



Plasminogen Activator Inhibitor-1 (PAI-1) in Steady State Sickle Cell Disease and Haemoglobin AA Phenotype Controls.

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SUMMARY

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Objective: Hypercoagulability in sickle cell disease may be responsible for an increased development of vascular occlusion in certain organs and acute pain episodes. Plasminogen Activator Inhibitor-1 (PAI-1) assay is a measure of hypercoagulability. This study aims to measure PAI-1 by Enzyme linked immunosorbent assay (ELISA).

Methods: A case-control, cross sectional study was carried out amongst sickle cell patients (cases) and blood donors (controls) at the sickle cell and blood donor clinics respectively of Lagos State University Teaching Hospital Ikeja. A total of 15mls of blood was collected per participant, shared into plain, Ethylene Diamine Tetra Acetic acid (EDTA) and citrated bottles. The sera were subjected to ELISA to determine concentration of PAI-1, full blood count was done on the EDTA samples and prothrombin and activated partial thrombin time were done on the citrated sample in all participants. A statistical software package SPSS version 17 was used for data analysis.

Results: A total of eighty (80) participants were enrolled into the study, consisting of forty (40) sickle cell anaemia (HbSS) patients (cases) and forty HbAA participants (controls). The overall mean plasminogen activator inhibitor concentration of cases and controls were 319.83±151.67 ng/ml and 236.80±120.32 ng/ml respectively. The overall mean prothrombin time (PT) and activated partial thrombin time (APTT) of cases were 18.1 ±1.6 seconds and 34.63±6.31 seconds respectively. The overall for controls were 18.0±1.9 and 38.0±6.3 seconds respectively.

Conclusion: PAI-1 concentration was elevated and APTT shortened in SCD compared with controls confirming evidence of hypercoagulable state in SCD patients.

INTRODUCTION

Sickle cell disease results from inherited abnormality of haemoglobin structure. The abnormal haemoglobin has valine substituting glutamine at position 6 of the beta-globin chain.[1] The haemoglobin variant, HbSS produced, polymerizes at low oxygen tension, causing the characteristic sickle deformity of the red cells, increased mechanical fragility, shortened survival, and chronic haemolytic anaemia.[2]

Sickle cell disease (HbSS) is common in Nigeria with prevalence values ranging from 2% to 3% of the 140 million populations.[2] Apart from the adequately documented haemolytic and vaso-occlusive complications, it is associated with increased frequency of thrombo-embolic complications.[3] Hypercoagulability in sickle cell disease may be responsible for the increased development of vascular occlusion in certain organs [4] and acute pain episodes.[5] Causes of hypercoagulability in sickle cell anaemia are

multifactorial, higher levels of PAI-1 was reported by Colombatti *et al* amongst Sickle cell disease SS-Sβ° patients compared with blood group matched controls and the high level was associated with cerebral small vessel vasculopathy.[4] Reduced levels of natural anticoagulants like proteins C and S5, increased levels of thrombin generation markers like thrombin-antithrombin (TAT) complexes/ protein fragment 1+2 (F1+2),[6] increased D-dimer complexes, circulating antiphospholipid antibodies, increased tissue factor expression and adherence of sickled erythrocytes to the vascular endothelium,[7, 8] have all been associated with incidence of thrombophilia in SCD.

Procoagulant markers like d-dimers and thrombin-antithrombin complexes (TAT) have correlated significantly with development of stroke and retinopathy respectively.[9] Plasminogen activator inhibitor (PAI) is produced by the endothelium, but is also secreted by other tissue types such as adipose tissue. PAI is a serine protease inhibitor (serpin).

There are two types, i.e. PAI-1 and PAI-2. PAI-2 is secreted by the placenta and only present in significant amounts during pregnancy.

