Increased Vasoocclusive Crises in “O” Blood Group Sickle Cell Disease Patients: Association with Underlying Thrombospondin Levels

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Competing interests: The authors have declared that no competing interests exist.

Abstract. Objectives: To explore the incidence of vaso-occlusive crisis (VOC) in Blood Group “O” sickle cell disease (SCD) patients, and correlate it with the blood group and thrombospondin (TSP) levels.

Methods: In 89 consecutive SCD patients, blood samples were obtained for von Williebrand factor (vWF:Ag) antigen, collagen binding activity (CBA), ristocetin binding activity (RCo), blood group typing, C-reactive protein (CRP), high performance liquid chromatography (HPLC), Serum TSP 1 and TSP 2 levels, complete blood counts (CBC), lactic dehydrogenase (LDH) levels, liver function (LFT) and renal function tests (RFT) during VOC episodes and in steady state conditions.

Results: In steady state SCD patients (n=72), “O” blood group patients (n=37) showed a significantly higher median serum TSP 1 and TSP 2 levels as compared to non-O blood group patients [n=35] [p <0.05, Mann-Whitney test]; with an inverse relation between vWF:Ag, Factor VIII:C and TSP levels. Furthermore, the serum TSP 1 and TSP 2 levels were significantly higher in patients presenting with acute VOC [n=17], as well as in those with repeated VOC’s (group 1, n=16), especially amongst blood group “O” patients [p, <0.05, Mann-Whitney test].

Conclusions: The study demonstrates an inverse relation between TSP and vWF levels, in blood group “O” SCD patients, with an upregulation of the TSP levels. Expectedly, during active VOC crisis, the TSP 1 and TSP 2 levels were significantly elevated.

Keywords: VOC; SCD; TSP 1; TSP 2; vWD; Blood groups.

Citation: Al Huneini M., Alkindi S., Panjwani V., Al Falahi K., Al Balushi B., Gravell D., Ho C.H., Krishnamoorthy R., Pathare A.V. Increased vasoocclusive crises in “O” blood group sickle cell disease patients: association with underlying thrombospondin levels. Mediterr J Hematol Infect Dis 2017, 9(1): e2017028, DOI: http://dx.doi.org/10.4084/MJHID.2017.028

Published: April 20, 2017 Received: December 21, 2016 Accepted: March 23, 2017

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Introduction. Sickle cell disease (SCD) is a condition with protean manifestations and demonstrates considerable clinical variability.¹² The disease constitutes one of the most frequent causes of hospitalizations in the Sultanate of Oman.³⁴ It is characterized by chronic hemolysis, frequent infections, recurrent occlusion of microcirculation; leading to painful crises, chronic organ damage and premature death. Intermittent painful episodes due to the vaso-occlusive crisis (VOC) is the most common clinical manifestation of SCD, but subclinical episodes also occur.⁵
The mechanisms by which VOC’s are initiated is complex and multifactorial. Sickle red blood cells (RBCs) contribute to the initial VOC process and play a significant part in nearly all the clinical manifestations of SCD. The pro-adhesive sickle cells bind to endothelial cell P-selectin, E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, CD36, leading to the complex process of endothelial activation. Polymerization of deoxy-HbS is an ongoing process in SCD and plays the most significant role in the process of sickling of RBCs in SCD. Thus, the end result of multiple episodic cycles of polymerization of deoxy-HbS with dehydration of the RBC is a dense, irreversibly sickled red cell. However, when oxygenated, an irreversibly sickle cell may contain no polymer but is nonetheless distorted in shape and may still contribute to vaso-occlusion. These features make negotiation of the microvasculature difficult, if not impossible, for these sickle erythrocytes.

