Sudan University of Sciences and Technology

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Evaluation of Serum Interleukin-8 Level in Sudanese Sickle Cell Disease Patients During Vaso-occlusive Crisis and the Steady State Conditions

تقويم مستوى انترليوكين 8 المصلي عند المرضى السودانيين بالأنيميا المنجلية خلال حالتي الازمة الوعائية و المستقرة

A dissertation Submitted in Partial Fulfillment of the Requirements for the Award M.Ss. Degree in Medical Laboratory Sciences (Hematology and Immunohematology)

Submitted by:

Reham Osman Yassin Ahmed

(B.Sc. in Medical Laboratory Sciences, Sudan University of Sciences and Technology, 2016)

Supervisor:

Dr: Kawthar Abdelgaleil MohammedSalih Ibrahim

(Assistant Professor, Sudan University of Sciences and Technology)

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بسم الله الرحمن الرحيم

الأية

وَاللَّهُ أَخْرَجَكُم مِّن بُطُونِ أُمَّهَاتِكُمْ لَا تَعْلَمُونَ شَيْئًا وَجَعَلَ لَكُمُ السَّمْعَ وَالْأَبْصَارَ وَالْأَفْئِدَةَ ۙ لَعَلَّكُمْ تَشْكُرُونَ (78)

صدق الله العظيم

سورة النحل الأية (78)

Dedication

To the candle which burn to light my life
My mother
To the source of confidence
My father
To those who have made it possible
Teachers and friends
To whom will find it beneficial work...

Acknowledgments

First of all, thanks to Allah who granted me the ability to accomplish this work.

I would like to express my sincere gratefulness and respect to Dr: Kawthar Abdelgaleil MohammedSalih, for her valuable guidance, kind supervision and great help. I was honored to be her candidate and to be guided all through this work by her kind and precious advices which helped me to present this work in its proper way.

Special thanks to staff of Institute of Endemic Diseases, Immunology and molecular biology department.

Abstract

This case-control study was aimed to evaluate the IL-8 level in Sudanese sickle cell anemia patients during vaso-occlusive crisis (VOC) and the steady state conditions and in control subjects in Khartoum state during the period from July to December (2018).

60 SCA patients were selected randomly, grouped into steady-state (n=30) and vaso-occlusion crisis (n=30) conditions. 28 of age and sex- matched control subjects were enrolled in this study. Venous blood sample (3ml) was collected in plain container from each subject. IL-8 concentration was measured using Enzyme Linked Immunosorbent Assay (ELISA) (Biolegend's ELISA MAX^{TM}) in Institute of Endemic Diseases. The data was analyzed using SPSS programme (Version 16) using one way ANOVA test and independent T-test for testing difference significance, correlation test for finding correlation.

The result showed that: means of IL-8 were 92.78±32.17, 148.45±165.72 and 90.48±24.94 in the steady state patients, VOC patients and control subjects respectively. IL-8 level was significantly elevated in VOC patients than the steady state group and control group (*P. values* were 0.033 and 0.029 respectively). There was no significant difference between IL-8 level in the steady state patients and controls (*p. value* 0.930). Hb level was significantly decreased in VOC group than the steady state group (*P. value* 0.000). Also there was no statistical correlation between IL-8 and age in the three groups (*P. values* 0.961 in steady state, 0.524 in VOC and 0.266 in controls). The difference in mean of IL-8 between males and females of the three groups was not statistically significant (*P. values* 0.116 in steady state, 0.37 in VOC, and 0.584 in controls).

The study concluded that in the studied population, IL-8 concentration may be a useful VOC marker.

