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Cytokine and chemokine signatures of steady-state sickle cell disease in African children: a cross-sectional study

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Running Title: Inflammatory markers of SCD in African children

Abstract

Background: Sickle cell disease (SCD) is characterized by chronic inflammation, yet the inflammatory landscape defining the clinically defined steady state remains poorly characterized, particularly in African populations where the disease burden is highest. Understanding baseline cytokine and chemokine signatures during the steady state is important for distinguishing physiological disease equilibrium from transitions toward acute complications.

Methods: We conducted a cross-sectional study of 129 children aged 2–17 years in Ghana, including 63 children with SCD in steady state and 66 healthy controls. Serum concentrations of 23 cytokines and chemokines were quantified using multiplex immunoassays alongside full blood count parameters. Comparative analyses and cytokine ratio assessments were performed to characterize steady-state SCD inflammatory signatures.

Results: Most pro-inflammatory cytokines and chemokines were significantly elevated in children with SCD compared with controls. However, IL-12p70 and IL-23p40 levels were significantly reduced in the SCD cohort, while CCL2 and IL-1β showed no significant differences between groups. Ratio analyses revealed lower IL-12p70/IL-4, IL-23p40/IL-4, and IL-1β/IL-10 ratios, alongside increased IL-1α/IL-4, IL-17A/IL-10, IL-3/IL-10, CCL3/IL-10, and GM-CSF/IL-10 ratios, indicating an imbalanced inflammatory milieu dominated by pro-inflammatory signals. Discriminant analyses using cytokine and hematological parameters further demonstrated clear separation between children with SCD and controls.

Conclusions: Steady-state SCD in African children is characterized by a distinct cytokine and chemokine profile marked by persistent pro-inflammatory activation alongside regulatory counterbalances. Reduced ratios of IL-12p70 and IL-23p40 relative to anti-inflammatory cytokines may contribute to maintaining the steady-state inflammatory equilibrium. These findings provide reference signatures that may support future studies investigating inflammatory biomarkers.

Introduction

Sickle cell disease (SCD) is a haematologic disorder that predominantly affects people of African descent. The sickle haemoglobin mutation is believed to have evolved as an adaptive response that provides protection against malaria.[1, 2] The disease remains highly prevalent in Africa, with sub-Saharan Africa accounting for approximately 80% of newborns worldwide born with the condition. In Ghana, about 2% of newborns are affected, with approximately 55% presenting with the homozygous form of the disease.[3] Individuals with SCD experience significantly reduced life expectancy globally, with even greater reductions in low-income countries.[4] The polymerization of deoxygenated haemoglobin leads to the formation of sickled red blood cells and subsequent vaso-occlusive crises (VOC), which are central features of disease pathophysiology.[5] In addition to vaso-occlusion, individuals with SCD experience increased haemolysis, anaemia, vasculopathy, oxidative stress, and chronic inflammation[5]

SCD exhibits substantial clinical heterogeneity. Some patients experience frequent vaso-occlusive crises and hospitalization,[6] whereas others remain largely asymptomatic for prolonged periods, commonly referred to as the steady state.[7] This variability suggests that environmental and endogenous factors influence disease severity and presentation. Neutrophils from individuals with SCD often display elevated levels of activating markers such as CD11B/CD18 and CD64,[8] which promote adhesion to the vascular endothelium and facilitate the attachment of sickled erythrocytes, contributing to the development of VOC. Inflammatory cytokines and monocytes are also implicated in the development of vaso-occlusive events.[9] Cytokines play complex roles in immune regulation; while essential for coordinating immune responses, excessive production can lead to tissue damage and inflammatory disease.[10] Individuals with SCD typically exhibit increased circulating immune cell populations, including monocytes, polymorphonuclear cells, and platelets, which are associated with elevated secretion of major pro-inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α.[11, 12] Dysregulated cytokine production has been implicated in a range of inflammatory conditions including cancer and atherosclerosis.[13] Chemokines, a subset of cytokines, mediate inflammation by promoting leukocyte recruitment and adhesion to the vascular wall through integrin-mediated mechanisms.[14] For example, IL-8 (CXCL8) enhances the adhesion of sickled red blood cells to the endothelium through activation of α4β1 integrins on sickle reticulocytes and endothelial fibronectin.[15, 16] Furthermore, cytokines such as IL-4, IL-1β, IL-6, IL-8, IL-10 and TNF-α contribute to the activation of leukocytes, platelets and endothelial cells, reinforcing a persistent pro-inflammatory state.[17, 18]

Increasing knowledge of cytokine biology in health and disease has led to interest in the balance between pro-inflammatory and anti-inflammatory cytokines as indicators of disease progression.[19-23] Several studies have suggested that the ratio of pro-inflammatory to anti-inflammatory cytokines may serve as an important indicator of how a disease develops and progresses.[21, 24-26] However, although cytokine dynamics during vaso-occlusive crisis have been investigated in several studies, the inflammatory characteristics that define the clinically defined steady state remain poorly understood.

In this study, we investigated the cytokine environment in children with SCD during the asymptomatic steady state and compared cytokine profiles with those of healthy controls. We further examined ratios of pro-inflammatory to anti-inflammatory cytokines to determine whether the steady-state immune environment is polarized toward pro-inflammatory or anti-inflammatory responses. We also compared cytokine levels and full blood count parameters among participants receiving disease management therapies including folic acid, multivitamins, vitamin C, and hydroxyurea. Finally, we developed a model based on cytokine levels and hematological parameters to assess their ability to discriminate between children with SCD and healthy controls.

