Original article:

Haemostatic alterations in patients of sickle cell trait and homozygous sickle cell disease – A hospital based case control study

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Date of Submission: 04 March 2014; Date of Publication: 11 June 2014

Abstract:
Background: Nearly every component of hemostasis is altered in patients with sickle cell disease (SCD). Though these patients are known to be in hypercoagulable state, increased risk of peri-operative bleeding complications has also been observed in these patients. Our aim was to determine the mean levels of platelet indices, Prothrombin time (PT), activated Thromboplastin time (APTT) and fibrinogen in patients with homozygous sickle cell disease (HbSS), sickle cell trait (HbAS) and normal controls (HbAA) and their role as prognostic markers.

Method & Materials: The study included 321 cases of sickle cell haemoglobinopathies (118 HbSS and 203 HbAS) and 321 normal controls. Platelet indices were determined by automated cell counter. PT, APTT and fibrinogen levels were estimated by using commercial agents and BK coagulometer. It was done by using student’s t-test and chi square tests by statistical software STATA version 9.0.

Results: Mean fibrinogen levels were 275.56, 357.37 and 522.24 mg/dl respectively in HbAA controls, HbAS and HbSS patients. The fibrinogen levels in HbSS patients were found to be raised even more in those in crisis. Mean platelet volume (MPV), Platelet distribution width (PDW) and PT and APTT values were also significantly prolonged in these patients.

Conclusions: Since, fibrinogen levels showed a higher increase in crisis, its estimation can be used as a parameter to monitor progression of sickle cell crisis. We obtained high MPV and PDW in HbSS patients as compared to controls; larger platelets are more thrombogenic, we propose a hypothesis that larger platelets in HbSS patients may predispose them to vaso-occlusive crisis.

Key words: Coagulation, fibrinogen, platelets, Sickle cell anaemia

Introduction
Sickle cell disease (SCD) is a common term for a group of haemoglobinopathies characterized by sickle cell anaemia, sickle beta thalassemia syndromes and other haemoglobinopathies in which HbS is in association with abnormal haemoglobin. Sickle cell anaemia (HbSS) results from homozgyosity for A-T substitution at codon 6 of β globin gene (GAG-GTG) leading to a glutamic acid to valine (Glu-Val) substitution in the β globin chain of human adult haemoglobin (Ingram 1958) [1,2]

Though the highest prevalence of HbS is in tropical Africa, more than 50% of world’s sickle cell anaemia cases are in India, second most common
haemoglobinopathy after thalassemia. In India, Vidarbha region of Central India and South India are considered as endemic areas for sickle cell haemoglobin. In a recent study by Deshmukh et al, prevalence of sickle cell haemoglobin in Wardha district was reported to be 2.9%. SCD is characterized by two cardinal pathological manifestations – chronic hemolytic anaemia and vaso-occlusive crisis. Though patients with SCD are characterized by a hypercoagulable state, nearly every component of hemostasis, including platelet function and the procoagulant, anticoagulant and fibrinolytic systems, is altered in this disease. Many studies have observed platelet activation and aggregation markers in homozygous SCD, but there is paucity in literature regarding the importance of simple parameters like Mean platelet volume (MPV) and Platelet distribution width (PDW) in this disease. Patients with homozygous SCD are in a hypercoagulable state, it is still debatable whether further increment occurs in this state during crisis. Also it remains to be elucidated whether this hypercoagulable state is involved in pathogenesis of crisis or is merely a secondary effect following crisis. This issue is further complicated by certain reports suggesting the presence of a hypercoagulable state in steady state sicklers and any evidence of hypercoagulability may be an early indication for an onset of crisis. In the current study we sought to estimate mean levels of various haemostatic parameters like platelet indices, mean prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen levels in patients with sickle cell trait (HbAS genotype) and homozygous SCD (HbSS genotype). These parameters were compared with those in normal HbAA controls.

Materials and Methods:
The present study was carried out in the Hematology division of Department of Pathology, at Mahatma Gandhi Institute of Medical Sciences, a teaching hospital in Central India from October 2007 to August 2009 under approval of the institutional ethics committee. The institute is located in the sickle cell endemic region and both cases and controls included in the study were residents of surrounding districts around the hospital.

