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Original Research Article

Evaluation of Procalcitonin Levels in Sickle Cell Disease Subjects in Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria

Manafa, P.O[1], Onyenekwe, C.C.[1], Okocha, E.C[2], Eze, S.C[1], Ekuma-Okereke, O[1], Chukwuma, G.O[1], Ibe N.C[1], Chukwuanukwu, R.C[1], Nwene, K.E[3], Mbachu N.A[4], Ebugosi, R.S[5], Manafa, V.I[6], Manafa, C.C[7]

[1]Department of Medical Laboratory Science, Faculty of Health Sciences & Technology, Nnamdi Azikiwe University, Nnewi Campus, Nigeria
[2]Department of Heamatology, College of Medicine, Nnamdi Azikiwe University, Nnewi Campus, Nigeria
[3]Center for Clinical research in Nigeria
[4]Department of Human Biochemistry, Nnamdi Azikiwe University, Nnewi Campus, Nigeria
[5]Department of Biochemistry, Tansian University, Anambra State, Nigeria
[6]Pathology Department, Clinical Biochemsitry, East Kent Hospital University NHS Foundation Trust, United Kingdom
[7]Royal Sussex County Hospital, Brighton

*Corresponding Author
Ekuma-Okereke, O

Abstract: **Background of study:** Sickle cell disease (SCD) is a genetic blood disorder affecting red blood cells with high morbidity and mortality rates. Serum procalcitonin has been used as a biomarker of prognosis in severe bacterial sepsis and septic shock. **Objective**: The aim of this study is to evaluate the serum level of procalcitonin among the different blood genotype group (homozygous sickle cell (HbSS); heterozygous sickle cell (HbAS) and normal subjects (HbAA). **Materials and methods:** A total of 90 subjects (consisting of 30 HbSS subjects in steady state, 30 HbAS individuals and 30 normal subjects (HbAA) as the control subjects) aged 17 to 60 years were randomly recruited for this study. All subjects with homozygous sickle cell disease in steady state were on vitamin C supplementation for at least six months prior to study. Hemoglobin genotype was determined using the electrophoresis method while procalcitonin were analyzed using the enzyme linked immunosorbent assay (ELISA) technique. The disease severity was evaluated using the severity scores. **Result:** The mean serum level of procalcitonin was significantly decreased ($P<0.05$) in HbSS disease subjects (0.86±0.28) compared with the control group (1.11±0.06). There was no significant difference ($P>0.05$) in the mean serum level of procalcitonin in HbAS (0.95±0.31) compared with HbSS (0.86±0.28) disease and the control group (1.11±0.06). A positive correlation was observed between the level of procalcitonin and the age of subjects with homozygous sickle cell disease ($r=0.099$; $P=0.638$) and heterozygous sickle cell disease individuals ($r=0.095$; $P=0.691$) while a negative correlation existed between the level of procalcitonin and age in the control group ($r=-0.056$; $P=0.816$). Also, a negative correlation was recorded when the mean level of procalcitonin was correlated with the disease severity in subjects with homozygous sickle cell disease ($r=-0.374$; $P=0.065$). **Conclusion**: The reduced mean value of procalcitonin in subjects with homozygous sickle cell disease suggests a decreased risk of bacterial septicaemia probably due to vitamin c supplementation.

Keywords: : Procalcitonin, Homozygous Sickle Cell Disease (HbSS), Heterozygous Sickle Cell Disease (HbAS), Vitamin C supplementation.

1. INTRODUCTION

Sickle cell anaemia (SCA) is a genetic disorder resulting in the production of abnormal sickle haemoglobin. Various factors like hemolysis, chronic inflammation and endothelial dysfunction culminate in acute vaso-occlusion which is responsible for much of the morbidity observed in SCA patients (Stuart *et al.*, 2004). Sickle cell disease (SCD) is a genetic blood disorder affecting red blood cells, with high morbidity and mortality rates. The United Nations has recognized SCD as a global public health concern, and the World Health Organization (WHO) recommends that 50% of member states will have established SCD control programs by 2020 (World Health Organization, 2006). It is typified by red blood cell (RBC) having shorter lifespan (less than 100-120 days), reduced oxygen