Methods

Study Participants

A cross-sectional study was conducted among children aged 2-17 years following consent by parents or guardians. Assent was additionally obtained from children aged 5 years and above in accordance with the approved ethical protocol. Children with SCD in steady state and healthy non-SCD children were selected. The selection of the steady state patient was based on the criteria employed by Akinola and colleagues who described the steady state as, “the period free of crisis extending from at least three weeks since the last clinical event and three months or more since the last blood transfusion, to at least one week before the start of a new clinical event”.[27]

The participants were recruited at the Ho Teaching Hospital (Children with SCD) and the Kodzobi Basic School (Control), both in the Volta Region of Ghana. A person with sickle cell disease was defined as one who tests positive for sickling of red cells under reduced oxygen tension and with the haemoglobin phenotype S, SC, or SF by cellulose acetate paper alkaline (pH 8.6) electrophoresis. Participants who tested positive for malaria or had febrile illness, fever, or Glucose-6-phosphate dehydrogenase deficiency were excluded from the study. Clinical data was

collected from the electronic folders of the participants with SCD and through interviews by the resident doctor. Data on the use of SCD disease management therapies such as folic acid, multivitamins, Vitamin C, and hydroxyurea were also retrieved from patient folders. Any participant who presented with a febrile illness or had a history of any febrile illness in the past week were also excluded. Following the exclusion, 63 children with sickle cell disease and 66 healthy children without SCD were selected for the study.

Sample Collection and Storage

A full blood count (28 parameters) was conducted using the Sysmex XN-550 (6-parts) analyser (Sysmex Corporation, Kobe, Japan at the Haematology unit of the Ho Teaching Hospital. Venous blood was collected with the BD vacutainer blood collection system by using aseptic techniques from any accessible ante-cubital vein. A tourniquet was applied on the upper arm to create stasis and then the area was disinfected. A vacutainer needle fitted to a holder was inserted into the vein and then about two (2) mL of the blood was collected into K_3-EDTA tubes for full blood count analysisand 3 mL in a serum separator tube which was allowed to clot. To collect the Serum, the clotted blood was centrifuged at 3000 ×g for 10 minutes at 4°C after which the separated serum was transferred into cryotubes. Flow cytometric analysis was performed on site (Hospital Laboratory) where samples were collected. The serum was transported immediately to the research the laboratory on ice and stored at -80°C for later analysis.

Determination of Sickling Status and Hb phenotype

The presence of HbS was confirmed using 2% (W/V) sodium metabisulphite as a reducing agent. Two (2) µL of blood was placed on a microscope slide, mixed with 50µl of 2% (W/V) sodium metabisulphite reagent and covered with a cover slip. With reduced oxygen tension, RBCs containing HbS turn sickle within 30 to 40 minutes of incubation at room temperature. The presence of sickled RBCs was confirmed by microscopy at x40 objective magnification [28].

Hb phenotype was determined using cellulose acetate alkaline electrophoresis (pH 8.6). Haemolysate (haemoglobin solution) was prepared by saline-washing of RBCs and lysing with 0.5% (v/v) Triton X-100 in 100 mg/L potassium cyanide. Electrophoresis was run on cellulose acetate membrane soaked in Tris EDTA Borate (TEB) buffer (pH 8.4 - 8.6) by passing direct current delivering 350V at 50mA through the electrophoresis tank for 25 minutes. Bands of haemoglobin travelled from the cathode (Haemoglobin is negatively charged at alkaline pH) to the anode and the haemoglobin phenotypes were determined based on a control showing

haemoglobins C, S, F, and A (from cathode to anode). The haemoglobins were separated based on net charge and molecular weight [29]. The HbSF category reflects electrophoretic phenotype classification based on cellulose acetate alkaline electrophoresis rather than molecularly confirmed genotype and may include individuals with HbSS and detectable elevated HbF.

Immunological Assays

A human 21-plex (CCL17, CCL2, CCL3, CCL4, G-CSF, GM-CSF, IFN-β, IFN-γ, IL-1α, IL-10, IL-12, IL-17/IL-17A, IL-18, IL-1B, IL-2, IL-3, IL-4, IL-6, IL-8, TNF-β, TNF-α) assay kit and 2-plex (CXCL-10 and IL-12p70) kit were used to quantify levels of the cytokines (R&D Systems, United States) in a 96-well plate using a magnetic bead-based multiplex assay. Reagents, standards, and sample dilutions were prepared according to the manufacturer's instructions. Serum samples were thawed on ice and diluted per manufacturer's recommendations before analysis. All blanks, standards and samples were run in duplicates on each plate. The plates were read using the LUMINEXR 200TM system, which runs on the Xponent 3.1 software. The concentration of all the analytes were within the limits of detection of the assay. Analysis of the results was done using the median fluorescent intensity (MFI) values. For further interrogation of the data, the cytokines were classified as follows: Pro-inflammatory (CCL2, CCL3, CCL4, CCL17, CXCL8/IL-8, CXCL-10, G-CSF, GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-17/IL-17A, IL-18, IFN-β, IFN-γ, TNF-β, TNF-α, IL-12p70, IL-23p40), and anti-inflammatory (IL-4, and IL-10) [6, 30].

Statistical Analysis

All data and statistical analyses were performed using R version 4.3.1 and the R-studio (2023.06.01). R packages such as ggpubr was used to draw the box plots comparing data between SCD and controls. Kruskal Wallis with multiple comparisons and Mann Whitney tests were undertaken. The Kruskal Wallis test was followed by Wilcoxon pairwise comparison post-hoc analysis to compare between the Hb phenotypes (HbS, HbSC, HbSF) and controls. Analysis of variance and independent sample t-test was used to compare normally distributed datasets. Corrplot and Hmisc in R were used for the correlation plots. The correlation cut-offs were based on the rule of thumb for interpreting the size of a correlation coefficient as suggested by Hinkle and colleagues [31]. Briefly, correlations were analyzed as follows: 0.90 to 1.00 (-0.90 to -1.00) - very high positive (negative) correlation; 0.70 to .89 (-0.70 to -0.89) - high positive (negative) correlation; 0.50 to 0.69 (-0.50 to -0.69) - moderate positive (negative) correlation; 0.30 to 0.49 (-0.30 to -0.49) - Low positive (negative) correlation; and 0.00 to 0.29 (0.00 to -0.29) - negligible correlation. Correlation analyses were exploratory and intended to identify potential associations