مستخلص البحث

أجريت هذه الدراسة (دراسة الحالات والشواهد) لتحديد مستوى انترليوكين 8 المصلي في المرضى السودانيين بالأنيميا المنجلية خلال الأزمة الوعائية والحالة المستقرة وفي الأفراد الطبيعيين بولاية الخرطوم في الفترة من يوليو حتى ديسمبر 2018. أختير60 مريض بالانيميا المنجلية عشوائيا ، 30 منهم خلال الأزمة الوعائية و 30 خلال الحالة المستقرة. أختير 28 متطوع سليم متشابهين في الجنس والعمر مع المرضى بالأنيميا المنجلية. سحبت عينة دم وريدية من كل مشارك (3 مل) في أنبوبة خالية من موانع التجلط. قيس مستوى انترليوكين 8 عن طريق فحص مقايسة الممتز المناعي المرتبط بالانزيم بمعهد الأمراض المتوطنة. حللت البيانات باستخدام برنامج الحزم الأحصائية للمجتمع (نسخة 16). كان الوسط الحسابي لانترليوكين 8 148.45±165.72، 92.78±32.17 و90.48±24.94 في المرضى بالأنيميا المنجلية خلال الأزمة الوعائية وخلال الحال الحالة المستقرة وفي الأفراد الطبيعيين بالترتيب. هنالك ارتفاع ذو دلالة احصائية في معدل انترليوكين 8 في المرضى بالأنيميا المنجلية خلال الأزمة الوعائية عند مقارنتهم بالمرضى في الحالة المستقرة والأفراد الطبيعيين (كانت القيم الاحتمالية 0.033 و 0.029 بالترتيب). بينما الفرق في معدل انترليوكين 8 بين المرضى في الحالة المستقرة والأفراد الطبيعيين لم يكن ذو دلالة احصائية (كانت القيمة الاحتمالية 0.930). أيضا معدل خضاب الدم كان منخفضا في المرضى خلال الأزمة الوعائية مقارنة بالمرضى في الحالة المستقرة(القيمة الاحتمالية 0.000). ليس هنالك علاقة بين معدل انترليوكين 8 والعمر في الثلاث مجموعات (القيم الاحتمالية0.961 في الحالة المستقرة ، 0.524في حالة الأزمة الوعائية و 0.266في الأفراد الطبيعيين). لاتوجد دلالة وصفية حسابية لانترليوكين 8 بين الرجال والنساء في الثلاث مجموعات (القيم الاحتمالية 0.116 في الحالة المستقرة، 0.37 في حالة الأزمة الوعائية و 0.584 في الأفراد الطبيعيين).

لقد توصلت الدراسة الى ان معدل انترليوكين 8 المصلي يمكن ان يستخدم كعلامة للأزمة الوعائية.

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List of abbreviations

APCs	Antigen presenting cells
ELISA	Enzyme linked immunosorbent assay
g/dl	gram/deciliter
Hb	Hemoglobin
HbA	Adult hemoglobin
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-9	Interleukin-9
IFNγ	Interferon gamma
KD	Kilo Dalton
N	Number
NO	Nitric oxide
P. value	Probability value
Pg/ml	Pico gram/milliliter
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCA	Sickle cell anemia
SD	Standard deviation
SCD	Sickle cell disease
HbS	Sickle hemoglobin
SPSS	Statistical package for social science
Th	T helper
TGF-β	Transforming growth factor – beta
TNF	Tumor necrosis factor
VOC	Vaso-occlusive crisis

Chapter One

Introduction and Literature review

Chapter One

Introduction and Literature review

1. Introduction:

Sickle cell disease (SCD) is an inherited disorder of hemoglobin (Hb) structure and synthesis, caused by a point mutation in the β - globin chain of Hb, causing the amino acid glutamic acid to be replaced with the hydrophobic amino acid valine at the sixth position (Keohane *et al*., 2016). 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 Hb which distorts red blood cells into a sickle shape and decreases their elasticity (Hoffbrand *et al*., 2016). Repeated episodes of sickling damage the cell membrane and decreases the cell's elasticity. As a consequence, these rigid blood cells are unable to deform as they pass through narrow capillaries, leading to vessel occlusion and ischemia (Obeagu *et al*., 2015). The disease characterized by chronic hemolysis, frequent infection and recurrent occlusion of microcirculation which cause painful crises and result in chronic organ damage and failure (Qazi *et al*., 2018). The term sickle cell crisis was introduced to describe a recurring attack of pain involving the skeleton, chest, abdomen, or all three (Greer *et al*., 2014). Steady state is the period between painful crisis (Makis *et al*., 2000). Intravascular hemolysis results in Hb release, which leads to an intense local and systemic inflammatory and cytokine response causing endothelial damage and sludging resulting in ischemia (Munker *et al*., 2007). Increased circulating levels of cytokines and chemokines lead to activation of vascular endothelium and adhesion of RBCs, leukocytes and platelets to the endothelium (Pitanga *et al*., 2013).

