



Sudan University of Science and Technology
College of Graduate Studies

Estimation of TNF- α Cytokine in Sudanese Sickle cell Disease Patients
During Vaso-occlusive Crisis and the Steady State Condition
In Khartoum state

تقدير مستوى المادة الخلوية عامل نخر الورم الفا عند المرضى السودانيين المصابين بالانيميا
المنجلية خلال حالة الازمة الوعائية والحالة المستقرة في ولاية الخرطوم

A research Dissertation Submitted in Partial Fulfillment of the
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Dedication

This is Dissertation dedicated to my parents who have given me the opportunity of an education from the best institutions and support throughout our lives. indebted to our best friends and many colleagues who have always supported us and for their companion and being such good friends.

Finally, cannot find words to express our gratitude to our families, parent, brothers and sisters, for their love and support during this work; they have always encouraged towards excellence.

Finally, dedicate this to all those who believe in the richness of learning.

Acknowledgment

God is our strength and our savior and He is with us every step of the way, and yes, there are a lot of times when only His footsteps can be seen. May God bless all our ways, guide and give the success have always longed for. It is with immense gratitude that we acknowledge the support and help of our supervisor Dr. Hisham Nour Aldayeem Altayeb Mohamed .His sage advice, insightful criticisms, and patient encouragement aided the writing of this thesis in innumerable ways.

Abstract

Sickle cell anemia is characterized by the presence of hemoglobin S where glutamic acid is replaced by valine, in low oxygen conditions which promotes polymerization of Hemoglobin which distorts RBCs into a sickle shape which leading to vessel occlusion the activations of leucocytes,RBCs and platelets lead to production of proinflammatory and anti-inflammatory cytokines. TNF- α cytokine stimulates increased expression of adhesion molecules on endothelial cells, contributing to leukocyte and RBC adhesion to endothelial cells and lead to the vascular inflammatory state that is present in Sickle cell anemia. This study aimed of estimate the serum level of TNF- α cytokine in Sudanese sickle cell disease patients during vaso occlusive crisis and the steady state condition.This study included 88 participants (patients and normal subjects); 28 as control 60 as cases(30 as steady state and 30 as vasoocclusive crisis) started from July(2018) to April (2019), the samples collection done by standard procedure in plain containers and then centrifuged to obtained serum for analysis in sandwich ELISA to estimated the level of TNF- α in sickle cell patients and control. The study revealed increased mean of TNF- α cytokine level in vasoocclusive crisis(5.51 ± 1.05) compared to steady state(1.76 ± 3.16) in Sickle cell patients and control group(2.30 ± 3.10), and no significant difference between groups. The study conducted that increased level of TNF- α cytokine in vasoocclusion crisis in comparison of steady state in sickle cell anemia and control group, demonstrates that the cytokine play important role in developed of vasoocclusive crisis in sickle cell anemia .

الخلاصة

فقر الدم المنجلي هو اضطراب غير متجانس يتميز بوجود الهيموجلوبين اس حيث يتم استبدالحمض الغلوتاميك بالحمض الأميني في ظروف منخفضة الأكسجين التي تعزز بلمرة الهيموجلوبين الذي يشوه خلايا الدم الحمراء إلى شكل منجل وهذه الخلايا تؤدي إلى انسداد الأوعية الدموية . تؤدي عمليات تنشيط الكريات البيضاء ، كريات الدم الحمراء والصفائح إلى إنتاج السيتوكينات المسببة للالتهاب والمضاد للالتهاب عامل نخر الورم الفا سيتوكين يعمل على تحفيز زيادة التعبير عن جزيئات الالتصاق على الخلايا البطانية ايضا يسهم في التصاق الكريات البيض و كريات الدم الحمراء على الخلايا البطانيةويؤدي إلى حالة التهابالأوعية الدموية الموجودة في مرضى الانيميا المنجلية.

اجريت هذه الدراسة لقياس مستويات المصل من السيتوكين عاملنخر الورم الفا في مرضى الخلايا المنجلية السودانية خلال أزمات انسداد الوعاء و الحالاتالمستقرتشملت هذه الدراسة 88 عينة دم 28 طبيعيين 60 كحالات مرضى (30 حالة ثابتة و 30حالة أزمات انسداد الوعاء) بدأت من يوليو2018() إلي ابريل (2019) جمع العينات تم وفق اسس ومعايير دقيقة ثم جمعها في حاويات خالية من مانع التجلط وتم الحصول على المصل بأستخدام قوة الطرد المركزي للتحليل استخدم جهاز مقايسة الممتاز المناعي المرتبط بالانزيم.

كشفت الدراسة عن زيادة متوسط مستواسيتوكين عامل نخر الورم الفا خلال أزمات انسداد الوعاء (5.51 ± 1.05)مقارنةمع الحالات الثابتة(1.76 ± 3.1)

و الحالات الطبيعية (2.30 ± 3.10)، ولا يوجد فرق كبير بين المجموعات.

وضحت الدراسة عن وجود زيادة متوسط مستواسيتوكينعاملنخر الورم الفا خلال أزمات انسداد الوعاءمقارنة مع حالات الثابتةو الحالات الطبيعية مما يوضح دور السيتوكين في تطور أزمات انسداد الوعاء في مرضى الخلايا المنجلية.

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List of abbreviations

<u>SCA</u>	<u>Sickle cell anemia</u>
<u>VOC</u>	<u>Vasoculic crisis</u>
<u>ACS</u>	<u>Acute chest syndrome</u>
<u>ROS</u>	<u>Reactive oxygen species</u>
<u>RNS</u>	<u>Reactive nitrogen species</u>
<u>NO</u>	<u>Nitric oxid</u>
<u>SCD</u>	<u>Sickle cell disease</u>
<u>HSCs</u>	<u>Haemopoietic stem cells</u>
<u>TSP</u>	<u>Thrombospondin</u>
<u>HU</u>	<u>Hydroxyurea</u>
<u>HB</u>	<u>Hemoglobin</u>

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Chapter One

Introduction

Chapter One

1.1.Introduction:

Sickle cell anemia (SCA), a heterogeneous disorder, is characterized by the presence of hemoglobin S where glutamic acid is replaced by valine at position six of beta globin chain (Schnog *etal.*,2004). SCA is prevalent in Sudan it range from 0.8% -30.4% depending on the geographical location (Sabahelzain andHamamy.,2014).

Under low oxygen conditions, the absence of a polar amino acid at position of six of the β - globin chain promotes the non - covalent polymerization of Hemoglobin which distorts red blood cells into a sickle shape and decreases their elasticity, These cells fail to return to normal shape when normal oxygen tension is restored and this rigid cell fail to pass through narrow capillaries, leading to vessel occlusion and ischemia(Obeagu *et al.*, 2015).A polymerization process that is considered the primary event leading to the pathogenesi,SCA patients have a heterogeneous clinical outcome characterized by painful vaso-occlusive crises, stroke, priapism, pulmonary hypertension, ACSand chronic organ injuries(Steinberg *et al.*,2001). Steady-state, the period between a painful crisis, in which the patient feels well, and vaso-occlusion, which is an ongoing subclinical process in these stable SCD patients (Pathare *et al.*,2004).The hemolysis that occurs in SCA lead to production of free hemoglobin has inflammatory and oxidant effects that lead to endothelium dysfunction, and other product include haem , ROS and RNS. Increased ROS and RNS levels and decreased NO levels contribute to the activation of RBCs, leukocytes, platelets and endothelial cells. This activation leads to increased production of proinflammatory and anti-inflammatory cytokines (Frenette*etal.*,2002),Increased circulating levels of cytokines and chemokines lead to further activation

of vascular endothelium and further adhesion of RBCs, leukocytes and platelets to the endothelium (Greeret *et al.*,2014).