RBCs in SCD also appear to have an increased binding affinity to the vascular endothelium. The degree of affinity correlates strongly with the severity of clinical disease. Several molecular interactions contribute to this endothelial affinity and are mediated by increased levels of integrin VLA-4 (α4β1) and membrane glycoprotein IV (CD36). VLA-4 mediates adhesion both to endothelial vascular cell adhesion molecule-1 (VCAM-1) and to fibronectin present in activated endothelium, whereas, CD36 mediates adhesion via thrombospondin to αVβ3 integrin on activated endothelium. Thrombospondin is normally present in platelet α granules and is released from activated platelets. Thrombospondin binds CD47 (integrin-associated protein) expressed on RBC membranes in addition to binding with CD36. The endothelial selectin activation by adhesion molecules expressed in sickle red cells, and their inhibition of endothelium-dependent vasorelaxation, by blocking the endothelium-derived relaxing factor (EDRF) contribute to worsening vaso-occlusion. Further, it has long been known that the microvasculature of patients with SCD may develop intimal hyperplasia. This creates irregular areas of endoluminal narrowing, which worsen vaso-occlusion by promoting thrombosis. This process has been documented in the cerebral and splenic vascular beds.

Many factors are known to affect the frequency of VOC, such as HbF concentration, sickle β haplotypes, and the presence of various adhesive substances, which enhance the sickle red cells adherence to the subendothelial structures. TSP and von Willebrand factor (vWF) are among the proteins that have been implicated as mediators of the adhesive interactions between sickle erythrocytes and the blood vessel wall. However, sickle erythrocytes were found more adherent to immobilized TSP than to vWF. Furthermore, TSP1 has been involved in the liberation of toxic membrane vesicles from RBCs, which contributes to the degradation of vascular function and promote vasoocclusion. Thrombospondin is known to bind CD47. However, although HbA and HbS RBCs express the same amount of CD47, adhesion of TSP to HbS RBCs is preferentially more, due to an upregulation of TSP in SCD patients which is mediated by VLA-4. Further, vWF also induces sickle erythrocyte adhesion by interaction with endothelial αVβ3 integrin. But, the binding of sickle RBCs to TSP was found to be inhibited by vWF. Therefore, the perturbation in the vascular endothelium, induced by sickle RBC’s, involves complex interactions between adhesive proteins, culminating in the VOC process and is orchestrated by the various cytokines, a mechanism quite different from thrombosis.

An increased risk of venous thromboembolism (VTE) is reported in non-O blood group patients, as well as in patients with sickle cell disease. Kostner et al reported that the odds ratio (OR) for VTE in individuals with non-O blood groups vs. “O” blood group individuals was 2.0 (95% CI, 1.4–2.9). After adjustment for factor VIII and VWF levels, the risk of VTE among non-O blood group carriers was still significantly high (OR 1.5; 95% CI, 1.0–2.2). Similar results were also reported by Tirado et al in 2005, and by Ahmed et al in 2015 specifically in sickle cell trait patients.

Interestingly, in a pilot study on consecutive SCD patients presenting with VOC, we observed a higher incidence in “O” blood group than the non-O blood group phenotype. Furthermore, since we know from the literature that vWF levels in blood group “O” subjects are on an average 25% lower than the non-O blood group subjects, we undertook this study, to see whether there is a relationship between blood group “O”, vWF and VOC levels and VOC occurrence in SCD patients.
Methods. 89 consecutive SCD patients (76-HbSS, 10-HbSββ0, 3-HbSβthal) were prospectively enrolled in this study after informed consent and approval by the Medical Research and Ethical Committee at the Sultan Qaboos University Hospital. 17 patients were recruited from the inpatient service during episodes of acute VOC’s, whereas the remaining 72 patients consented at the outpatient haematology clinics in the steady state. vWF antigen, ristocetin cofactor activity, collagen binding activity, vWF multimer analysis, blood group typing, CRP, HPLC, Thrombospondin [TSP 1], TSP 2, CBC, LFT, LDH, and RFT were recorded at enrollment. Severity and number of VOC’s/year were assessed by stratifying these patients into 2 groups. Group 1 consists of patients with a history of significant VOC’s, [with ≥4 VOC’s/year needing inpatient care] and Group 2 consisting of SCD patients with non-frequent VOC’s [with <4 VOC’s/year].