The PAI-1 which is the main inhibitor of the plasminogen activators i.e. tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), tPA and uPA convert plasminogen to plasmin. Increased level of plasmin enhances fibrinolysis by converting fibrin to fibrin degradation products (FDPs). Increased FDPs is associated with haemorrhage. Elevated level of PAI-1 effectively inhibits tPA activity levels thereby blocking conversion of plasminogen to plasmin and impairing fibrinolysis which leads to thrombosis. Elevated levels of PAI-1 are therefore linked with venous or arterial thrombosis. Increased levels of PAI-1 are also stimulated by cytokines, growth factors and lipoproteins common to global inflammatory response.[10,11]

The frequency of pain episodes in sickle cell disease patients has been positively correlated with the plasma concentration of D-dimers in non-crises steady state, suggesting d-dimers levels may predict frequency of acute pain crises [12]. Plasminogen activator 1 gene mutation has also been strongly associated with avascular necrosis of the head of femur and hypofibrinolysis. [13] There is increasing evidence that sickle cell disease (SCD), is characterized by a hypercoagulable state [14]. In addition to increased thrombin and fibrin generation, increased tissue factor activity, and increased platelet activation, patients with sickle cell disease manifest thrombotic complications, including venous thromboembolism, in situ pulmonary thrombosis and stroke[14]. Furthermore, the risk of thromboembolic complications appears to be higher following splenectomy.

Structure of plasminogen activator inhibitor

Plasminogen activator inhibitor-1 (PAI-1) [15,16] is an inhibitor of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) [17] and is a member of the serpin family of protease inhibitors. [18]

PAI-1 could spontaneously folds into a stable inactive state without cleavage; this state is termed 'latent' because inhibitory activity can be restored through denaturation and renaturation. The three-dimensional structure reveals that residues on the N-terminal side of the primary recognition site are inserted as a central strand of the largest β sheet, in positions similar to the corresponding residues in the cleaved form of the serpin α 1proteinase inhibitor (α 1-P1). [20]

Residues C-terminal to the recognition site occupy positions on the surface of the molecule distinct from those of the corresponding residues in cleaved serpins [21] or in the intact inactive serpin homologue, ovalbumin [22] and its cleavage product, plakal-bumin.[23] The structure of latent PAI-1 is similar to one formed after cleavage in other serpins, and the stability of both latent PAI-1 and cleaved serpins may be derived from the same structural features. [23]

Genetic of thromboembolism Venous thromboembolism is believed to be caused by genetic and acquired risk factors. The inherited hypercoagulable syndromes primarily affect veins, and only rarely cause arterial thrombosis. The acquired hypercoagulable states, such as the antiphospholipid antibody syndrome, are more commonly implicated in arterial stroke. [24]

Fewer studies on the association of PAI -1 association and thromboembolism are available in the literature compared with studies on Factor V Leiden mutation, prothrombin G20210A, and methylene tetrahydrofolate reductase (MTHFR) C677T and their association with incidence, and recurrence of thromboembolism among SCD patients.

This study aims to determine alteration in levels of Plasminogen activator 1 (PAI-1) in sickle cell disease patients in steady state from age-matched haemoglobin phenotype AA controls.

MATERIALS AND METHODS

Study Population

A case-control, cross sectional study was carried out amongst sickle cell patients (cases) and blood donor (controls) at the sickle cell and blood donor clinics respectively of Lagos State University Teaching Hospital Ikeja. All consenting sickle cell disease patients with Haemoglobin phenotype SS and Haemoglobin phenotype AA prospective blood donors were recruited consecutively into the study after obtaining the institution's research and ethical committee's approval. Participants were asked and aided to fill the structured questionnaires.

Collection of Samples

After an informed consent, fifteen milliliters of blood was collected from those who consented to participating in the study. Samples were shared into plain, Ethylene Diamine Tetra Acetic acid (EDTA) and citrated bottles. Serum from each subject was stored at -20°C on the day of collection until the required sample size was obtained. The sera were subjected to enzyme linked immunosorbent assay (ELISA) to determine concentration of PAI-1 in the cases and controls. ELISA kit was obtained from Biovendor Laboratoni Medicina a.s of the Czech Republic.

Determination of Human PAI-1 Concentration

Using the absorbance of each sample, the concentration of circulating PAI-1 concentration for the cases and controls were determined by extrapolation from a standard curve plotted using the absorbance at 450nm on the ordinate and the concentrations of diluted PAI-1 standards on the abscissa, standards were provided by the manufacturers.

