronic inflammatory disease (24-26). Interleukin-27 of the IL-12 family and IL-37 of the IL-1 family have received special attention due to their potent anti-inflammatory and potential therapeutic use in patients with atherosclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis, ankylosing spondylitis and others (24, 25, 27-29). Dendritic cells and macrophages produce IL-27 after stimulation via TLR2, TLR4 and TLR9 (30, 31). Naive CD4+ T cells in the presence of IL-27 or TGF-β differentiates into IL-10 producing Tr1 cells, a potent suppressor of inflammation. In addition, IL-27 is capable of inhibiting the differentiation of naive CD4+ T cells in Th17 and/or Treg Foxp3+ (32). IL-27 has a potential inhibitory role in some inflammatory diseases such as colitis, multiple sclerosis, atherosclerosis, autoimmune disorders and others (33-35). Most of these studies focus on the role of IL-27 in regulating adaptive immune cells, but more recently, researchers have demonstrated that IL-27 and IL-37 modulate the functions of neutrophils thereby suppressing the neutrophil activation and production of pro-inflammatory cytokine (36, 37). The biological role of IL-37 is still unclear, but its marked ability to inhibit inflammation is worth evaluating in SCA (38, 39). The expression of IL-37 in macrophages and epithelial cells decreases the secretion of pro-inflammatory cytokines, whereas the silencing of the IL-37 gene in human cells led to a greater expression of these cytokines (38). Based on the potential anti-inflammatory

role of IL-27 and IL-37 in chronic inflammatory disorders and lack of studies evaluating their role in SCA, this study aimed to evaluate the serum levels of IL-27, IL-37 and other cytokines in Brazilian HU-naive SCA patients compared with HU-treated patients and normal HbAA controls. In addition, to demonstrate the effect of IL-27 and IL-37 on *in vitro* production of IL-8 in monocyte and neutrophil cultures stimulated with heme.

2. Materials and methods

2.1. Study participants

The study was a cross-sectional study comprising eighty-two (82) previously diagnosed adult HbSS patients on follow up at the Hematology and Hemotherapy Center of Pernambuco (HEMOPE), Recife. The control group consisted of forty-nine (49) healthy adult (HbAA) individuals from the same region and with same ethnical characteristics. Patients who had been previously diagnosed with HbSS by alkaline electrophoresis, High Performance Liquid Chromatography (HPLC) and solubility test were included, if in steady state. Steady state was defined as stable clinical state for three (3) months, without signs or symptoms of infection, pain, other acute episode suggestive of crisis or blood transfusion. Those individuals with any of the following were excluded: pregnancy; infections; acute or chronic inflammatory disease or use of immunosuppressant in the last two weeks; malignancies and SCA in acute crisis. The participants who fulfilled the above criteria were grouped as follows: HU-naive group (HbSS) – SCA patients who were not taking hydroxyurea; HU-treated group (HbSSHU) – SCA patients on hydroxyurea; and Hemoglobin AA control group (HbAA control) – Hemoglobin AA individuals without SCA or trait.

All participants signed the consent form and the questionnaire was filled adequately by interview and from patient's notes. Some of the details obtained based on the questionnaire were bio-data and complications of SCA suffered. Subsequently, 5mL of venous blood was collected into plain sample tube from each patient and control in addition to the routine blood samples for complete blood count. The samples were

centrifuged and the sera were stored in aliquots at -80ºC until analyzed. When the collection was complete, the samples were then transported in dry ice to UNICAMP, Campinas, southeast Brazil and were stored at -80ºC until cytokines were assayed.

The study was approved by ethical committees of both UNICAMP, Campinas and HEMOPE, Recife (CAAE: 52941315.6.0000.5404) and all participants gave written consent.

2.2. Hematological analysis

Hematological analysis was performed using an automated cell counter (Abbott cell Dyn Ruby analyzer, Illinois, U.S.A.). The hemoglobin S, F and A2 were determined by HPLC (BIO-RAD variant II, CA, U.S.A.).

2.3. Quantification of plasma concentrations of pro-and anti-inflammatory cytokines

The serum levels of the IL-1β, TNF-α, IL-6, IL-8, TGF-β, IL-27 and IL-37 were quantified by enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D Systems, Minneapolis, MN or Biolegend, San Diego, CA, USA) according to manufacturer's instructions.

2.4. Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood was collected from five (5) healthy controls (80-100mL) into sodium heparin tubes. The samples were centrifuged on a ficoll-hypaque solution (density 1.077; 400g for 30 minutes at room temperature) and the mononuclear cells (layer over the ficoll-hypaque solution) were transferred to another 15mL falcon tubes. PBMCs were washed twice with RPMI 1640 medium (300g for 10 minutes at 4℃) and re-suspended in supplemented RPMI 1640 medium (10% fetal bovine serum + L-glutamine [2mM] + gentamicin [5µg/mL] + sodium pyruvate [1µM]). Cells were quantified and subjected to monocyte isolation. The buffy coat layer over the red blood cells was transferred to a 15mL falcon tube for neutrophil isolation.

2.5. Isolation of monocytes

Monocytes were isolated using the Monocyte Isolation Kit II (Miltenyi Biotec, Germany) kit according to the manufacturer's instructions. Briefly, PBMCs were re-suspended at a concentration of $1x10^7$ cells in 80µL of PBS-B-E buffer (PBS-BSA [0.5%] + EDTA [2mM]) and incubated for 10 minutes at 4°C with a mixture of biotinylated antibody against CD16 (neutrophils and NK cells), CD19 (B lymphocytes), CD56 (NK cells), CD123 (dendritic cells and basophils) and CD235a (Glycophorin A, Erythrocytes). After incubation, the anti-biotin antibody coupled to magnetic beads were added and the cells incubated for 15 minutes at 4°C. After washing with PBS-B-E (300g, 10 minutes, 4°C), the cells were re-suspended in 500µL of buffer and passed through a magnetic separation LD (Miltenyi Biotec, Germany) column. The fraction not retained by the column (monocytes) was collected, centrifuged (300g, 10 minutes, 4°C) and re-suspended in supplemented RPMI 1640 medium at a concentration of $1x10^6$ cells/mL. Monocytes obtained after separation were used in the experiments described below. The purity of the isolated monocytes was evaluated by flow cytometry and was always >95%.

2.6. Isolation of peripheral blood neutrophils

After the supernatant containing mononuclear cells, plasma and Ficoll solution has been removed, the polymorphonuclear cells located in the buffy coat layer were transferred to a 15mL falcon tube. Then, the cells were re-suspended in red blood cell lysis buffer (NH_4Cl 155mM, $KHCO_3$ 10mM and EDTA 1mM) and incubated at room temperature for 10 minutes. After a second wash, the number of cells was estimated and re-suspended in supplemented RPMI 1640 medium. Cell suspensions at a concentration of $1x10^6$ cells per well were used for subsequent experiments.

2.7. Neutrophils and monocytes cell cultures

Immediately after purification, neutrophils and monocytes in separate cultures were suspended in supplemented RPMI-1640 medium. Separate cultures of neutrophils and monocytes were subjected into 3 groups - cells without pre-stimulation; cells with 4 hours of heme pre-stimulation followed by addition of cytokines (recombinant human, IL-27 or IL-37) and cells with 4 hours of cytokine (IL-27 or IL-37) pre-stimulation and followed by addition of heme. Cell cultures were treated without or with 30μM heme (Sigma-Aldrich Co, U.S.A), 5ng/mL rhIL-27, 5ng/mL rhIL-37 and 10ng/mL rhTNF-α (all from R&D Systems, Minneapolis, MN, U.S.A). Neutrophil and monocyte cultures were incubated for 18 hours and 24 hours respectively at 37°C, in a 5% CO_2 atmosphere. After incubation, cell cultures were centrifuged and the supernatant was collected for the measurement of IL-8 (a marker of inflammation) by ELISA according to manufacturer's instructions.

2.8. Statistical analysis

GraphPad Prism Program, version 6 for Windows (San Diego, California, U.S.A) was used for data analysis. The normal distribution of the quantitative variables was verified by the Kolmogorov-Smirnov and Shapiro-Wilk tests. Continuous variables that had a non-normal distribution were expressed in median with interquartile range (IQR) and analyzed by the Kruskal-Wallis with Dunn's multiple comparisons post-test for three or more independent groups. One-way ANOVA for repeated measures with Tukey post-test was used to compare parameters obtained from *in vitro* experiments. Correlations were determined by Spearman's rank correlation coefficient and the results were considered statistically significant when the probability was less than 5% ($p<0.05$).

3. Results

3.1. Demographic and hematological parameters of the participants

One hundred and thirty-one (131) adult participants were enrolled. The mean ages of the 35 hydroxyurea-naive SCA (HbSS) patients, (31.94±9.37years) and 47 hydroxyurea-treated SCA (HbSSHU) patients, (30.64±8.26years) were higher than that of the 49 HbAA controls (24.73±4.66years). There were more males in the HbSS and HbSSHU groups. As expected, the red blood cells, hematocrit and hemoglobin were significantly higher in the HbAA controls than HbSS and HbSSHU groups (p<0.0001 for each comparison). The leukocytes, neutrophils, lymphocytes, monocytes and platelets were significantly higher in the HbSS group than in the HbAA controls. The demographic and hematological parameters are summarized in Table 1.

Table 1: Demographic and hematological parameters of participants

Parameters	HbSS (N=35)	HbSSHU (N=47)	HbAA (N=49)	*p* value		
	A	B	C	A vs B	A vs C	B vs C
Age (Years)	31.94 ± 9.37	30.64 ± 8.26	24.73 ± 4.66	NS	**< 0.001**	**< 0.001**
Male/female	23 / 12	24 / 23	18 / 31	NS	NS	NS
RBC (x10^6/µL)	2.46 ± 0.72	2.48 ± 0.43	4.79 ± 0.60	NS	**< 0.0001**	**< 0.0001**
Hemoglobin (g/dL)	7.54 ±1.56	8.9 ± 1.4	13.8 ± 1.5	**<0.05**	**< 0.0001**	**< 0.0001**
Hematocrit (%)	21.19 ± 4.54	24.25 ± 5.14	41.63 ± 4.42	NS	**< 0.0001**	**< 0.0001**
WBC (x10^3/µL)	10.72 ± 3.81	8.70 ± 2.84	6.72 ± 2.81	**<0.05**	**< 0.0001**	**<0.05**
Neutrophils (x10^3/µL)	5.94 ± 2.75	4.56 ± 2.32	3.67 ± 2.13	NS	**0.0007**	NS
Lymphocytes (x10^3/µL)	3.42 ± 1.51	3.03 ± 0.98	2.30 ± 0.82	NS	**< 0.0001**	**< 0.01**
Monocytes (x10^3/µL)	1.14 ± 0.60	0.92 ± 0.45	0.59 ± 0.90	NS	**< 0.0001**	**< 0.0001**
Platelets (x10^3/µL)	431.2 ± 168.2	335.2 ± 120.0	256.2 ± 53.82	NS	**< 0.001**	**< 0.001**
Hemoglobin F (%)	5.86 ± 4.47	7.74 ± 5.07	ND	NS	**-**	**-**
Hemoglobin S (%)	86.05 ± 5.14	88.29 ± 6.24	ND	NS	**-**	**-**
Hemoglobin A2 (%)	3.53 ±0.86	3.05 ±1.21	ND	NS	**-**	**-**
Reticulocytes (x10^3/µL)	275.40 ±116.40	271.10 ± 99.0	ND	NS	**-**	**-**
LDH (U/L)	704.10 ± 404.90	618.90 ± 370.20	ND	NS	**-**	**-**

The results expressed as mean ± SD. NS= not significant; ND= not done; LDH = Serum lactate dehydrogenase, WBC = White blood cells, RBC = Red blood cells.