Interleukin-8 (IL-8) is a pro-inflammatory member of the CXC chemokine family that produced by many types of cells such as macrophages, dendritic cell and vascular endothelial cell (Owen *et al*., 2013). IL-8 contribute to the chronic inflammatory state that is present in various inflammatory diseases, including acute respiratory distress syndrome, psoriasis, chronic obstructive pulmonary disease, rheumatoid arthritis (Qazi *et al*., 2011) and SCD (Pitanga *et al*., 2013) . This cytokine induce the adhesion of RBC and leukocytes to the vascular endothelium and this adhesion can cause vaso-occlusion and local hypoxia (Keikhaei *et al*., 2013). In 2013 Elzubeir and his colleagues estimate the serum IL-6 level in Sudanese SCD patients and the findings indicated that serum level of IL-6 may contribute to susceptibility to many

crisis and may use as useful marker for early broadcasting tool for SCD crisis (Elzubeir *et al*., 2013).

1.2. Literature review:

1.2.1. Blood:

Blood is specialized liquid connective tissue (Chauhan, 2013). , pumped by the heart through arteries and veins reaching all body's cells (Mehta and Hoffbrand, 2014). It transport and distribute oxygen, nutrients, hormones and waste products, regulate pH, osmotic pressure and body temperature, control blood loss by assistance of platelets and coagulation factors and involves in body's immune response which mediated by leukocytes. (Cheesbrough, 2005)**.** It consists of cells (erythrocytes and leukocyte) and cell fragments (platelets), surrounded by liquid extracellular matrix called plasma (Chauhan, 2013).

1.2.2. Erythropoiesis:

Erythropoiesis is a continuous and dynamic process by which erythrocytes are generated from multipotent hematopoietic stem cells (HSCs) (Zhang *et al*.,2015).It is occur in special anatomical site called erythroid island (niches) in which erythroid precursors proliferate, differentiate, and enucleate(Chasis and Mohanads, 2008).

1.2.3. Red blood cells (RBCs):

RBCs are a discoid shape have specialized membrane flexibility which provide large surface areas for gas exchange, and allow repeated passes through narrow capillaries (Blann and Ahmed, 2014). RBCs lack nuclei and other organelles. These unique differences enabling maximal cytoplasmic occupation by hemoglobin (Hb) (Palis, 2014).

The majority of erythrocytes (90%) are phagocytized and destroyed by the spleen (extravascular hemolysis) after complete their lifespan 120 days. The hemoglobin is broken down into the heme ring and the globin proteins. The iron is removed from the heme ring and either returns to the bone marrow to be inserted into new erythrocytes or enters the iron storage pool (Kern, 2002).

1.2.4. Hemoglobin (Hb):

Hb molecule is composed of iron containing pigment called (heme) and protein (globin) (Bain and Gupta, 2003).It binds efficiently to oxygen molecules, and somewhat less efficiently to carbon dioxide molecules, thereby functioning in the transport of gases through the bloodstream (Palis, 2014). Different hamoglobins are synthesized in the embryo, fetus and adult, each adapted to their particular oxygen requirements. They all have a tetrameric structure made up of two different pairs of

globin chains, each attached to one heme molecule (Blann and Ahmed, 2014) Table (1-1).

Hemoglobin abnormalities result either from Synthesis of an abnormal haemoglobin that arise from synthesis of an α or β chain with an amino acid substitution such as Hb S, C, D and E or from reduced rate of synthesis of normal α- or β-globin chains (α- and β-thalassaemias)((Hoffbrand and Moss, 2016).