Cases were selected from patients whose blood samples were submitted to the hematology section for hemoglobin electrophoresis, which was either advised by their treating doctor or was performed to confirm a positive sickling test. All confirmed patients of sickle cell haemoglobinopathy diagnosed by presence of Hemoglobin ‘S’ band on hemoglobin electrophoresis (performed on cellulose acetate strip at alkaline pH) constituted our cases. They were further subdivided into two groups: Sickle cell trait (patients whose electrophoresis showed presence of both Haemoglobin ‘A’ band and Haemoglobin ‘S’ band – HbAS genotype) and Homozygous Sickle cell disease patients (patients whose electrophoresis showed presence of Haemoglobin ‘S’ band with or without Haemoglobin ‘F’ band – HbSS genotype). Homozygous patients were further categorized into crisis and steady state depending upon the presence or absence of acute symptoms like pain in any body part mainly joints, breathlessness, headaches, sign and symptoms of anemia, priapism, acute chest syndrome, splenic sequestration crisis. Informed consent was sought from all cases for further haemostatic evaluation. Those cases where informed consent could not be obtained or sufficient blood...
sample could not be collected for coagulation analysis were excluded from the study.

Selection of Controls:
We recruited a control for each case. These controls were randomly sampled from patients referred for hemoglobin electrophoresis to hematology laboratory and whose hemoglobin electrophoresis pattern was AA type. A peripheral blood smear of randomly selected controls was screened for the presence of spherocytes, sickle cells, target cells, fragmented cells and nucleated red cells. If any evidence of hemolytic anemia was detected on peripheral smear, another eligible control was selected. All eligible controls were then contacted and explained about the study procedure. Patients in whom sufficient amount of blood could not be collected for coagulation analysis or in whom consent was not obtained were excluded from being a control.

Study variables and investigations
We collected various demographic (age, gender), hematologic (hemoglobin, red cell indices, platelet counts, hemoglobin electrophoresis), and haemostatic variables (PT, APTT, fibrinogen levels) from cases as well as controls. To eliminate any bias in the measurement and interpretation of these results, all samples were coded so that the technicians were not aware of the case and control status of the samples. From each case and control, two venous blood samples were collected (1-2 ml blood in EDTA anticoagulant for hematological investigation and 1.8 ml blood in sodium citrate anticoagulant for coagulation studies). Complete blood count (CBC) was done by automated haematology cell counter (Beckman coulter Act – Diff 2 model) within 1-3 hours of collection of blood sample. Peripheral blood smear was stained by Leishman stain. Haemolysate was prepared from blood obtained in EDTA anticoagulant and haemoglobin electrophoresis was performed using cellulose acetate paper at alkaline pH 8.4. Samples obtained in sodium citrate anticoagulant (1.8 ml blood + 0.2 ml sodium citrate) were used for coagulation studies. The samples were centrifuged at 1200g for fifteen minutes and platelet poor plasma (PPP) was prepared which was then used for coagulation studies. All coagulation studies were performed by BK coagulometer using commercial reagents and following standard procedures for each test. Thromboplastin reagent (Uniplastin, ISI – 1.05) for PT estimation and Liqueceline for APTT estimation was supplied by Tulip diagnostics, Goa. Both PT and APTT tests were carried out in duplicate on the patient’s plasma and the control plasma. Results were expressed as the mean of the duplicate readings in seconds. Fibrinogen assay was done by using Fibroquant kit manufactured by Tulip diagnostics, Goa which uses Clauss technique for fibrinogen estimation. The fibrinogen concentration was read off directly by interpolating the mean clotting time obtained at 1:10 dilution of the sample, from the calibration curve plotted on the graph paper provided with the Fibroquant kit.

All coagulation tests were performed on the day of collection, else the platelet poor plasma was frozen in deep freezer maintained at the temperature of -70°C. Since this is a pilot study, no formal sample size calculation was done and all eligible and consenting cases were included during the study period.

In this study, all procedures done were in accordance with the ethical standards of the IEC.
Statistical analysis
All the quantitative variables were collected and analyzed as a continuous variable and mean was used as measure of central tendency and distribution. Sickle cell disease patients (Hb SS type) are known to have different haematological parameters as compared to those with sickle cell trait (Hb AS type), we analyzed these groups separately. We performed a descriptive statistical analysis of various demographic, haematologic and coagulation parameters between these subgroups and controls. In addition we performed a subgroup analysis by comparing these parameters between steady state and crisis patients of HbSS group. The difference between the continuous and discrete variables was compared using student’s t-test and chi square tests respectively and p<0.01 was used to classify difference between two groups as significant. All statistical analysis was done by using statistical software STATA version 9.0.