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carrying capacity, vaso-occlusion, inflammation as well as impairment in iron metabolism (Agasa *et al.*, 2010).The prevalence of SCD is very high in Central Africa, Mediterranean region, Eastern countries and in certain parts of India (Kate, 2001). The highest prevalence of sickle-cell trait (SCT) in Africa occurs between the latitudes of 15_ North and 20_ south, where the prevalence ranges between 10% and 40% of the population (Agasa *et al.*, 2010). The high mortality rates in Sub-Saharan Africa are influenced by multiple factors including limited resources leading to poor access to care, and lack of comprehensive SCD management programs. Interventions that have been effective in reducing mortality among SCD patients in high resource countries such as newborn screening, and prophylactic penicillin administration are not available in most low resource countries (Odame, 2010). Serjeant, (1997) stated that sickle cell anaemia results from a point mutation in the genetic code such that glutamic acid is replaced by at position six of the beta globin chain of haemoglobin (Hb). This substitution transforms normal adult hemoglobin (HbA) into sickle hemoglobin (HbS). Patients with sickle cell anemia (homozygous hemoglobin S) usually suffer from intermittent clinical or hematologic crisis. The former, also called painful crisis are characterized by musculoskeletal or abdominal pain attributed to "log-jams" of sickled erythrocytes in small blood vessels. Bone and pulmonary infarction, renal dysfunction, priapism, leg ulcer and a variety of neurologic sequelae are well known complications of associated tissue anoxia, thrombosis and necrosis. Accelerated anemia due to red cell sequestration or hyperhemolysis may complicate clinical crisis, but hematologic crises typically appear separately and are due to transient depression of bone marrow (Smits *et al.*, 1968).

Bacterial infections are a major cause of morbidity and mortality in children with sickle-cell anemia. Several organisms, including *Streptococcus pneumonia*, *Haemophilus influenza*, and non-typhi *Salmonella* species, have been identified as important causative agents through studies undertaken in the USA (Barrett, 1971). The introduction of penicillin prophylaxis and immunization with conjugate vaccines directed against *S pneumonia* and *H influenza* type b have led to substantial improvements in the prognosis of patients born with sickle-cell anemia in developed countries (John *et al*., 1984).

Procalcitonin (PCT), the precursor peptide of calcitonin, a hormone involved in calcium homeostasis, is present in normal subjects in extremely low serum levels (0.1 to 0.5 ng/ml) (Sexton *et al.*, 2008). PCT is synthesized physiologically by thyroid C cells but in sepsis has an extrathyroidal origin. After intravenous injection of endotoxin from *Escherichia coli* to healthy volunteers, serum PCT becomes detectable at 4 hrs, maintaining a plateau through 8 and 24 hrs, following an increase of proinflammatory cytokines (Dandona *et al.*, 1996). PCT was first found elevated in sepsis in 1993 (Assicot *et al.*, 1993). PCT normalizes more rapidly than CRP. Whether PCT is more specific for infection than cytokines is still debatable. Presently, a number of studies point out that PCT is a superior marker than CRP for diagnosis of sepsis and/or infection, but some authors disagree (Gattas *et al.*, 2003). An updated meta-analysis of studies is therefore needed. In response to stimulation due to bacterial infection, serum procalcitonin rises substantially and its role in inflammatory response includes chemotactic function, modulation of inducible nitric oxide synthase and induction of cytokines, among others (Hagiwara *et al.*, 2012*).* Serum half-life of PCT is about 20-24 hours and PCT has a high stability in serum and plasma *ex vivo* which making it particularly suitable for routine laboratory parameters of infection (Hagiwara *et al.*, 2012). Furthermore, circulating serum PCT levels halve daily when the infection is controlled by host immune system or antibiotic therapy, PCT also had been recommended for monitoring disease course and response to treatment (Hagiwara *et al.*, 2012**).** Several studies have shown that the measurement of procalcitonin is highly sensitive and specific for distinguishing systemic inflammatory response syndrome (SIRS) from sepsis, bacterial pneumonia of other inflammatory lung conditions and pancreatic necrosis from septic pancreas necrosis. Serial PCT assessments have been used to determine the time of treatment of pneumonia and other infections (Briel *et al.*, 2008). More recently, PCT has been used as a biomarker of prognosis in severe sepsis and septic shock, it is ubiquitously expressed in sepsis (Becker *et al.*, 2004).