Cytokines Proteins that are produced and secreted by many different cell types, and mediate inflammatory and immune reactions. (Abbas *et al.*, 2015). CD4+ T cells are further divided into subsets by their function and pattern of cytokine secretion (Raghupathy *et al.*, 2000).Th1 subset secretes IL-2, IFN- γ and TNF- α , and is responsible for many classic cell-mediated functions, including activation of cytotoxic T lymphocytes and macrophages (Owen *et al.*,2013).

The pro- inflammatory cytokine TNF- α is produced mainly by monocytes /macrophages and Tcell (Ikramet *et al.*,2004). Biological responses to TNF- α are mediated by two groups of receptors, TNFR55 and TNFR 75, which are present on the membrane of several types of cells, excluding RBCs (Popa*et al.*,2007).

TNF- α stimulates increased expression of adhesion molecules on endothelial cells, contributing to leukocyte adhesion. And stimulate RBC adhesion to endothelial cells. In addition, induce neutrophil degranulation, capillary leak and vasoconstriction , and TNF- α inhibits cell proliferation and induces cell death. Also contribute to the vascular inflammatory state that is present in various inflammatory diseases, including SCA(Pathare*et al.*,2004).

Blood transfusion is used in the treatment and prevention of acute and chronic complications of SCD. The main side effects of blood transfusion in patients with SCD are alloimmunization, haemolytic transfusion reactions, iron overload and transfusion transmitted infection (Masteret *et al.*, 2016).

Hydroxyurea is the only effective drug proven to reduce the frequency of painful episodes. It raises the level of HbF and the haemoglobin level most common short-term hydroxyurea toxicity in patients with SCA is transient and reversible myelosuppression, primarily neutropenia (Agrawal *et al.*, 2014). SCA patients who are treated with hydroxyurea display an altered profile of TNF- α levels. studies showed that HU did not significantly increase or decrease plasma TNF- α concentrations (Pitanga *et al.*, 2013). Other studies show Hydroxyurea was associated with important decreases in the levels of TNF α (Colela *et al.*, 2012).

1.2. Rationale:

Vasooocclusive crisis is the main clinical manifestation and cause of hospitalization, organ damage, and death in sickle cell patient .Cytokines are a play important role of development of vasooculssive crisis , and role as biomarkers of the crisis and steady states. No published studies in Sudan documented the estimation of TNF- α cytokine in sickle cell patient in Sudan,so this study conducted to estimate level of TNF- α cytokine in sickle cell patients

1.3. Objectives:

1.3.1. General objective:

-To estimate the serum level of TNF- α cytokine in Sudanese sickle cell disease patients during vaso occlusive crises and the steady state condition .

1.3.2. Specific objectives:

- To measure serum level of TNF- α in SCD patients during vasoocclusive crises and the steady state in sickle cell disease using ELISA Kits.

-To compare the results during vasoocclusive crises and steady state

Chapter Two
Literature review

Chapter two

2.1.Literature review

2.1.1.Hb synthesis:

Erythropoiesis is the generation of red blood cells carrying the respiratory pigment haemoglobin, for the transport of oxygen to the tissues. This process, from the erythroid commitment of multipotent haemopoietic stem cells (HSCs), Primitive and definitive erythropoietic cells are distinguished by their morphology, cytokine responsiveness, growth kinetics, transcription factor programmes, epigenetic programmes and patterns of gene expression. The individual components of the haemoglobin synthetic pathway (iron, free porphyrins, haem and monomeric globin chains) are all toxic to the cell, and feedback loops have evolved to ensure that cells are not damaged by these intermediates. In particular, the synthesis of globin is inaccurately matched with the synthesis of haem, in which some steps occur in the cytoplasm and others in the mitochondria (Higgs *etal.*, 2016).

Two β -globin protein chains combine with two α globin protein chain and heme to form predominant hemoglobin found in human adult, the types of haemoglobin produced are quite distinct in embryonic (Hb Gower I $\zeta\epsilon_2$, Hb Gower II $\alpha_2\epsilon_2$ and Hb Portland $\zeta_2\gamma_2$), fetal (HbF $\alpha_2\gamma_2$) and adult (HbA $\alpha_2\beta_2$ and HbA2 $\alpha_2\delta_2$) erythroid cells. These specific patterns of globin expression provide critical markers for identifying the developmental stages of erythropoiesis (Higgs *etal.*, 2016).

2.1.2.p-globin gene:

The p-globin gene is located on the short arm of chromosome 11. It is a member of the globin gene family, a group of genes involved in oxygen transport. Over 475 p-globin gene variants exist, and several result in life-threatening illness (Ashley-Koch *et al.*, 2000).

2.1.3.Hb S:

Hb S is produced by Point mutation (GAG > GTG) occurs in the sixth codon of the beta globin gene (HBB) and causes valine to replace glutamic acid in the sixth amino acid of the beta (β) globin chain of the hemoglobin molecule (Pitanga *et al.*,2013). Individuals of African descent exhibit the highest frequency of at risk genotypes associated with Hb S. The term "sickle cell disease" refers to a collection of autosomal recessive genetic disorders characterized by the Hb S variant of the P-globin gene. Individuals who are affected with sickle cell anemia have two copies of this variant (Hb SS), and the primary hemoglobin present in their red blood cells is sickle hemoglobin. Individuals affected with other types of sickle cell disease are compound heterozygotes. They possess one copy of the Hb S variant plus one copy of another (β -globin gene variant, such as Hb C or Hb P-thalassemia. Carrier individuals have one copy of the sickle variant and one copy of the normal β -globin gene (Hb AS), producing a mixture of sickle hemoglobin and normal hemoglobin. The carrier state for sickle cell disease is often referred to as "sickle cell trait." All individuals who are homozygous or compound heterozygous for Hb S exhibit some clinical manifestations of sickle cell disease (Ashley-Koch *et al.*,2000).

2.1.4.Beta globin cluster haplotype :

A cluster of several other globin genes is located on chromosome 11 near the β -globin gene. Thus, this region is referred to as the β -globin cluster region. DNA markers in the β -globin cluster region are highly variable. The combination of DNA markers observed on

a particular chromosome form what is called a haplotype. While many haplotypes exist for the P-globin cluster region, only specific haplotypes are found on chromosomes that carry the Hb S variant. These haplotypes are named for the geographic regions of Africa and the Middle East where they predominate (Ashley-Koch *et al.*,2000).

The beta globin gene haplotype have been correlated with clinical features of SCA patient ,the CAR haplotype may be associated with more sever symptome ,while the SEN haplotype correlate with better prognosis. Despite common genetic background the phenotypic expression in SCA patient varies widely from mild clinical symptoms with survival into 60 – 70 years of age to very sever clinical symptom with multi oragan damage and early mortality (Cajado *etal.*,2011).

2.1.5.Sickle cell anemia:

Patients with sickle cell anemia (HbSS) suffer from acute, painful vaso-occlusion crises, infections, and life- threatening acute chest syndrome. The period between the painful crises, during which the patient is symptom free, is considered the steady state (Akohoue*et al.*,2007). Symptoms usually appear within the first 6 months of life, but there is considerable variability in the severity of the disorder (Ashley-Koch *et al.*,2000).