Blood was obtained by venipuncture into vacutainer tubes with Ethylene diamine tetra acetic acid anticoagulant, 3.2% sodium citrate, and plain tubes. Complete blood counts were performed with an electronic cell counter (Abbott CELL-DYN® Sapphire, Abbott Diagnostics, Abbott Park, IN). A fresh hemolysate was prepared from each sample and subjected to cation-exchange HPLC (Bio-Rad VARIANT, Bio-Rad Laboratories, Hercules, CA) to study the sickle phenotype. Serum was separated from a clotted tube sample at 1,000g at 4°C for 10 min and stored at -70°C for Thrombospondin [ELISA] and other biochemical assays. CRP was estimated by rate nephelometry, in addition to the various biochemical parameters of renal and liver function.

Whole blood samples were collected for coagulation studies in 3.2% sodium citrate (at a ratio of 9:1 v/v) and centrifuged the same day within 2 hours of collection, aliquoted, and stored at -70°C for Thrombospondin [ELISA] and other biochemical assays. CRP was estimated by rate nephelometry, in addition to the various biochemical parameters of renal and liver function.

Whole blood samples were collected for coagulation studies in 3.2% sodium citrate (at a ratio of 9:1 v/v) and centrifuged the same day within 2 hours of collection, aliquoted, and stored at -70°C. Plasma activities of fibrinogen (Claus assay), coagulation assays for prothrombin time, activated partial thromboplastin time were performed on the same day [Dade Behring reagents]. Platelet poor plasma samples [5 aliquots of 1 ml plasma each] were frozen to perform the vWD phenotypic studies.

The vWF: Ag assay was performed by the Dade Behring vWF Ag latex agglutination method for quantitative determination of vWF Ag in human plasma by immunoturbidometry, according to the procedure supplied by the manufacturer (Dade Behring vWF Ag kit). The vWF: RCo was measured by platelet aggregometry using normal lyophilized platelets with CHRONO PAR [ristocetin] for the CHRONO-LOG aggregometer. The vWF: CBA was measured by an ELISA for the determination of vWF function in the human plasma using Life Diagnostic ELISA kits in duplicate, according to the manufacturer’s instructions. Factor VIII: C levels were measured using a one-stage assay. [Dade Behring reagents]

TSP 1 and TSP 2 concentrations in serum were determined using the Quantikine Human Thrombospondin Immunoassay kits [R & D systems, Minneapolis, MN]. This assay employs the quantitative sandwich enzyme immunoassay technique and contains NS0-expressed recombinant human Thrombospondin.

Statistical Analysis. Data was analyzed with IBM Statistical Package for the Social Sciences software (SPSS, version 19.0; SPSS, Chicago, Illinois, USA). Continuous variables were expressed as mean ±SD, whereas categorical variables were expressed as numbers (percentages). Means of continuous and categorical variables were compared using Mann-Whitney U and Fisher’s exact tests respectively. The vWF: Ag, vWF: CBA, FVIII: C and TSP levels showed a skewed distribution and were expressed as median values with interquartile ranges. A p value <0.05 was considered as statistically significant.

Results. The mean age ±SD of this SCD patient cohort was 23.8 ± 6.3 (range 15 to 48 years). 50 patients were males (56%) and 17 (19%) presented with acute VOC’s; whereas 16 (18%) (Group 1) comprised of patients with a history of significant VOC’s [≥4 VOC’s/year]. 37 patients (42%) were on stable hydroxyurea therapy. 72 patients (81%) were enrolled in “steady state” defined as “no acute illness or crisis or infection in the past 3 months” when they visited the clinic, with a prior appointment as an outpatient. Amongst these, 37 (51%) had “O” blood group phenotype, whereas, 35 had non-O blood group [A group -18 patients; B group -14 patients and AB group -3 patients]. None of the study participants enrolled were on a chronic exchange transfusion program.

