Relationship between heamatological indices and pro-inflammatory proteins (TNF-α and SAA) among children and adults with SCD

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ABSTRACT

Inflammatory process in sickle cell disease (SCD) is chronic and progressive, waxing and waning. Clinical episodes occur at critical levels of inflammatory proteins such as Tumour necrosis factor-alpha (TNF-α) and serum amyloid A (SAA). This critical level can be investigated and used as diagnostic targets in the management of the SCD patient. The aim of this research was to determine the relationship between haematological indices and pro-inflammatory proteins (TNF-α and SAA) in the serum of SCD patients. There were three groups of 45 subjects each: SCD patients in steady state, SCD in VOC (which constituted the cases) and normal controls with HbAA. These participants had their blood samples analyzed for full blood count and their serum for TNF-α and SAA using the enzyme linked immunosorbent assay method (ELISA). Results were analyzed with the SPSS version 20. There were 135 respondents, with age ranging from 4 to 38 years; 78 were males and 57 were females. Out of the135, 90 had SCD, with 53 children and 37 adults. A positive correlation was observed between SAA, neutrophil and reticulocyte counts and a negative correlation with haemoglobin concentration and lymphocyte count. The results of this study support the role of inflammation and inflammatory mediators in SCD. Serum amyloid A and TNF-α together can be useful as markers for assessing the disease status. Further investigations of these two proteins, on a large scale in SCD patients are recommended.

Keywords: Heamatological indices, pro-inflammatory proteins, children and adults.

INTRODUCTION

Sickle cell disease is a multi-organ disease characterized by sickled red cells, premature destruction of red cells (haemolysis), a susceptibility to infection and recurrent blood vessel obstruction causing tissue ischaemia with infarction. The latter is the underlying pathology of recurrent acute episodes of pain which is the hallmark of the disease. Pain in SCD can be acute or chronic [1]. There is the acute pain of recurrent vessel occlusion, ischaemia and infarction occurring at variable rates in the steady state; the chronic pain associated with avascular necrosis of bone at joints (hips, shoulders, ankles and spine), leg ulcers and other degenerative joint diseases (rheumatoid or osteo- arthritis). Tumour necrosis factor-alpha (TNF-α) and Serum amyloid A(SAA) proteins are acute phase proteins up-regulated in response to traumatic and excitotoxic tissue injury. TNF-α stimulates the liver to increase acute phase protein production, while SAA levels have been known to be exceptionally high in clinically severe pain episodes and organ damage [2]. Both proteins are potent chemoattractants for leucocytes, and high baseline white cell count is a major risk factor for SCD severity. TNF-α also up regulates cell adhesion molecules expression and increases vascular permeability, this response is usually localized and temporary, but high systemic concentrations can induce shock-like symptoms and cause vaso-occlusion in the micro vessels. Vaso-occlusive crisis is one of the most important, commonly encountered clinical features of SCD. The purpose of this study is to observe the relationship between haematological indices and pro-

inflammatory proteins (TNF-α and SAA) among children and adults with SCD [3]. This may validate their potential role in the prediction of an acute pain episode, with the view of recommending their use for assessing disease status in the steady state SCD patients. These may also be potential targets in the treatment of this disease. The degree of inflammation can be assessed in serum, and used for diagnostic and prognostic purposes. SAA protein is an acute inflammatory signaling molecule, implicated in the pathogenesis of many chronic inflammatory diseases; TNF-α is also an important inflammatory and immune response cytokine. They have been used to assess and monitor the degree of inflammation, tissue destruction and disease progression. They enable reliable predictions of acute illness and complications in SCD and therefore are potential therapeutic targets [4].

AIM AND OBJECTIVES

To determine the relationship between heamatological indices and pro-inflammatory proteins (TNF-α and SAA) among children and adults with SCD.

MATERIALS AND METHODS
Study Area

This study was carried out in Port Harcourt, using patients recruited from the University of Port Harcourt Teaching Hospital (UPTH), the Braithwaite Memorial Specialist hospital (BMSH), St Martin's Hospital, and the Palmers' Hospital. Port Harcourt is in the South-South zone of Nigeria. These hospitals are major hospitals that render services to patients within the state and neighboring states including Akwa Ibom, Bayelsa, Imo and Abia.

STUDY DESIGN

This was a hospital-based case control study.