3.2 Clinical data of participants

Out of the 82 SCA patients, most, 72 (87.8%) had experienced bone pain crisis in their lifetime. Other complications reported were osteonecrosis, 17 (20.7%); acute chest syndrome, 6 (7.3%); pulmonary hypertension, 5 (6.1%); sequestration crisis, 6 (7.3%); gallstone, 56 (68.3%); stroke, 16 (19.5%); persistent splenomegaly, 7 (8.5%);

chronic leg ulcers, 24 (29.3%); pneumonia, 36 (43.9%). In addition, 77 (93.3%) of them had had blood transfusion in the past. Of the 47 male SCA patients, 11 (23.4%) had experienced priapism in the past.

3.3 Anti-inflammatory cytokine levels in sickle cell anemia patients and controls

The serum levels of the IL-27, IL-37 and TGF–β among the HU-naive SCA patients (HbSS), hydroxyurea-treated SCA patients (HbSSHU) and the control individuals (HbAA) are shown in Figure 1A-C. Interleukin 27 was significantly higher in the HbSS group than the HbAA controls (median [IQR]: 966.7 [677.9 - 11,154.0] vs. 738.1 [470.0 – 1,706.0] pg/mL). Serum IL-27 was not significantly different between the HbSS and HbSSHU groups (median [IQR]: 966.7 [677.9 - 11,154.0] vs. 677.9 [490.2 – 3,214.0] pg/mL). Similarly, the serum IL-37 level was significantly higher in the HbSS group than the HbAA control group (median [IQR]: 225.0 [33.9 – 1,648.0] vs. 55.76 [22.63 – 179.0] pg/mL). There was no significant difference in IL-37 level between HbSS and HbSSHU (median [IQR]: 225.0 [33.9 – 1,648.0] vs. 111.2 [34.72 – 569.4] pg/mL, respectively). Serum levels of both cytokines were not significantly different between HbSSHU and HbAA groups (IL-27: 677.9 [490.2 – 3,214.0] vs. 738.1 [470.0 – 1,706.0] pg/mL and IL-37: 111.2 [34.72 – 569.4] vs. 55.76 [22.63 – 179.0] pg/mL, respectively). There was no significant difference in TGF-β levels among the HbAA, HbSS and HbSSHU (54,612.0 [45,274.0 – 69,600.0], 55,506.0 [41,717.0 – 73,497.0] and 53,839.0 [41,614.0 – 69,359.0] pg/mL respectively).

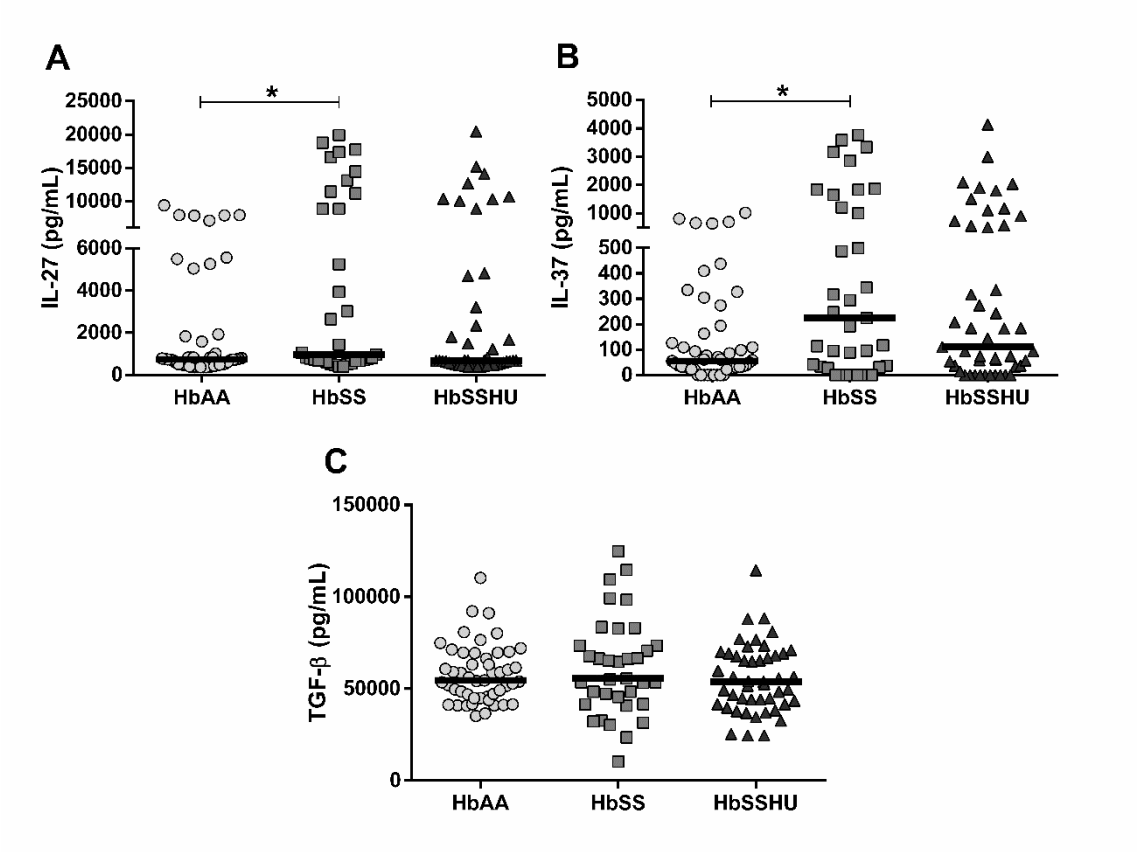

Figure 1. Serum levels of anti-inflammatory cytokines IL-27 (A), IL-37 (B) and TGF-β (C) in control individuals (HbAA control, n=49), hydroxyurea-naive (HbSS, n=35), hydroxyurea-treated (HbSSHU, n=47) patients. The horizontal lines represent median. Kruskal-Wallis with Dunn's multiple comparisons post-test; * $p<0.05$

3.4 Pro-inflammatory cytokine levels in sickle cell anemia patients and controls

The serum levels of TNF-α, IL-1β, IL-6 and IL-8 among the HbSS patients HbSSHU and the HbAA groups are shown in Figure 2A-D. Interleukin-1β, IL-6 and IL-8 were significantly higher in the HbSS (median [IQR]: 0.0 [0.0 - 17.53], 8.62 [4.7 – 57.08] and 26.48 [14.38 – 226.1] pg/mL respectively) than in the HbAA group (median [IQR]: 0.0 [0.0 – 0.0], 3.59 [2.65 – 7.31] and 0.0 [0.0 – 38.72] pg/mL respectively). In addition, IL-8 was significantly higher in the HbSSHU (median [IQR]: 17.09 [12.97 – 40.12] pg/mL)

than in the HbAA group (median [IQR]: 0.0 [0.0 - 38.72] pg/mL). TNF-α, IL-1β and IL-6 did not show significant difference in between HbSSHU and HbAA groups. Similarly, there was no significant difference in serum TNF-α level among the three groups.

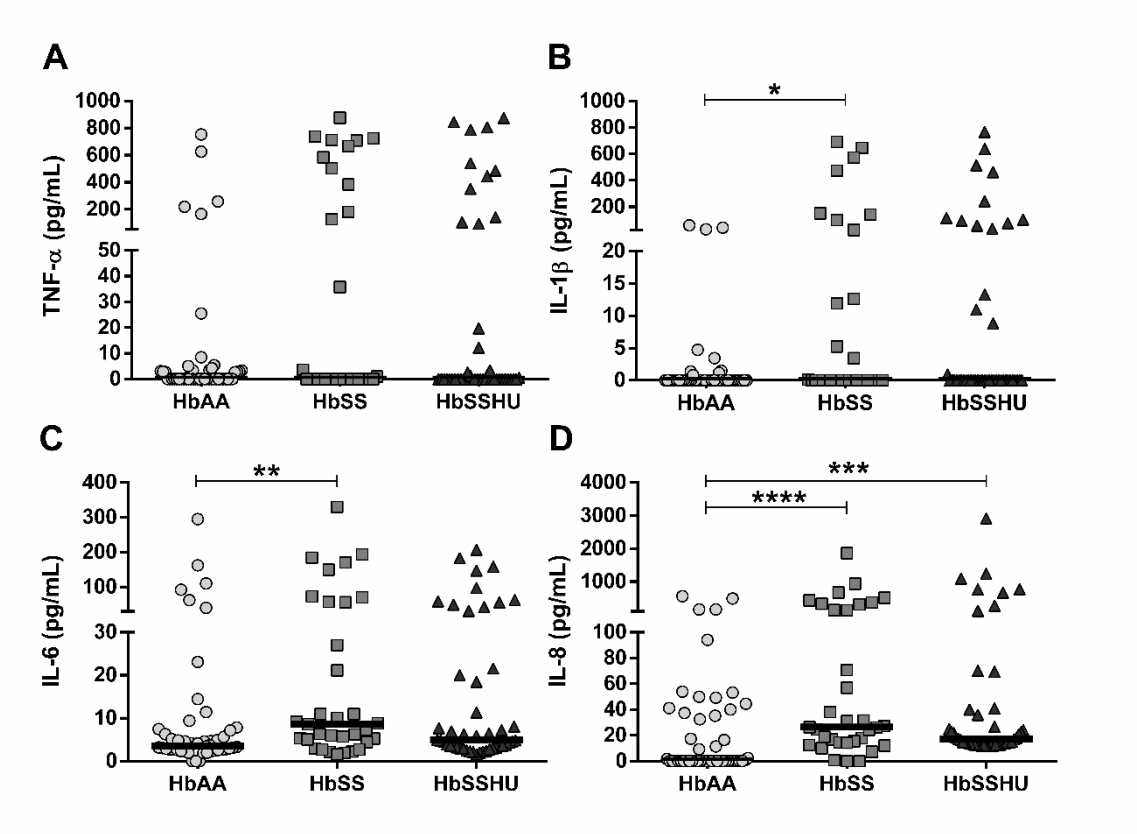

Figure 2. Serum levels of pro-inflammatory cytokines TNF-α (A), IL-1β (B), IL-6 (C) and IL-8 (D) in control individuals (HbAA control, n=49), hydroxyurea-naive (HbSS, n=35), hydroxyurea-treated (HbSSHU, n=47) patients. The horizontal lines represent median. Kruskal-Wallis with Dunn's multiple comparisons post-test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

3.5. Correlations of serum IL-37 and IL-27 in sickle cell anemia patients

Serum levels of IL-37 and IL-27 were strongly and positively correlated ($p<0.0001$ and r=0.8639) in the SCA patients (HbSS and HbSSHU) (Figure 3).