When compared with healthy controls (HbAA), patients with HbSS are hypermetabolic and show several abnormalities of the immune system, including high WBC counts, elevated serum levels of acute phase proteins such as CRP and of proinflammatorycytokines. Sickled red blood cells from Hgb deoxygenation are a significant

source of reactive oxygen species (free radicals) , which may be involved in the pathogenesis of vasoocclusion and acute chest syndrome in sickle cell disease (Akohoue *et al.*,2007).

Other clinical outcome include stroke, priapism, pulmonary hypertension and acute chest syndrome and chronic organ injuries. As a result of this mutation, deoxygenated hemoglobin molecules undergo a polymerization process that is considered the primary event leading to the pathogenesis of SCA. Sickled red blood cells, as well as leukocytes, platelets and the vascular endothelium, are elements that obstruct vessels and trigger vasoocclusive crises. The hemolysis that occurs in SCA can be both extravascular and intravascular. Intravascular hemolysis occurs when red blood cells (RBCs) rupture and release free hemoglobin into the plasma. Free hemoglobin has inflammatory and oxidant effects that lead to endothelium dysfunction. Other hemolysis products, including haem, reactive oxygen species (ROS) and reactive nitrogen species, are also released into the bloodstream, where they cause increased oxidative stress and decreased plasma levels of the vasodilator nitric oxide (NO). Increased ROS and RNS levels and decreased NO levels contribute to the activation of RBCs, leukocytes, platelets and endothelial cells. This activation leads to increased production of proinflammatory and anti-inflammatory cytokines, which gives SCA the characteristics of a chronic inflammatory disease . Several cytokines, such as interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α), are associated with the activation of leukocytes, particularly monocytes and neutrophils, in SCA. Several other cytokines are

also involved in the chronic inflammatory state that is present in SCA(Pitanga*et al.*,2013).

2.1.6.The role of cytokine in sickle disease:

Cytokines Proteins that are produced and secreted by many different cell types, and mediate inflammatory and immune reactions(Abbaset *al.*, 2015).

Occlusion of the microcirculation (clinical and sub clinical), infection,and hemolysis are important factor (inflammatory and non inflammatory)that stimulate the production of cytokine and acute phase proteins,as consequence of chronic hemolytic anemia,an increased number of reticulocyte is often present in circulation of SCD patient.on their surface reticulocyte express the integrin complex $\alpha 4\beta 1$,which binds to plasma and endothelial membrane fibronectin and to an adhesive molecule on fibronectin and to an adhesive molecule on the surface of the endothelial cell V-CAM-1. The above interaction are enhanced ,especially after the activation of endothelial cell by inflammatory cytokine such as tumor necrosis ,interleukin1 and interleukin 8. Red blood cells from SCD patient and endothelial cells express on their surface glycoprotein IV (CD36) which acts as a receptor for thrombospondin (TSP).TSP is produced by activated platelets and endothelial cells with the mediation of cytokine .cytokines seem to be involved in pathogenesis of vasoocclusive phenomena in SCD by several possible mechanism ,endothelial activation ,induction of red cell adhesiveness to endothelium ,induction of neutrophil adhesiveness to endothelium and to plasma fibronectin ,development of vascular intimal hyperplasia ,platelet activation

,endothelin production and dysgranulation of apoptosis ,also thought to be involved in regulation of hemopoiesis and inhibition of immune functions and development of growth deficits in SCD patients . Studies give varied informations about level of cytokines during steady state and crisis of SCD patients (Makis*etal.*,2000).

2.1.7.TNF- α cytokine :

The pro- inflammatory cytokine TNF- α is produced mainly by monocytes /macrophages , but other cells, such as T-cells, smooth muscle cells, adipocytes and fibroblasts, can also produce this cytokine. TNF- α is named for its ability to stimulate tumor necrosis and regression in vivo Biological responses to TNF- α are mediated by two groups of receptors, TNFR55 and TNFR 75, which are present on the membrane of several types of cells, excluding RBCs,also stimulates increased expression of adhesion molecules on endothelial cells, contributing to leukocyte adhesion and stimulate RBC adhesion to endothelial cells In addition, the cytokine induce neutrophil degranulation, capillary leak and vasoconstriction and inhibits cell proliferation and induces cell death. Also contribute to the vascular inflammatory state that is present in various inflammatory diseases, including SCA. As mentioned previously (Pitanga*etal.*,2013).

Several studies have shown that patients display higher levels of a number of cytokines, although SCA patients in VOC had higher levels of TNF- α than patients in the steady-state group, (Tavakkoli*etal.*,2004).

2.1.8.Hydroxyurea :

This treatment improving both clinical complications and mortality in SCA and has several well-defined beneficial effects that could contribute to inhibition of the hypercoagulability state in SCA. These include a reduction in phosphatidyl serine exposure by erythrocytes, a reduction in the adhesive properties of erythrocytes and leukocytes, endothelial activation, nitric oxide depletion, platelet activation, and adhesive properties(Colela *etal.*,2012). SCA patients who are treated with hydroxyurea (HU) display an altered profile of TNF- α levels. Studies showed that HU did not significantly increase or decrease plasma TNF- α concentrations(Pitanga*etal* .,2013). Other studies show Hydroxyurea was associated with important decreases in levels of TNF- α (Colela *etal.*,2012).

2.1.9.Sickle cell anemia in Sudan:

Distribution of sickle cell anemia among the Sudanese, studies showed that sickle cell gene frequencies vary from region to another in Sudan as well as within the same region (Ahmed*etal.*, 2017). The first case of sickle cell anemia in Sudan was reported in 1926 and that was the first reported case in Africa(Ahmed *etal* .,2015).SCA is prevalent in Sudan it range from 0.8% -30.4% depending on the geographical location(Sabah elzain and Hamamy.,2014).And considered as the major haemoglobinopathy seen Khartoum,.This may be due to the migration of tribes from western Sudan as a result of drought and desertification in the 1970s and 1980s, and the conflicts in Darfur in 2005. These tribes are with the highest rate of (SCD)in Western Sudan particularly

Messeryia tribes in Darfur and Kordofan regions (Federal Ministry of Health., 2013). Northern Sudan show low frequency of SCA,Eastern Sudan Showing that sickle cells trait Hb AS was found in approximately 35% of study subjects in Hausa and 24% in Massaleet, where asHbSS was reported as 6% and 5% in Hausa and Massaleet respectively, in Western Sudan The presence of HbS is already well documented among Kordofan and Darfur region inhabitants, Sickle cell disease in Messeryia of Darfur and Messeryia Hummer of Kordofan showed a prevalence of 30.4% and 18% respectively (Ahmed*etal.*,2017).

2.2.Previous studies

In 1998 Michaels and his colleagues measured serum level of TNF- α by sandwich enzyme-linked immunoassay in three groups of children aged 2 to 18 years: 30 well children with SCD, 21 with SCD in pain crisis, and 20 healthy age-matched controls. There was no significant difference in TNF- α levels across all groups (Michaels *et al.*, 1998).

Other study done in Oman by Pathare and co-authors in 2004. They measured TNF- α level by ELISA SCD patients (n = 60); in steady state (n = 26) and in painful crisis (n = 34) and compared with nonanemic age- and sex-matched normal Omani controls (n = 20). The mean serum TNF- α level in the control group and steady Group patients were similar at 72.5 pg/mL (range 48–93 pg/mL) and 70.42 pg/mL (range 37–110 pg/mL), respectively, but there was an increase in crisis Group patients at 77.38 pg/mL (range 53–111 pg/mL) when compared with the steady Group patients, but this increase was not statistically significant (P 0.092) (Pathare *et al.*, 2004).