between inflammatory and hematological biomarkers. Given the large number of comparisons performed, findings were interpreted cautiously due to the potential for false positive associations. Boxplots were used to present comparative tests, whilst correlation plots were used to visualize correlations between the biomarkers. MASS and tidyverse packages in R were used for exploratory linear and quadratic discriminant analyses to assess separation between SCD cases and controls based on selected cytokine and full blood count variables. These analyses were intended as exploratory within-dataset assessments rather than validated predictive modelling, as no independent training or validation cohort was available. Comparisons by heatmaps were generated using ComplexHeatmap in R. A test with *p*-value less than 0.05 was considered statistically significant.

Results

Demographic and Patient Characteristics

A total of one hundred and twenty-nine (129) children aged 2-17 years were recruited to the study comprising 63 children with SCD in steady state and 66 healthy non-SCD children. The proportions according to the disease state are provided in Table 1. The mean ages of the participants in the SCD case group was 9.2±4.4 (females 11.8±3.9 and males 9.6±3.0) years and of the control group, 11±3.7 (females 10.5±5.5 and males 8.1±4.1) years. The mean age difference between cases (9.24±4.41) and controls (10.76±3.66) was weakly significant (p=0.046). Haemoglobin phenotypes across the study participants were A, AC, AS (Controls), S, SC, and SF (SCD) with proportions of 40.3%, 7.8%, 3.1%, 23.3%, 11.6% and 14.0%, respectively. The mean ages of the participants did not differ statistically between the Hb phenotypes, F (5, 129) = 2.08, p = .072, and use of hydroxyurea (HU), F (1, 63) = 0.01, p = .937. Some participants with SCD were on regular multivitamins, folic acid, Zincovit, Vitamin C, and Hydroxyurea. Table 1 below summarizes the information above. HU induces production of HbF and in the very young, HbF may also be present in the early days of life. In our study however, the participants with HbF included older participants. Out of the 18 participants with the HbSF phenotype, only 9 were receiving hydroxyurea. Of these, one had been on treatment for 1 month and another for 3 months (15–20 mg/kg/day), while the remaining 7 had received hydroxyurea for 12–20 months (5 mg/kg/day). Across the wider SCD cohort, 12 participants were receiving hydroxyurea, of whom 3 were aged 5–10 years and 9 were aged 11–13 years. Quantitative HbF levels were not measured in this study.

Table 1

Full blood count and inflammatory cytokine levels in the steady state of SCD

The erythrocytes (RBC), haemoglobin (HGB), haematocrit (HCT) and mean platelet volume (MPV) were decreased in the SCD group whereas all other full blood count parameters were increased in the SCD group (Figure 1a) compared to the control group. Table 2 and The levels of the pro-inflammatory cytokines (CCL3, CCL4, CCL17, CXCL8/IL-8, CXCL-10, G-CSF, GM-CSF, IL-1α, IL-2, IL-3, IL-6, IL-17/IL-17A, IL-18, IFN-β, IFN-γ, TNF-β, TNF-α) and anti-inflammatory cytokines (IL-4, and IL-10) were increased (p<0.01 for all) during steady-state disease. Levels of CCL2 and IL-1β were found not to differ significantly (p>0.05) between the SCD group and controls. The pro-inflammatory cytokines IL-23p40 and IL-12p70 were lower among the SCD group compared to the controls (p<0.01 in both).

Table 2

Figure 1

Steady-state SCD is characterized by an imbalanced cytokine milieu polarized towards pro-inflammation.

Ratios of the measured pro-inflammatory cytokines against IL-4 and IL-10 were determined (Figures 2). The ratios of TNFα, TNFβ, IL-8, IL-2, IL-18, IFN-β, IFN-γ (Suppl. Figure A1), CCL4, CCL17, CXCL10 (Suppl. Figure 1C), and G-CSF to IL-4 (Suppl. Figure 1B) were found to be similar among the SCD subgroups and the controls (p>0.05). Ratios of IL-12p70, IL-23p40, IL-1β, IL-6, and CCL2 to IL-4 (Figure 2A1) were found to be lower (p<0.05) while ratios of IL-1α, IL-17A, IL-3 (Figure 2A1), CCL3 (Figure 2C), and GM-CSF (Figure 2B) to IL-4 were higher (p<0.05) among the SCD group compared to the controls.

The ratios of IL-6, TNFα, TNFβ, IL-2, IL-18, IFN-γ (Suppl. Figure 1A2), CCL2, CCL17, CXCL10 (Suppl. Figure 1C), and G-CSF against IL-10 (Suppl. Figure 1B) were found to be similar among the SCD group and the controls (p>0.05). Ratios of IL-12p70, IL-23p40, and IL-1β to IL-10 (Figure 2A2) were found to be lower (p<0.01) while ratios of IL-1α, IL-17A, IL-3, IL-8 (Figure 2A2), CCL3, CCL4 (Figure 2C), and GM-CSF against IL-10 (Figure 2B) were higher (p<0.05) among the SCD group when compared to the controls. Although the IL-8/IL-10 ratios (Figure 2A2) were higher among the SCD group, only those with HbS had higher ratios (p<0.01) compared to the controls while levels were the same between those with HbSC, HbSF, and controls (p>0.05). Although the IL-23p40/IL-10 ratio (Figure 2A2) was generally lower among the SCD group than the controls, no significant difference was observed in the IL-23p40/IL-10 ratios between HbSF and controls (p>0.05). However, the ratios were significantly lower between HbS and controls (p<0.01) and HbSC and controls (<0.05). The IL-23p40/IL-10 ratio was also significantly lower among HbS compared to HbSC. The cytokine ratio analysis suggests a relative pro-inflammatory skew involving IL-1α, IL-17A, IL-3, CCL3, and GM-CSF in children with SCD.