The better diagnostic tests for sepsis have long been sought, and among the most prominent candidates in this respect are PCT, as a novel biomarker of infection, which has become increasingly popular and several studies have highlighted the usefulness of monitoring PCT levels for identifying infectious processes (Aikawa *et al.*, 2005). Isolated determinations of serum PCT have shown variable results (Karlsson *et al.*, 2010). Most studies say that it is not possible to predict the outcome of critically ill patients based on high levels of PCT. Encouraging results were obtained from studies involving small numbers of patients and showed that serial determinations of PCT correlated with prognosis (Claeys *et al.*, 2002).

2. MATERIALS AND METHODS

Materials

- Zip Zone electrophoresis chamber and EV 243 power supply (Helena Biosciences, UK).
- Spectrophotometer (Mindray MR-96A)
- Human PCT (Procalcitonin) ELISA Kit

Study Site

This research was carried out at Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra State, Nigeria.

Study Design:

A total of 90 subjects were randomly recruited for this pilot study comprising of 30 homozygous sickle cell (HbSS) subjects in steady state on vitamin c supplementation for at least six months (test group), 30 heterozygous sickle cell (HbAS) subjects and 30 normal subjects (HbAA) (control). The selection of the steady state group was based on subjects not experiencing crisis for at least two weeks and not having blood transfusion for at least 1 month prior to the study.

Inclusion Criteria:

- Homozygous (HbSS) sickle cell disease subjects in steady state on vitamin c supplementation for at least six months.
- Heterozygous (HbAS) sickle cell disease subjects and normal healthy subjects (HbAA, control).
- Individuals within the age range of 17 to 60 years of age.

Exclusion Criteria:

The following were excluded from the study:

- Individuals with homozygous sickle cell disease on supplementations other than vitamin c.
- Subjects with other sickle cell syndromes such as HbSβ-thalassemia, HbSE, HbSC, HbSD-Punjab and others.
- Individuals outside the age range of 17 to 60 years of age.
- Patients who were a part of special program/trial that may have affected their clinical, haematological, biochemical status and other variables.

Ethical Approval:

The ethical approval for this research was obtained from the Nnamdi Azikiwe University Teaching Hospital (NAUTH) ethical committee; and in accordance with the Helsinki Declaration by the World Medical Association (WMA) on the ethical principles for medical research involving human subjects, informed consent was obtained from the participants prior to study.

Specimen collection:

About 5ml of whole venous blood was collected aseptically from each subject through venipuncture and 2ml dispensed into an EDTA container for the determination of genotype and full blood count. The remaining 3ml were dispensed into plain containers and centrifuged at 5000rpm for 5 minutes. The serum was extracted and used for the estimation of procalcitonin level.

METHODS:

Determination of Haemoglobin Genotype:

The method of Daniel (1999) as modified by Manafa *et al.* (2017) was used for cellulose acetate paper haemoglobin electrophoresis.

Principle:

Charged particles when in an electric field migrate to their counter electrodes. In an alkaline pH (8.2-8.6), haemoglobin (Hb) is a negatively charged molecule and will migrate towards the anode. The various Haemoglobins move at different rates depending on their net negative charge, which in turn is controlled by the composition (amino acids) of the Hb molecule (globin chain). These appear as bands on cellulose acetate membrane. The samples are run with known controls.

Estimation of procalcitonin levels:

The levels of procalcitonin were estimated using the sandwich ELISA method described by Brahms (2008).

Test principle:

The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to PCT. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for PCT and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain PCT, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) will be measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of PCT.

Estimation of full blood count (FBC)

Full blood count was estimated by the method as described by Buttarello and Plebani (2008). This is essentially a Sysmex procedure.

Principle:

The aspirated blood sample was measured to a predetermined volume, diluted at the specified ratio and then fed into each transducer chamber which has a minute aperture and also contain electrodes in which direct current flows. Blood cells suspended in the diluents pass through the aperture causing electrical resistance between the electrodes and the blood cell size was detected as electric pulses. Blood cells count was calculated by counting the pulses and the histogram determined by the pulse sizes.

Severity scoring system in sickle cell disease

The determination of disease severity score was performed according to the method described by Manafa *et al*. (2017).