In Iran Keikhaei and his co-authors in 2013. Measured serum level of TNF- α in 54 SCA patient evaluated in two groups as follows; group A consisted of 39 VOC patients and group B comprised 15 Steady state patients and 19 healthy volunteers were included as controls measured used ELISA. The mean compared between three group patient in VOC show higher mean than thoses in steady state condition but this difference was insignificant the mean and SD were 17.8 ± 6.33 12.03 ± 0.85 12.17 ± 0.55 respectively the *p*-value were 0.13 between VOC and steady insignificant , 0.009 between VOC and control significant and 0.42 between control and steady insignificant (Keikhaei *et al.*, 2013).

In Brazil Colella and his colleagues estimated the effect of hydroxyurea on TNF- α level. Were selected steady-state SCA patients with no history of painful crisis, hospitalizations or blood transfusions in the preceding 3 months and 25 healthy age- matched controls. Patients were subdivided into two groups according to the use (SS-HU group, n = 23) or not (SS group, n = 15) of hydroxyurea. Compared mean and p value between three group, TNF-a serum levels (2.7 pg mL⁻¹ [SS] vs. 0 pg mL⁻¹ [controls], P < 0.0001) were observed in SS patients in comparison with controls. TNF-a, 2.7 pg mL⁻¹ [SS] vs. 0.3 pg mL⁻¹ [SS- HU], P < 0.0001) Hydroxyurea was associated with important decreases of TNF- α levels (Colella *et al.* ,2012).

TNF- α have been evaluated by Francis and his co-authors in 1992. Were measured using enzyme-linked immuno- sorbent assay in 59 plasma samples from 34 adult subjects with sickle cell disease. Plasma TNF- α was elevated on at least one occasion in 18 of the 21 steady-state sickle cell subjects and in 13 of the 19 crisis subjects. Tumor necrosis factor was undetectable in all eight healthy control subjects. Tumor necrosis factor levels ranged from <60 to 780 pg/mL in the steady state and from <60 to 725 pg/mL in crisis. Mean TNF- α was 150 \pm 171 (SD) pg/mL in the steady state and 151 \pm 181 pg/mL in crisis(Francis *et al.*,1992).

Study was done in Saudi by Alsharif in 2017. Measured serum level of TNF- α in Fifty sickle cell anemia Saudi patients in stable state and fifty age and sex matched healthy as control group. The mean and SD were 5.73 \pm 1.52 in steady state, 4.16 \pm 3 1 in control group. there was significant difference between two group p value (P < 0.05)(Alsharif.,2017).

Other study done by Gonzalez and his co-authors in 1998 measured TNF- α level by ELISA in blood samples were obtained from a group of 11 comparably aged African- American consenting volunteers who were documented to be negative for sickle or C hemoglobin. These samples served as age- and race-matched healthy controls. For this study, blood was drawn from 13 consecutive consenting adult patients (20 to 50 years of age) with known homozygous sickle cell disease presenting in acute pain crisis. Compare mean between control and crisis 44.41 +- 15.68 ,61.21 +- 5.62 respectively not different between healthy controls and sickle cell patients ($P > 0.05$), although a TNF- α remained higher in crisis than controls. No difference was observed between the levels in crisis and those of the same patients at post crisis follow-up ($P > 0.05$) (Gonzalez *etal.*, 1998).

In Brazil Veiga and his co-authors in 2013 evaluated of serum TNF- α level in 25 Brazilian children of African descent were involved in this study and divided in two groups: SCA (n=10) and control (n=15) age and sex matched. Analysis show higher level of TNF α in SCD group than control ($p < 0.05$) (Veiga *etal.*, 2013).

Other study done by Tavakkoli and his colleagues in 2004 to evaluate the effect of hydroxyurea in TNF- α level. Plasma collected from SCA patient during steady state and vasoocclusive crisis (VOC) and healthy adults as control. After analysis show TNF- α found greater in SCD than control ($P < 0.05$) and TNF- α in steady group was not significantly different than in VOC, however the plasma TNF- α tended to be greater in VOC group ($p > 0.1$) (Tavakkoli *etal.*, 2004).

Chapter Three
Materials and Methods

Chapter three

3. Materials and Methods:

3.1. Study design:

Designed as analytical case control study.

3.2. Study Area and duration:

This study was conducted in Al-Khartoum Locality at GaafarIbn-Auf Paediatric Tertiary Hospital, Albulk Hospital and Ahmed Gassim hospital during the period from July to April (2019).

3.3. Study population:

Sudanese sickle cell patients and apparently health subjects with different age and gender.

3.4. Inclusion criteria:

All Sudanese sickle cell patients as case group and healthy volunteer as control group.

3.5. Exclusion criteria:

All sickle cell patients with other disease that affect cytokines levels such as autoimmune diseases and infection.

3.6. Sample size:

A total of 60 sickle cell patients (30 during crisis and 30 during steady state) and 28 as healthy volunteer.

3.7. Sampling Techniques:

collected venous blood in a plain container (3ml) and allow to clot at room temperature. Then the sample was centrifuged and serum was be

collected in a sterile container and stored at -20°C until analysis. Serum levels of cytokine was measured using BioLegend's ELISA kits.

3.8. Data collection

Structural questionnaire sheet for all our patients to obtain the demographic data and lab investigations to obtain clinical information.

3.9. Principle of the ELISA

BioLegend's ELISA MAX™ Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human TNF- α specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and TNF- α binds to the immobilized capture antibody. Next, a biotinylated anti-human TNF- α detection antibody is added, produced an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB Substrate Solution, produced a blue color in proportion to the concentration of TNF- α present in the sample. Finally, the Stop Solution changed the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate reader (www.biolegend.com).

3.10.Lab method :

Day 1:

100 μL of diluted capture antibody solution was added to each well of plate, the plate was sealed and incubated overnight between 2C° and 8C° .

Day 2:

1. Plate was washed 4 times and blocked by added 200 μL 1x Assay diluent A to each well, then plate was sealed and incubated at room

temperature for 1 hour with shaking on plate shaker (500 rpm with 0.3cm circular orbit).

2. Plate was washed 4 times, then 100 μ L from diluted standards and sample were added to the appropriate wells.

3. Plate was sealed and incubated at room temperature for 2 hours with shaking.

4. Plate was washed 4 times, then 100 μ L of diluted detection antibody solution was added to each well then sealed the plate and incubated at room temperature for 1 hour with shaking.

5. Plate was washed 4 times, then 100 μ L of diluted Avidin-HRP solution was added to each well, then the plate was sealed and incubated at room temperature for 30 minutes with shaking.

6. Plate was washed 5 times and soaked for 3 seconds to 1 minute per wash, 100 μ L of freshly mixed TMB substrate solution was added to each well and incubated in dark place for 15 minutes.

7. 100 μ L of stop solution was added to each well. Then the absorbance was readed at 450 nm and 570 nm within 15 minutes.

3.11. ELISA reader principle:

White light produced by the lamps is focused into a beam by the lens and passes through the sample. Part of the light is absorbed by the sample and the remaining light is transmitted. It is filtered by interference filters and focused onto the photodiodes. The photodiode converts the received light into an electrical signal which is transformed into a digital form, from which the microprocessor calculates the absorbance, taking in account of the blank and dichromatic selection (www.diasource.be).

