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Article

# Predicting sickle cell vaso-occlusion by microscopic imaging and modeling

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## SUMMARY

**Sickle cell disease (SCD) is a hereditary condition in which red blood cells become rigid and develop a sickle shape due to abnormal hemoglobin S. This leads to obstructed microvasculature and causes vaso-occlusive crises (VOCs). These blockages result in severe complications like pain crises, acute chest syndrome, and strokes. The physical mechanisms behind VOCs are not fully understood, hindering the development of predictive diagnostic tools. Previous studies using microfluidic devices and mathematical modeling to analyze the transit of sickle red blood cells through microvasculature-mimicking slits found that increased viscosity is positively related to longer transit times, which can predict disease severity. Since blood viscosity is positively correlated with the frequency of sickle red cells, we hypothesized that a higher percentage of sickle cells in peripheral blood would correspond to a greater likelihood of VOCs in SCD patients. To test this hypothesis, we used the software ImageJ to analyze blood smear images from 24 SCD patients to quantify the percentage of sickle cells. We found a positive correlation between sickle cell frequency and the incidence of VOC events. These findings suggest that blood smear imaging combined with microfluidic analysis and mathematical modeling could serve as a rapid, non-invasive diagnostic tool to predict pain crises in SCD patients. This approach has significant clinical implications, offering a potential method to predict VOC events for personalized treatment strategies, ultimately aiming to reduce hospital admissions and improve outcomes in SCD patients.**

## INTRODUCTION

Sickle cell disease (SCD) is an inherited red blood cell (RBC) disorder and one of the leading causes of anemia, predominantly affecting the African American population in the United States (1). Patients with SCD often show symptoms such as pain crisis, acute chest syndrome, stroke, and jaundice. Normal RBCs have a characteristic bi-concave disk shape with the flexibility to deform and squeeze through microvasculature much smaller in diameter than the RBCs (2-7). These features of normal RBCs are disrupted in SCD.

RBCs from healthy adults contain globin genes that produce normal hemoglobin A protein to carry oxygen (4). Hemoglobin A protein is composed of two alpha-globin and two beta-globin proteins, which form a complex with heme (2). The RBCs of patients with SCD inherit abnormal beta-globin genes from both parents, which leads to the generation of abnormal beta-globin protein called globin S (2). The beta-globin gene is located on chromosome 11, with the mutant globin S form resulting from the replacement of thymine by adenine (2). Consequently, glutamic acid is replaced by valine during translation, leading to abnormal hemoglobin S (2,6). The polymerization or aggregation of hemoglobin S causes mature RBCs to become insoluble, less deformable, stickier, and sickle-shaped (2, 6, 8-16). The frequency of sickle cells between patients varies, consistent with the fact that fetal hemoglobin is generated in these patients to compensate for anemia (1). The frequency of sickle cells is also known to be correlated with the oxygenation status of the patients (8). Patients with SCD experience periodic pain crises that require urgent treatment (1, 8, 12, 16-19). These episodes can be triggered by factors like dehydration, infection, or stress, and often require prompt pain management and supportive care (16-19). The pain crises are mainly due to the occlusions of microvasculature, or vaso-occlusion (VOC), by the sickle cells, since these cells cannot deform to migrate through small vessels (20). These episodes can affect multiple organ systems, and their clinical manifestations range from localized limb pain to life-threatening complications such as acute chest syndrome or splenic sequestration (1). VOCs can recur with variable frequency across patients (1, 8). The underlying pathophysiology involves not only mechanical blockage by rigid, sickle cells but also inflammation, endothelial activation, and cell adhesion events (17-19). Although VOC is well known, the physical mechanisms of how these sickle RBCs block microvascular circulation are unclear and continue to be studied (19).

Microfluidic devices are *in vitro* platforms that manipulate small volumes of fluids in microscale channels, allowing controlled simulation of a biological environment. These devices have been increasingly applied to mimic and study biological processes that are difficult to observe *in vivo* (21). In this respect, they are ideal for studying RBC physiology, given the unique and complex membrane structures of healthy red cells. RBCs comprise a lipid bilayer cytoplasmic membrane connected to an inner cytoskeleton (2, 4). The red cell cytoskeleton is highly flexible and possesses the capacity to migrate through microvasculature and slits between the endothelial cells lining the vessels (4, 7, 21).