Results
This study was conducted between 1\textsuperscript{st} June 2007 and 30\textsuperscript{th} May 2009. A total of 2238 haemoglobin electrophoresis tests were performed during this period, and 452 of these had sickle cell haemoglobinopathy. Of these we could not contact 84 patients, 37 were repeat tests and 10 patients refused consent. A total of 321 remaining eligible and confirmed cases of sickle cell haemoglobinopathy (124 [38\%] males and 197 [62\%] females) were included in the study. Out of these, 118 patients (36\%) belonged to HbSS genotype and 203 (64\%) to HbAS genotype. In total 118 cases of HbSS genotype, 66 were in steady state and 52 were in crisis. All 321 controls (one for each case) were randomly sampled from the remaining 1786 patients who had HbAA pattern on haemoglobin electrophoresis.

The baseline characteristics between cases and controls are compared in Table 1. Patients with sickle cell trait belonged to a higher age group when compared to those with disease. In trait significantly higher proportion of females (142/203) were seen as compared to diseased group (55/118). Mean haemoglobin, Red Blood Corpuscle (RBC) count, Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCHC) and Packed Cell Volume (PCV) values were significantly lower in sickle cell patients when compared with normal controls. Mean MCV values were higher in HbSS patients as compared to normal controls. Table 2 compares the mean values of platelet parameter, PT, APTT and Fibrinogen between the three groups. No significant difference was noted between the platelet counts of sickle cell patients and control group, mean values for Mean Platelet Volume (MPV) and Platelet Distribution Width (PDW) were found to be significantly higher for sickle cell patients as compared to controls.

We found significantly higher PT and APTT values in HbSS patients (Mean 16.32 sec and 34.06 sec respectively) as compared to HbAS patients (Mean 14.38 sec and 30.56 sec respectively) and normal HbAA controls (Mean 13.11 sec and 29.01 sec respectively). The values of these parameters were also found significantly higher for HbAS patients as compared to HbAA controls. Mean fibrinogen level was significantly higher for HbSS patients (Mean 522.24 mg/dl) as compared to HbAS patients (Mean 357.37 mg/dl) and normal HbAA controls (Mean 275.56 mg/dl). (Figure 1) the difference between
fibrinogen levels in sickle cell patients and normal controls was also found to be statistically significant. In the group of sickle cell disease, only values of APTT (34.56 sec and 33.66 sec respectively) and fibrinogen (574.32 mg/dl and 481.21 mg/dl respectively) significantly different in those with or without crisis (Table 3, Figure 2).

Discussion

In this study, we found significantly high fibrinogen, PT, APTT, MPV and PDW levels in HbAS and HbSS patients as compared to normal HbAA controls. These findings reflect the wide spectrum of haemostatic abnormalities seen in these patients. Another important observation has been the significantly high mean fibrinogen levels in sickle cell crisis patients as compared to those in steady state. Our finding of significantly raised fibrinogen concentration among patients with sickle cell haemoglobinopathy as compared to HbAA control subjects has also been reported by other workers. Most authors have linked the hypercoagulable state in HbSS patients to increased levels of fibrinogen concentration. Though the basic physiological function of fibrinogen in hemostasis is the formation of a fibrin network, it is also a major determinant of whole blood viscosity. This effect on viscosity is more pronounced at low shear rates as is the microvasculature in sickle cell crisis and hence it has been proposed that raised fibrinogen concentration may be a contributory factor to red cell sludging seen during crisis. However some authors do not consider the elevated concentrations of fibrinogen to be specific. Fibrinogen is also a acute phase reactant, and its levels may rise in any condition that causes inflammation or tissue damage. Buseri et al suggested that the raised fibrinogen level observed in HbSS patients may be due to increased production, as a reactive process probably in response to the chronic hemolytic process.

Our study has certain strengths and limitations. This is one of the largest coagulation studies among patients with sickle cell disease, where cases and controls belong to the same study base and have been carefully selected. All investigations were done in blinded manner, and subjectivity in their interpretation was avoided. Our study also has certain limitations. We did not have cases of other haemoglobinopathies associated with sickle cell disease. Our cases and controls were not age matched, however haemoglobin electrophoresis is usually ordered among young adults and children so our patients were from similar age groups. Patients with sickle cell disease (SS type) were younger than those with trait (AS type); however we do not feel that this difference could have affected coagulation tests. We did not rule out presence of hepatic disease in cases and controls, neither did we screen them for vitamin K deficiency or lupus anticoagulants. This however would have affected only a minority of cases and controls and we do not suspect this to be a major limitation either.