Table 1 summarizes the vWF parameters and TSP 1 and TSP 2 levels in the all the steady state
SCD patients [n=72]. Amongst these, in the “O” blood group patients [n=37], the median serum TSP 1 and TSP 2 levels were significantly higher than the non-O blood groups SCD patients [n=35] [p<0.05, Mann-Whitney U test]. Furthermore, there was an inverse correlation between the TSP levels and Factor VIII: C levels. The inter-assay and intra-assay CV for thrombospondin assay were 6.3% and 5% respectively.

Table 2 summarizes the vWF parameters and TSP 1 and TSP 2 levels in the two subgroups of the study participants, namely those in steady state [n=72] and those with acute VOC’s [n=17]. In the SCD patients admitted for VOC’s, the median serum TSP 1 and TSP 2 were significantly higher than those in steady state SCD [p<0.05, Mann-Whitney U test]. Furthermore, all the vWF parameters studied were significantly lower in the painful crisis patients. The number of SCD patients with “O” blood group were relatively higher in the painful crisis group (65%) but was not statistically significant. However, the number of SCD patients on HU were significantly higher in the painful crisis group (64%), although the HbF levels were similar. The median serum TSP 1 and TSP 2 were higher in the “O” blood group subsets comparing steady state group and “acute” crisis group [p<0.05, Mann-Whitney U test].

Table 3 summarizes the vWF parameters and TSP 1 and TSP 2 levels in groups 1 and 2, namely those with a history of frequent VOC’s [n=16] and those with infrequent VOC’s [n=73]. The median

| ABO Blood Group | Age, yrs. Mean±SD | vWF:Ag, iu/mL Median | vWF:CBA, iu/mL Median | vWF:III:C, iu/mL Median | TSP 1, ng/mL Median | TSP 2, ng/mL Median |
|----------------|------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| “O” [n=37]     | 23.35, 46.29     | 1.14, 0.86-1.37     | 1.3, 0.9-1.53       | 1.27, 1.1-1.6       | 31, 25.2-49         | 939, 828-1064       |
| Non-O [n=35]   | 24.85, 46.14     | 1.35, 0.84-1.77     | 1.6, 1.2-1.94       | 1.4, 1.1-1.8       | 24.8, 16.8-28.4     | 922, 824.5-960.75   |
| p value        | 0.26*            | 0.09*               | 0.036**             | 0.16*              | 0.045**             | 0.042**             |

** p<0.05 - Significant, Mann-Whitney; NS* – Not significant.

**Table 1.** vWF:Ag, vWF:CBA, FVIII:C, Thrombospondin [TSP 1 and TSP 2] levels [Median, IQ range] with Age [Mean±SD] & ABO blood Group distribution in SCD patients in steady state[n=72].

**Table 2.** Age, [Mean±SD], Clotting times, Fibrinogen, vWF:Ag, vWF:CBA, FVIII:C, Thrombospondin [TSP 1 and TSP 2] levels [Median, IQ range] in the SCD patients in steady-state and VOC’s in “O” blood group patients.

**Table 3.** Age, [Mean±SD], Clotting times, Fibrinogen, vWF:Ag, vWF:CBA, FVIII:C, Thrombospondin [TSP 1 and TSP 2] levels [Median, IQ range] in the SCD patients in steady-state and VOC’s in “O” blood group patients.
Tables 1