3.12. Data Analysis:

Statistical analysis was performed using statistical package for social science (SPSS) software program Version 21, The data was displayed as (mean SD) for analyzing concentration, and was selected one way ANOVA and independent (T test). The probability value (PV) less than 0.05 was considered significant.

3.13. Ethical Consideration

This study was approved by college of medical laboratory science department of hematology and immunohematology, permission of hospital manager was taken before beginning and informed consent was taken from every patient.

Chapter Four

Results

Chapter four

4.Result :

In this study eightyeight plasma samples were obtained with standard procedure from eighty eight participants as sixty sickle cell patients (Cases) divided into two groups thirty in vasoocclusion crisis (21 with musculoskeletal pain, 4 with hemolytic crisis ,4 with acute chest syndrome and 1 with retionpathy) and thirty in steady state, and twenty eight healthy people as control. All serum were investigated for TNF- α cytokine level. Results in (tables 1 -2) and figure (1 – 2)

4.1. Gender Distribution:

Of the total 60 patients, 34 were males and 26 were females. In VOC 53% were males and 47% were females ,in steady state 60% were males and 40% females, among the total 28 control group, 36% of them were males and 61% were females. figures 3-5.

4.2. Age Distribution:

The ages of patients ranged from 4 to 17 years with the control group ranged from 4 to 17 years old. figure 6

the Table 1:mean \pm SD of TNF- α of all sickle cell disease patients (N = 60) grouped into steady-state and vaso-occlusion crisis (VOC) conditions and healthy controls

Study group	Mean \pmSD of TNF-α(pg/ml)	P-value
Steady state group	1.76 \pm 3.16	0.073
VOC group	5.51 \pm 1.05	
Control group	2.30 \pm 3.10	

Table 2:mean of HB in sickle cell patient and control

Group	Mean of HB g/dl
VOC	5.9
Steady state	7.3
Control	N/D

N=number N/D =not determined

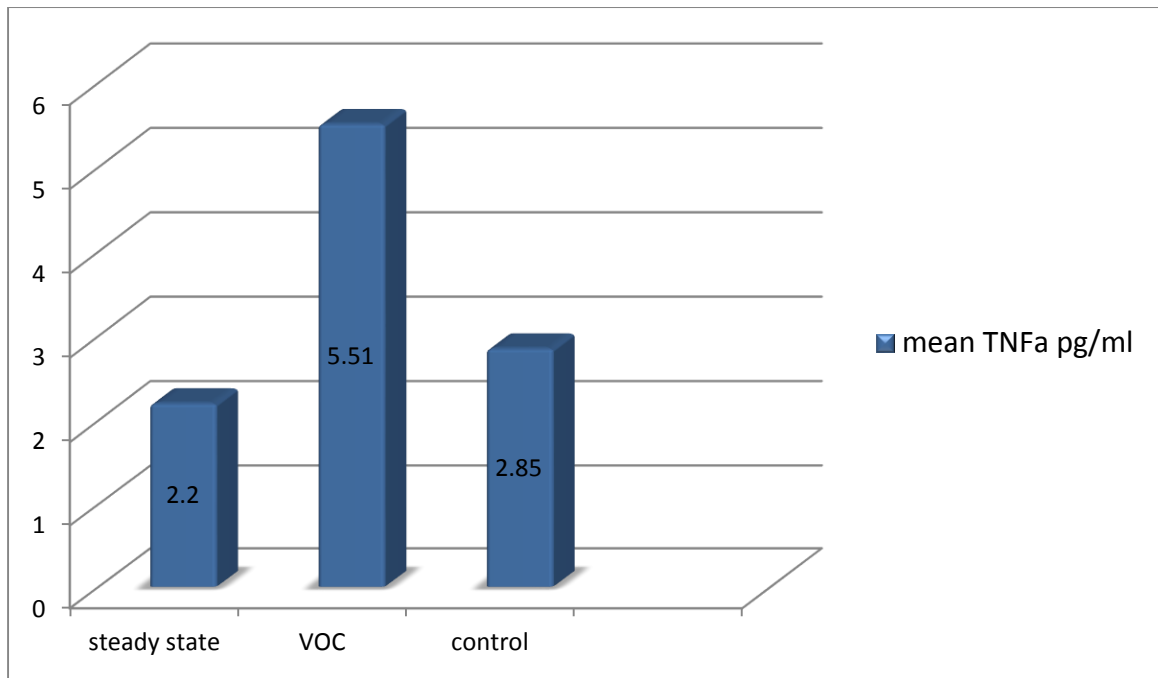


Figure 1: Mean of TNF- α level in the steady state patients, VOC patients and control group

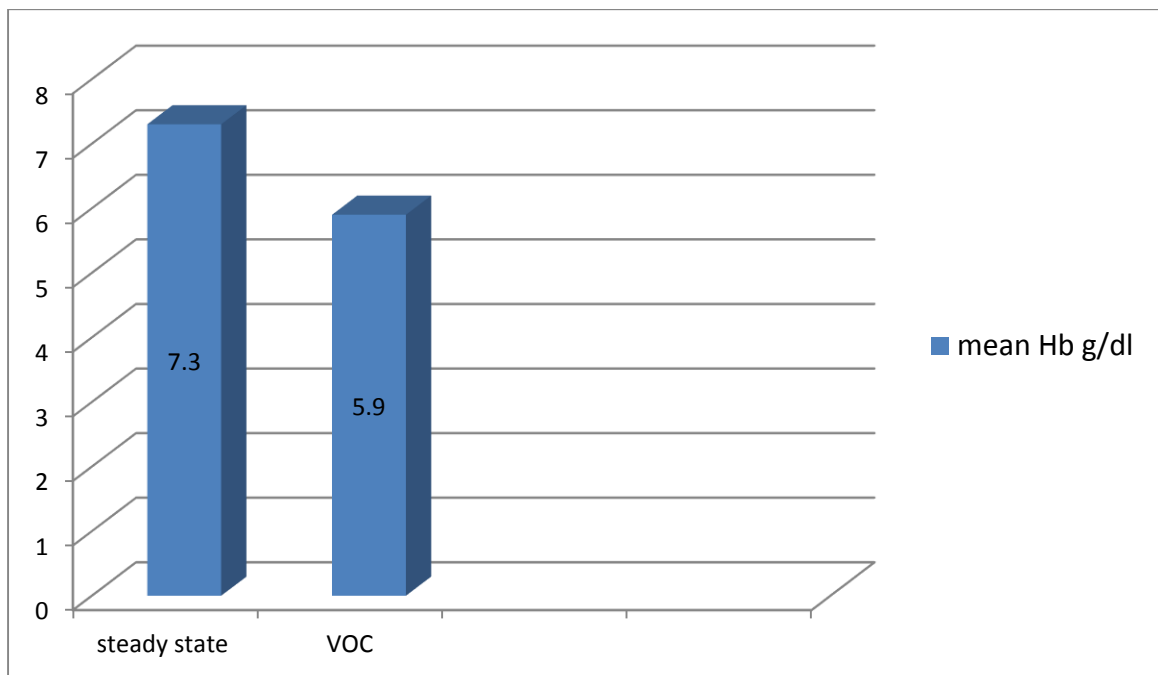


Figure 2: Mean of Hb between steady state and VOC there was significant difference in Hb level in two groups (p value 0.00)