Among sickle cell patients, we found fibrinogen values to be significantly increased in those with crisis when compared to those in steady state. A significant rise in fibrinogen concentration during pain crisis has also been reported by Gordon et al. We also found increased fibrinogen levels in HbAS patients when compared to normal HbAA controls. Fibrinogen may or may not be a contributory factor to the pathogenesis of vaso-occlusive crisis, but since we observed significant increase in the levels of fibrinogen with increasing severity of the disease, we
agree with Buseri et al that fibrinogen level estimation can be done to monitor the progression of sickle cell pain crisis.\cite{5}

Our findings of increased mean PT and APTT levels in patients with sickle cell haemoglobinopathy are in concordance with the findings of Wright et al, Raffini et al and Buseri et al; who also reported either elevated PT or both PT and APTT levels in HbSS patients in their studies.\cite{5,9,14} There are certain possible explanations for the significantly increased PT and APTT values observed in our study. The first explanation could be hepatocytic dysfunction frequently observed in sickle cell disease.\cite{9} Decreased synthesis of clotting factors has also been reported in HbSS patients.\cite{5,9,14} Though we did not measure factor concentration in our study, neither did we perform LFT in all the patients, subclinical hepatic injury resulting into decreased synthesis of clotting factors or synthesis of dysfunctional clotting factors may be a possible reason for prolonged PT and APTT values observed in our study. Increased consumption of coagulation factors is another alternative plausible explanation for the prolonged coagulation results.\cite{5,9} The third possible explanation for the abnormal PT and APTT values found in our study could be an associated vitamin K deficiency due to decreased dietary intake, intrahepatic cholestasis or chronic antibiotic use frequently practiced in sickle cell patients.\cite{9} We did not screen for the presence of anti-phospholipid antibodies in our study, but they may be present in patients with sickle cell haemoglobinopathy.\cite{15} These can contribute to thrombophilia seen in these patients, but their presence may also lead to prolongation of phospholipid dependent tests like PT and APTT.

An interesting observation of our study has been the prolonged PT and APTT values despite significantly raised fibrinogen levels (suggesting a hypercoagulable state). Similar findings were also observed by Buseri et al and they hypothesized that sickle cell patients are in a hypocoagulable state in steady state and any evidence of hypercoagulability is an early indicator of crisis.\cite{5} Our findings do not support this hypothesis as we observed both significantly higher APTT and fibrinogen levels in SCD with crisis as compared to those in steady state.

Another unique finding of our study is that we observed significantly prolonged PT and APTT values in parallel to the degree of disease severity amongst sickle cell genotypes. So far only limited data is available regarding the integrity of coagulation pathway in sickle cell trait.\cite{16} Though we observed a statistically significant difference between PT and APTT values of controls and sickle cell patients and also between HbAS and HbSS patients, the clinical significance of these findings yet remains to be determined. None of our patients presented with bleeding clinically. Since most of the PT and APTT values were found to be only mildly elevated in our study, there was less likelihood of development of bleeding complications. However, our study was not designed to evaluate the risk of bleeding in patients with sickle cell anaemia and abnormal coagulation studies. To determine whether patients with sickle cell anaemia have an increased risk of bleeding, and to evaluate whether this is influenced by abnormal coagulation studies, further prospective study is warranted.

Patients with sickle cell haemoglobinopathy were found to have larger and more variably sized platelets in our study as compared to the healthy control.
Larger platelets are metabolically and enzymatically more active than their smaller counterpart as they are denser, aggregate more rapidly with subendothelial collagen, produce more thromboxane A2 and express more glycoprotein Ib and glycoprotein IIb/IIIa receptors. Since larger platelets are more thrombogenic, it has been hypothesized that in patients with atherosclerosis they may lead to acute thrombotic occlusion of coronary artery resulting into unstable angina, myocardial infarction or sudden cardiac death.\[17\]

Using the same corollary, it is likely that large platelets in patients with sickle cell disease may predispose these individuals to vaso-occlusive crisis. Though we obtained significantly higher MPV values in HbSS patients as compared to HbAA controls, we observed only a non-significant rise in these values during crisis. It can be argued that for our hypothesis to be true, we should have observed a substantial increase. However, since MPV is affected by a number of factors (including drugs) and the timing of collection of blood sample during crisis has not been constant in our study, we recommend that more prospective studies should be carried out to explore the association between high MPV and vaso-occlusive crisis. Increased PDW in HbSS patients as compared to normal HbAA controls has been reported in only one prior study and the authors hypothesized that PDW can be used as a simple marker to assess the severity of vaso-occlusive crisis.\[8\]

To conclude, in our study HbAS and HbSS patients were found to have significantly higher mean fibrinogen levels as compared to HbAA controls and crisis had even higher mean fibrinogen levels as compared to steady state, fibrinogen level estimation can be used as a parameter to assess the progression of disease. In present study we observed a cut off value of 550 mg/ dl will be able to differentiate most cases of crisis from steady state. There are only few outliers from steady state beyond this value and only one case of crisis had value less than this figure. Moreover this value may also be used to monitor response to treatment in patient of sickle cell crisis who had raised levels prior to start of treatment. Future research is required to study the clinical significance of increased PT and APTT seen in these patients and also to look for a possible role of larger platelets in genesis of vaso-occlusive crisis.