Recent biophysical studies using *in vitro* biomimetic microfluidics and multiscale modeling have confirmed that

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healthy RBCs can pass through submicron-wide inter-endothelial rigid slits at body temperature (22). These features of RBCs can be replicated in circumstances of migration through microvasculature. Unlike the slits that have a narrow range of sizes in the spleen and bone marrow, microvasculature in other parts of the body contain small vessels with a variety of diameters, lengths, and widths (8, 23-27). During their roughly 120-day life span, RBCs undergo constant deformation processes, which demand a dynamic and highly flexible plasma membrane and cytoskeleton network (28). These features are compromised in sickle RBCs (29). Understanding the biophysical mechanisms of sickle cell VOCs is important to predict the occurrence of pain crises and avoid complications.

Previously, we applied the microfluidic device and mathematical RBC modeling to analyze sickle cell VOCs under physiological conditions (22). We found that the viscosity of the sickle cells is positively related to the transit time of the slit, which could predict the disease severity of the patients (22). Based on these findings, we hypothesized that a higher percentage of sickle cells in peripheral blood would correspond to a greater likelihood of VOCs in SCD patients, as sickle RBCs are less deformable and more adhesive, and thus may lead to mechanical blockage of small vessels and impaired microvascular circulation. In this study, we analyzed the blood smears from SCD patients using the software ImageJ and correlated them with the patients' clinical presentations and VOC events. We found a positive correlation between sickle cell frequency and the incidence of VOC events. This study has a potential clinical impact on developing an inexpensive and rapid diagnostic method to predict pain crises.

## RESULTS

We obtained images of peripheral blood smears from 24 patients with SCD. Three images of each patient's sample were taken from different areas of the smear to ensure that the quantification of the sickle cell frequencies was representative (**Figure 1**). Through these images, we examined the correlation between sickle cell frequency and severity of VOCs.

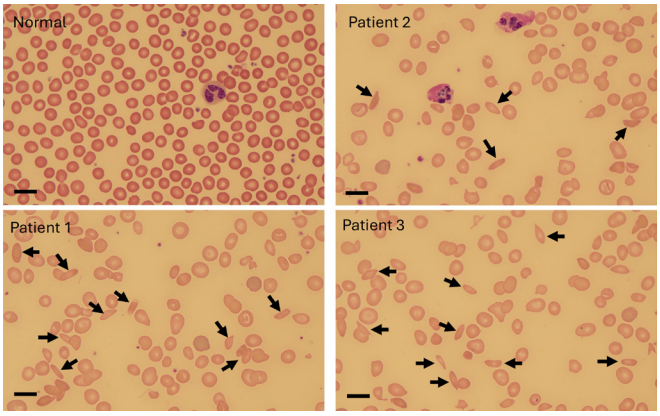


**Figure 1: Representative images of sickle cells in peripheral blood smears.** Peripheral blood smears from a normal, healthy individual and from three patients with sickle cell disease. The sickle cell images were obtained from a cohort of 72 image samples derived from 24 patients, with three images collected per patient. The smears were stained with Wright-Giemsa stain to highlight cellular morphology. Arrows point to the sickle red blood cells identified using ImageJ software based on pre-defined sickle cell parameters. Scale bars: 10 μm.