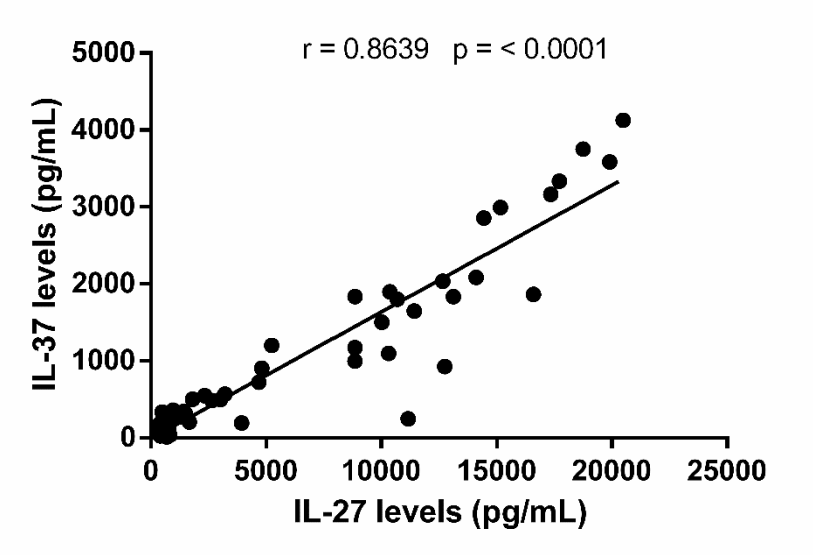

Figure 3. Correlation between circulating IL-27 and IL-37 levels among sickle cell anemia patients (Hydroxyurea- naive and treated patients) (n=82); Spearman correlation for non-parametric data; r = correlation coefficient.

3.6. Effects of heme and cytokines on IL-8 production by neutrophils and monocytes

Using ELISA based quantitation of IL-8 (as a marker of inflammation) in the culture supernatant; we investigated the inflammatory effect of heme, IL-27, IL-37 and TNF-α (positive control), Figure 4. Separate neutrophil and monocyte cultures were stimulated with heme, IL-27, IL-37 and TNF-α for 18 and 24 hours, respectively. In monocyte cultures (Figure 4B), heme induced a significantly higher concentration of IL-8 compared to the control (unstimulated, $p<0.01$). Neither IL-27 nor IL-37 induced the secretion of IL-8 from both neutrophils and monocytes.

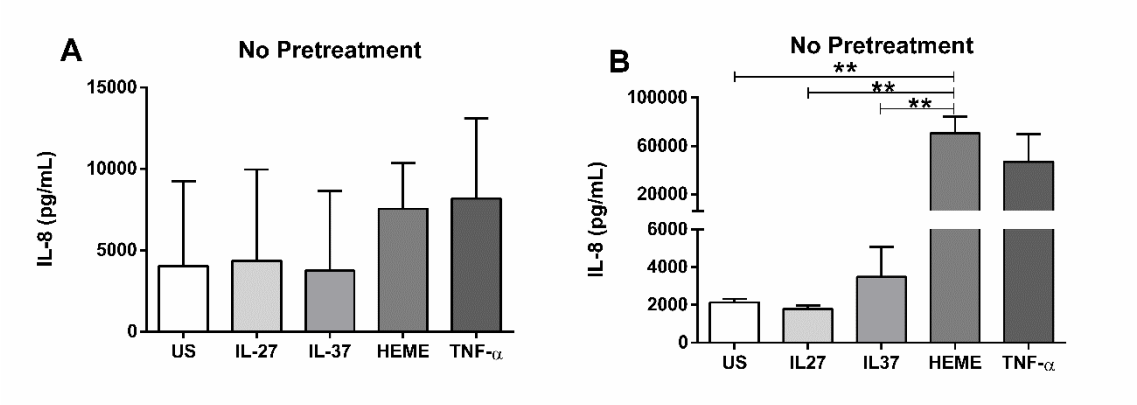

Figure 4: *In vitro* production of IL-8 by neutrophils (A) and monocytes (B) from control individuals (n=5) after stimulation with heme, IL-27, IL-37 and TNF-α. US- unstimulated/culture medium; HEME-30µM; IL-27- 5ng/mL; IL-37- 5ng/mL. One-way ANOVA for repeated measures with Tukey post-test; *p <0.01.

3.7. Effects of IL-27 and IL-37 on IL-8 production by heme pre-stimulated neutrophils and monocytes

We investigated the inhibitory effect of IL-27 and IL-37 in neutrophil and monocyte cultures, shown in Figure 5. After 4 hours of pre-stimulation of neutrophils with heme, IL-37 significantly suppressed IL-8 production by the neutrophils (p<0.05) but IL-27 did not reach statistical significance, Figure 5A. Neither IL-27 nor IL-37 significantly suppressed the inflammatory response induced by heme in monocyte cultures, Figure 5B.

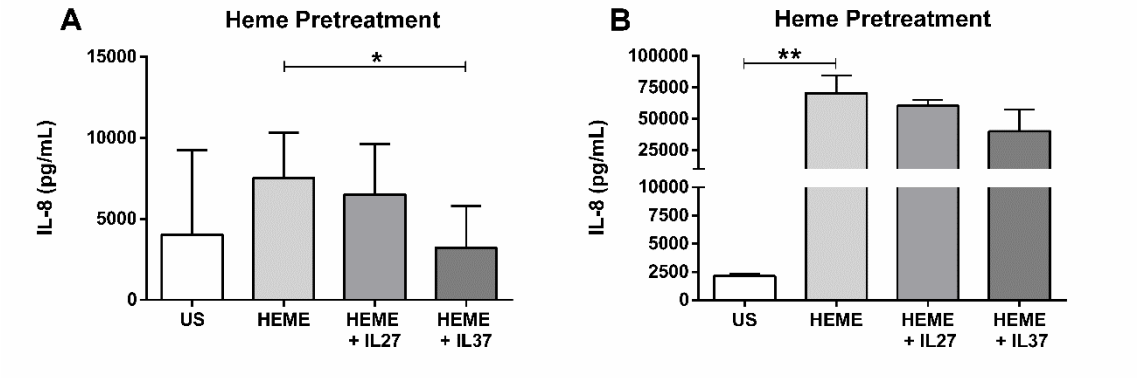

Figure 5. Effect of IL-27 and IL-37 on IL-8 production by heme pre-stimulated neutrophils (A) and monocytes (B) from control individuals (n=5). US-

unstimulated/culture medium; HEME-30µM; IL-27- 5ng/mL; IL-37- 5ng/mL. One-way ANOVA for repeated measures with Tukey post-test; *p <0.05; ** p<0.01.

3.8. Effects of heme on IL-8 production by IL-27 and IL-37 pre-stimulated neutrophils and monocytes

Following 4 hours of pre-stimulation of neutrophil cultures with IL-27, heme was added to cultures over 18 hours. There was significant reduction of IL-8 secretion in neutrophil cultures compared to heme-stimulated neutrophils (Figure 6A). Similar reduction of IL-8 production was observed with IL-37 pre-stimulated neutrophils.

In monocyte cultures, Figure 6B, IL-37 pre-stimulation inhibited the production of IL-8 by heme. IL-27 pre-stimulation yielded similar pattern in monocyte cultures.

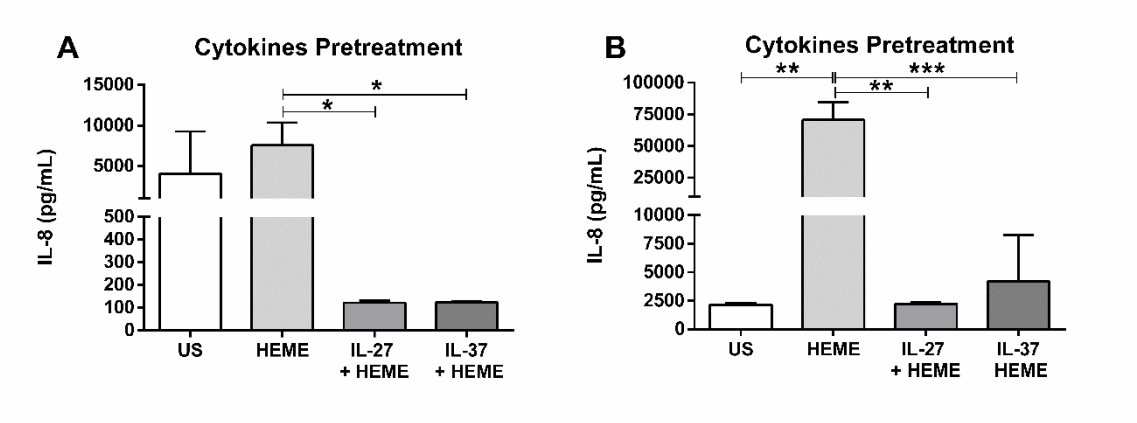

Figure 6: ***Effect of heme on IL-8 production by IL-27 and IL-37 pre-stimulated*** **neutrophils (A) and monocytes (B) from control individuals (n=5).** US-unstimulated/ culture medium; HEME (30µM); IL-27 (5ng/mL); IL-37 (5ng/mL). One-way ANOVA for repeated measures with Tukey post-test; *p<0.05; **p<0.01;***p<0.001.

