

In vitro evidence of complement activation in patients with sickle cell disease

Sickle cell disease (SCD) remains a devastating and painful condition leading to significant morbidity and mortality in the era of hydroxycarbamide.¹ Current understanding of disease pathophysiology has focused not only on the interaction between sickle red blood cells and neutrophils, platelets or endothelial cells in small blood vessels,² but also on the effects of red blood cell adhesion and hemolysis³ resulting in vaso-occlusive crisis. An important component of this pathophysiological mechanism may also be complement activation, a rather neglected entity in SCD. We studied, for the first time in SCD, novel markers that have been shown to reliably detect complement activation in the serum of patients with thrombotic microangiopathies. Interestingly, we found increased complement activation in a portion of patients, especially older patients and those with higher HbS levels.

Today, there is a greater understanding of SCD pathophysiology. However, despite this, the last decades have not seen any dramatic change in the management of SCD and acute vaso-occlusive episodes. Older studies have suggested activation of the alternative pathway of complement (APC) in SCD, especially during acute vaso-occlusive episodes. Sickle red blood cells seemed more sensitive in binding C3⁴ and C5b-9 or membrane attack complex (MAC),⁵ causing increased APC-mediated cell killing *in vivo*. More recent reports have shown that free hemoglobin from hemolysis secondary to the SCD crisis interacts with C3 and results in increased soluble C5b-9.⁶ However, little is known about APC activation in the clinical setting of SCD, possibly due to the complexity of complement diagnostics. In the field of complement diagnostics, functional assays have recently been shown to distinguish complement-related thrombotic microangiopathies from other thrombotic microangiopathies.^{7,8} Therefore, we hypothesized that APC activation could be detected in the sera of SCD patients in steady state using these functional assays. Furthermore, we investigated if complement activation is associated with routine clinical parameters and if it can be blocked by *in vitro* complement inhibition.

Consecutive SCD patients in steady state were enrolled prospectively from November 2016 to March 2017. Our study was approved by the Institutional Review Board and was conducted in accordance with the Declaration of Helsinki.

Patients' history, and clinical and laboratory data were recorded. Complement activation was detected in patient sera using the modified Ham test, a cell proliferation assay based on the susceptibility of a paroxysmal nocturnal hemoglobinuria (PNH)-like cell line to complement activated serum. Methods were as previously described.⁷ Normal human serum (NHS) from 10 age- and sex-matched healthy Caucasian volunteers was used as a negative control and lipopolysaccharides (LPS)-incubated normal serum as a positive control. All samples were tested twice in triplicates.

To further investigate complement activation in patient sera, we also measured soluble human C5b-9 using a commercially available ELISA kit (AMSBio, Abingdon, UK). The assay has a sensitivity of 1 pg/mL and a coefficient of variability of 4.4%. Eculizumab containing serum was collected from a PNH patient within 60 minutes after the infusion and used to test complement blockade by eculizumab in the modified Ham test. Statistical analysis

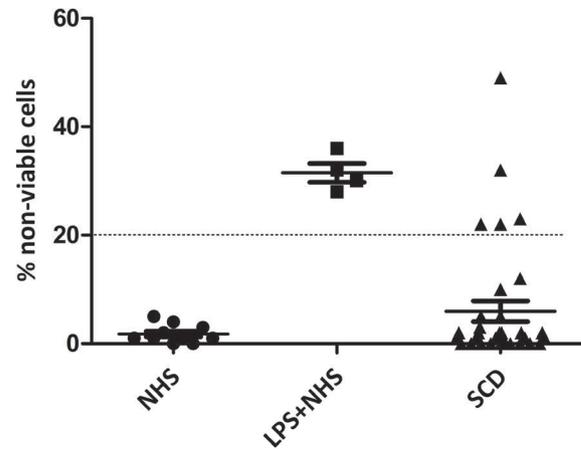


Figure 1. Increased complement-mediated cell killing (>20% of non-viable cells) in a portion of patients with sickle cell disease (SCD) using the modified Ham test. Normal human serum (NHS) was used as a negative control and lipopolysaccharide (LPS)-incubated normal serum as a positive control.

was performed using the Statistical Package for Social Sciences (SPSS) 20.0 for Windows (SPSS, Chicago, IL, USA). Results are presented for continuous variables as mean±Standard Deviation or (for skewed variables) as median±interquartile range, and for qualitative variables as frequencies. Statistical analyses were carried out using the independent samples Student *t*-test or the Mann Whitney U test to compare differences between mean values of two groups. Pearson or Spearman correlation coefficients were used according to the distribution of the variable. $P \leq 0.05$ was considered statistically significant.