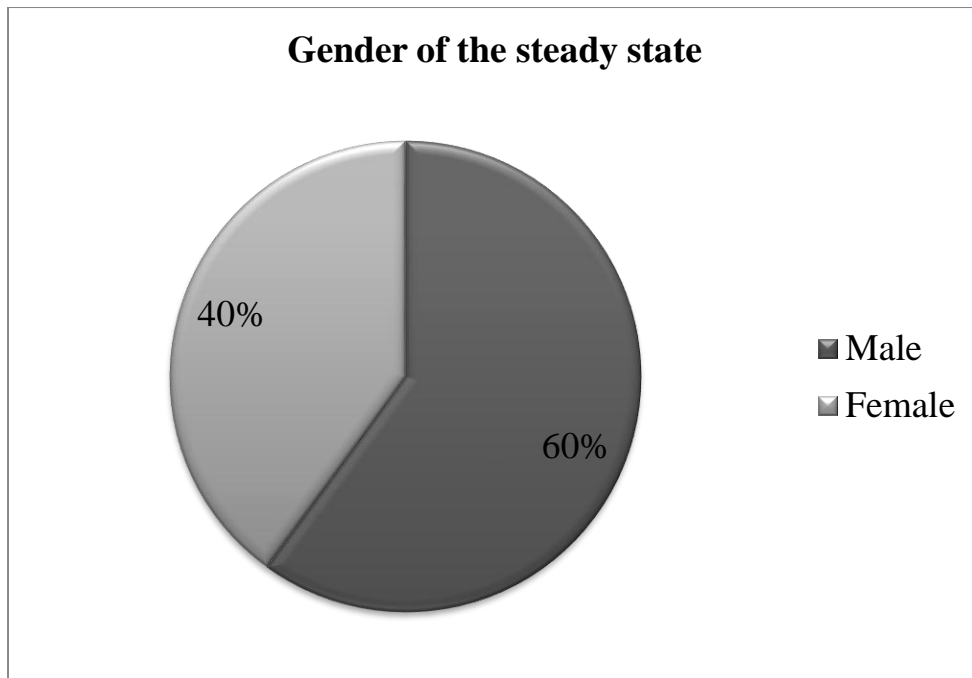


Figure 3:percentages of male and females in the steady state patients

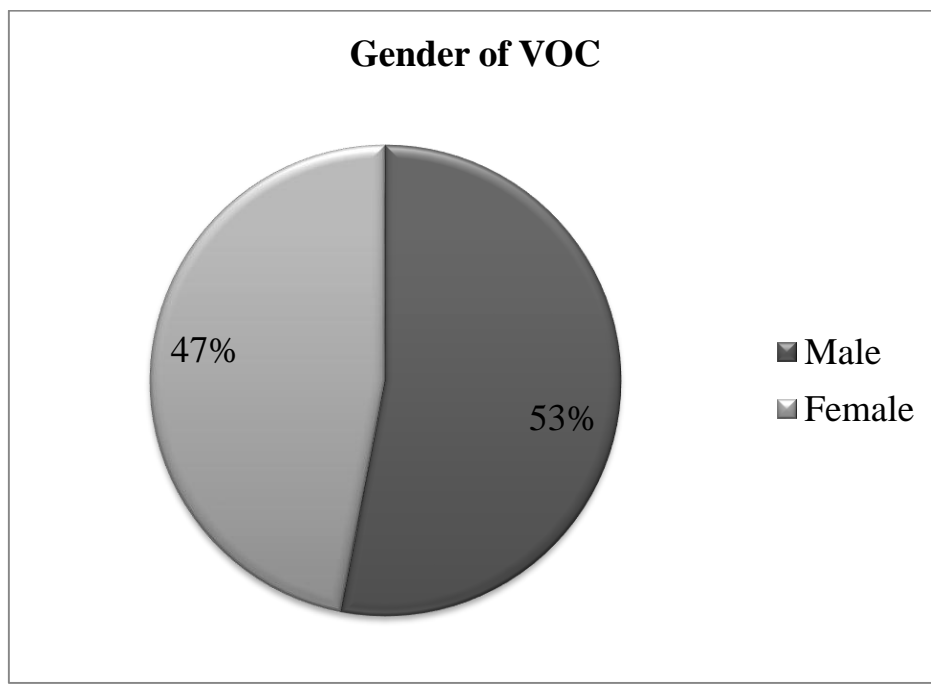


Figure 4:percentages of male and females in theVOCpatients

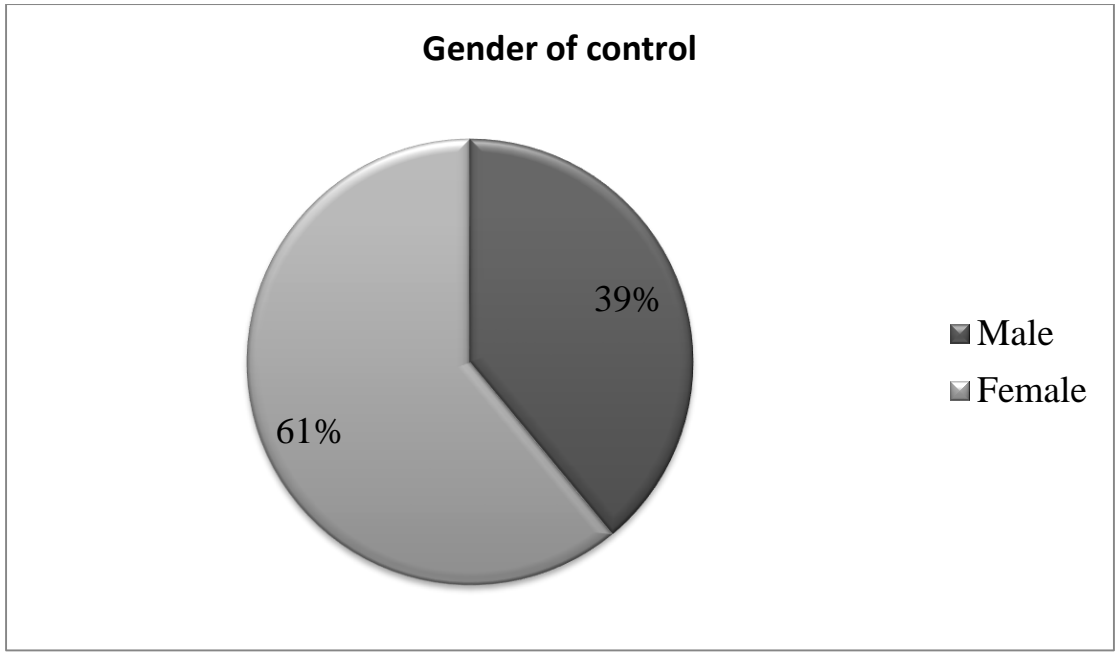


Figure 5: percentages of male and females in the control group

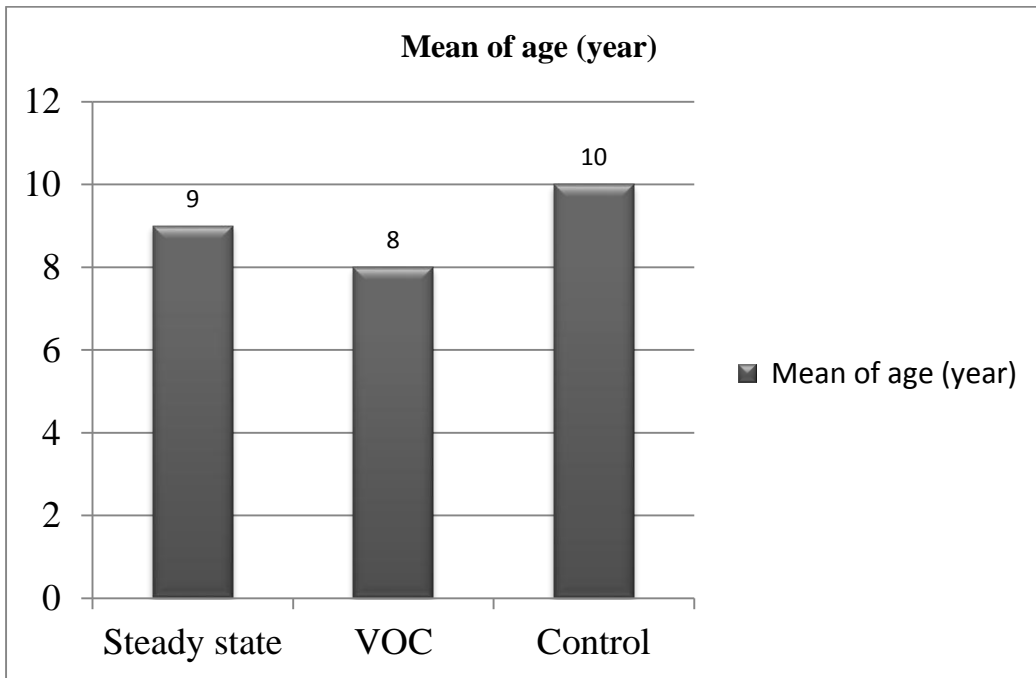


Figure6 :Mean of age in steady state ,VOC and control

Chapter Five
Discussion, Conclusion and
Recommendations

Chapter five

5.1. Discussion :

Sickle cell disease, particularly in the homozygous state, characterized by significant morbidity and early mortality (Elzubeir *et al.*, 2017). Major factors thought to contribute to high mortality rate among children with Sickle cell disease in Africa are the cultural background, lack of medical education and limited healthcare facilities. (Daaketal ., 2016). Several studies have demonstrated that the cytokine play important role in vasoocclusion and pathophysiology of Sickle cell disease (Keikhaei *et al.*, 2013).

TNF- α contribute to the vascular inflammatory state condition by stimulates increased expression of adhesion molecules on endothelial cells, contributing to leukocyte and RBC adhesion to endothelial cells (Pitanga*et al.*, 2013).

The result demonstrated increased mean of TNF- α level in sickle cell patients during vasoocclusion crisis compared with sickle cell patients during steady state but this difference was statistically insignificant (p -value= 0.073) and this result parallel with other studies done by Michaels *et al.*, 1998; Pathare *et al.*, 2004; Keikhaei *et al.*, 2013; Gonzalez *et al.*, 1998 and Tavakkoli *et al.*, 2004 whom showed increased mean of TNF- α in VOC patients compare to steady state SCA patients but this differences was statistically insignificantly.

And compared mean of TNF- α cytokine level in vasoocclusion crisis SCA and control group showed increased mean of TNF- α in VOC to control group but this difference was statistically insignificant (p - value =0.073)

and this result parallel with other studies done by Pathare *etal.*, 2004; Michaels *etal.*, 1998 and Gonzalez *etal.*, 1998 another studies done by Keikhae *etal.*,2013;Veiga *etal.*,2013andTavakkoli *etal.*, 2004showed significant differences between VOC and control group. Also in this study compared mean of TNF- α level in steady state SCA and control group and was statistically insignificant (p - value= 0.073) and this parallel with studies done by Pathare *etal.*, 2004; Michaels *etal.* ,1998and Keikhaei *etal.*, 2013 showed insignificant differences of TNF- α level between steady and control. Other studies done by Veiga *etal.*, 2013; Alsharif *etal.*, 2017; Colella *etal.*, 2012and Tavakkoli *etal.*, 2004 showed significant diferances between steady state and control. In the comparison of mean of Hb in steady state (7.3) and VOC (5.3) difference between group was significant (p - value=0.00) and this not parrallel with study done byKeikhaei *et al.*, 2013.