4. Discussion

Interleukin-37 and IL-27 are two (2) recently characterized cytokines with anti-inflammatory activity. As far as we know, the role of both cytokines is yet to be studied in SCA or other hemolytic anemias. Therefore, for the first time, we give a report that serum IL-27 and IL-37 are elevated in SCA patients especially those who are hydroxyurea-naive compared to HbAA controls. This is consistent with previous studies in which IL-37 and IL-27 were elevated in patients with various chronic inflammatory diseases such as ankylosing spondylitis, rheumatoid arthritis, colitis and atherosclerosis (25, 29, 40-42). The findings from this study show that the studied anti-inflammatory cytokines as well as the pro-inflammatory cytokines were markedly produced in SCA patients. Elevated white cell count, neutrophils, lymphocytes, monocytes and platelets in both groups of SCA patients may have partly contributed to the elevated circulating levels of IL-27 and IL-37 as well as the pro-inflammatory cytokines. This is corroborated by other researchers who reported that leukocytes including neutrophils, monocytes/macrophages are important sources of cytokines in addition to other cell types (5, 8).

Serum IL-27 and IL-37 were weakly and positively correlated with pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α; results not shown), which other researchers have described to be important in the pathogenesis of SCA (4, 11, 13, 18). Yang *et al*. found a positive association between serum IL-37 and pro-inflammatory cytokines; and Lai *et al*. found that elevated serum IL-27 were positively correlated with disease severity in patients with rheumatoid arthritis (42, 43). However, there was no positive correlation between IL-27 or IL-37 and the clinical complications in our patients. The different etiologies in these disease entities may be accountable for the disparity. While SCA is purely an inherited monogenic disorder in etiology, rheumatoid arthritis have an autoimmune component (43, 44). Contrary to other researchers, who found significant reduction in the levels of TNF-α and IL-1β in HU-treated patients compared to

the HU-naive patients, the use of HU was not associated with significant alteration in the levels of both pro- and anti-inflammatory cytokines in our cohort (15). Difference in the dose of HU, the duration of use of HU or some other modulators of the disease may explain this discordance. Hydroxyurea, the only approved hypomethylating agent, has previously been suggested to suppress inflammation (45, 46). The anti-inflammatory effect of HU could be supported by lack of significant difference in the serum level of all the pro-inflammatory cytokines (except IL-8) between the HU-treated SCA and the control group. It would be reasonable to suggest that HU helps to maintain the inflammatory milieu at a state that is similar to the baseline whereas inflammation is significantly augmented in the absence of HU. The anti-inflammatory cytokines in a similar way was not significantly different in the HU-treated patients compared to the controls. The pattern observed between HU-treated patients and HbAA controls could be corroborated by the weak positive correlation between the pro- and anti-inflammatory cytokine (results not shown).

A positive correlation between serum IL-27 and IL-37 in the SCA patients may suggest that the production of both cytokines may be induced by similar damage-associated molecular pattern (DAMP), a group of endogenous molecules derived from damaged cells and extracellular matrix degradation capable of promoting and exacerbating immune responses (27). DAMPs such as free hemoglobin, heme or other breakdown products of RBC may have a role to play in the production of both groups of cytokines. Studies have shown that plasma levels of heme ranges from 20-600μM in patients with intravascular hemolysis such as SCA and paroxysmal nocturnal hemoglobinuria (PNH) (27, 47). Therefore, the effect noted in this study at a heme concentration of 30μM could be considered pathological. This effect may be extended to other conditions with raised heme levels for example cardiopulmonary bypass, hemolytic blood transfusion reactions (48, 49). Continuous hemolysis as is seen in patients with hemolytic anemias, of which SCA is an example, is associated with elevated IL-8 levels

as seen in both groups of SCA patients. *In vitro* experiments in the present study showed that heme stimulates pro-inflammatory response as evidenced by increased production of IL-8 in both neutrophil and monocyte cultures. The effect on other pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α did not reach statistical significance (data not shown). Elevated heme levels overwhelm hemopexin, a potent heme scavenger; heme oxygenase; and stimulate the circulating monocytes and neutrophils, which secret IL-8 (5, 44). In addition, the neutrophils produce IL-8, which self-activate in an autocrine fashion. Secreted IL-8 is responsible for neutrophilic chemoattraction to sites of endothelial injury as the neutrophils represent the most abundant innate immune cells. IL-8 may be important in SCA pathophysiology as earlier reported by Gonçalves *et al.* who suggested same as a maker of VOC in SCA patients (14). Clinical effects of IL-27, IL-37 or their antagonists on monocytes/macrophages and neutrophils as immunotherapy in patients with sickle cell disease and/ or hemolytic anemias may give desired results by ameliorating disease complications mediated by inflammation. These agents may affect other cells *in vivo* such as T, B, NK cells, dendritic cells and macrophages , which express the IL-27R and IL-18R complexes if used in vivo (50, 51). Hence, the possible side effects that may emanate from the use of these agents or their targets/antagonists may be predictable.

The anti-inflammatory role of both IL-27 and IL-37 was further strengthened by inhibition of inflammatory response induced by heme in culture pre-stimulated with the IL-27 or IL-37. From the foregoing therefore, the use of IL-27 or IL-37 before sudden rise of heme concentration in hemolytic anemia could be protective and may ameliorate clinical complications mediated by innate immune response. This may be beneficial in acute episodes as well as chronic end organ morbidities. However, the inhibitory response of IL-27 and IL-37 was not obvious when these cytokines were added to heme pre-stimulated neutrophils and monocytes. This suggests that the presence of heme induces inflammatory pathways that could not be reversed or inhibited by the addition of

either IL-27 or IL-37. It could be inferred that the chronic inflammatory state induced by heme may play a positive feedback in inducing secretion of the pro-inflammatory cytokines and a negative feedback in inducing secretion of IL-37 and IL-27 (27, 29). Elevated levels of IL-37 and IL-27 maybe potent enough to regulate the excessive effect caused by the pro-inflammatory cytokines in a tendency to maintain the homeostatic balance thus, prevent excessive tissue damage. However, their potential inhibitory effect may not be sufficient to neutralize the effect of heme, as the latter is an inciting DAMP that initiated the inflammation cascade in these patients. Therefore, the probable use of IL-27 or IL-37 before sudden rise in circulating heme level may be preventive rather than therapeutic.

5. Conclusions

Our results show that IL-27 and IL-37 levels are positively correlated in SCA patients and serum IL-27 and IL-37 are elevated in HU-naive patients compared to the controls. Furthermore, the use of HU did not significantly alter the secretion of both anti- and pro-inflammatory cytokines (except IL-8) in the HU-treated SCA patients compared to controls. This support the anti-inflammatory action of HU. In *in vitro* studies, heme at a concentration similar to pathological levels seen in hemolytic anemias such as SCA is a potent stimulus of IL-8 in neutrophils and monocytes. The marked upregulation of the pro-inflammatory pathway(s) leading to secretion of IL-8 in neutrophils and monocytes could be significantly suppressed by the use of IL-27 or IL-37 before sudden rise in the concentration of heme. IL-27 and IL-37 may play a regulatory role on the pro-inflammatory pathways, as suggested by the *in vitro* studies. This role is probably sufficient to prevent further cellular or tissue damage but not potent enough to prevent inflammation. These cytokines may be immunotherapeutic targets in such scenarios. However, these findings should be interpreted with caution, as there are many modulating factors in SCA patients.

Limitation of the study: Children were not enrolled as participants. The findings would be more representative because children have less end organ damage due to chronic hemolysis and inflammatory response compared to adults.

Highlights:

- ✓ This study represent the first report on the role of IL-27 and IL-37 in sickle cell Anemia.
- ✓ The level of these anti-inflammatory cytokines are elevated in hydroxyurea-naïve SCA patients and are positively correlated with pro-inflammatory cytokines.
- ✓ The level of these cytokines in the circulation may not be sufficient to neutralize the effects of pro-inflammatory cytokines in SCA patients.
- ✓ These anti-inflammatory cytokines may be potential immunotherapeutic target for ameliorating complications in SCA patient

Competing interests

The authors declare that they have no competing interests.

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Author contributions

All authors read and approved of the manuscript.

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4. DISCUSSION

Interleukin-37 and Interleukin-27 are two (2) recently characterized cytokines with anti-inflammatory activity. From literature search, the role of both cytokines is yet to be studied in sickle cell anemia or other hemolytic anemias. Hence, for the first time, we give a report that serum IL-27 and IL-37 are elevated in SCA patients especially those who are hydroxyurea-naive compared with HbAA controls. This is consistent with previous studies in which IL-37 and IL-27 were elevated in various chronic inflammatory diseases such as ankylosing spondylitis, rheumatoid arthritis, colitis, atherosclerosis (53, 57, 70-72). The findings from the present study show that IL-27 and IL-37 as well as the pro-inflammatory cytokines were markedly produced in HU-naïve SCA patients. Elevated white cell count, neutrophils, lymphocytes, monocytes and platelets in both groups of SCA patients may have partly contributed to the elevated circulating levels of IL-27 and IL-37 as well as the pro-inflammatory cytokines. This is buttressed by other researchers who reported that leukocytes including neutrophils, monocytes/macrophages are important sources of cytokines in addition to other cell types (19, 34). Rudloff *et al.* observed that constitutive IL-37 secretion by dendritic cells might serve to maintain an anti-inflammatory milieu at steady state, whereas IL-37 is stored in monocytes/macrophages to be available for rapid release upon inflammatory encounters thus, acting as a novel anti-inflammatory alarmin (73). Similarly, macrophages and dendritic cells are the primary sources of IL-27 (58). These may partly explain the strong positive correlation between the serum IL-37 and IL-27 noted in our patients. More so, the secretion of both cytokines require the activation of Toll-Like Receptors (TLR) by

various pro-inflammatory stimuli, such as IL-1β, IL-18, TNF, IFN-gamma, or TLR agonists.