ETHICAL APPROVAL

This study was approved by the UPTH Research and Ethical Board. The approval was presented to the other peripheral hospitals (Appendix I). An informed consent was obtained from all participants of the study.

STUDY POPULATION

The study was composed of a total of 66 children and 69 adults. Study participants were selected in a systematic manner and were divided into 3 subpopulations.
a) SCD patients in steady state who were recruited from the paediatric- (25 from UPTH and 5 from BMH) and adult- (11 from UPTH and 4 from BMH) haematology outpatient clinics of the participating hospitals
b) SCD patients in Vaso-occlusive crisis recruited through the Accidents and Emergency units and children emergency wards of the participating hospitals. There were 20 children recruited from UPTH and 3 from BMH; 13 adult patients from UPTH, 5 from BMH, 2 from Palmers' and 2 from St. Martin's.
c) Normal healthy adult volunteers attending the blood donor unit in UPTH and healthy children of health care workers.
A questionnaire was filled for participants by the researcher after consent was obtained
Data in the questionnaire included: participant number, age, gender, Hb phenotype, brief history of any acute illness, the presence of bone pain and characterization (site, severity, character and duration), history of any current medication and the test results of this study.

INCLUSION CRITERIA OF CASES

1. All cases with confirmed SCD by Hb electrophoresis.

2. Consenting SCD child caregiver and adult SCD patients in steady state, (patients with 2 or more month's

history of no crisis or blood transfusion)**Error! Bookmark not defined.·Error! Bookmark not defined.·Error! Bookmark not defined.**

(OR)

3. SCD patients in vaso-occlusive crisis (patients admitted for severe bone pain crisis).

EXCLUSION CRITERIA OF CASES

1. People with Hb AS phenotype.
2. Subjects with SCD complications, or chronic infections, or chronic inflammatory conditions, (for example, leg ulcers, hypertension).
3. Subjects positive for transfusion-transmissible infections (HIV, hepatitis, and syphilis).
4. Refusal to give consent.
5. Pregnant SCD patients.

CONTROL SELECTION PROCESS

Adult controls who were 18 years and above were recruited from the blood donor clinic. Healthy adults with known HbAA phenotype were selected in a systematic manner. Children used for controls were those less than 18 years who were children of healthcare workers in the participating hospitals. These control individuals were not age- and sex- matched with the SCD patients group, but were healthy individuals with normal haemoglobin profiles and lacked a history of anaemia, inflammatory conditions, and haematological diseases.

SAMPLE SIZE DETERMINATION

Sample size was calculated using the prevalence of SCA (2.9%) in the University of Port Harcourt Teaching Hospital**Error! Bookmark not defined.** and the formula $N= (Z^2pq) /d^2)$. [i]

N= desired sample size

Z= standard deviation at 1.96 (95% confidence interval)

P= proportion of the target population, estimated to have the particular characteristics (2.9% or 0.029)

q =1-p

d = degree of precision used (0.05)

This gives a minimum sample size of 43.3; approximately 45 for each subgroup of participants.

Each of the subgroups (subjects in VOC, in steady state and normal controls), had a sample size of 45 giving a total of 135.

EQUIPMENT AND MATERIALS

(A) Reagents used:

1. Human TNF-α and SAA micro plate
2. Sealing tapes
3. Standard solutions of TNF-α and SAA
4. Biotinylated antibodies against TNF-α and SAA
5. MIX diluents concentrate
6. Wash buffer concentrate
7. Streptavidin-Peroxidase (SP) conjugate
8. Aperoxidase-chromogen substrate (tetramethylbenzidine)
9. A stop solution- 0.5 N hydrochloric acid
10. Rapid immunochromatographic test strips for serological screening (Determine® HIV-1/2, Diaspot® HBsAg, ABON® Biopharm HCV and Syphilis)
11. Tris-EDTA-borate buffer

(B) Materials used:

1. Calibrated pipettes
2. Stop watch
3. Calibrated beakers
4. Deionized or distilled water
5. Test tubes
6. EDTA sample bottles and plain bottles
7. Sample applicator (for the electrophoresis)

(C) Equipment

1. 6800 Autohaematology analyzer system (Mindray® product).
2. A computer system and a printing machine
3. Sample mixing machine
4. Micro plate reader
5. Electrophoretic tank
6. Power supply

SPECIMEN COLLECTION, PREPARATION AND STORAGE

For every participant, a total of 6mls of venous blood was collected by venepuncture using aseptic techniques.