Reduced IL-12p70, IL-23p40, and IL-1β ratios relative to IL-4 and IL-10 are features of the steady-state inflammatory profile in children with SCD.

The pro-inflammatory to anti-inflammatory cytokine ratio analysis (Figure 2) showed reduced ratios of CCL2, IL-12p70, IL-23p40, and IL-1β relative to IL-4 (p<0.05 for all). Reduced ratios of IL-12p70, IL-23p40, and IL-1β relative to both IL-4 and IL-10 were also observed in the SCD

cohort. These findings suggest that selected inflammatory pathways may be differentially regulated within the steady-state inflammatory environment of children with SCD.

IL-4 and IL-10 could regulate inflammation during steady-state SCD

IL-4 and IL-10 were increased in the patients with SCD as opposed to the controls. The IL-4/IL-10 ratio (Figure 2D) suggests that in the child with steady-state SCD, increased IL-4 and IL-10 levels may play significant roles in regulating inflammation and maintaining the steady state.

Figure 2

Correlations between cytokines and full blood count biomarkers of SCD patients

Some low and moderately positive and negative correlations were observed between some of the biomarkers assayed (figure 3). A low reciprocal correlation and a low positive correlation (Figure 3ii) was observed among the SCD group between CCL3 and MCV (r= 0.32 $p<0.05$,) and CXCL10 and MCV (r=0.31, $p<.05$) respectively. The low positive correlations were observed between PLT and IL-17A (r=0.33, $p<0.05$); IL-23p40 (IL-12) and IFN-γ (r=0.36, $p<0.05$); basophil counts and G-CSF (r=0.43, $p<0.05$); eosinophil counts and CXCL10 (r=0.45, $p<0.05$); neutrophil count and IL8 (r=0.31, $p<0.05$); basophil count and IL8 (r=0.43, $p<0.05$); eosinophil counts and IL18 (r=0.31, $p<0.05$); basophil and eosinophil counts (r=0.40, $p<0.05$); basophil and lymphocyte counts (r=0.41, $p<0.05$); and basophil and monocyte counts (r=0.40, $p<0.05$). On the other hand, high positive correlations were observed between basophil and lymphocyte counts (r=0.81, $p<0.05$), and basophil and monocyte counts (r=0.84, $p<0.05$). There was also a high positive correlation between lymphocyte and monocyte counts (r=0.71, $p<0.05$).

Exploratory discriminant analysis of cytokine and hematological parameters

Linear discriminant analysis was initially applied to distinguish between cases and controls using the untransformed data; however, the Box's M test for equality of covariance matrices was not satisfied ($p<0.0001$). Linear and quadratic discriminant analyses were therefore performed using normalized CCL3, IFN-β, IL-2, IL-4, IL-8, TNF-β, neutrophil (NEUTABS), lymphocyte (LYMPHABS), monocyte (MONOABS), eosinophil (EOSABS), and basophil (BASOABS) counts, as well as IL-10, IL-17A, TNF-α, G-CSF, GM-CSF, CCL17, and CCL4. These exploratory

analyses demonstrated separation between SCD cases and controls within the study dataset. Because no independent training or validation cohort was available, these analyses should be interpreted as exploratory within-dataset assessments rather than validated predictive models.

Canonical Discriminant Function Coefficients

The canonical discriminant function coefficients describe the relative contribution of variables within the fitted exploratory discriminant model.

D1 = 0.018 + 0.572CCL3 - 0.426IFN_B + 0.307IL_2 + 0.628IL_4 - 0.278IL_8 + 0.23TNF_B + 0.604NEUTABS + 0.419LYMPHABS - 0.199MONOABS + 0.281EOSABS + 0.033BASOABS - 0.326IL_10 + 0.249IL_17A + 0.253TNF_A - 0.118G_CSF + 0.276GM_CSF+ 0.083CCL17 + 0.414CCL4

Within the fitted model, IFN-β, IL-8, MONOABS, IL-10, and G-CSF had negative parameter estimates, whereas the remaining variables had positive parameter estimates. Positive and negative coefficient directions reflect variable behaviour within the fitted model and should not be interpreted as standalone diagnostic indicators.

Cytokines and haematological parameters associated with gender, use of hydroxyurea, folic acid, multivitamins, and vitamin C among children in steady state SCD.

Male children with SCD were shown to be associated with lower IL-1α, IL-4, and TNF-β, and higher PDW values compared to the females (p<0.05 in all) (Table 3). The use of hydroxyurea was associated with reduced IL-12p70, IL-23p40, and RDW. Conversely, an increased IL-17A, MCH, and MCV were associated with the use of hydroxyurea in children with asymptomatic SCD (p<0.05 in all). Folic acid was also associated with an increased MCV but also with a decreased MPV (p<0.05 in both cases). The use of multivitamins was found to be associated with an increased IL-18 (p<0.05). Finally, the use of vitamin C was associated with an increase in IFN-γ (p<0.05).

Table 3

Discussion

Altered levels of several inflammatory molecules during the steady state of SCD have been reported.[18, 30, 32-36] In this study, we observed significant increases in most cytokines and blood cell populations among children with SCD compared with healthy controls. These findings are consistent with previous reports describing persistent inflammatory activation in individuals with SCD.[30, 34] However, our study also observed reduced levels of IL-12/IL-23p40 and IL-12p70 in the SCD cohort compared with controls, together with similar levels of CCL2 and IL-1β between the two groups.[37] These findings suggest that although steady-state SCD is characterized by elevated inflammatory activity, specific cytokine pathways may be selectively regulated to maintain the clinically defined steady state.