Table (1-1) Hemoglobin variants: (Horton-Szar *et al.*, 2012; Kawthalkar, 2013**)**

Developmental stage	Hemoglobin types	Chains
Embryonic	Hb Gower I Hb Gower II Hb Portland	ζ2ε2 a2ε2 ζ2γ2
Fetal	HbF (80%) HbA (20%)	α2γ2: higher affinity for O2
Adult	HbA (97%) HbA2 (2.5%) HbF (0.5%)	α2β2: Principal Hb of adult Hb α2δ2

1.2.5. Anemia:

1.2.5.1. Definition:

Anemia is defined operationally as a reduction in the hemoglobin content of blood that can be caused by a decrease in RBCs, hemoglobin, and hematocrit below the reference interval for healthy individuals of similar age, sex, and race, under similar environmental conditions (Porwit *et al*., 2011). Functionally defined as reduction in the oxygen carrying capacity of the blood (d'Onofrio and Zini, 2015).

1.2.5.2. Classification:

A pathophysiologic approach divides anemia into three categories:

1.2.5.2.1. Anemia due to blood loss: Acute and chronic (Porwit *et al*., 2011).

1.2.5.2.2. Impaired production: anemia of chronic disease, aplastic anemia, disorders of iron metabolism and heme synthesis (iron deficiency, sideroblastic

anemia and hemochromatosis), megaloblastic anemia (vitamin B12 or folic acid deficiency), myelodysplasia and myelophthisic (Beck, 2009).

1.2.5.2.3. Increased destruction (hemolytic): extrinsic to RBCs (autoimmune or isoimmune, infections and physical or chemical agents), Intrinsic to RBCs (enzyme deficiencies, hemoglobinopathies due to amino acid substitutions,chain synthesis defects or combinations and Membrane defects) (Porwit *et al*., 2011)

1.2.6. Sickle cell disease:

Sickle cell disease is a term for a group of disorders that includes homozygous sickle cell anemia (HbSS), sickle cell hemoglobin C disease (HbSC), sickle cell thalassemia disease (S/thal) and other compound heterozygous conditions. They are all characterized by the presence of the mutated β globin gene, and all cause clinical disease (Arceci *et al*., 2006). It's a common disorder of people from African descent; however, it is also seen in people of Mediterranean, South and Central American and East Indian ancestry (Schmaier and Lazarus, 2012).

1.2.6.1. Normal Hb (Hb A) and Sickle Hb (Hb S):

The normal adult haemoglobin molecule is a tetramer composed of 2 α-chains and **2** β-chains. Each chain is provided with a heme molecule which reversibly binds oxygen (Blann and Ahmed, 2014). The oxygenated molecule is called oxy-hemoglobin and the deoxygenated one, deoxy-hemoglobin (Kern, 2002). Normal hemoglobin is soluble in both its *oxy* and deoxy forms (ciesla, 2012). Sickle hemoglobin (Hb S) result from point mutation in the β - globin chain gene, causing the amino acid glutamic acid to be replaced with the hydrophobic amino acid valine at the sixth position in the β-globin chain of Hb (Mehta and Hoffbrand, 2005). Hb S is less soluble in the deoxygenated form, leading to the characteristic polymer formation which produces the sickle cell (Thachil and Quentin, 2014).

1.2.6.2. Genetics and classification:

Sickle cell anemia is a beta chain variant, and beta chains are located on chromosome 11, which has one location for the inheritance of a normal beta chain or an abnormal beta chain (Ciesla, 2012). Sickle cell anemia is an autosomal co-dominant inherited in simple mendelian fashion (Hastings *et al*., 2012).The genetic defect is a point mutation in codon 6 of the β - globin gene (GTG for GAG) that results in the

substitution of a hydrophobic valine residue for a hydrophilic glutamic acid residue of beta globin chain (Greer *et al*., 2014).

The common sickling disorders consist of the homozygous state for the sickle cell gene that is, sickle cell anaemia (Hb SS) and the compound heterozygous state for the sickle cell gene and that for either Hb C (another β chain variant) or β thalassaemia (termed Hb SC disease or sickle cell β thalassaemia) (Proven, 2003).