5.2.Conclusion:

The study showed increased mean of TNF- α cytokine level in vasoocclusion crisis SCA in compare of steady state SCA and control group but this difference was insignificant. May need to increase sample size to demonstrate effect of TNF- α cytokine in developpe of vasoocclusion crisis in SCA

5.3.Recommendations:

All sickle cell patients should be examined for TNF- α cytokine level.

References

References:

Abbas, A.K., Lichtman, A.H. and Pillai, S. (2015). Cellular and molecular immunology, 8th ed, Elsevier Saunders, Canada, p469, 472

Ahmed, F.E., Gaboli, H.O., Attalla, B. (2015). Pica among Sudanese children with sickle cell anemia, Basic research journal, 4(1) pp.01-07

Ahmed, N.H.M., Mohammed, A.A., Abdelgyoum, A.M (2017). Evaluation of Abdominal Organs in Children with Sickle Cell Anemia using Ultrasonography, Scholars Journal of Applied Medical Sciences, 5(3C):920-925

Agrawal, K., Patel, R.K., Shah, V., Nainiwal, L. and Trivedi, B. (2014). Hydroxyurea in Sickle Cell Disease: Drug Review. Indian J Hematol Blood Transfus 30(2):91-96

Akohoue, S.A., Shanker, S., Milng, G. J Morrow, K. Chen, W.U. Ajayi, AND Buchowski, M.S. (2007). Energy Expenditure, Inflammation, and Oxidative Stress in Steady-State Adolescents With Sickle Cell Anemia. International Pediatric Research Foundation. 61(2):233-238

Alsharif, M.F. (2017). Immunology and Systemic Inflammation Biomarkers Among Saudi Patients with Sickle Cell Anemia in Asymptomatic Steady State Condition. Current Research in Diabetes and Obesity Journal 2 (3):478-483

Ashley-Koch, A., Yang, Q, and Olney, R.S (.2000). Sickle Hemoglobin (Hb S) Allele and Sickle Cell Disease. American Journal of Epidemiology 9(151):839-845

Cajado, C., Cerqueira B.A., Couto, F.D., Moura, J.B. Vialas-Boas .W., Dorea, M.J., Lyra, I.M (2011). TNF-alpha and IL8 serum level and gene polymorphism (-308>A and -251A>T) are associated with classical biomarker and medical history in children with sickle cell anemia, Elsevier .56.312-317

Colella, M.P., Depaula, E.V, Conran, N., Machadoneto, J.A., Annichin, B.J.M., Costa, F.F., Saad, S.T.O and Traina, F. (2012). Hydroxyurea is associated with reductions in hypercoagulability markers in sickle cell

anemia. International Society on Thrombosis and Haemostasis. **10**, 1967-1970

Daak A.A. (2016) Sickle cell disease in western Sudan. *Tropical Medicine and International Health*. **21**(5) 642–65

El-Hazmi MA, Al-Hazmi AM,(2011). Warsy AS. Sickle cell disease in Middle East Arab countries. *Indian J Med Res.* **134**(5):597-610.