Serum IL-27 and IL-37 levels were positively correlated with pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α; appendix 2, figure 3-6) in both HU-naïve and HU-treated patients. Most of these cytokines have been described to be important in the pathogenesis of SCA (37,39, 44,74). Indeed, there is an association between the serum IL-27 or IL-37 levels and serum levels of pro-inflammatory cytokines; and between these cytokines and severity of the disease. Yang *et al*. reported a positive association between serum IL-37 and pro-inflammatory cytokines; and Lai *et al*. found that elevated serum IL-27 were positively correlated with disease severity in patients with rheumatoid arthritis (70-72, 75). Zhao *et al*. found that the plasma level of IL-37 was positively correlated with pro-inflammatory cytokines (IL-17A, TNF-α) and disease activity in rheumatoid arthritis patients (56). The anti-inhibitory role of IL-37 could be explained by two hypothesis from existing studies: either circulating IL-37 inhibits the actions of pro-inflammatory cytokines or their receptors, or IL-37 translocate to the nucleus where it interacts with SMAD3 pathway to interrupt transcription of pro-inflammatory cytokine genes (68, 76-78). A positive correlation between the pro- and anti-inflammatory cytokines suggests that these anti-inflammatory cytokines, IL-27 and IL-37 may have the role of reducing or limiting production and/or activities of the pro-inflammatory cytokine in SCA patients. However, there was no positive correlation between IL-27 or IL-37 and the clinical complications in the present study. The different etiologies in these disease entities may be accountable for the disparity. While sickle cell anemia is purely an inherited monogenic disorder in etiology, rheumatoid arthritis have an autoimmune

component (18, 75). Contrary to other researchers, who found significant reduction in the levels of TNF-α and IL-1β in HU-treated patients compared to the HU-naive patients, use of HU was not associated with a significant alteration in the levels of both pro and anti-inflammatory cytokines in the present studied (41). Difference in the dose of HU, the duration of use of HU or some other modulators of the disease may explain this discordance. Hydroxyurea, the only approved HbF-increasing drug has previously been suggested to suppress inflammation (79, 80). However, this study found that HU-treated patients had lower prevalence of leg ulcers thus, supporting previous report that include leg ulcers as an indication for use of HU (80). While most of our patients used a total dose of 1000mg per day, a few were on 500mg or 1500mg per day (appendix 2, figure 1 and 2). This finding suggests that the dose of HU required to give significant difference in each complication cannot be generalized rather may need to be individualized for each complication. This could be exemplified by Saad *et al*., who observed that the use of hydroxyurea for prevention of priapism in SCD patients was successful. However, it was importantly pointed out that the dose required to stop priapism was higher than that normally used in their clinic for frequent painful crisis treatment, which is usually lower than 20 mg/kg (81). The anti-inflammatory effect of HU could be supported by lack of significant difference in the serum level of pro-inflammatory cytokines (except IL-8) between the HU-treated SCA and the control groups. It would be reasonable to suggest that HU helps to maintain a balanced milieu at a state that is not significantly different from the baseline whereas in the absence of HU, inflammation is significantly enhanced. The anti-inflammatory cytokines in a similar way was not significantly different in the HU-treated patients compared to the controls. The pattern of the

two groups of cytokines observed between HU-treated patients and controls in the present study could be corroborated by positive correlation between the pro- and anti-inflammatory cytokine (appendix 2, figure 3-6).

There was a positive correlation between IL-27 and IL-37 in the 2 groups of SCA separately (appendix 2, figure 3A, 5A), hence both groups were analyzed together (figure 3). This indicates that HU may not significantly change the pattern or relationship between these 2 cytokines. A positive correlation between serum IL-27 and IL-37 in the SCA patients may suggest that the production of both cytokines may be induced by similar damage-associated molecular pattern (DAMP), a group of endogenous molecules derived from damaged cells and extracellular matrix degradation capable of promoting and exacerbating immune responses (55). DAMPs such as heme, free hemoglobin or other breakdown products of RBC may have a role to play in the production of both pro-inflammatory and anti-inflammatory cytokines. Heme and other products of hemolysis are elevated in patients with SCA and other hemolytic anemias. Studies have shown that plasma levels of heme ranges from 20-600 μM in patients with intravascular hemolysis such as SCD and paroxysmal nocturnal hemoglobinuria (PNH) (82). Therefore, the effect noted in the *in vitro* experiments in this study at a heme concentration of 30 μM could be considered pathological. This effect may be extended to other conditions with raised heme levels for example cardiopulmonary bypass, hemolytic blood transfusion reactions (83, 84).

Heme has a pro-inflammatory property as consistently reported by other researchers (85). Continuous hemolysis seen in patients with hemolytic anemias is associated with elevated IL-8 levels similar to the observation in both groups of SCA patients. In the i*n vitro* experiments in the present study, it was shown

that heme stimulates pro-inflammatory response, evidenced by increased production of IL-8 in both human neutrophils and monocytes. The secretion of other quantified pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α did not reach statistical significance (appendix 2, figure 7A-C and 8A-C). Though TNF-α and IL-1β were reported as important cytokines in the pathogenesis of SCD by some researchers who found elevated levels in SCD, some others did not find elevated levels in their cohort (41, 44). This supports the fact that elevated TNF-α level may be consistent with the acute pain crisis rather than the use of HU when compared with the steady state. Moreover, pre-stimulation of either neutrophils or monocytes with heme or rhIL-37 or rhIL-27 did not alter the concentration of these cytokines. Probably, heme alone may not be sufficient to enhance the secretion of this cytokines or that the threshold concentration necessary to significantly alter their secretion in neutrophils or monocytes was not reached. Elevated heme levels in hemolytic anemia and related conditions overwhelm hemopexin, a potent heme scavenger. This would subsequently overwhelm intracellular heme oxygenase and stimulate the upregulation of IL-8. Furthermore, excess heme stimulate monocytes to secret IL-8 with the major function of chemotaxis (18, 19). In addition, neutrophils produce IL-8, which help to stimulate self and other neutrophils in an autocrine and paracrine fashion respectively, hence contributing to acute inflammation. Interleukin-8 is responsible for neutrophilic chemoattraction to sites of endothelial injury. Neutrophils represent the most abundant innate immune cells and thereby play critical role in acute response of inflammation. IL-8 may play an important role in sickle cell anemia pathophysiology as earlier reported by Gonçalves *et al.* who suggested same as a maker of vaso-occlusion in SCA patients (40).

The anti-inflammatory role of both IL-27 and IL-37 was further strengthened by inhibition of inflammatory response induced by heme in cultures pre-stimulated with the rhIL-27 or rhIL-37. It is interesting to emphasize that the concentration of rhIL-27 and rhIL-37 added to the cultures were very high (in nanograms) compared to the median concentrations of both cytokines (in picograms) found in the SCA patients. This suggests that the circulating levels in these patients are lower than the concentration that may be sufficient to reduce or nullify the inflammatory effect of heme thus, emphasizing a probable role for these anti-inflammatory cytokines as immunotherapies in mitigating complications associated with SCA. From the foregoing therefore, the use of IL-27 or IL-37 before sudden rise of a heme concentration in hemolytic anemia could be protective and may ameliorate clinical complications mediated by innate immune response. This may be beneficial in acute episodes as well as chronic end organ morbidities. However, the inhibitory response of IL-27 and IL-37 was not obvious when these cytokines were added to heme pre-stimulated neutrophils and monocytes. This suggests that the presence of heme induces inflammatory pathways that could not be reversed or inhibited by the addition of either IL-27 or IL-37. Putting this together, it could be inferred that the chronic inflammatory state induced by heme and other products of hemolysis in patients with SCA and other hemolytic anemias may play a positive feedback in inducing secretion of the IL-8, a pro-inflammatory chemokine, which addition of IL-27 or IL-37 could not reverse (55, 57). Elevated levels of IL-37 and IL-27 maybe potent enough to regulate the excessive effect caused by the pro-inflammatory cytokines in a tendency to maintain the homeostatic balance and so prevent excessive tissue damage. However, their potential inhibitory effect at the

circulating concentration may not be sufficient to neutralize the effect of heme, as the latter is an inciting DAMP that initiated the inflammation cascade in these patients. Therefore, the probable use of IL-27 or IL-37 at a high concentration before sudden rise in circulating heme level may be preventive rather than therapeutic.

5. CONCLUSIONS

Our results show that IL-27 and IL-37 levels are positively correlated in SCA patients and serum IL-27 and IL-37 are elevated in HU-naive patients compared to the controls. Furthermore, the use of HU did not significantly alter the secretion of the both anti- and pro-inflammatory cytokines except IL-8 in the HU-treated SCA patients compared to controls thus, supports the anti-inflammatory action of HU. In *in vitro* studies, heme at a concentration similar to pathological levels seen in hemolytic anemias such as SCA is a potent stimulus of IL-8 in neutrophils and monocytes. The marked up regulation of the pro-inflammatory pathway(s) leading to secretion of IL-8 in neutrophils and monocytes could be significantly suppressed by the use of IL-27 or IL-37 before sudden rise in the concentration of heme. IL-27 and IL-37 may play a regulatory role on the pro-inflammatory pathways, as suggested by the *in vitro* studies. This role is probably sufficient to prevent further cellular or tissue damage but not potent enough to prevent inflammation. These cytokines may be immunotherapeutic targets in such scenarios.

Limitation of the study: The study is a cross-sectional study hence caution must be taken in interpreting the results. In addition, because of the numerous modulators of SCA, there is need to carry out similar study in other group of patients with a different ethnic / genetic and environmental characteristics; preferably as a prospective study.

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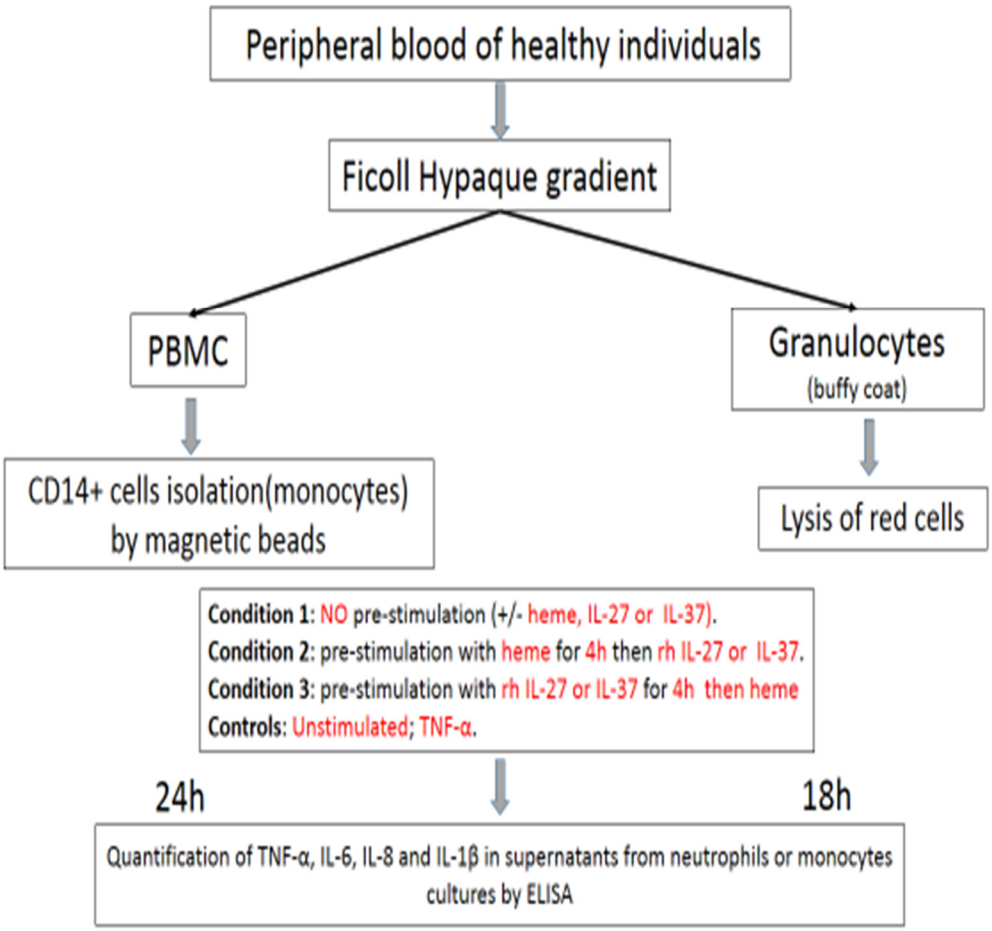
7. APPENDIX

Appendix 1: Materials and Methods

Figure 1. Flow chart for *in vitro* assay



PBMC Peripheral blood mononuclear cells.