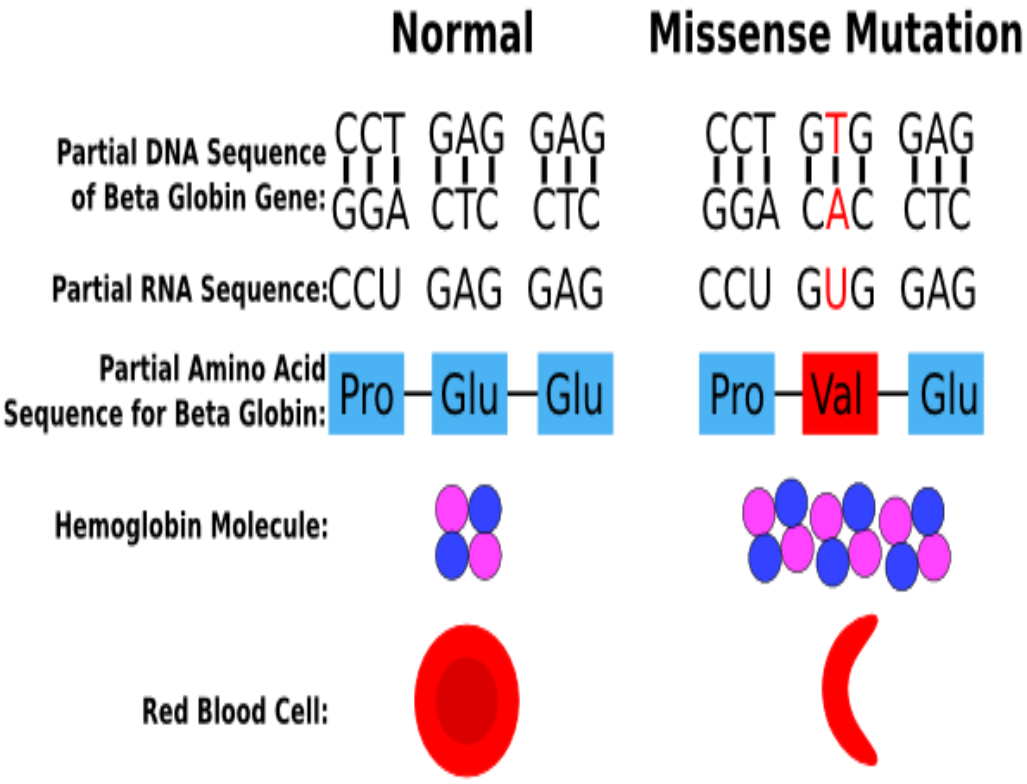

Figure (1-1): Molecular biology of HbS. A point mutation in codon 6 of the β - globin gene (GTG for GAG) that results in the substitution of a hydrophobic valine residue for a hydrophilic glutamic acid residue of beta globin chain. www.wordpress.com