1) Three millilitres of venous blood was dispensed into ethylenediamine tetra-acetic acid (EDTA) bottles for full blood count (FBC) consisting of haemoglobin, total white cell count and differentials, platelet count and absolute reticulocyte count; and serological screening for hepatitis B and C, syphilis and HIV 1&2. The bottles were filled appropriately to ensure a proper blood to anticoagulant ratio. The samples were mixed immediately by gentle inversion to ensure adequate mixing of blood with the anticoagulant.

2) Another 3mls of venous blood was collected simultaneously and dispensed into serum separator tubes or plain sterile bottles. Clot was allowed to form and the tubes centrifuged at 2500rpm for 10 minutes. [ii] Supernatant serum was then transferred to tubes for storage at -20°C.

METHODOLOGY

Full Blood Count was carried out using a 5 part auto analyzer (BC 6800 Autohaematology analyzer system Mindray® product). The samples were placed on the sample mixing machine. The power supply, connections of the analyzer to the reagents or diluents, to the waste and pneumatic unit were checked. The auto-analyzer, loaded with all its reagents for the analysis, and the PC software were switched on and allowed to boot (analyzer automatically performed a self test procedure, background cycle and initialized the system). The control samples were run first. A clean uncapped EDTA tube was presented to the sample probe, making sure the probe goes deep into the bottom of tube to avoid spills, and bubbles. The aspirate button was pressed to start dispensing the diluents; the tube was removed when buzzer sounds. The machine analyzed a sample in 1.5 minutes; the machine automatically displays the results, which is printed from the printer. [iii]

Serology: Anticoagulated whole blood was centrifuged at 2500rpm to separate plasma, and serological screening for HIV, hepatitis B & C and VDRL were carried out using the rapid test strips (Determine HIV-1/2, Diaspot HBsAg and ABON Biopharm HCV and Syphilis). Two drops of patients plasma was applied to the sample application area or pad. Test was read after 15 minutes. A positive result was shown by a pink line in both the control and test areas (areas were clearly indicated on strips) and a negative result was shown by a pink line in the control area only.

Hb Phenotype: Hb electrophoresis for Hb phenotype was carried out on all samples. This was determined using cellulose acetate electrophoresis in alkaline buffer. Cellulose acetate strips are gently blotted after soaking in Tris buffer. 2 microlitre of haemolysate (control and test samples) were applied in a line midway between the centre and the cathodic end of each strip. The strips were quickly transferred to the electrophoretic tank. Current was applied and electrophoresis was carried out for about 30 minutes at 220 volts across the strip. At the end of separation, the strips were removed using forceps and dried in a hot air oven. The test bands were inspected and interpreted in relation to the controls.

Enzyme-Linked Immunosorbent Assay: Commercial assay kits: Assay Max® Human SAA and Human Tumour Necrosis factor (TNF)-alpha Enzyme-Linked Immunosorbent Assay (ELISA) kits. Catalog No. EA8001-1 and ET2010-1 respectively, manufactured by Assaypro LLC®, St. Charles Missouri, USA, were used to determine SAA and TNF-α levels in human serum. [iv] The kit manufacturer's instructions were followed strictly.

Measurement principle: Assay employs a quantitative sandwich enzyme immunoassay technique, measuring TNF-α in 5 hours and SAA in about four hours. The micro plate wells were pre-

coated with a monoclonal antibody specific for TNF-α and polyclonal antibody specific for SAA. Standards and samples were sandwiched by the immobilized antibodies and the biotinylated antibodies specific for these molecules, which were then recognized by a streptavidin-peroxidase (SP) conjugate. Unbound materials were washed off and a peroxidase-enzyme substrate was added. The colour development was stopped and colour intensity measured.[v]

TEST PROCEDURE

All frozen serum samples and reagents were brought out of the freezer to defrost at room temperature before analysis.