Appendix 2: Results

Figure 2A: The duration of treatment with hydroxyrea in the hydroxyrea-treated SCA patients. Of the 47 HbSS patients on hydroxyurea, a majority 83% (39) had been on Hu for more than 12 months, 11% (5) for 3-6 months.

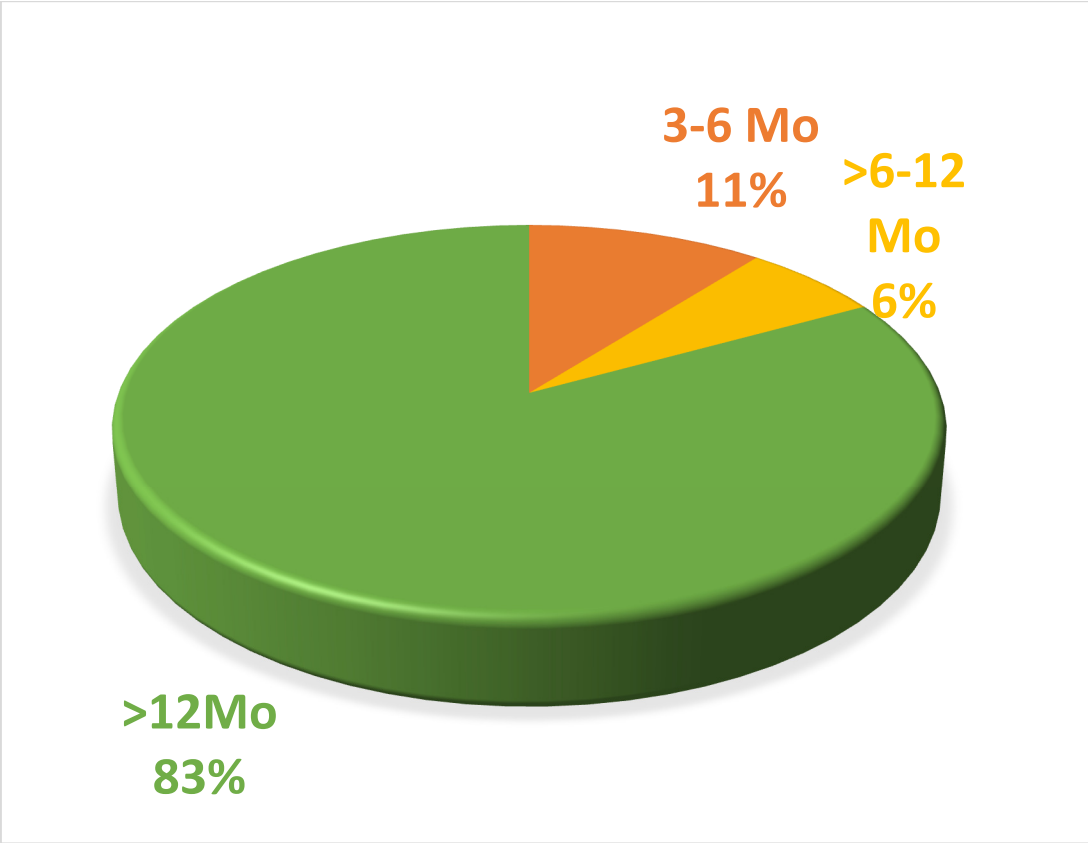


Figure 2B: The dose of hydroxyrea used by the Hydroxyurea-treated SCA patients. Of the 47 HbSS patients on hydroxyurea, a majority 74% (35) had been regular on oral 1000g of HU, daily and 22% (10) were on 500g of HU daily.

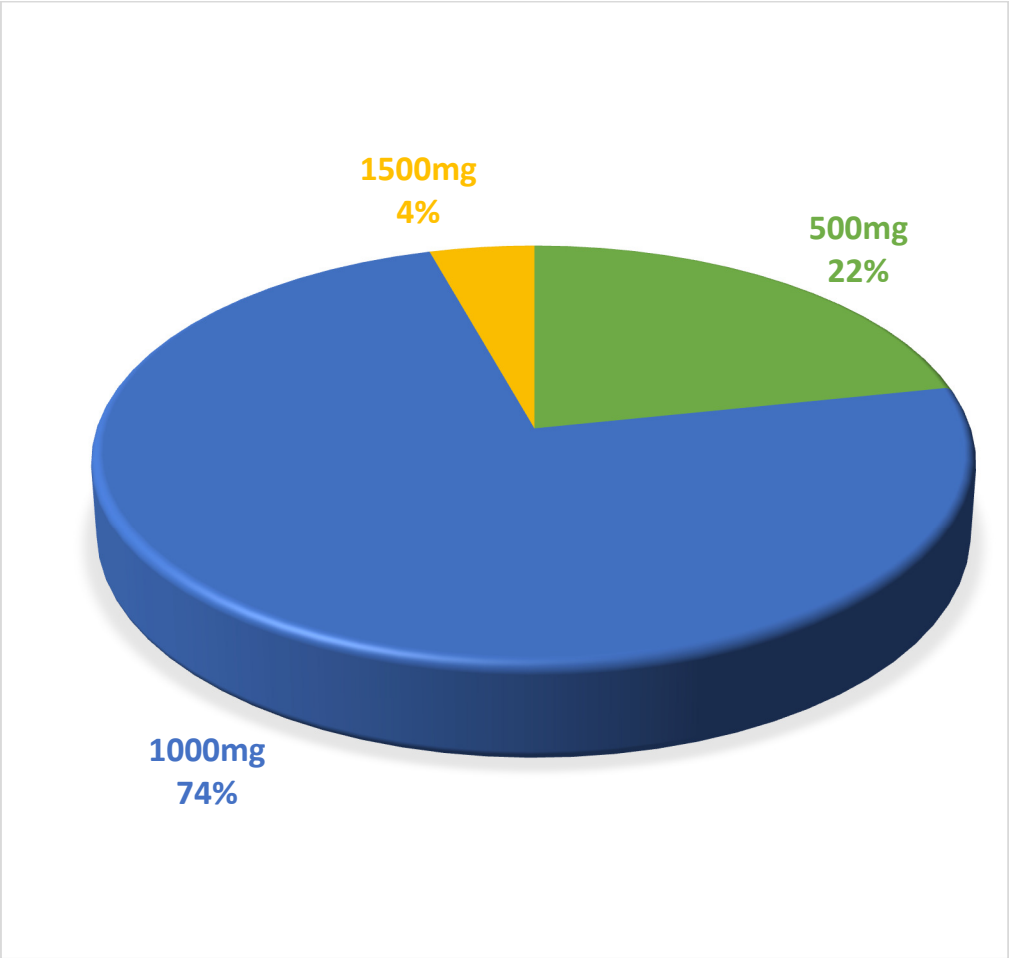


Figure 3: Correlation of serum IL-37 with IL-27, IL-1β, IL-6, IL-8, TNF-α and TGF-β in the HbSS patients. n=35; Spearman correlation for non-parametric data.

Serum IL-37 and IL-27 were strongly and positively correlated, p<0.0001 and r=0.831among the 35 hydroxyurea-naïve SCA patients (figure A). Serum IL-37 level was positively correlated with TNF- α level, IL-1 β , IL-6 and IL-8 ($p = 0.0004$, $p < 0.0001$, $p = 0.0007$, $p = 0.0001$ respectively; figure B-E). However, the correlation of IL-37 with TGF- β was not significant (p=0.412; figure F)

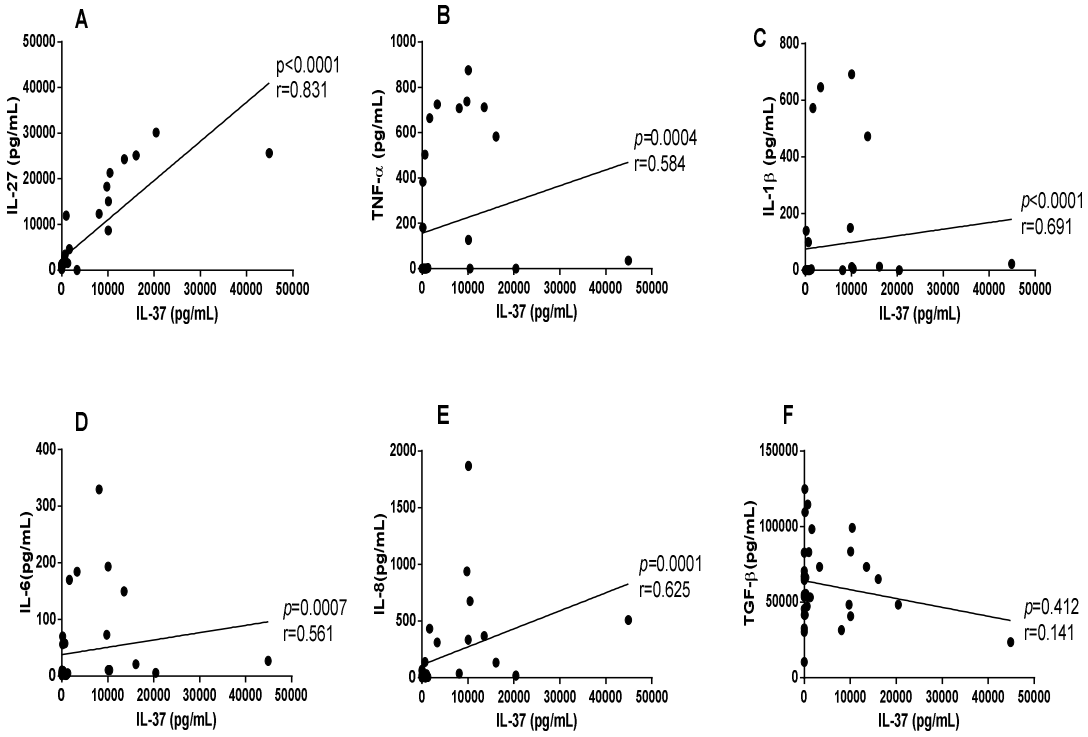


Figure 4: Correlation of serum IL-27 with IL-1β, IL-6, IL-8, TNF-α and TGF-β in the HbSS patients. n=35; Spearman correlation for non-parametric data.