Relationship between TSP levels

| SCD patients [n=89] | Group 1 [n=16] | Group 2 [n=73] | Group 1-“O” group [n=10] | Group 2-“O” Group [n=38] |
|---------------------|----------------|----------------|-------------------------|-------------------------|
| Age, yrs. | 23.19 ± 5.05 | 23.9 ± 6.5 | 24.6 ± 5.5 | 22 ± 19.26 |
| vWF:Ag, iu/mL, Median Interquartile Range | 1.14 (0.8-1.6) | 1.14³ (0.87-1.67) | 1.15 (0.78-2.01) | 1.15 (0.87-1.5) |
| vWF:CBA, iu/mL, Median Interquartile Range | 1.4 (0.9-1.9) | 1.4² (1.04-1.68) | 1.26 (0.88-1.93) | 1.3 (0.9-1.5) |
| FVIII:C, iu/mL, Median Interquartile Range | 1.15 (0.9-1.37) | 1.3¹ (0.96-1.68) | 1.23 (1.07-1.4) | 1.3 (1.0-1.59) |
| TSP 1,iu/mL, Median Interquartile Range | 27.2 (20.9-31.2) | 23.4⁴ (15.49-49.4) | 41.4⁵ (17.8-74.2) | 30.8⁶ (21.8-46) |
| TSP 2,iu/mL, Median Interquartile Range | 877 (789-1030) | 920 (808-1035) | 924⁷ (828-1136) | 93⁸³ (849-1096) |
| % SCD patients with “O” Blood Group | 62.5% | 52% | | |
| HbF levels,% in SCD patients on HU Mean ± SD | 6.1 ± 6.5 | 6.6⁹ ± 5.7 | | |
| % SCD patients on HU | 68.75% | 36%³ | 26/72 | 55 | 100+55

p<0.05, Chi square, *Not significant. ³p>0.05, Mann-Whitney [Group 1 v/s Group 2], ⁴p<0.05, Mann-Whitney [O v/s non-O blood group patients]

Discussion. This study documents that both serum TSP 1 and serum TSP 2 were higher in the “O” blood group subsets in both groups 1 and 2 [p<0.05, Mann-Whitney U test]. Furthermore, there was an over representation of “O” blood group in Group 1 SCD patients (62.5%), but this was not statistically significant. However, the number of SCD patients on HU were significantly higher in the group 1 (68.75%), although the HbF levels were similar.

serum TSP 1 and TSP 2 were higher in the “O” blood group subsets in both groups 1 and 2 [p<0.05, Mann-Whitney U test]. Furthermore, there was an over representation of “O” blood group in Group 1 SCD patients (62.5%), but this was not statistically significant. However, the number of SCD patients on HU were significantly higher in the group 1 (68.75%), although the HbF levels were similar. It, therefore, appears that the increased presence of markers of platelet activation such as β-selectin, platelet factor-4, beta thromboglobulin, soluble CD40 and platelet microparticles seen during VOC’s is representative of the underlying inflammatory state.

In this study, we observed that “O” blood group was overrepresented in SCD patients presenting with VOC’s, in comparison to the non-O blood group SCD patients. This was documented both in patients who were frequently admitted with VOC’s in group 1 (62.5%) as well as in patients who were enrolled in the study as in-patients (65%). Since it is known that vWF levels are lower in “O” blood group than non-O blood group subjects, we investigated whether vWF levels would play a contributory role in the occurrence of VOC’s in SCD patients. We observed that there was an inverse relationship between TSP levels and vWF and FVIII: C during active VOC’s, with the TSP1 and TSP2 levels being significantly elevated (Tables 1 and 2). Interpreting this observation in the light experimental evidence that sickle erythrocyte adhesion to immobilized TSP is inhibited by vWF, implies that sickle RBC adhesion is significantly influenced by the relative concentrations of TSP and vWF in the vascular wall. Thus in “O” blood group SCD patients with a relatively lower basal vWF levels, the relative rise in the TSP levels could promote VOC’s more easily in comparison to the non-O blood group SCD patients.
Interestingly, ABO blood group has been shown to have a profound influence on the incidence of VTE, with plasma levels of vWF, being approximately 25% higher in individuals who have non-O blood group rather than “O” blood group. Several case-control studies have consistently shown that non-O blood group patients have an increased risk for venous thrombosis with the AB blood group having a two-fold higher risk for thrombotic vascular disease.

Thus, the important point this paper raises is that although “O” blood group SCD patients are at a lower risk for VTE, they were actually overrepresented amongst SCD patient with VOC’s. This implies that mechanistic differences in pathways leading to VTE and VOC’s in SCD patients are likely to explain the dissimilarities seen with different underlying risk factors. In fact, VOC’s, as opposed to VTE’s, is an inflammatory condition with the clinical manifestations and complications reflecting an interplay of several biomarkers.