1. All reagents (MIX diluents, wash buffer standard, biotinylated antibodies and SP conjugate) were prepared as instructed in the manual.
2. Standard curve preparation: the 2.5ng of human TNF-α (HTNF-α) standard was reconstituted with 2.5 ml of MIX diluents to give a 1ng/ml standard stock solution. This was then serially diluted to obtain standard points: 0.5, 0.25, 0.125, 0.0625, 0.0313 and 0.0156ng/ml solutions. Likewise the standard stock solution (16μg/ml) of human SAA (HSAA) was serially diluted, first by 1:8 ratios to give a standard point of 2μg/ml, then sequentially by 1:2 to give 1, 0.5, 0.25 and 0.125 μg/ml solutions. The MIX diluents served as the blank or zero standards for both procedures (0μg/ml).
3. Fifty microlitres (50μl) of HTNF-α / HSAA standards and samples were added to the wells and covered with the sealing tape and incubated for 2 hours.
4. After 2 hours wells were washed with wash buffer manually and 50μl of biotinylated antibody added per well and incubated for 1 hr (SAA) and 2hrs (TNF-α).
5. Step 5 was followed by another wash and then the addition of 50μl SP conjugate per well and incubated for 30 minutes and excess conjugate washed off.
6. Chromogen substrate (50μl) was added per well and incubated for 15mins (SAA), 30mins (TNF-α) for optimal blue color density.
7. Stop solution (50μl) was added per well with a color change to yellow
8. Wells were then read immediately at 450nm on the micro plate reader.

Normal plasma SAA level is less than 6μg/ml and TNF-α level is 48pg/ml (0.048ng/ml).

STATISTICAL ANALYSIS

Data was entered and analyzed using the IBM statistical package for social sciences software (SPSS) version 20. Descriptive statistics (mean, standard deviation, percentages and charts) were used to summarize the variables and characterize the demographics. Student's t- test and Analysis of variance (ANOVA) were used to compare the differences in means between two and three groups respectively. Post hoc test was performed using Scheffe test to explore significant mean differences across groups. Chi Square or Fishers exact tests were used to compare differences in proportions across groups. Pearson's correlation coefficient was used to examine the correlation between TNF, SAA and the haematological parameters. P-values <0.05 were considered statistically significant.

RESULTS
Demographics of the Study Participants

There was a total of 135 study participants, 66 were children, 53 had sickle cell disease (SCD) with 13 HbAA controls; 37(56.1%) were males and 29(43.9%) were females. Their ages ranged between 4 and 17 years. (Table1)

There were also 69 adults, 37 SCD and 32 HbAA controls; 41(59.4%) were males and 28(40.6%) were females. Their ages ranged between 18 and 38 years. (Table 2)

Correlation of SAA, TNF-α and haematological parameters: Children

The Pearson's correlation analysis revealed a significant positive correlation between SAA and the white cell count (p < 0.001), SAA and the neutrophil count (p= 0.035). A significant negative correlation between SAA and the Hb (p=0.025); SAA and lymphocyte count (p < 0.001); SAA and monocyte count (p= 0.047) and between SAA and eosinophil counts (p= 0.005). (Table 3)
There was no significant positive or negative correlation between TNF and any of the indices in children. (Table 3)

Correlation of SAA, TNF-α and haematological parameters: Adults

The Pearson's correlation analysis revealed a significant positive correlation between SAA and the reticulocyte count (p < 0.001), SAA and the neutrophil count (p< 0.001), SAA and basophil count (p=0.031). A significant negative correlation between SAA and the Hb (p<0.001); SAA and lymphocyte count (p < 0.001); SAA and eosinophil count (p< 0.001) and between SAA and platelet counts (p= 0.037). (Table 4.12) While TNF showed a significant positive correlation with the reticulocyte count (p=0.012) and neutrophil count (p=0.002). It had a significant negative correlation with the lymphocyte count (p= 0.002). (Table 4)

Table 1: Age and sex distribution of children among the study groups

Variables	VOC n =23(%)	Steady State n =30(%)	Control n =13(%)	Total n =66(%)
Age (years)				
4-10 years	13 (56.5)	19 (63.3)	6 (46.2)	38 (57.6)
11-17 years	10 (43.5)	11 (36.7)	7 (53.8)	28 (42.4)
	Chi square =1.112; p value=0.574			
Sex				
Male	13 (56.5)	15 (50.0)	9 (69.2)	37 (56.1)
Female	10 (43.5)	15 (50.0)	4 (30.8)	29 (43.9)
	Chi square =1.365; p value=0.505			

Table 2: Mean age of children among the study groups

	VOC n =23	Steady State n =30	Control n =13
Mean age ± SD	*9.4 ± 3.2*	*9.2 ± 3.6*	*10.8 ± 4.0*