Serum IL-27 level was positively correlated with TNF- α level, IL-1 β , IL-6 and IL-8 (p = 0.017, p <0.002, p = 0.013, p =0.001 respectively; figure A-D). However, the correlation of IL-27 with TGF- β was not statistically significant (p=0.575; figure E).

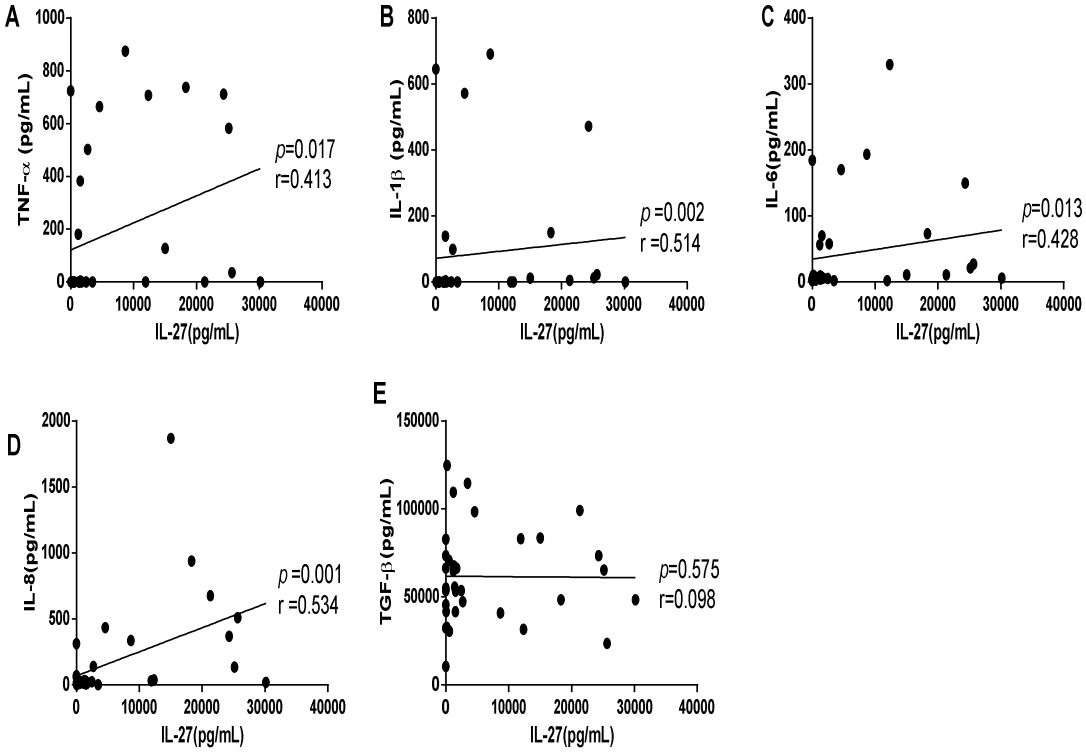


Figure 5: Correlation of serum IL-37 with IL-27, IL-1β, IL-6, IL-8, TNF-α and TGF-β in the HbSSHU patients. n=42; Spearman correlation for non-parametric data.

Similar to the hydroxyurea-nafor non-parametric data. IL-8, TNF-GF-β in the HbSS patientsients;5(115).5).md IL-27 among the hydroxyurea-treated SCA patients, *p*<0.0001 and r = 0.872 (figure A). TNF-α, IL-1β, IL-6 and IL-8, were positively correlated with IL-37 (*p*<0.0001, *p*=0.002, *p*=0.0003 and *p*=0.0009 respectively; figure B-E).

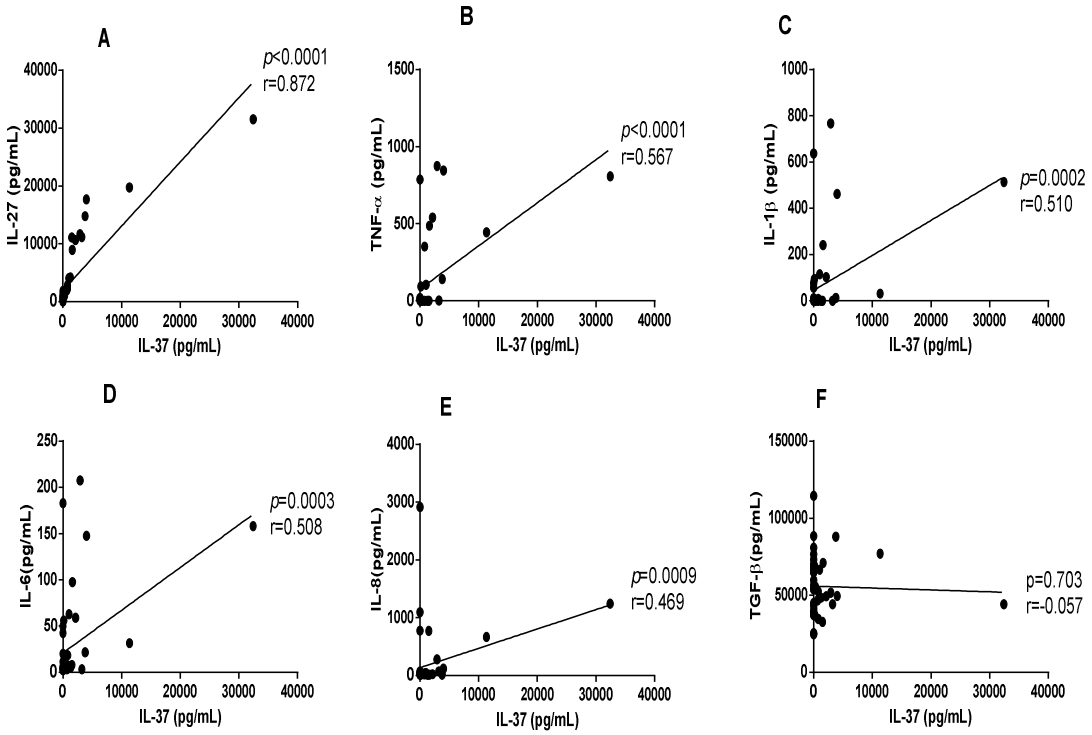


Figure 6: Correlation of serum IL-27 with IL-1β, IL-6, IL-8, TNF-α and TGF-β in the HbSS patients. n=42; Spearman correlation for non-parametric data.

When IL-27 was correlated with TNF- α , IL-1 β , IL-6 and IL-8 in the hydroxyurea-treated sickle cell anemia patients, there was a significant positive correlation between IL-27 and TNF- α (p<0.017, figure A). Correlation of IL-27 and IL-1 β , IL-6 and IL-8, was positive (p=0.002, p<0.013 and p=0.001 respectively; figure B-D).

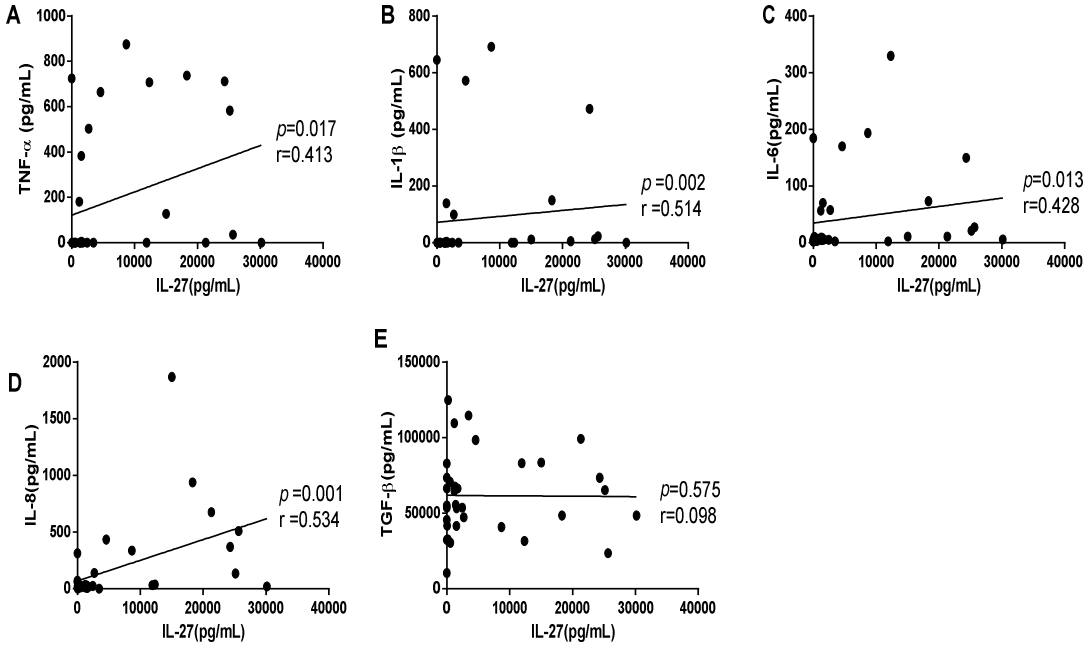


Figure 7. Effect of IL-27 and IL-37 on heme- induced production of pro-inflammatory cytokine in neutrophils (n=5).

In neutrophil cultures, 4 hours pre-stimulation with rhIL-27 or rhIL-37 before heme was added, yielded a significantly higher secretion of TNF-α compared with incubation with heme alone, p= < 0.01 in each case (figure A). There was no significant difference in the secretion of IL-1β and IL-6 (figure B and C). HEME/IL27, 4-hour pre-stimulation before addition of rhIL-27; HEME/IL37, 4-hour pre-stimulation with heme before addition of rhIL-37; IL27/HEME, 4-hour pre-stimulation with rhIL27 before addition of heme; IL37/HEME, 4-hour pre-stimulation with rhIL37 before addition of heme; TNF/HEME, 4-hour pre-stimulation with rhTNF-α before addition of heme.*p < 0.05, **p < 0.01, ***p < 0.001.