Anaemia score

- Hb ≥ 10g/dl → 0
- Hb ≥ 8g/d < 10g/dl → 1
- Hb ≥ 6 < 8g/dl → 2
- Hb ≥ 4 < 6g/dl → 3
- Hb< 4g/dl → 4

Complications score

The complications included stroke, retinopathy, acute chest syndrome, nephropathy, priapism, leg ulcer, pulmonary hypertension, liver failure, and anaemic heart failure.

Each complication was scored 1 except

- Nephropathy – 2
- Stroke – 2

WBC score

- Count < 9×10^9 cells/µl → 0
- Count ≥ 9 < 11×10^9 cells/µl → 1
- Count ≥ 11 < 15×10^9 cells/µl → 2
- Count ≥ 15×10^9 cells/µl → 3

Transfusion score

Life Transfusion Rate = $\frac{\text{Total Number of Blood Pint}}{\text{Age}}$

Approximate to the nearest whole number

Hospital Admission

No of hospital admission per year = $\frac{\text{No of hospital admission in the last 3 years}}{3}$

Approximate to the nearest whole number

Disease severity scores

- ≤ 3 – mild
- > 3 – ≤ 7 moderate
- > 7 – severe

Statistical Analysis:

The statistical analysis was performed using the student's t-test and the Analysis of Variance (ANOVA). Values were deemed significant at P<0.05. Correlation of the parameters with disease severity was determined using the Pearson's correlation coefficient. The statistical analysis was done using SPSS version 21.0.

3. RESULTS

Table 1: Mean ± SD of procalcitonin levels in different blood genotype groups:

There was a significant difference (P<0.05) in the mean serum levels of procalcitonin among different blood genotype groups.

Table 1: **Mean ± SD of procalcitonin levels in different blood genotype groups:**

Parameter	Groups	Number (n)	Mean±SD
Procalcitonin (ng/ml)	HbSS	30	0.86±0.28
	HbAS	30	0.95±0.31
	HbAA	30	1.11±0.06
F-value			4.3450
P-value			0.017

Table 2: Variations of the serum levels procalcitonin in HbSS, HbAS and HbAA Subjects:

The post-hoc analysis showed a significantly decreased mean serum levels of procalcitonin in subjects with homozygous sickle cell (HbSS) (0.86±0.28) compared with the control subjects (HbAA) (1.11±0.06) (P<0.05). However, there was no significant difference (P>0.05) in the mean serum levels of procalcitonin in heterozygous sickle cell subjects (HbAS) (0.95±0.31) compared with that of normal control group (1.11±0.06). The same pattern was observed when the mean serum levels of procalcitonin in heterozygous sickle cell anemia was compared with that in homozygous sickle cell (HbSS) subjects (P>0.05).

Table 2: Variations of the serum levels procalcitonin in HbSS, HbAS and HbAA Subjects:

Groups	P-value
HbSS Vs HbAA	0.014
HbSS Vs HbAS	0.843
HbAS Vs HbAA	0.252

Table 3: Relationships between mean serum level of procalcitonin and age in HbSS, HbAS and HbAA Subjects:

Table 3 shows a positive correlation between the level of procalcitonin and age in homozygous sickle cell (HbSS) disease subjects (r=0.099; P=0.638). A positive correlation existed between the levels of procalcitonin and age in heterozygous sickle cell (HbAS) disease subjects (r=0.095; P=0.691). However, a negative correlation existed between the levels of procalcitonin and age in HbAA subjects (r=-0.056; P=0.816)

Table 3: Relationships between mean serums level of procalcitonin and age in HbSS, HbAS and HbAA Subjects:

Parameters	r-value	P-value
HbSS		
Procalcitonin vs Age	0.099	0.638
HbAS		
Procalcitonin vs Age	0.095	0.691
HbAA		
Procalcitonin vs Age	-0.056	0.816

Figure 4.1: Correlation of the levels of procalcitonin with disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state

A negative correlation existed between the levels of procalcitonin and disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state (r=-0.374; P=0.065).