References

Table 1 – Baseline characteristics of normal controls, HbAS and HbSS patients

<table>
<thead>
<tr>
<th></th>
<th>Sickle cell haemoglobinopathy</th>
<th>Controls (n=321)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HbSS type (n=118)</td>
<td>HbAS (n=203)</td>
<td></td>
</tr>
<tr>
<td>AGE (years)</td>
<td>20.05 (11.16)</td>
<td>31.08 (16.78)</td>
<td>22.57 (10.35)</td>
</tr>
<tr>
<td>Haemoglobin (gram/deciliter)</td>
<td>7.53 (1.97)</td>
<td>9.31 (2.25)</td>
<td>12.18 (1.33)</td>
</tr>
<tr>
<td>RBC count (x 10^6/microliter)</td>
<td>2.66 (0.80)</td>
<td>3.72 (0.88)</td>
<td>4.32 (0.52)</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>24.04 (5.96)</td>
<td>28.64 (5.74)</td>
<td>35.79 (4.21)</td>
</tr>
<tr>
<td>MCV (femtoliter)</td>
<td>86.20 (13.21)</td>
<td>78.52 (12.17)</td>
<td>83.13 (9.33)</td>
</tr>
<tr>
<td>MCH (%)</td>
<td>28.90 (4.64)</td>
<td>26.30 (6.56)</td>
<td>28.17 (3.10)</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>32.20 (1.89)</td>
<td>32.30 (2.41)</td>
<td>33.85 (1.70)</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>21.45 (4.82)</td>
<td>18.74 (4.33)</td>
<td>15.47 (2.05)</td>
</tr>
</tbody>
</table>

*All values are expressed as Mean (Standard Deviation)

Table 2 – Comparison of mean values of platelet derived parameters, PT, APTT and fibrinogen levels among controls, HbAS and HbSS patients

<table>
<thead>
<tr>
<th></th>
<th>Sickle cell haemoglobinopathy</th>
<th>Controls (n=321)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HbSS type (n=118)</td>
<td>HbAS (n=203)</td>
<td></td>
</tr>
<tr>
<td>Platelet count (x 10^3/µl)</td>
<td>316.67 (180.83)</td>
<td>271.27 (125.12)</td>
<td>290.43 (85.47)</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>7.98 (1.31)</td>
<td>8.10 (1.28)</td>
<td>7.48 (0.83)</td>
</tr>
<tr>
<td>Pct (%)</td>
<td>0.245 (0.121)</td>
<td>0.217 (0.099)</td>
<td>0.213 (0.060)</td>
</tr>
<tr>
<td>PDW</td>
<td>17.30 (1.10)</td>
<td>17.25 (1.14)</td>
<td>16.66 (0.92)</td>
</tr>
<tr>
<td>PT (Seconds)</td>
<td>16.32 (2.00)</td>
<td>14.38 (2.10)</td>
<td>13.11 (0.79)</td>
</tr>
</tbody>
</table>
Table 3 – Comparison of mean values of platelet derived parameters, PT, APTT and fibrinogen levels in steady state sicklers versus those in crisis

<table>
<thead>
<tr>
<th></th>
<th>HbSS (Steady state) (n=66)</th>
<th>HbSS (Crisis) (n=52)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet count</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x 10^3/µl)</td>
<td>326.19 (178.93)</td>
<td>304.59 (184.23)</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>MPV (fl)</strong></td>
<td>7.89 (1.13)</td>
<td>8.10 (1.51)</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>Pct (%)</strong></td>
<td>0.251 (0.124)</td>
<td>0.238 (0.118)</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>PDW</strong></td>
<td>17.25 (1.17)</td>
<td>17.38 (1.00)</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>PT (Seconds)</strong></td>
<td>16.46 (2.49)</td>
<td>16.14 (1.09)</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>APTT (Seconds)</strong></td>
<td>33.66 (2.07)</td>
<td>34.56 (1.16)</td>
<td>0.0063</td>
</tr>
<tr>
<td><strong>Fibrinogen (mg/dl)</strong></td>
<td>481.21 (47.10)</td>
<td>574.32 (0.52)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*All values are expressed as Mean (Standard Deviation)
Figure 1 - Box-plot depicting median fibrinogen values and their range in controls, sickle cell trait (HbAS) and sickle cell disease (HbSS) patients.

Figure 2 - Box-plot depicting median fibrinogen values and their range in sickle cell disease patients (steady state versus crisis).