Full Blood Count Analysis

Five mls of EDTA sample from each participant was used for full blood count (FBC) analysis. FBC was done by Sysmex KN-21N (manufactured by Sysmex Corporation Kobe, Japan. A three-part auto analyzer able to run 19 parameters per sample including packed cell volume (PCV), white blood cell (WBC) and platelets.

Well mixed blood sample was aspirated by letting the equipment sampling probe into the blood sample and then pressing the start button. Approximately 20 μ L of blood was aspirated by the auto analyzer. The result of analysis was displayed after about 30 seconds. A print out copy of result was released on the thermal printing paper.

Determination of Prothrombin Time and Activated Partial Thrombin Time

Kits for PT and APTT were obtained from Randox

Laboratories, manufactured at United Kingdom. Four and half mls of citrated sample was run for PT, APTT on the same day of collection. Plasma sample from each participant was made platelet poor by two centrifugations; initial supernatant was re-centrifuged to get a plate-poor plasma. A total of 100 μ L was aspirated into a glasstube in a water bath at 37°C, equal volume of tissue thromboplastin was added, stopwatch started immediately. Clotting time for each sample was noted. Similarly, 100 μ L sample from each participant was subjected to incubation for 10 minutes at 37°C in a water bath, after adding equal volume of APTT reagent. Equal volume of calcium chloride was added, stopwatch turned on and clotting time noted for each sample.

Sample size determination

There is no known prevalence of PAI-1 in SCD patients in Nigeria. Due to lack of known local prevalence of PAI-1 in SCD patients in Nigeria, a 50% was used to calculate the sample size for this study using the formula $n = z^2pq/d^2$ [25], where $Z = 1.96$ normal deviate representing the 95% confidence limit, $d = 0.05$ as acceptable margin of error, $p =$ seroprevalence of 50% (0.5), q which is the probability of the event not occurring, which will be $1 - p = 0.5$ to obtain a minimum sample size of 384.

However, because of cost, only one kit of Human PAI-1 ELISA could be obtained, so 96-well kit could accommodate only 40 sickle cell anaemia, 40 blood donor controls and 16 standards.

Statistical analysis

A statistical software package SPSS version 17 was used for data analysis. The descriptive data were given as percentages. The Pearson chi-square test, correlation test and analysis of variance were used for analytical assessment and the differences considered to be statistically significant when the p value obtained is less than 0.05.

RESULTS

A total of eighty (80) participants were enrolled into the study, consisting of forty (40) sickle cell anaemia (HbSS) patients (cases) and forty HbAA participants (controls). The cases consisted of 22 (55%) males and 18 (45%) females (Table 1), while the controls were 32 (80%) males and 8 (20%) females (Table 1).

The overall mean ages of cases and controls were 23.72 \pm 8.6 (Table 1) and 31.45 \pm 6.9 years (Table 1) respectively. The minimum and maximum ages of both cases and controls were 14 / 40 years and 20/49 years respectively. The mean age of male cases was 21 \pm 6.79 years and female cases was 23.72 \pm 8.67 years, while the mean ages of male and female controls were 31.47 \pm 6.41 and 31.38 \pm 9.03 years respectively. The ages of participants in groups are shown in table 2.

The overall mean age of diagnosis of sickle cell anaemia was 8.4 \pm 7.9 years and the overall annual mean number of crises was 8.4 \pm 7.44 times. The mean age of diagnosis for male and female cases were 10.40 \pm 9.28 and