1.2.6.3. Pathophysiology

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 haemoglobin, which distorts red blood cells into a sickle shape and decreases their elasticity (Hoffbrand *et al*., 2016). The loss of red blood cell elasticity is central of the patho physiology of sickle cell disease (Makis *et al*., 2000). In sickle cell disease, low oxygen tension promotes red blood cell sickling and repeated episodes of sickling damage the cell membrane and decreases the cell's elasticity (Thachil Quentin, 2014). Rigid blood cells are unable to deform as they pass through narrow capillaries, leading to vessel occlusion and Ischemia (Obeagu *et al*., 2015). Hb S molecules within the RBCs become less soluble, forming liquid crystals of Hb S polymers that grow in length beyond the diameter of the RBC, causing sickling (Munker *et al*., 2007), which is promoted by low oxygen tension, low pH, increased 2,3-diphosphoglycerate, high cellular concentration of hemoglobins, loss of cell water, Hb C, and Hb O–Arab. Sickling is retarded by Hb A, Hb F (at least 30%), Hb J, and a-thalassemia (Turgeon, 2012). The blood becomes more viscous when polymers are formed and sickle cells are created (Mehta and Hoffbrand, 2005). Increased blood viscosity and sickle cell formation slow blood flow (Keohane *et al*., 2016). The sickling of red cells in the circulating blood has two major pathological effects: (i) the distorted and rigid cells block small blood vessels, impairing flow and causing ischemia and infarction; and (ii) repeated 'sickle-unsickle' cycles lead to loss offragments of red cell membrane, and the cells become spherocytic and fragile, removed prematurely by the reticulo endothelial system, and to a lesser extent destroyed in the circulation resulting in both extravascular and intravascular hemolysis (Sexena and Pati, 2013).Intravascular hemolysis lead to release of free Hb , heme, reactive oxygen species , (ROS), and reactive nitrogen species (RNS) into the bloodstream, where they cause increased oxidative stress and decreased plasma levels of the vasodilator nitric oxide (NO) (Conran *et al*., 2009). As a consequence RBCs, leukocytes, platelets and endothelial cells are activated and produce pro inflammatory cytokines such as IL-8, IL-6, IL-1, TNF (Pitanga *et al*., 2013). 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 (Greer *et al*., 2014).