| Patient | Sex | Age | Average % sickle cell | Clinical symptoms and presentations |
|---|---|---|---|---|
| 1 | F | 25 | 8.02 | Left flank pain, hemolysis, stroke |
| 2 | F | 27 | 11.60 | Moyamoya disease, RBC exchange every week, acute ischemic stroke, headache, acute ischemic stroke, headache |
| 3 | M | 34 | 13.99 | Renal transplant, eight units RBC every quarter |
| 4 | F | 49 | 2.41 | Pain in leg and arms, possible chest pain |
| 5 | F | 63 | 0.53 | Headache, generalized body pain |
| 6 | F | 24 | 4.95 | Generalized body pain, back and leg pain |
| 7 | F | 52 | 0.98 | Generalized body pain, superficial venous thrombosis |
| 8 | F | 27 | 10.23 | Moyamoya disease, RBC exchange every week, acute ischemic stroke, headache |
| 9 | F | 32 | 6.93 | Internal jugular vein thromboembolism, acute transverse sinus thrombosis, moyamoya disease |
| 10 | F | 49 | 3.25 | Pain in leg and arms, possible chest, |
| 11 | F | 40 | 5.10 | Moyamoya disease, CVA, acute CVA |
| 12 | F | 63 | 2.01 | Headache, generalized body pain |
| 13 | F | 32 | 5.28 | Internal jugular vein thromboembolism, acute transverse sinus thrombosis, moyamoya disease |
| 14 | F | 34 | 1.22 | Generalized body pain |
| 15 | F | 25 | 0.28 | Bilateral hip and leg pain, low back pain |
| 16 | F | 64 | 1.68 | Generalized body pain |
| 17 | F | 49 | 1.48 | Pain in leg and arms, possible chest pain |
| 18 | F | 25 | 3.81 | Left flank pain, hemolysis, stroke |
| 19 | M | 52 | 5.16 | CVA, CKD, moyamoya disease, neurocognitive disorder, PE/DVT, bilateral shoulder AVN, history of acute chest pain |
| 20 | F | 63 | 0.74 | Headache, generalized body pain |
| 21 | M | 34 | 15.40 | Left side chest pain, Sickle cell painful crisis, hemolysis, priapism |
| 22 | F | 63 | 3.02 | Headache, generalized body pain |
| 23 | F | 62 | 2.74 | No significant VOCs |
| 24 | M | 62 | 30.08 | Acute VOC, acute chest syndrome, bilateral hip avascular necrosis, bilateral edema |

**Table 1: Sickle cell frequencies and clinical presentation.** The sickle cell frequency of each patient was the average of three independent blood smears. The sickle cell frequency of each blood smear was measured using ImageJ software from ten random fields. M: Male; F: Female. Age in years.

The average percentage of sickle RBCs among these patients showed marked variability, ranging from as low as 0.28% to as high as 30.08% (**Table 1**). This wide range highlights the diverse severity of SCD manifestations within the patient population. Patient 15 exhibited the lowest average sickle cell percentage at 0.28%, indicating a possibly milder form of the disease or effective management of symptoms. Similarly, Patients 5 and 7 had low average sickle cell percentages of 0.53% and 0.98%, respectively. Patients 4, 6, 10, 12, 14, 16, 17, 18, 22, and 23 displayed moderate sickle cell percentages, with averages ranging from 1% to 5%. In contrast, increased averages between 5% and 30% were seen in patients 1, 2, 3, 8, 9, 13, 19, 21, and 24, with patient 24 having the highest average sickle cell percentage at 30.08%.

We next reviewed the clinical presentations of these patients. We first categorized the severity of sickle cell percentages as low (<5%), moderate (5-10%), and high (>10%). Indeed, patients with low sickle cell percentages generally reported milder symptoms, such as headaches and generalized body pain. For example, Patient 5, with a sickle cell percentage of 0.53%, experienced headaches and generalized body pain, and Patient 15, with a sickle cell percentage of 0.28%, reported bilateral hip and leg pain and

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| Symptoms | Disease severity score |
|---|---|
| No significant VOCs | 0 (low) |
| Generalized body pain | 1 (low) |
| Chest pain, moyamoya disease | 2 (moderate) |
| Acute VOC | 3 (moderate) |
| Stroke | 4 (high) |
| Renal transplant, RBC exchange | 5 (high) |

**Table 2: Sickle cell disease clinical severity score.** Disease severity scores were assigned based on each patient's most clinically significant symptom(s), as determined from their medical records. If a patient exhibited symptoms that spanned multiple categories, the highest applicable score was assigned to reflect the most severe manifestation of disease. The scoring system was developed internally to stratify patients into low (scores 0–1), moderate (2–3), and high (4–5) severity categories based on VOC-related complications.

low back pain. Patients with moderate sickle cell percentages (5–10%) experienced more pronounced symptoms, including pain crises and specific complications. Patient 6, with a percentage of 4.95%, suffered from generalized body pain, especially in the back and legs, and Patient 9, at 6.93%, had internal jugular vein thromboembolism, acute transverse sinus thrombosis, and moyamoya disease. In contrast to these patients with low to moderate blood sickle cell frequencies, those with high sickle cell percentages (>10%) often had severe complications and frequent VOC events. For instance, Patient 24, with the highest percentage of 30.08%, presented with acute VOC, acute chest syndrome, bilateral hip avascular necrosis, and bilateral edema. Patient 21, at 15.40%, experienced left-sided chest pain, painful crises, hemolysis, and priapism. Additionally, Patient 3, with a percentage of 13.99%, underwent a renal transplant and requires regular RBC transfusions. Patients 2, 8, 9, 13, and 19 reported severe conditions such as moyamoya disease, strokes, thromboembolism, and frequent pain crises (**Table 1**).