We observed lower levels of IL-12p70 and IL-23p40 in the SCD group compared with controls. Taylor and colleagues reported undetectable IL-12 levels in both healthy controls and steady-state SCD participants and attributed this observation to the fact that significant IL-12 production is often associated with microbial infection or altered immune activation.[38] Pitanga and colleagues also suggested that IL-12 levels should be monitored during VOC to rule out microbial infection.[35] Despite the reduced IL-12p70 levels observed in our study, IFN-γ levels were increased in the SCD group compared with controls. While IL-12 is a recognised inducepr of IFN-γ production,[42] IFN-γ expression can also be driven through alternative inflammatory pathways, including activation of NK cells, T lymphocytes, and other cytokine-mediated immune stimulation within chronically inflamed environments such as SCD.[6, 40] The positive correlation observed between IL-12p70 and IFN-γ within the cohort suggests that the biological relationship between these cytokines remains present, but that IL-12 may not be the sole determinant of IFN-γ levels in this setting. These findings therefore likely reflect the complexity of cytokine network regulation during steady-state SCD rather than a simple linear regulatory relationship. Nitric oxide is an important regulator of vascular homeostasis, and its bioavailability has been linked to the degree of vasculopathy and complications observed in SCD.[43-45]

We observed elevated levels of several pro-inflammatory cytokines, including IL-3, IL-6, TNF-α, and GM-CSF in children with SCD. Tumour necrosis factor-α (TNF-α) has consistently been reported to be elevated in SCD and is known to contribute to vaso-occlusive processes.[6, 8, 46]. TNF-α promotes the expression of adhesion molecules on both neutrophils and endothelial cells, thereby enhancing leukocyte adhesion and contributing to vascular occlusion. In addition, TNF-α can influence nitric oxide levels, further affecting vascular tone and vaso-occlusive risk.

IL-6 is a pleiotropic cytokine involved in coordinating immune and inflammatory responses.[47, 48] Although IL-6 has recognized context-dependent immunomodulatory functions, including effects on regulatory pathways, it is also widely associated with chronic inflammatory activation and synergistic interactions with pro-inflammatory mediators such as TNF-α. The concurrent elevation of IL-6 and IL-10 in our cohort may therefore reflect the complex interplay between persistent inflammatory activation and compensatory regulatory responses during steady-state SCD rather than a direct regulatory role of IL-6 in maintaining the steady-state condition.

The study also observed increased levels of the anti-inflammatory cytokines IL-4 and IL-10 in the SCD group compared with controls. Previous studies have reported elevated IL-4 levels during vaso-occlusive crisis, whereas comparable levels were observed between steady-state patients and healthy controls.[30] Similar observations have been reported in other cohorts.[7] IL-10 has been suggested to play a protective role by suppressing the production of pro-inflammatory cytokines including GM-CSF, G-CSF, IL-8, TNF-α, IL-1α, IL-1β, and IL-6.[49] The elevated IL-10 levels observed in our cohort may reflect compensatory anti-inflammatory responses within the chronically activated immune environment of steady-state SCD rather than direct evidence of protection against vaso-occlusive events.

The balance between pro- and anti-inflammatory cytokines has been proposed as an important determinant of disease outcomes. Cytokine ratios have been used as indicators of inflammatory balance in several disease conditions. Our findings show that ratios of IL-12p70, IL-23p40 and IL-1β relative to IL-4 and IL-10 were reduced in children with SCD, whereas ratios of IL-1α, IL-17A and IL-3 relative to IL-4 and IL-10 were increased. These observations suggest that the steady state is characterized by a regulated inflammatory environment in which pro-inflammatory activity is counterbalanced by regulatory cytokines.

The study also observed increased levels of several chemokines including CCL3, CCL4, CCL17, CXCL8/IL-8 and CXCL10. These chemokines play important roles in leukocyte recruitment and chronic inflammation.[37] All white blood cell populations were increased among participants with SCD, supporting the involvement of these chemokines in the disease process. CCL3 and CCL4 are known to recruit inflammatory cells to sites of tissue injury.[50, 51] CCL17 has also been associated with the differentiation of haematopoietic progenitor cells and may influence the continued supply of blood cells to the circulation.[52] Increased CXCL10 levels have also been reported in SCD and may reflect enhanced leukocyte recruitment and activation.[53-55]

We also examined associations between cytokine levels and supportive therapies, used by participants. Participants receiving vitamin C exhibited higher IFN-γ levels compared with those not receiving vitamin C. Vitamin C functions as a potent antioxidant and plays a role in immune regulation.66 Vitamin C has been shown to stimulate dendritic cells to produce IL-12 and promote differentiation of naïve CD4+ T cells into Th1 cells.[56, 57] This may explain the higher IFN-γ levels observed among children receiving vitamin C in our cohort.

We also observed increased IL-17A levels among children receiving hydroxyurea. Hydroxyurea therapy promotes the production of fetal haemoglobin and reduces the frequency of acute pain crises, acute chest syndrome, hospitalization, and transfusion requirements in SCD.[6, 58] The relationship between hydroxyurea therapy and IL-17A levels requires further investigation.

Finally, exploratory discriminant analysis suggested separation between SCD cases and controls within this dataset when cytokine and hematological parameters were considered together. However, because no independent validation was performed, these findings should be interpreted cautiously and primarily as hypothesis-generating observations.In conclusion, our study demonstrates that children with SCD in steady-state exhibit a distinct inflammatory profile that differs from that of healthy individuals. Selected pro-inflammatory cytokines including IL-1α, IL-17A, IL-3, CCL3, and GM-CSF were associated with the inflammatory profile observed during steady-state SCD, whereas reduced ratios of IL-12p70, IL-23p40, and IL-1β relative to IL-4 and IL-10 may reflect regulatory features of this steady-state inflammatory environment. Although the cross-sectional design limits causal interpretation, these findings provide insight into the immunological landscape of steady-state SCD and may help inform future studies investigating inflammatory biomarkers and disease progression.