Elzubeir A.M, Alobied A, Halim H, Awad M, Elfaki S, Mohammed M, Saddig O.A (2017) Estimation of Serum Interleukin-6 level as a Useful Marker for Clinical Severity of Sickle Cell Disease among Sudanese Patients. *International Journal of Multidisciplinary and Current Research.* **5**, 642-644

Francis R.B, Haywood J, Losanges M.D.(1992) Elevated immunoreactive TNF α and IL2 in sickle cell disease .the journal medical association **84**(7);611-615

Federal Ministry of Health.(2013). Development of a national package for management of Sickle Cell Disorders. 8 Apr 2013. Khartoum.

Frenette, P.S. (2002) Sickle cell vaso-occlusion: Multi- step and multicellular paradigm. *Current Opinion Hematology*, **9**, 101-106.

Gonzalez E.G, Doherty J.C, Eric W. Bergreen, Organ G, Telfer M. and McMillen M.A.(1998), Plasma Endothelin-1, Cytokine, and Prostaglandin E2 Levels in Sickle Cell Disease and Acute Vaso-Occlusive Sickle Crisis, *American society of hematology* .**92**: 2551-2555

Greer, J.P., Arber, D.A., Glader, B., List, A.F., Means, R.T., Paraskevas, F., Rodger, G.M. (2014). *Wintrobe's Clinical Hematology*, 13th ed, Lippincott Williams and Wilkins, Philadelphia, p830

Higgs D.R, Roy N, Hay D (2016). *Erythropoiesis. postgraduate hematology seventh edition* 11-21

Ikram, N., Hassan, K. and Tufail, S. (2004) Cytokines. *International Journal of Pathology*, **2**, 47-58.

Keikhaei B., Mohseni A.R, Norouzirad R., Alinejadi M, Ghanbari S, Shiravi F, Solgi G.(2013) Altered levels of pro-inflammatory cytokines in

sickle cell disease patients during vaso-occlusive crises and the steady state condition, Eur. Cytokine Netw. Vol. **24**(1): 45-52.

Makis, A.C., Hatzimichael, E.C, Bourantas, K.L .(2000).the role of cytokine in sickle cell disease .Ann Hematol**79**(5):407-413

..

Master, M., Ong, M. and Mansour, R.P. (2016). Blood Transfusion in Adult Patients with Sickle Cell Disease: Incidence of Alloimmunization Blood.**128**(22):4859

Michaels L.A., -Frempong, K.O, Zhao, H, and Steven D. Douglas, (1998) .Serum Levels of Substance P Are Elevated in Patients With Sickle Cell Disease and Increase Further During Vaso-Occlusive Crisis, American society of hematology **92**(9):3148-3151

Obeagu, E.I., ocheei, K.C.N, Wachukwn, B.N, and Nchuma, B.O. (2015) Sickle cell anemia .A review journal of applied medical sciences 3(6B);22444-22522

Owen, J.A., Punt, J. and Stranford, S.A. (2013). KUBY Immunology, 7th ed, W. H. Freeman and Company, New York, 370

Pathare, A., Al Kindi, S., Alnaqdy, A.A., Daar, S., Knox-Macaulay, H. and Dennison, D. (2004). Cytokine Profile of Sickle Cell Disease in Oman. American Journal of Hematology **77**(4):323–328

Popa, C., Netea, M.G., van Riel, P.L., van der Meer, J.W. and Stalenhoef, A.F. (2007) The role of TNF-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. Journal of Lipid Research, **48**, 751-76

Pitanga, T.N., Boas, V.W, Veloso, B.A, Seixas, M.O, Barbosa, C.G, Adorno, A.V Goncalves, M.S (2013). Cytokine profiles in sickle cell anemia: Pathways to be unraveled. Advances in Bioscience and Biotechnolog. **4**(7A1) 6-12

Raghupathy, R., Haider, M.Z., Azizieh, F., D'Souza, T.M. and Adekile, A.D.(2000) . Th1 and Th2 cytokine profiles in sickle cell disease. *ActaHaematol* **103**(4): 197-202.

Rees.D.C, Williams T.N, Gladwin M.T.(2010) Sickle-cell disease. *Lancet*: 376

Robert. B., Francis, Jr, MD, and L. Julian Haywood, MD Los Angeles, California (1992)Elevated Immunoreactive Tumor Necrosis Factor And Interlukin-1 in Sickle Cell Disease,*Journal of the National medical association*,. **84**(7):611-615

Sabahelzain M., Hamamy H. The ethnic distribution of sickle cell disease in sudan.*panafr Med J*.2014:18:13.

Schnog J. B., Duits A. J., Muskiet F. A., ten Cate H.,Rojer R. A., Brandjes D. P. Sickle cell disease:general overview. *Neth J Med* 2004 Nov: 62 (10)

Steinberg, M.H. and Rodgers, G.P. (2001) Pathophysiol- ogy of sickle cell disease: Role of cellular and genetic modifiers. *Seminars in Hematology*, 38, 299-306

Stuart M.J, Nagel R.L. Sickle cell disease. *Lancet* (2004); 364: 1343-60

Tavakkoli, F.,Nahavandi, M., Wyche, M.Q. and Perlin, E. (2004) Plasma levels of TNF-alpha in sickle cell patients receiving hydroxyurea. *Hematology*, **9**, 61-64.)

Veiga, P.C., Schroth, R.J., Guedes, R., Freire, S.M. and Nogueira-Filho, G. (2013) Serum cytokine profile among Brazilian children of African descent with periodontal in- flammation and sickle cell anaemia. *Archives Oral Biol ogy*, **58**, 505-510

Appendix (1)

Sudan University of Sciences and Technology

College of Graduate studies

College of Medical Laboratory Sciences

Department of Hematology and Immunohematology

Estimation of TNF- α Level in Sudanese Sickle Cell Disease Patients
During Vaso-occlusive Crisis and the Steady State Condition

تقدير مستوى المادة الخلوية عامل نخر الورم الفا عند المرضى السودانيين المصابين بالانيميا
المنجلية خلال حالة الازمة الوعائية والحالة المستقرة

_Date: / /2018

_ID . Number:

_Age:Years

_Gender:

Male :{ }

Female: { }

_Clinical remarks:.....pain and
site of pain.....

_Acute chest syndrome.....

_leg ulcer.....

_Other.....

Date:

Signature:

Appendix (2)

استمارة موافقة مشارك

تقييم مستوى المادة الخلوية عاملنخر الورم الفاعند
المرضى السودانيين بالانيميا المنجلية خلال حالة
الازمة الوعائية والحالة المستقرة

أنيميا الخلايا المنجلية هي أحد أنواع فقر الدم وهي تصيب كريات الدم الحمراء وهي من أشهر أمراض الدم الوراثية الإنحلالية التي تسبب تكسر كريات الدم الحمراء. من مضاعفات المرض السكتة الدماغية، متلازمة الصدر الحاد، إرتفاع الضغط الدموي وتضرر الاعضاء.

هل توافق على المشاركة في البحث العلمي عن مرضى أنيميا الخلايا المنجلية , التابع لجامعة السودان للعلوم والتكنولوجيا كلية علوم المختبرات الطبية , وأنت على علم تام بمحتوى البحث , مشاركته عن طريق التبرع بعينه من دمك , مشاركته إختياريه من دون إكراه و من دون أي مقابل مادي ؟

.....
.....
.....
.....

اسم المشارك والتوقيع:

.....
رقم تلفون المشارك.....

هذه المعلومات لغرض البحث العلمي فقط , وتتمتع بالسريه الكامله.

اسم الباحث والتوقيع:.....

رقم تلفون الباحث.....

Appendix (3)

ELISA Washer