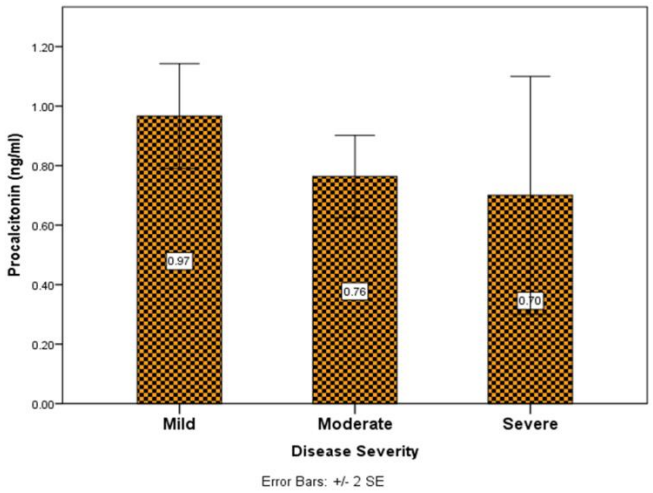

Figure 4.1: Correlation of the levels of procalcitonin with disease severity in homozygous sickle cell (HbSS) anemia subjects in steady state.

4. DISCUSSION

Sickle cell disease (SCD) is a collective term for a number of genetic disorders in which hemoglobin is structurally abnormal, resulting in the episode formation of sickle shaped red blood cells and a wide range of clinical manifestations (Stuart *et al.*, 2004). Historically, infection is a major cause of mortality in SCD particularly in children and it was implicated in 20-25%of deaths in prospective cohort studies over the last twenty years (Catherine *et al.*, 2010). Since the association of procalcitonin with severe bacterial infection was reported (Assicot *et al.*, 1993), studies have primarily assessed the value of procalcitonin in its ability to discriminate between different microbiological etiologies, infectious and non-infectious causes of systemic inflammatory response syndromes and to predict outcomes in critical illness.

In this study, there was a significant difference in the mean serum levels of procalcitonin in the different blood genotype groups. Post hoc analysis showed a significantly decreased mean serum level of procalcitonin in subjects with homozygous sickle cell (HbSS) disease compared with that in control group. However, no significant difference was observed in the mean serum levels of procalcitonin in heterozygous sickle cell subjects (HbAS) compared with the control groups (HbAA) and homozygous sickle cell disease subjects. These data indicates that blood group genotypes apparently influence the levels of serum procalcitonin in relation to the baseline status of patients with sickle cell disease (HbSS) which in turn are clearly less elevated than serum procalcitonin levels in the control group (HbAA).

Nylen *et al.* (1997) reported an increase in the mean serum levels of procalcitonin in sickle cell disease patients with sepsis. Serum procalcitonin are also increased in other severe infections and inflammation such as pancreatitis, appendicitis (Kafetzis *et al.*, 2005), burns (Von Heimburg *et al.*, 1998), heat stroke (Nylén *et al.*, 1997), multitrauma (Maier *et al.*, 2009) and extensive surgery (Meisner *et al.*, 1998). While specific assay of serum Procalcitonin in the healthy subject (control group) is less than 10 pg·mL-1, it is not uncommon for levels to exceed 100 000 pg·mL-1 (Bassim *et al.*, 2008). Fluids other than blood can also manifest increased levels of Procalcitonin. For example, salivary levels of this prohormone are increased in periodontitis (Bassim *et al.*, 2008). Also, in persons with wartime extremity injuries, the Procalcionin in the wound exudate is significantly increased in those patients whose wounds dehisce when compared with wounds that subsequently heal (Forsberg *et al.*, 2008). Serum PCT levels below 500 pg·mL-1 are relatively uncommon in patients with classic sepsis symptomatology, but values below this level may indeed occur (Becker *et al.*, 2007). Clinically, the daily determination of PCT in sepsis is most useful (Hochreiter *et al.*, 2009). During the course of a septic process, there may be a marked increase in serum PCT, often indicating an exacerbation of the illness. Moreover, a decreasing level often is a favorable sign (Jensen *et al.*, 2006). However, it should be emphasized that during the course of a septic process, complications may occur, such as hypotension, shock, heart failure, respiratory insufficiency or disseminated vascular coagulation. These conditions greatly influence the course and ultimate outcome of the disease without necessarily, in themselves, affecting PCT levels (Kenneth *et al.*, 2010).