To illustrate the relationship between the percentages of sickle cells and the severity of VOC events, we classified VOC events into self-defined risk categories (**Table 2**). The disease severity scores we defined inherently incorporate VOC frequency and severity, along with other clinical manifestations. We found that patients in the high-risk groups

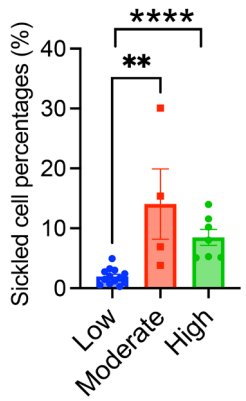


**Figure 2: Sickle cell percentages and disease severity score.** The sickle cell percentage was calculated as the average from three independent blood smears per patient using ImageJ analysis of ten random fields each, and the disease severity score was assigned based on documented clinical symptoms using the criteria in Table 2. Each dot represents a patient. The comparison among multiple groups was evaluated with one-way ANOVA tests. ** p<0.01. **** p<0.0001 ns: non-significant. Low means disease scores 0 and 1. Moderate means disease scores 2 and 3. High means disease scores 4 and 5.

had a significantly higher percentage of sickle cells (8.79 ± 1.51%) than those in the low-risk group (2.10 ± 0.47%), highlighting a statistically significant difference between the two groups (p<0.0001) (**Figure 2**). We also found a positive correlation between sickle cell percentage and the frequency of VOC events (Pearson correlation, R=0.5543, p=0.0049) (**Figure 3**). This correlation indicates that as the percentage of sickle RBCs increases, the severity and frequency of VOC events also rise, supporting the idea that sickle cell burden is linked to clinical complications in patients with SCD.

## DISCUSSION

In this study, we hypothesized that a higher percentage of sickle cells in peripheral blood would correspond to a greater likelihood of VOCs in SCD patients. We focused on developing a non-invasive, rapid, and affordable method to predict VOC risk associated with SCD. We measured the percentages of sickle cells in patients with SCD and found that the frequency of sickle cells in peripheral blood was positively correlated

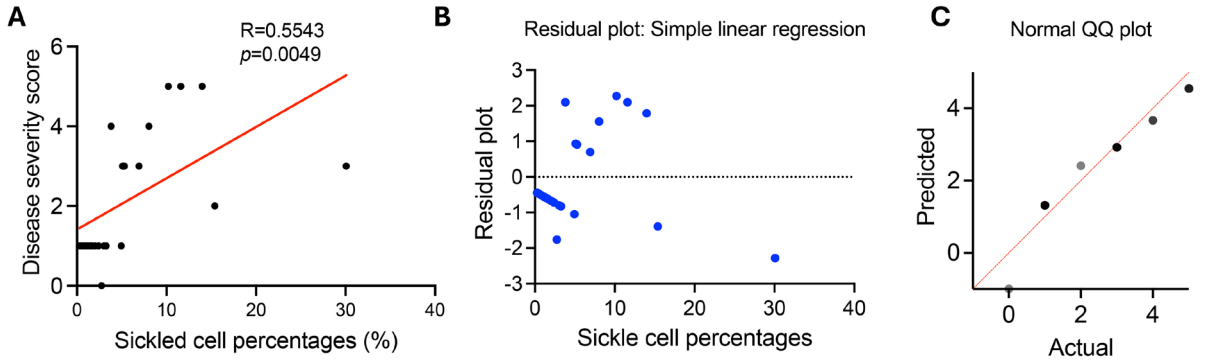


**Figure 3: Sickle cell percentages and disease severity score are positively correlated. (A)** Linear regression analysis was performed using sickle cell percentage as a continuous predictor and disease severity score as the outcome (Pearson correlation, R = 0.5543, p-value = 0.0049 < 0.05). Each dot represents one patient (n = 24), with the sickle cell percentage quantified via ImageJ analysis of blood smears and the disease severity score assigned based on clinical symptoms categorized in Table 2. **(B)** Residual plot from a simple linear regression of sickle cell percentages. **(C)** Normal Q-Q plot comparing predicted values to actual values.