Statements & Declarations

Funding

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Competing Interests

PWA has served on the advisory boards of K36 Therapeutics and CommBio Tx. All others declare no competing interests.

Author Contributions

Conceptualization: EAA, KHAA, PWA, KOD; Data curation: EAA, JAA, HO, EN; Formal Analysis: EAA, DA, AAB, KOD; Funding acquisition and resources: EAA, PWA, KOD; Methodology and Investigation: EAA, DA, JAA, HO, AF, EN, JKAT, HKB, GSK, KHAA; Visualization: EAA, DA, KOD; Writing – original draft: EAA, DA, AF, AAB, JG, KOD; Writing – review & editing: All Authors; Supervision: PWA, LM, KOD

Data Availability

Relevant data has been reported in the manuscript. Any other data where needed will be made available upon a reasonable request from the corresponding author. Contact email: Contact Email: kwabena.duedu@bcu.ac.uk

Ethics Approval

This study was performed in line with the principles of the Declaration of Helsinki. The study was approved by the Research Ethics Committee of the University of Health and Allied Sciences, Ho with protocol reference numbers UHAS-REC A.3 [1] 18-19 and UHAS-REC A.12 [42] 20-21. Permission was sought from the Research Directorate of the Ho Teaching Hospital (HTH/RPPME/19/1) and the Adaklu District Directorate of the Ghana Education Service (GES/VR/ADEO/48/VOL.1/31).

Consent to Participate

Written informed consent was obtained from parents or guardians for participants younger than 16 years. Assent was obtained from children aged 5 years and above, in accordance with ethics approval requirements. For participants aged 16 years and above, informed consent was obtained directly from the participant in addition to parental/guardian consent where required.

Consent to Publish

Not Applicable

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Figure Legends

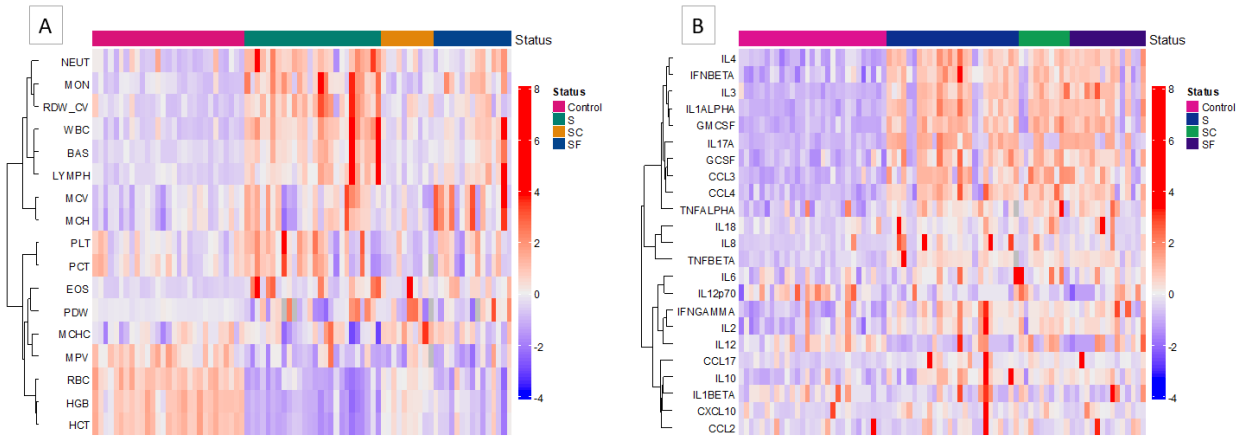

Figure 1: Heatmap of levels of full blood count and inflammatory biomarkers in study participants groups by cases (SF, S, SC) and the non-SCD controls (1A) compares the parameters of the full blood count among the control group and the individual SCD haemoglobin phenotypes. There is a wide variation of the parameters within the SCD group compared to the controls. The participants with SCD presented with significantly lower values for the RBC parameters and higher values for the WBC parameters compared to the control group. However, significantly lower values for RBC parameters and higher values for the WBC parameters are seen for participants with HbS compared to the other SCD phenotypes and controls. (1B) compares the serum cytokine profile between the SCD phenotypes and controls. Wide variations are seen between groups. Most participants with SCD are presenting with higher cytokine values compared to the controls. However, slight variation is seen for IL12, IL-12p70, IL-1β, and CCL2 between participants with SCD and controls.