Furthermore, a positive correlation was observed between the levels of procalcitonin and age of subjects with homozygous sickle cell (HbSS) disease and heterozygous sickle cell individuals while a negative correlation existed between the levels of procalcitonin and age of the control group. These findings are supported by Behrooz *et al.* (2017) who reported that the patients' age and serum procalcitonin levels did not correlate significantly in the case groups. In addition, Behrooz also examined the diagnostic performance of serum procalcitonin separately in adults (18-65 years) and the elderly (over 65 years). Based on his findings, at optimal cut-off values of 0.09 ng/ml for adults and 0.08 ng/ml for the elderly, serum procalcitonin was accompanied with a better diagnostic performance in the former (sensitivity and specificity of 82.6 and 82% in adults versus 69.1 and 70% in elderly, respectively). The usefulness of serum procalcitonin to manage patients with suspected bacterial septicaemia has rarely been examined in the elderly (Lai *et al.*,

2010). It is still not clear how the age of patients may affect serum levels of procalcitonin during bacterial sepsis (Behrooz *et al*., 2017). Whenever sepsis occurs, the innate immune response is activated by releasing various cytokines such as interleukin 1, 6 and 8, tumor necrosis factor -α and interferon - γ from the endothelial cells and macrophages (Russel, 2006). When this reaction is extensive and diffused, endothelial cell damage may ensue, which in turn may cause hemodynamic changes and organ failure (Lai *et al*, 2010). Among the elderly and immunocompromised patients, however, the classic signs of sepsis may be missing because of decreased inflammatory response in such patients (Steichen *et al*, 2009). This can explain why serum procalcitonin is more reliable indicator of bacterial septicaemia less than 65 years of age (Behrooz *et al*, 2017).

This present study recorded a negative correlation between the level of procalcitonin and disease severity in homozygous sickle cell disease subjects. This is in variance to some previous studies which showed that serum procalcitonin correlated positively with severity of the disease. Becker *et al*. (2007) observed that serum procalcitonin correlate with the severity of the condition and remain elevated for the period of the inflammatory process. However, these studies may not be comparable since the HbSS subjects were all in steady state rather than in crisis. It is also possible that the above discrepancies may be due to previous antibiotic treatment, host immune system and/or effective management of subjects with high severity. This is supported by Hagiwara *et al*. (2012) when they observed that circulating serum procalcitonin levels reduces when infection is controlled by host immune system or antibiotic therapy. On the surface, it may seem to make sense that a positive correlation should exist between the levels of procalcitonin and severity of disease since it would seem that increased serum procalcitonin levels correlates positively with the severity of inflammatory response to infections and therefore disease severity as reported by Meisner *et al*. (2002). However, a closer look at this hypothesis reveals flaws in the light of the question; is procalcitonin just a biomarker or does it play a casual role in the pathogenesis of bacterial sepsis associated with sickle cell disease? Masia *et al.* (2005) observed that patients with high severity scores had high procalcitonin levels associated with mortality and complications, however, Beoric *et al* (2005) found no association between procalcitonin and pneumonia severity index score.

On the other hand, Atis *et al*. (2017) reported high procalcitonin levels associated with patients who had high scores in the scoring system. Also, Sheriff *et al*. (2013) observed that increasing severity related to sickle cell disease was associated with a pronounced gradual increase in the levels of serum procalcitonin. However, most studies say that it is not possible to predict the outcome of disease severity in homozygous sickle cell disease patients based on high levels of procalcitonin (Claeys *et al*., 2002) Moreover, the eventual outcome is influenced by the precipitating cause, as well as the clinical care. Thus, clinical severity-of-illness scores or prognostic scores, some of which involve parameters such as age or concomitant illness, for example, Acute Physiology and Chronic Health Evaluation score (Claeys *et al*., 2002), multiple organ failure (MOF) scores (Hensler *et al.*, 2003), sequential organ assessment score (Castelli *et al.*, 2004) or simplified acute physiology score II (Cheval *et al.*, 2000) often correlate with serum PCT levels, albeit only approximately.

5. CONCLUSION

The reduced mean value of procalcitonin in subjects with homozygous sickle cell disease suggests a decreased risk of bacterial septicaemia probably due to vitamin c supplementation. This is because; supplementation with vitamin C has been demonstrated to offer a reasonable protection to subjects with severe complications of sickle cell disorder. Regular supplementation with vitamin C is therefore recommended in the management of subjects with sickle cell disease. However, a multi-centre study with a larger study population is advised.

6. CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest regarding the publication of this paper.

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