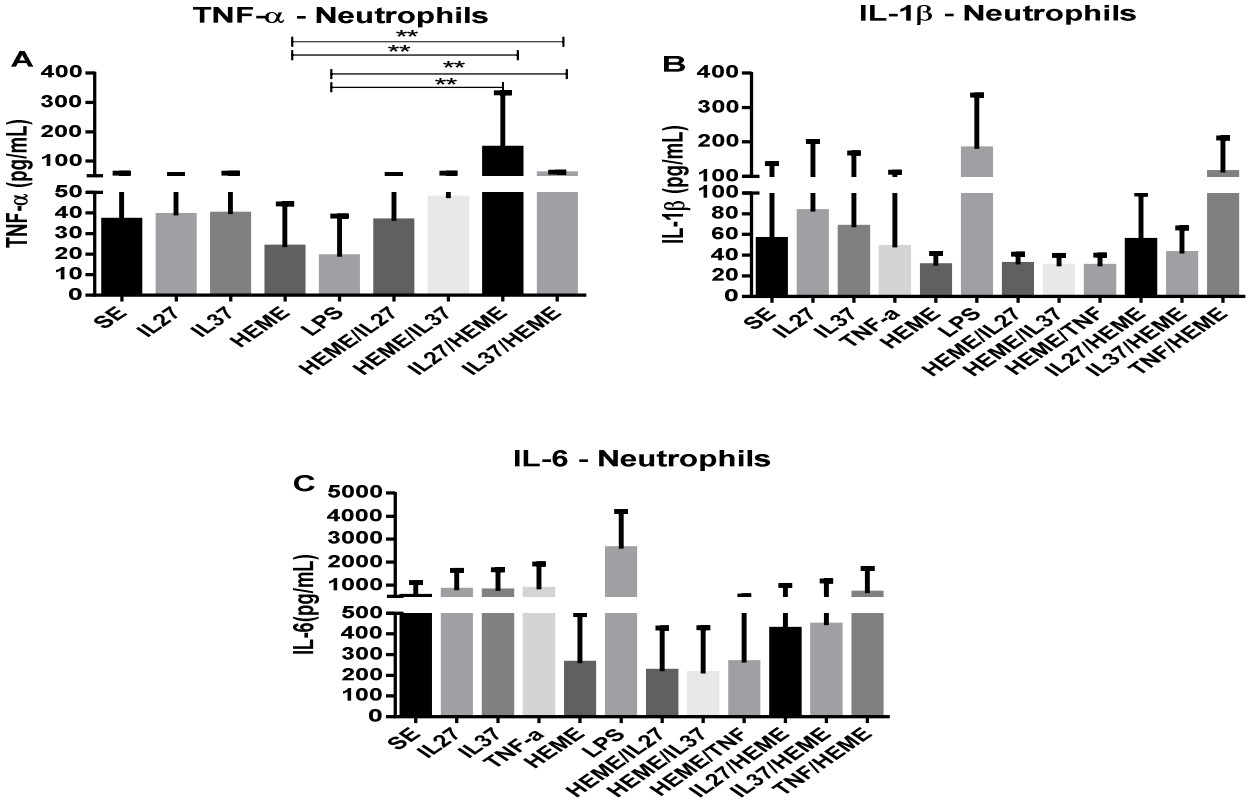
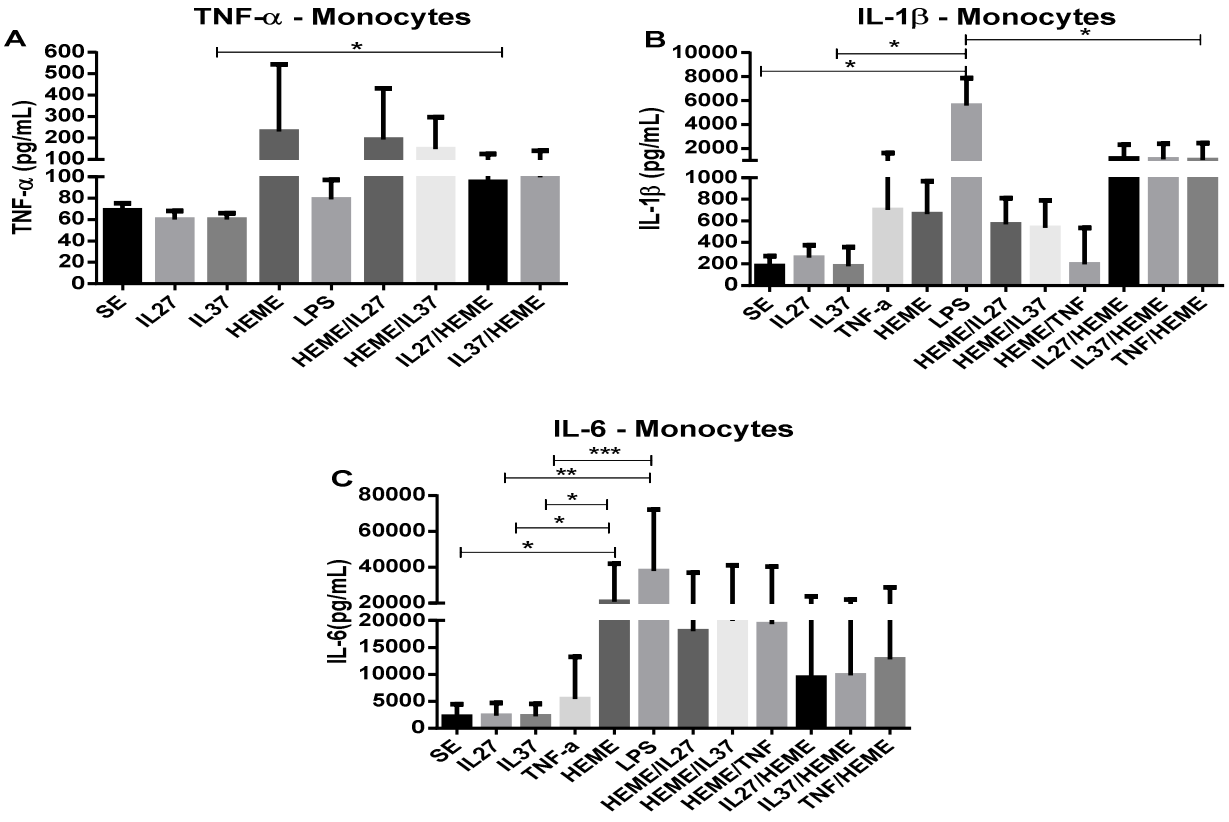
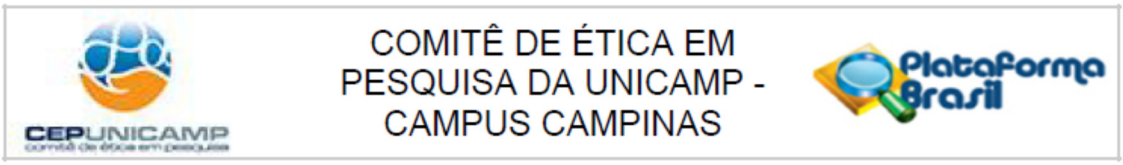


Figure 8. Effect of IL-27 and IL-37 on heme- induced production of pro-inflammatory cytokine in monocytes (n=5).

In monocyte cultures, 4 hours pre-stimulation with rhIL-27 before heme was added, yielded a significantly higher secretion of TNF-α compared with incubation with rhIL-37 alone, $p = < 0.05$ in each case (figure A). Pre-stimulation with heme before addition of rhIL-27 or rhIL-37 and pre-stimulation with cytokines before addition of heme did not yield significant alteration in the concentration in TNF-α, IL-1β and IL-6 (figure A, B and C). HEME/IL27, 4-hour pre-stimulation before addition of rhIL-27; HEME/IL37, 4-hour pre-stimulation with heme before addition of rhIL-37; IL27/HEME, 4-hour pre-stimulation with rhIL27 before addition of heme; IL37/HEME, 4-hour pre-stimulation with rhIL37 before addition of heme; TNF/HEME, 4-hour pre-stimulation with rhTNF-α before addition of heme. .*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$.



Appendix 3: Ethical approvance



PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Avaliação da resposta anti-inflamatória em pacientes com anemia falciforme: o papel da IL-27 e IL-37

Pesquisador: Adekunle Emmanuel Alagbe

Área Temática:

Versão: 3

CAAE: 52941315.6.0000.5404

Instituição Proponente: Faculdade de Ciências Medicas - UNICAMP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.479.495

Apresentação do Projeto:

Trata-se de uma emenda para exclusão da instituição co-participante Fundação de Hematologia e Hemoterapia do Estado de Pernambuco - HEMOPE.

Objetivo da Pesquisa:

Os objetivos não foram alterados em relação ao projeto original.

Avaliação dos Riscos e Benefícios:

Os riscos e benefícios não foram alterados em relação ao projeto original.

Comentários e Considerações sobre a Pesquisa:

Não há comentários adicionais em virtude desta emenda.

Considerações sobre os Termos de apresentação obrigatória:

Foi apresentado o arquivo com as Informações Básicas do projeto "PB_INFORMAÇÕES_BÁSICAS_685376_E1.pdf 23/03/2016 13:49:50"

Recomendações:

Endereço: Rua Tessália Vieira de Camargo, 126
Bairro: Barão Geraldo **CEP:** 13.083-887
UF: SP **Município:** CAMPINAS
Telefone: (19)3521-8936 **Fax:** (19)3521-7187 **E-mail:** cep@fcm.unicamp.br

Conclusões ou Pendências e Lista de Inadequações:

Aprovada a exclusão da instituição co-participante HEMOPE.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_685376 E1.pdf	23/03/2016 13:49:50		Aceito
Outros	Resposta_ao_Comite_de_Etica_em_Pesquisa_da_Unicamp.pdf	09/03/2016 15:49:43	Adekunle Emmanuel Alagbe	Aceito
Projeto Detalhado / Brochura Investigador	projeto.pdf	09/03/2016 15:49:05	Adekunle Emmanuel Alagbe	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_paciente_corrigido.pdf	09/03/2016 15:48:46	Adekunle Emmanuel Alagbe	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_controle.pdf	09/03/2016 15:48:34	Adekunle Emmanuel Alagbe	Aceito
Folha de Rosto	FR_Adekunle.pdf	22/12/2015 15:56:18	Adekunle Emmanuel Alagbe	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Biorrepositorio_mest_Adekunle.pdf	22/12/2015 15:55:12	Adekunle Emmanuel Alagbe	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CAMPINAS, 06 de Abril de 2016

Assinado por:
Renata Maria dos Santos Celeghini
(Coordenador)

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