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with the VOC events.

Our data show a positive correlation between the percentage of sickle RBCs in peripheral blood and the severity of VOC events in patients with SCD. Patients with higher sickle cell percentages are more likely to experience severe VOCs and related complications, such as strokes, organ damage, and frequent pain crises. Conversely, those with lower sickle cell percentages generally exhibit milder symptoms and encounter fewer severe events. This correlation suggests that a higher proportion of sickle cells increases the likelihood of vascular blockages, leading to more intense and recurrent symptoms. These findings have significant implications for clinical practice. Regular assessment of sickle cell percentages can be valuable in predicting the risk of VOC events, enabling healthcare providers to monitor patients more effectively. Understanding this correlation aids in tailoring personalized treatment plans, including the consideration of interventions like RBC exchange or the adoption of more aggressive management strategies for high-risk patients. Furthermore, this underscores the need for additional research to explore the underlying mechanisms and identify other contributing factors to VOC severity, which could lead to improved therapeutic approaches and patient outcomes.

However, while this general trend of increasing sickle cell percentage correlating with more severe VOCs holds true, individual variations exist due to factors such as age, overall health, genetic differences, and treatment regimens. Some patients with lower sickle cell percentages still report significant pain or complications, indicating that the sickle cell percentage is only one of several factors influencing VOCs. These exceptions highlight the multifactorial nature of SCD, where environmental influences and individual patient responses play critical roles. Therefore, a comprehensive approach considering all these factors is essential for accurately assessing disease severity and tailoring effective treatment strategies.

The reliability and clinical relevance of our findings were enhanced by the close alignment between experimental data and mathematical modeling, validating the study's methodological approach. This alignment not only underscores the robustness of the predictive model but also highlights the potential for clinical application in predicting patient-specific risk of VOC. A previous study has established a modeling system for healthy and sickle RBC, demonstrating that transit time is negatively related to pressure drop and positively related to cell volume and viscosity (22). Building on this framework, our current results suggest that the Matlab-based modeling system, when integrated with blood smear analysis and ImageJ software quantification, could serve as a practical and non-invasive method for predicting pain crises in SCD patients. By delineating the conditions precipitating microvascular blockage, our research lays the foundation for a potential new strategy in SCD management—moving from reactive treatment to proactive prevention.

The SCD cases represent a range of clinical severity, from lower-risk cases with few sickle cells and VOCs to higher-risk cases with frequent VOCs and higher percentages of sickle RBCs. Twenty-four patients with blood smears performed were selected out of the initial 62 SCD patients. The selection was aimed to reflect the variability observed in routine clinical practice and was not predetermined by sickle cell burden. This approach provided a comprehensive selection of cases to facilitate comparison across mild, moderate, and severe sickling profiles. However, the sample size is small, which reduces the power of the statistical analyses and limits the generalizability of the findings to a broader SCD population. Additionally, the male-to-female ratio was skewed with more female patients being studied, which may also limit generalizability. Future studies should aim to include a larger, more diverse cohort and incorporate additional variables such as treatment regimens and socio-economic factors.

Overall, this research represents an innovative approach to the understanding and management of SCD. By providing a novel predictive tool for VOC risk, our work opens potential avenues for early intervention and personalized treatment strategies, aiming to substantially reduce the frequency of hospital admissions and the economic burden associated with SCD. Ultimately, the study holds the possibility not only to improve patient outcomes but also to address the broader socio-economic and health disparities faced by the SCD patient community, marking a transformative step in the fight against this debilitating disease.

## MATERIALS AND METHODS

### Case selection

The study was approved by the Institutional Review Board of Northwestern University. We enrolled 24 patients with confirmed SCD. Cases were chosen from SCD patients with peripheral blood samples collected between January and June 2024 at Northwestern Memorial Hospital. Eligible cases included patients with at least one recorded VOC event and a documented percentage of sickle RBCs, varying across cases from mild to severe sickling percentages. We categorized the sickle cell percentages as low (<5%), moderate (5–10%), and high (>10%). Patient demographics, including age, sex, and total RBC count, were recorded, along with specific clinical features related to VOCs, such as pain locations, hemolysis, moyamoya disease, renal complications, and thrombotic events. Each patient's medical history of emergency department visits due to VOCs was also considered to understand the frequency and intensity of VOC occurrences.