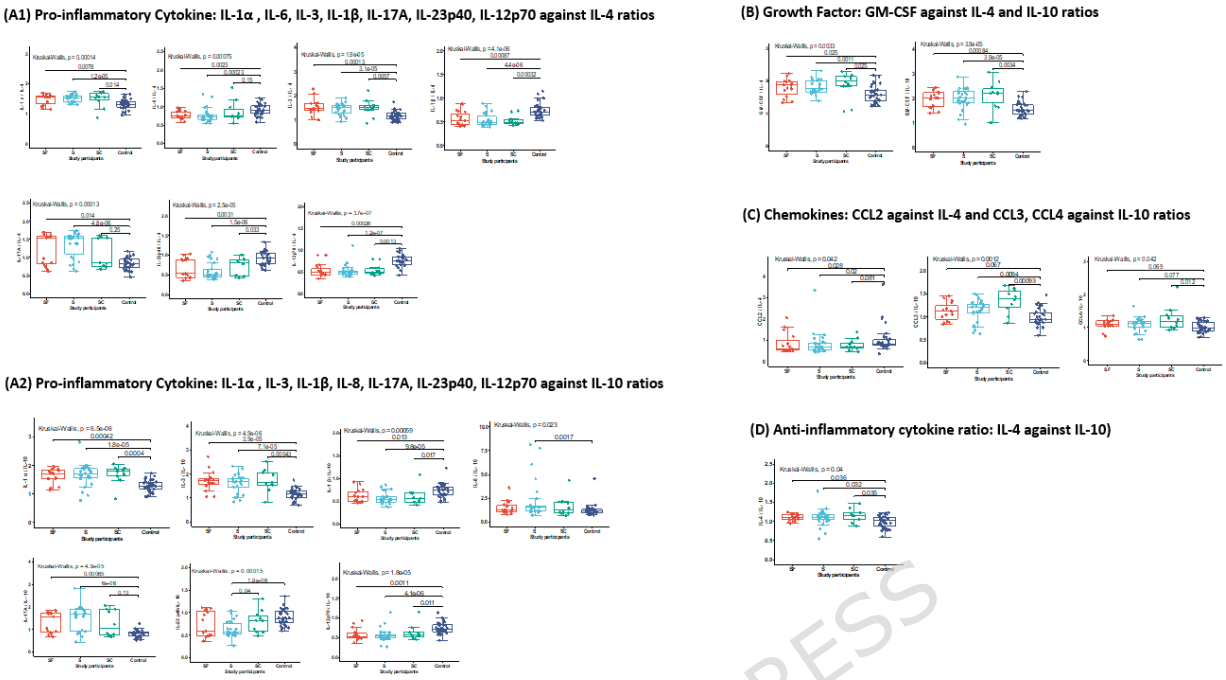

Figure 2: Comparison of statistically significant inflammatory biomarker ratios between SCD cases categorized by genotype (SF, S, SC) and non-SCD controls. (2A1 and 2A2) compares ratios of pro-inflammatory cytokines among the panel assayed against IL-4 and IL-10, respectively. Ratios of IL-12p70, IL-23p40, IL-1β, IL-6 (2A1), and CCL2 (2C) to IL-4 were found to be lower ($p<0.05$) while ratios of IL-1α, IL-17A, and IL-3 to IL-4 (2A1) were higher ($p<0.05$) among the SCD group compared to the controls. Ratios of IL-12p70, IL-23p40, and IL-1β to IL-10 were found to be lower ($p<0.01$) while ratios of IL-1α, IL-17A, IL-3, and IL-8 against IL-10 were higher ($p<0.05$) among the SCD group when compared to the controls (2A2). (2B) compared ratios of growth factor, GM-CSF against IL-10 and IL-4. The ratio of GM-CSF to IL-4 and IL-10 was higher ($p<0.05$) among the SCD group compared to the controls. (2C) compares ratios of chemokines against IL-10 and IL-4. The ratio of CCL2 to IL-4 was found to be lower ($p<0.05$) and CCL3 against IL-4 was higher ($p<0.05$) among the SCD group compared to the controls. Ratios of CCL3 and CCL4 against IL-10 were higher ($p<0.05$) among the SCD group when compared to the controls. (2D) shows an increased IL-4 to IL-10 (anti-inflammatory cytokines) ratio among participants with SCD compared to controls.

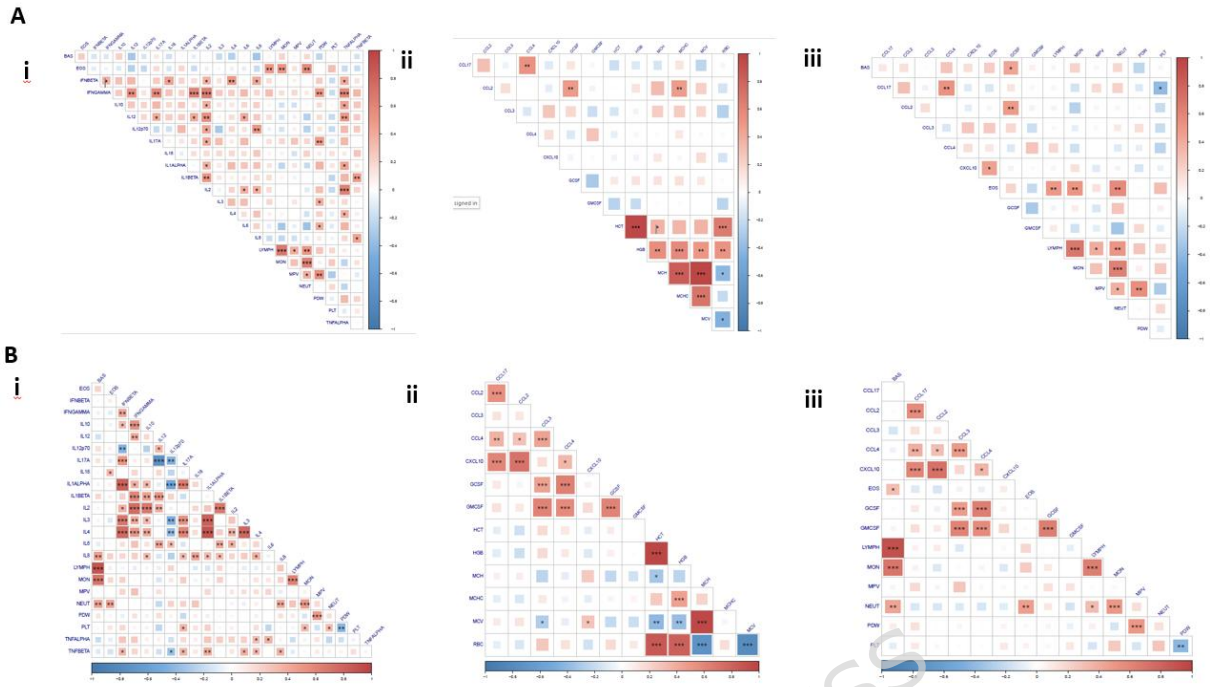

Figure 3: Correlations (Spearman's) between the various full blood count and inflammatory biomarkers in the SCD patients. Stars (***) represent significant *p*-values at the following levels: *p*-value<0.001 (***), *p*-value<0.01 (**), *p*-value<0.05 (*). Deep blue colour signifies a strong positive correlation whereas deep red colour signifies strong negative correlations. Dim Correlations are those that were not statistically significant.