We studied 34 Caucasian SCD patients (36.8±11.1 years of age; 17 male, 17 female). Patients' characteristics are shown in Table 1. Based on previous studies^{7,9} and results of internal controls, percentage of non-viable cells higher than 20% was considered a positive modified Ham test, indicating increased APC activation in 5 SCD patients (Figure 1). We compared patients' characteristics as shown in Table 1 in positive compared to negative SCD patients in the modified Ham test. Positive patients had significantly increased HbS levels (80.0±9.4 vs. 61.6±5.0%; $P=0.014$), while 2 of 5 were homozygous (HbS/S) and 3 of 5 double heterozygous (HbS/β-Thal). The majority of positive patients (4 of 5) were not on hydroxycarbamide. Percentage of non-viable cells in the modified Ham test showed a trend towards a significant association with HbS percentage ($r^2=0.310$, $P=0.074$), lactate dehydrogenase (LDH) levels ($r^2=0.319$, $P=0.066$), and platelets ($r^2=0.319$, $P=0.073$).

Regarding soluble C5b-9 levels, their median value in SCD patients was 256 ng/mL (interquartile range 108 ng/mL). This value was significantly increased compared to normal human serum (median 175 ng/mL, interquartile range 123 ng/mL; $P<0.001$). Soluble C5b-9 levels were not associated with patients' characteristics, except for age. A significant positive association was shown between age and soluble C5b-9 levels in SCD patients ($r^2=0.439$, $P=0.012$). We then evaluated *in vitro* the efficacy of complement inhibition by eculizumab in the modified Ham test. Mixing eculizumab-containing serum with complement-activated sera abrogated complement-mediated cell killing in a dose-dependent relationship that was consistent across the 3 patients tested.

Table 1. Patients' characteristics.

Characteristics	Patients (n=34)
Age (years)	36.8 ± 11.1
Hemoglobin (g/dL)	9.5 ± 1.1
Hemoglobin S (%)	64.8 ± 15.9
Hemoglobin F (%)	11.4 ± 9.1
HbS/S (n) – Hb/S (n)	9 - 25
Platelets (10 ⁹ /L)	355.9 ± 240.8
White blood cell count (10 ⁹ /L)	10.2 ± 4.8
Lactate dehydrogenase (U/L)	420.5 ± 142.9
Bilirubin (mg/dL)	1.8 ± 1.6
Splenectomy (n)	18
Hydroxycarbamide treatment (n)	21
Antithrombotic/antiplatelet treatment (n)	14
History of vaso-occlusive crises (n)	12

Continuous variables are expressed as mean±Standard Deviation. n: number; Hb/S: hemoglobin S; HbS/S: homozygous HbS.

There have only been a limited number of studies in the field, and our study provides only preliminary *in vitro* results in a rather small number of patients. In addition, our real-world patient population might differ from SCD patients in other countries. Interpretation of results should take into account the relatively high number of Hbβ/S patients and previous or current treatments (splenectomy, antithrombotic or antiplatelet therapy and hydroxycarbamide).

In line with our data, Chapin *et al.* reported similarly increased soluble C5b-9 levels in patients with SCD.¹⁰ In addition, age that was significantly associated with C5b-9 in our population, has recently been found to be an important predictor of mortality in adult patients with SCD.¹ Interestingly, HbS percentage (a major indicator of disease in SCD patients) was associated with evidence of systemic complement dysregulation in our population for whom we used a cell-based functional assay. The modified Ham test was originally developed for atypical hemolytic uremic syndrome (aHUS), a disease of excessive complement activation.⁵ Except for aHUS, the modified Ham test has also been shown to detect complement activation in other hemolytic anemias.^{9,11} As our understanding of complement-related disorders evolves, it seems that cell-based assays may better reflect complement activation *in vitro*, since activation is caused as a result of defective interaction with cells.¹²

In conclusion, our results suggest that complement dysregulation might be an additional factor in the complex pathophysiology of SCD in steady state. Further studies with larger series of patients are needed in order to elucidate the role of APC activation during painful or vaso-occlusive crisis, and evaluate the role of different functional assays. While more SCD patients undergo hematopoietic cell transplantation, SCD patients may be at risk of complement-mediated vascular injury, not only due to their underlying hemolytic disease, but also due to other triggers, such as transplant-associated thrombotic

microangiopathy.¹³ Early recognition of patients with increased complement activation may facilitate prompt intervention. Future prospective studies are needed to better understand the role of complement in the pathophysiology and therapeutics of SCD.

Eleni Gavriilaki,¹ Maria Mainou,² Ioanna Christodoulou,² Eudoxia-Evaggelia Koravou,¹ Aggeliki Paleta,¹ Tasoula Touloumenidou,¹ Apostolia Papalexandri,¹ Anastasia Athanasiadou,¹ Chrysa Apostolou,² Philippos Klonizakis,² Achilles Anagnostopoulos¹ and Efthymia Vlachaki²

¹Haematology Department BMT Unit, G. Papanicolaou Hospital, Thessaloniki and ²Adults Thalassaemia Unit, 2nd Department of Internal Medicine, Aristotle University of Thessaloniki, Hippokraton General Hospital of Thessaloniki, Greece

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Correspondence: elenicelli@yahoo.gr
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