### Preparation of blood smear

A blood smear was prepared by first collecting a small volume of fresh peripheral blood directly from the patient using standard aseptic techniques. To ensure optimal cell morphology and avoid clotting, the blood was collected without anticoagulant and used immediately. A clean glass slide was selected to prevent interference from any debris or residue that could distort the smear. A small drop of blood, approximately 2–3 mm in diameter, was carefully placed near one end of the slide using a pipette. A second slide, a spreader, was held at a 30–45° angle to the slide containing the blood drop. The spreader slide was pulled back until it touched the blood drop, allowing the drop to spread along its edge by capillary action. Then, in one smooth, continuous motion, the spreader slide was pushed forward, distributing the blood evenly across the surface of the primary slide to create a monolayer of cells. The slide was then left to air dry completely at room temperature without any external heat or airflow. The drying time was monitored to ensure the smear was fully dry before fixation and staining.

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### Staining of blood smear

For staining the blood smear, the fully air-dried slide was first fixed by immersing it in methanol for 1–2 minutes. After fixation, the smear was allowed to air dry before applying the stain. Wright-Giemsa stain (Sigma) was prepared per the manufacturer's instructions at a stain-to-buffer ratio of 1:1 with a pH of 6.8–7.0. The stain was applied to cover the entire smear. After staining for 5–10 minutes, an equal volume of buffer solution was added to dilute the stain on the slide, creating a thin film over the smear. This film was left undisturbed for 5–7 minutes to allow the stain components to interact with the cellular elements. The slide was then gently rinsed with distilled water, followed by air-drying. A small drop of the mounting medium was applied over the stained area. A clean, thin coverslip was lowered onto the mounting medium. The coverslip was gently pressed to create a sealed and permanent preparation.

### Analyzing sickle cell frequency using ImageJ

To analyze the percentage of sickle RBCs from a blood smear using ImageJ software, the prepared smear slide was first digitally imaged at high resolution (Leica Aperio). The image was opened in ImageJ, where it was converted to grayscale to enhance contrast between cells and the background. Using the "Threshold" tool, the grayscale was adjusted to isolate RBCs based on shape and intensity, making them distinguishable from the surrounding area. The "Analyze Particles" function was then applied to identify individual RBCs, allowing ImageJ to automatically count the total number of cells in the selected area. To differentiate sickle cells from normal RBCs, we defined specific shape thresholds using two quantitative parameters: aspect ratio (AR) and circularity. Sickle cells typically exhibit an elongated or irregular morphology, whereas normal RBCs are generally round with smooth contours. In our analysis, we classified cells as sickle if they met both of the following criteria: aspect ratio >2.0 and circularity <0.6. These thresholds were empirically determined based on a visual assessment of a representative training set and implemented in ImageJ using the Shape Descriptors plugin to automatically filter and count sickle cells. Normal RBCs were excluded from the count if their aspect ratio was ≤2.0 and circularity was ≥0.6. The final count of sickle cells was then compared to the total RBC count, yielding the percentage of sickle RBCs.

Patients with no significant VOCs were assigned a score of 0 (low). Patients experiencing generalized body pain were scored 1 (low). A score of 2 (moderate) was assigned to patients with symptoms such as chest pain or moyamoya disease. Acute VOC episodes were categorized with a score of 3 (moderate). Patients who experienced strokes were given a score of 4 (high), while those requiring renal transplant or regular RBC exchange were assigned the highest score of 5 (high).

### Statistical analyses

To assess the relationship between sickle cell burden and VOC incidence, we used linear regression with sickle cell percentage as a continuous predictor variable and disease severity score as the dependent variable. The regression model was tested for linearity, homoscedasticity, and normality of residuals to ensure valid statistical assumptions.

For comparisons involving categorical outcomes, we applied one-way ANOVA where appropriate, ensuring normality and equal variance assumptions were met. All statistical analyses were performed using GraphPad Prism (v9.0) and R (v4.2.0), with $p < 0.05$ considered statistically significant.

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