Table 1: Demographic, supplement and Hb phenotype description of the study participants

Characteristics	N (%)	Characteristics	N (%)
SCD Status		**Multivitamins (SCD)**	
Control	66 (51.2)	Yes	31 (49.2)
SCD	63 (48.8)	No	31 (49.2)
		Missing	1 (1.6)
Gender			
Male (SCD)	33 (52.4)	**Folic Acid (SCD)**	
Female (SCD)	30 (47.6)	Yes	50 (79.4)
		No	12 (19.0
Male (Control)	31 (47.0)	Missing	1 (1.6)
Female (Control)	35 (53.0)		
		Zincovit (SCD)	
Hb Phenotype		Yes	24 (38.1)
A	52 (40.3)	No	38 (60.3)
AC	10 (7.8)	Missing	1 (1.6)
AS	4 (3.1)		
S	30 (23.3)	**Vit C intake (SCD)**	
SC	15 (11.6)	Yes	10 (15.9)
SF	18 (14.0)	No	52 (82.5)
		Missing	1 (1.6)
	Mean Age±S.D		
S	8.9±4.5	**Hydroxyurea (SCD)**	
SC	8.3±4.2	Yes	12 (19.1)
SF	10.7±4.2	No	50 (79.3)
A	10.9±3.8	Missing	1 (1.6)
AC	9.8±2.9		
AS	11.5±4.2		

S.D - Standard Deviation

Table 2: Comparing levels (MFI) of cytokines between participants with SCD and healthy controls.

Cytokine	Healthy Control	SCD	P-value (T-test)
CXCL10	132.74±151.25	205.24±256.06	0.004
IL-12p70	6.42±1.02	5.96±0.85	0.012
CCL17	8.40±2.03	13.19±8.86	<0.001
CCL2	8.70±4.47	9.56±5.53	0.449
CCL3	8.56±1.19	12.30±2.56	<0.001
CCL4	8.68±0.80	11.76±2.09	<0.001
G-CSF	8.23±0.98	10.50±1.51	<0.001
GM-CSF	13.52±1.56	21.20±4.58	<0.001
IFN-β	8.35±0.85	10.13±1.10	<0.001
IFN-γ	8.19±1.35	9.87±1.49	<0.001
IL-1α	11.17±1.26	17.50±3.62	<0.001
IL-10	8.84±1.35	10.75±2.36	<0.001
IL-12/IL-23P40	7.80±1.31	7.23±2.34	0.022
IL-17/IL-17A	7.02±1.20	14.82±5.47	<0.001
IL-18	30.84±17.36	49.18±45.78	0.009
IL-1β	6.32±0.98	6.22±1.46	0.096
IL-2	7.67±1.48	9.38±1.66	<0.001
IL-3	10.06±1.62	17.47±4.54	<0.001
IL-4	8.61±1.04	11.60±1.54	<0.001
IL-6	7.86±1.28	8.96±2.00	0.017
IL-8	10.93±6.62	20.42±16.88	<0.001
TNF-β	9.84±1.21	13.91±5.25	<0.001
TNF-α	8.74±3.06	12.16±3.31	<0.001

P-value computed using the independent sample T-test.

Table 3: Comparison of cytokine, red cell, and platelet indices based with significant differences between gender, use of Hydroxyurea, Folic Acid, Multivitamins and Vit C among participants with SCD.

Variables	N	Mean ± SD	P-value
Gender			
IL-1α (Female)	30	18.52±3.03	0.044
IL-1α (Male)	32	16.53±3.92	
IL-4 (Female)	30	12.48±1.87	0.0126
IL-4 (Male)	32	11.06±2.19	
PDW (Female)	30	14.30±2.75	0.0083
PDW (Male)	32	16.45±3.31	
TNF-β (Female)	30	15.33±6.93	0.0177
TNF-β (Male)	32	12.55±2.25	
Hydroxyurea			
IL-12p70 (Hydroxyurea)	12	5.53±1.00	0.044
IL-12p70 (No Hydroxyurea)	50	6.06±0.78	
IL-23p40 (Hydroxyurea)	12	5.50±0.94	0.0026
IL-23p40 (No Hydroxyurea)	50	7.65±2.39	
IL-17_IL-17A (Hydroxyurea)	12	18.08±3.42	0.0342
IL-17_IL-17A (No Hydroxyurea)	50	14.03±5.61	
MCV (Hydroxyurea)	12	98.83±16.48	0.0031
MCV (No Hydroxyurea)	50	83.27±11.70	
MCH (Hydroxyurea)	12	32.22±5.27	0.0023
MCH (No Hydroxyurea)	50	26.95±3.83	
RDW_CV (Hydroxyurea)	12	15.55±1.76	0.0161
RDW_CV (No Hydroxyurea)	50	17.67±3.43	
Folic Acid			
MCV (Folic Acid)	50	88.05±14.42	0.037
MCV (No Folic Acid)	12	78.93±9.76	
MPV (Folic Acid)	50	8.85±0.77	0.0164
MPV (No Folic Acid)	12	9.55±1.02	
Multivitamins			
IL-18_IL-1F4 (Multivitamins)	31	46.14±57.78	0.0026
IL-18_IL-1F4 (No Multivitamins)	31	52.87±25.30	
Vitamin C			
IFN-γ (vitC)	10	11.03±1.82	0.0232
IFN-γ (No vitC)	52	9.59±1.27	

*Note: *One patient did not have adequate sample for the cytokine assay and hence total SCD patients reported in this table is 62. P-values computed using the independent sample T-test*