S.D – Standard deviation; F test – 15.400, p value=0.0001*

* Statistically significant

Table 3: Relationship between heamatological indices and pro-inflammatory proteins (TNF-α and SAA) among children with SCD

Heamatological indices	TNF-α		SAA	
	Correlation Co-efficient (r)	P-value	Correlation Co-efficient (r)	P-value
Hb	-0.057	0.649	-0.276	0.025*
Reticulocyte count	0.235	0.058	0.113	0.366
WBC	0.081	0.520	0.612	0.0001*
Neutrophil	0.031	0.802	0.260	0.035*
Lymphocyte	-0.101	0.418	-0.423	0.0001*
Monocyte	-0.012	0.923	-0.245	0.047*
Eosinophil	-0.005	0.971	-0.343	0.005*
Basophil	-0.011	0.932	0.155	0.213
Platelet	-0.049	0.696	-0.208	0.094

* Statistically significant

Table 4: Relationship between heamatological indices and pro-inflammatory proteins (TNF-α and SAA) among Adults with SCD

Heamatological indices	TNF-α		SAA	
	Correlation Co-efficient (r)	P-value	Correlation Co-efficient (r)	P-value
Hb	-0.171	0.160	-0.460	0.0001*
Reticulocyte count	0.301	0.012*	0.521	0.0001*
WBC	0.026	0.830	0.141	0.249
Neutrophil	0.360	0.002*	0.671	0.0001*
Lymphocyte	-0.363	0.002*	-0.681	0.0001*
Monocyte	-0.158	0.194	-0.199	0.102
Eosinophil	-0.179	0.141	-0.492	0.0001*
Basophil	-0.131	0.283	0.259	0.031*

| Platelet | -0.027 | 0.826 | -0.252 | *0.037** |

* Statistically significant

DISCUSSION

Serum amyloid A induces production of granulocyte colony stimulating factor (GCSF) important in granulopoiesis, and IL-8, a powerful chemo attractant for neutrophils and may have contributed to the significantly raised neutrophil counts in VOC [1]. The children had significantly higher SAA levels compared with the adults, again hinting at age as a risk factor for VOC [2].

A serum SAA level of 6µg/ml was defined as the threshold beyond which inflammatory episodes become significant. In the steady state about 10% of the children and 26.7% of the adult cases were clinically asymptomatic, with SAA values of 6µg/ml and above. This was assumed to reflect an adaptation to the chronic inflammatory state characteristic of the steady state. The sensitivity and specificity of SAA was thus calculated as 91.3% and 90% respectively for children and 59.1% and 73.3% respectively in adults. SAA can then be said to be a highly sensitive, as well as specific screening tool in children for the early detection of steady state patients at risk of crisis in the near future. A significant positive correlation was observed between SAA and neutrophil count in both children and adults; white cell count in children and reticulocyte in adults. A significant negative correlation was observed in both children and adults between: SAA and Hb level; SAA and lymphocyte count.

In children there was no observed correlation between TNF and any of the haematological indices. But in adults a significant positive correlation was observed between: TNF and reticulocyte count; TNF and neutrophil count. A significant negative correlation with the lymphocyte count was observed also. TNF is a potent pyrogen, an important haemopoietic cytokine particularly for myeloid component and a chemo attractant for neutrophils. It therefore contributes to the acute elevations in neutrophil counts. Musa et al in the Zaria study inferred that reticulocytes having the propensity for adherence, due to surface adhesion molecules such as CD36, contribute to elevated cytokine levels in patients in VOC due to sequestration of cells to sites of inflammation and causing also reduction in cell count.

A serum TNF level of 0.05ng/ml was defined as the threshold beyond which inflammatory episodes become significant. In the steady state about 10% of the children were clinically asymptomatic, 0% in adults; they had TNF levels of 0.05ng/ml and above, This also is an attempt at adaptation to the chronic inflammatory state characteristic of the steady state. The sensitivity and specificity of TNF was thus calculated at 8.7% and 90% respectively in children; 18.2% and 100% in adults. TNF is thus not a sensitive screening tool but may be utilized for its high specificity to rule in the risk for VOC and complications once it tests positive. The sensitivity and specificity are characteristics of a test tool which are reproducible in any size of population.

CONCLUSION

This study, showed serum levels of SAA and TNF in SCD children and adults and their relationship to the inflammatory cells, as well as their usefulness as assessors of SCD in children and adults

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