6.0 \pm 5.32 years respectively and an annual mean number of crisis for males and females were 2.67 \pm 1.67 and 2.31 \pm 1.37 times. The overall mean haematological parameters of cases were, white blood cell count value was 11.89 \pm 5.08 $\times 10^9$ /L, packed cell volume value was 23.83 \pm 6.1%, platelets count value was 385.32 \pm 202.04 $\times 10^9$ /L, the mean haematological parameters of male cases were, white blood cell count value was 12.21 \pm 2.8 $\times 10^9$ /L, packed cell volume value was 23.12 \pm 5.02% and platelets count value was 391.68 \pm 151.20 $\times 10^9$ /L and females cases were white blood cell count value was 11.53 \pm 6.88 $\times 10^9$ /L, packed cell volume value was 24.74 \pm 7.37%, and platelets count value was 377.76 \pm 254.92 $\times 10^9$ /L. The overall mean haematological parameters of controls were white blood cell count value was 6.25 \pm 7.1 $\times 10^9$ /L, packed cell volume value was 42.50 \pm 3.8% and platelets count value was 182.12 \pm 74.14 $\times 10^9$ /L, for male controls were, white blood cell count 4.82 \pm 1.0 $\times 10^9$ /L, packed cell volume value was 42.98 \pm 3.76% and platelets count value was 185.17 \pm 70.79 $\times 10^9$ /L and for females, white blood cell count value was 5.20 \pm 1.06 $\times 10^9$ /L, packed cell volume value was 38.86 \pm 2.36%, platelets count value was 158.77 \pm 112.49 $\times 10^9$ /L.

The overall mean plasminogen activator inhibitor concentration of cases and controls were 319.83 \pm 151.67 ng/ml and 236.80 \pm 120.32 ng/ml respectively. The mean for male cases was 338.17 \pm 169.45 ng/ml and for female cases 292.33 \pm 122.05 ng/ml. The mean for male and female controls were 266.59 \pm 110.11 and 117.63 \pm 82.28. (Table 3).

The overall mean prothrombin time (PT) and activated partial thrombin time (APTT) of cases were 18.1 \pm 1.6 seconds and 34.63 \pm 6.31 seconds respectively. The mean time for male cases were 18.61 \pm 1.71 and 34.17 \pm 7.68 seconds respectively and for female cases 17.57 \pm 1.34 and 35.23 \pm 4.06 seconds respectively. The overall for controls were 18.0 \pm 1.9 and 38.0 \pm 6.3, for male controls were 18.21 \pm 1.88 and 37.6 \pm 6.6 seconds respectively, for female controls were 17.6 \pm 2.0 and 40.66 \pm 3.51 seconds respectively. (Table 4).

Cross tabulating age with plasminogen activator inhibitor concentration of cases was not statistically significant p value = 0.095 but age positively correlated with the concentration $R = 0.069$. Gender of cases was also not statistically significant with plasminogen activator inhibitor concentration p value = 0.417. Number of crises of cases did not reach a statistically significant value when compared with plasminogen activator inhibitor concentration p value = 0.413 but correlated positively with the concentration $R = 0.54$.

Only one of forty cases (2.5%) took alcohol regularly, the same number admitted to being HIV positive, and gave positive history of previous episode of stroke. Half (50%) of the cases gave a positive history of hip pain and limping. The same number admitted to previous history of acute chest pain. While 3 of 40 (7.5%) cases has had hip surgery. Majority of the cases (30 of 40) 75% were on routine drugs like folic acid, vitamin B complex, paludrine only while 25% admitted to be on other drugs like tegretol, cafergot, antiknocks apart from their routine drugs.

Table 1. Demographic data of cases and controls

	Males	Females	Overall	P value	T value
Cases					
Number	32(80%)	8(20%)	40(100%)		
Mean age(years)	31.47±6.41	31.38±9.03	31.45±6.92	0.15	0.16
Controls					
Number	32(80%)	8(20%)	40(100%)		
Mean age(years)	31.47±6.41	31.38±9.03	31.45±6.92	0.15	0.16

Table 2. Age of cases and controls in groups Ages in years

Number	Percent	Cases
14-20	19	47.5
21-30	13	32.5
31-39	8	20.0
Controls		
20-30	17	42.5
31-40	19	47.5
41-50	4	10

Table 3. The Mean Plasminogen Activator Inhibitor Concentration of participants

Participants	Cases (ng/ml)	Controls (ng/ml)	P Value	T Value
Males	338.17±169.45	266.59±110.11	0.07	0.10
Females	292.33±122.05	117.63±82.28	0.22	0.10
Overall	319.83±151.67	236.80±120.32	0.05	0.10