The up-regulation of P-selectin in endothelial cells and platelets contributes to the cell-cell interactions that are involved in the pathogenesis of VOC’s and sickle cell-related pain. Ataga et al., recently demonstrated that therapy with crizanlizumab, an antibody against P-selectin, resulted in a significantly lower rate of VOC’s as compared to placebo. In transgenic humanized SCD mice, Bennewitz et. al., recently demonstrated that microembolism of precapillary pulmonary arteriolar vessels by neutrophil-platelet aggregates, causing acute chest syndrome cleared following infusion of platelet P-selectin antibody. Usefulness of both these therapeutic approaches demonstrates the role of selectin as an important adhesive protein that plays a significant role in the pathogenesis of sickle VOC’s.

Selectin is important in the activation of platelets, which is another mechanistic pathway active in sickle VOC’s. Al Najjar et. al., demonstrated that patients with SCD have increased expression of E-selectin and P-selectin and play an important role in the pathogenesis of VOC’s. Annarapu et. al., demonstrated that free plasma hemoglobin present following intravascular hemolysis in SCD binds to glycoprotein 1ba, inducing the activation of platelets. Wu et. al., in a double blind, randomized study showed that prasugrel, a third-generation thienopyridine, was able to decrease platelet activation biomarkers and reduce sickle-cell VOC pain as compared to placebo. Although it is believed that platelets mediate intercellular adhesion during sickle cell VOC’s, Heeney et al., in an international multicenter study utilizing prasugrel, failed to show a statistically significant reduction in VOC’s, although there was a trend to show a reduction in the VOC pain.

Low molecular weight heparins (LMWH) have been used to control the hypercoagulability associated with sickle cell VOC’s. In a randomized study using Tinzaparin, Qari et. al., showed reduced severity and duration of acute crisis in sickle cell anemia. However, well-designed placebo-controlled studies with different LMWH, and enrolling participants with different genotypes of sickle cell disease are lacking. Telen et al. demonstrated that sevuparin, a heparin-derived polysaccharide, reduced sickle cell related VOC’s. The efficacy of sevuparin is believed to be due to its anti-adhesive properties, as it binds to P-and L-selectins, TSP, fibronectin, and VWF, all of which are involved in the sickle cell VOC’s.

Lastly, it has also been reported that a high level of extracellular hemoglobin plays an important role in SCD patients since nitric oxide (NO) quenching mechanism are compromised. The free hemoglobin binds not only to vWF multimers but also with ADAMTS-13, leading to an acquired ADAMTS-13 deficiency, blocking appropriate proteolysis of vWF, causing the accumulation of ultra-large vWF multimers. However, using real-time fluorescence intravital microscopy, Barazia et al. showed that plasma nitric oxide levels could be normalized by using hydroxyurea therapy.

Overall, therefore, it is quite apparent that SCD is actually a well-recognized state of chronic indolent inflammation and there indeed exist several lines of evidence demonstrating the mechanistic differences in VOC pathways as against VTE pathways. Adhesive proteins like selectins and TSP decelerate sickle red cells and the platelet-leukocytes interactions in the circulation, facilitating endothelial adhesion and other cell-cell interactions, ultimately leading to vascular occlusion in sickle VOC’s. However, the occurrence of VTE depends on its predisposing risk factors.
The major drawback of this study is the small number of evaluable patients. Although the study prospectively enrolled consecutive patients for almost 2 years, we were able to get only a total of 89 SCD patients. Nevertheless, our data are valuable as they show observations in an ethnic SCD population that have not been reported before.

Conclusions. Abnormal adhesive interactions between sickle erythrocytes and vascular endothelial cells and/or subendothelial matrix play a significant role in the initiation of sickle VOC’s. Selectins, TSP and vWF are important mediators of the adhesive interactions between sickle erythrocytes and the blood vessel wall. Our study showed an inverse relation between TSP and vWF levels, in blood group “O” SCD patients with elevated TSP levels during active VOC’s.

Acknowledgements. We wish to thank the Hospital Administration for allowing the use of hospital data. This work was supported by a research grant no. IG/MED/HAEM/10/02.

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