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New insights into red cell rheology and adhesion in patients with sickle cell anaemia during vaso-occlusive crises

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Painful vaso-occlusive crisis (VOC) represents the most frequent complication encountered by SCA patients. In animal models, increased red blood cell (RBC) adhesion to the endothelium has been demonstrated to initiate VOC by increasing the transit time of RBC through the microcirculation, predisposing them to sickle and occlude the vessels (Kaul *et al.*,

1996). Correlation between RBC adhesion and disease severity has been reported (Hebbel *et al.*, 1980a) and patients with frequent VOC are characterized by increased blood viscosity compared to patients with few VOC (Connes *et al.*, 2016). However, while most studies assessed RBC adhesiveness or blood rheology in patients at steady state, only few analysed changes in RBC properties during and after a VOC in the same patients (Ballas and Smith 1992). The present study was devoted to compare blood rheology, the percentage of irreversibly sickled cells (ISCs) and RBC adhesion in the same SCA patients at steady state and during acute VOC.

We included 32 SCA adults (12 females/20 males, median age 27 years; IQR: 18–38 years) regularly followed by the Sickle Cell Centre of Guadeloupe and admitted to the emergency department for painful VOC (Hierso *et al.*, 2017). Blood was sampled after admission in the emergency department for a VOC episode and before any treatment/medication provided. At least 3 months after their release of the emergency department, blood samples were collected from the same patient at steady-state (i.e., without any transfusion in the previous 3 months and without any acute sickle complication in the previous and the following 2 months). Blood viscosity at native haematocrit (Hct), RBC deformability (elongation index), RBC aggregation (Aggregation Index; AI) and the RBC disaggregation threshold were measured as previously described (Baskurt *et al.*, 2009).

RBC adhesion experiments were performed in monolayers of TNF α -activated (TrHBMECs) endothelial cells with blood samples of 11 patients available both at steady-state and during VOC. For adhesion assays, perfusion of RBC at 1.5% Hct in Hanks solution with a shear stress of 0.2 dyn/cm² for 10 min and washes of 5 min were performed (0.5, 1, 1.5, 2 and 3 dyn/cm²). Data collection and analysis were performed using the AxioVision 4 analysis (Carl Zeiss). Two μ l of packed RBC were suspended in 200 μ l of ID-CellStab (Biorad) and 50,000

events were acquired using an Imagestream ISX MkII flow cytometer (Amnis Corp, EMD Millipore). ISCs were quantified using the IDEAS software (version 6.2) as previously described (Lizarralde Iragorri *et al.*, 2018). Results are presented as median and interquartile range (IQR). Wilcoxon test was used for comparisons between VOC and steady-state. Spearman correlations were performed to test the associations between different parameters.

RBC elongation index decreased and RBC aggregation increased during VOC compared to steady state (Figure 1). The RBC disaggregation threshold tended to be higher during VOC than at steady-state, while no difference was observed for blood viscosity.

No statistically significant difference in the median of ISC was detected between VOC (median 4.335, IQR: 1.450-13.906) and steady state (median 4.160, IQR: 1.50-10.60). RBC adhesion during VOC was not higher compared to steady state (median 61, IQR: 4.160-848.0 RBC/mm² and median 86.5, IQR: 848.0-806.0RBC/mm², respectively, $p = 0.096$). However, during VOC, we observed a positive correlation between RBC adhesion and the %ISCs, (Fig 2A) although no association was observed between RBC adhesion and the percentage of reticulocytes (Fig 2B). In contrast, at steady state, no correlation was observed between RBC adhesion and the %ISCs (Fig 2C) but a positive correlation was found with the percentage of reticulocytes (Fig 2D).

This study analysing RBC morphological properties and function is one of the few comparing the same patients during VOC (before any treatment) and in steady state condition. We observed a decrease in RBC deformability and showed that RBC aggregation increased during VOC compared to steady-state condition. The decrease of RBC deformability during VOC is in agreement with the hypothesis of Ballas (Ballas and Smith 1992) who suggested that the most deformable sickled RBC are sequestered within the microcirculation during

VOC because of their maximal adhesiveness to the endothelium. The slightly but significantly higher RBC aggregation during VOC is consistent with a previous report showing increased erythrocyte sedimentation rate in SCA patients during VOC (Lawrence and Fabry 1986). These RBC aggregates tended to be stickier/robust during VOC, which could slow down blood flow at the entry of capillaries. Sticky sickled RBC aggregates would preferentially deposit at bifurcation level in vascular networks, which could increase the risks for VOC (Loiseau *et al.*, 2015). Surprisingly, we did not observe any change in blood viscosity during VOC. While patients with high blood viscosity are at risk for frequent VOC (Connes *et al.*, 2016), our results suggest that blood viscosity does not further increase during a VOC episode.

ISCs are a subpopulation of poorly deformable dense cells formed during prolonged deoxygenation, which contribute to the pathophysiology of VOC. Our findings demonstrated no difference in the %ISCs between VOC and steady state but rather we observed a significant correlation between ISCs and adherent cells at VOC. The lack of %ISC changes between VOC and steady-state could be explained by the fact that some high density sickle erythrocytes would have been trapped into the microcirculation during VOC and thus not available in peripheral blood for analysis (Fabry *et al.*, 1989). In contrast to a previous study indicating that enhanced adhesion was related to VOC (Hebbel *et al.*, 1980b), no difference in RBC adhesion was observed between VOC and steady state.

Unfortunately, it is difficult to conclude definitively due to the limited number of patients from whom the adhesion experiments were investigated, even though it was checked carefully that the studied sample was representative of the whole cohort (Hierso *et al.*, 2017). However, one interesting and new data from this study is that enhanced RBC adhesion to endothelial cells is most probably mainly related to reticulocytes at basal state while during VOC, adhesion events involve preferentially ISCs RBC sub-population. Further studies focusing on

the comparison of reticulocyte and ISC characteristics between asymptomatic patients and those who suffer from VOC will provide more input to this topic.

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Author contributions: All the authors met the criteria for contributing authors. CL, PC, SEH, MR and MDHD conceived and designed the experiments. CL, PC, SEH, KC, MR and MDHD performed the experiments. CL, PC, RH, MR and MDHD analysed the data. RH, NL contributed to the collection of the clinical data. CL, PC, SEH, JE, CLVK, MR and MDHD wrote the paper. All authors revised and approved the paper.

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Figure legends

Figure 1: Selected rheology parameters at steady state and during vaso-occlusive crisis in SCA patients. A, Blood viscosity; B, RBC deformability; C, RBC aggregation index; D, RBC aggregates strength

Figure 2: Relationships between ISCs, reticulocytes and adherence during vaso-occlusive crisis and at steady-state in a SCA patients. (A,C) correlations between ISCs; (B, D) correlations between reticulocytes and adherent SS-RBCs during vaso-occlusive crisis and at steady-state.