Table 4. The mean values of Coagulation Profiles of Participants

Participants	Cases		Controls		P value	Tvalue
	PT	APTT	PT	APTT		
Males	18.61±1.71	34.17±7.68	18.21±1.88	37.6±6.6	0.07	0.07
Females	17.57±1.34	35.22±4.06	17.6±2.0	40.66±3.51	0.18	0.18
Overall	18.1±1.6	34.63±6.3	18.09±1.9	38.06±6.30	0.05	0.10

DISCUSSION

A multi factorial process seems to be responsible for the myriad of complications associated with sickle cell anaemia. The pathophysiology of numerous complications like avascular necrosis of femoral head, acute chest syndrome, vaso-occlusive crises etc may be due to vascular occlusion resulting from ischaemia and organ damage. Some of these processes include, increased blood viscosity and reduced red cell deformability,[26] abnormal adhesive properties of the red cells, [27,28] intimal proliferation,[29] bone marrow or fat embolism [30] and chronic hyper coagulability.[31,32]

This study highlights one of the many causes of chronic hypercoagulability in sickle cell anaemia viz; comparing plasma level of plasminogen activator inhibitor -1 concentration in SCD with HbAA controls. Unlike a reduction in anticoagulant protein (tPA), procoagulants proteins like fibrinogen, fibrinopeptide A and D dimer were elevated in Nigerian- sickle cell anaemia patients compared to HbAA controls as reported by Ekwere *et al.*[33] However, this study was embarked on because PAI-1 concentration has not been well studied amongst Nigerians.

PAI-1 being a pro coagulant protein is expected to be increased in sickle cell anaemia patients compared with HbAA controls. In keeping with this study, all previous researches have demonstrated elevation of the PAI-1 concentration in sickle cell disease over normal controls [34,35]. Furthermore, causes of elevation of PAI-1 in sickle cell anaemia are not known. Elevated PAI expression was observed in pulmonary endothelial cells and macrophages of sickle mice. Placenta growth factor (PIGF) released at high concentration in sickle erythroblast was thought to be responsible for the elevated PAI concentration. It was also reported that there was low levels of PAI-1 in PIGF knock-out mice and sickle-PIGF knock out mice and over expression of PIGF in normal mice increased circulating PAI-1.[35]

Expectedly in this study, overall mean APTT value was lower for sickle cell disease patients compared with HbAA controls, however, almost similar values were obtained for PT in both the sickle cell disease and controls. Ajuwon *et al* [36] also reported a lower APTT and PT values in Sickle cell anaemia patients compared with controls. Elevated PAI-1, plasminogen and fibrinogen concentrations and reduction in APTT, PT and tissue plasminogen levels are

prothrombotic and antifibrinolytic features of sickle cell anaemia which may account for vasculopathy and complications like avascular necrosis of femoral head, acute chest syndrome, and vaso-occlusive crises.

Platelets count in this study doubles value reported for HbAA controls, this determines the degree of predisposition to chronic hypercoagulability. This is in consonance with previous study amongst Nigerian sickle cell anaemia patients, as reported by Akinbami *et al.*[37] In some studies, it was found that reduced or absent splenic sequestration of platelets as a result of hyposplenism in sickle cell anaemia contributes significantly to a higher mean platelet counts compared with controls.[38]

Also, a negative feedback effect on erythropoietin production in subjects as a result of anaemia could also account for the thrombocytosis. Also, erythropoietin has a structural homology with thrombopoietin, roughly half of thrombopoietin has identity with or similarity to erythropoietin at the N-terminal region. [39]

The numbers of crises which occur in these cases per annum were not statistically significant with the PAI-1 concentration in this study. This could be due to the fact that other causes apart from elevated PAI-1 could be responsible for crisis in sickle cell disease. Possible limitations of this study are reliability on information provided by the sickle cell anaemia participants in the questionnaires, particularly the number of crisis they have had during the year and their drug profile information. Also cases and controls were not aged matched, because majority (47.5%) of the controls were in their 4th decade unlike 47.5% cases that were mostly in their 3rd. Majority of blood donors in the centre used were in their 4th decade of life. This could possibly have impacted on the PAI-1 results obtained

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Conflict of Interest

The research was self-sponsored, there is no conflict of interest declared.

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