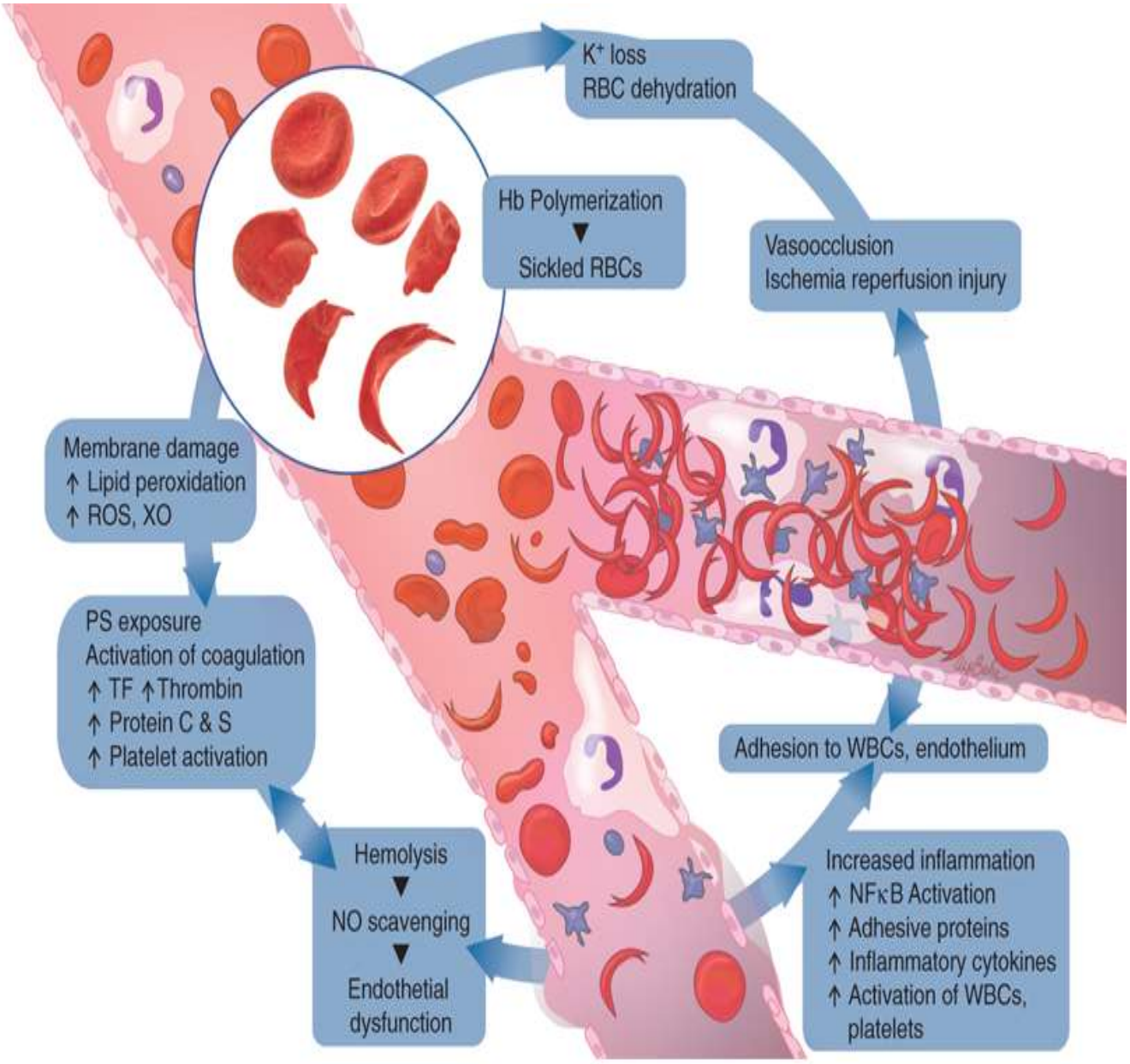

Figure (1-2): Pathophysiology of sickle cell disease (Kaushansky *et al*., 2016)

1.2.6.4. Clinical features:

Sickle cell anemia is not apparent until approximately 6 months of age, when HbF levels fall (Hoffbrand and Moss, 2016). The most severely affected individuals have either Hb SS or $S\beta^0$ (no normal beta-globin production) (Wahed and Dasgupta, 2015).Individuals with sickle-cell trait are usuall asymptomatic (Horton-Szar, 2012) and those who are double heterozygotes for sickle-cell and haemoglobin C disease usually have clinically milder forms of the disease (Hoffman *et al*., 2016). Sickle cell disease characterized by periodic episodes of acute vascular occlusion (painful crisis) that have their onset in the first or second year of life (Hoffman *et al*., 2000). Painful crisis may be precipitated by events that impair tissue oxygenation, perfusion, or acid - base status. Infection, especially pneumonia, and systemic dehydration are common precipitation events (Turgeon, 2012). Severe impairment of organ perfusion, leading to bone necrosis, acute chest syndrome stroke, priapism and skin ulcers result from vascular occlusion (Ballas, 2010). Recurrent splenic infarction results in complete splenic involution at a young age, also known as autosplenectomy (Ballas, 2010). Patients with SS disease are especially susceptible to infection with encapsulated bacteria due to autosplenectomy (Schmaier and Lazarus, 2012).

Aplastic crisis occur as a result of infection with parvovirus or from folate deficiency and are characterized by a sudden fall in hemoglobin and reticulocytes, usually requiring transfusion (Proven *et al*., 2004). Hemolytic crisis characterized by an increased rate of hemolysis and fall in hemoglobin but rise in reticulocytes and usually accompany a painful crisis (Hoffbrand *et al*., 2016).

1.2.6.5. Laboratory diagnosis:

In Hb SS, haemoglobin levels in the range of 6-8g/dl with a high reticulocyte count. In other forms of sickle cell disease, Hb levels tend to be higher (Horton-Szar *et al*., 2012). Blood picture shows a reticulocytosis with a varying number of sickle cells and target cells (Rozenberg, 2011). Features of splenic atrophy (e.g. Howell–Jolly bodies) may also be present (Mehta and Hoffbrand, 2005). Sickle solubility test, a mixture of Hb S in a reducing solution (such a sodium dithionite) gives a turbid appearance, whereas normal Hb gives a clear solution (Obeagu *et al*., 2015). Sickling in whole blood by the addition of a reducing agent such as sodium dithionite to blood which enhance sickling process (Howard and Hamilton, 2013). Hb electrophoresis at alkaline pH, haemoglobin is a negatively charged protein and when subjected to

electrophoresis will migrate toward the anode (+). Structural variants that have a change in the charge on the surface of the molecule at alkaline pH will separate from Hb A. (Bain *et al*., 2011). Hb S >90%; Hb A2 <3.5%; Hb F <10%; no Hb A (Wahed and Dasgupta, 2015).

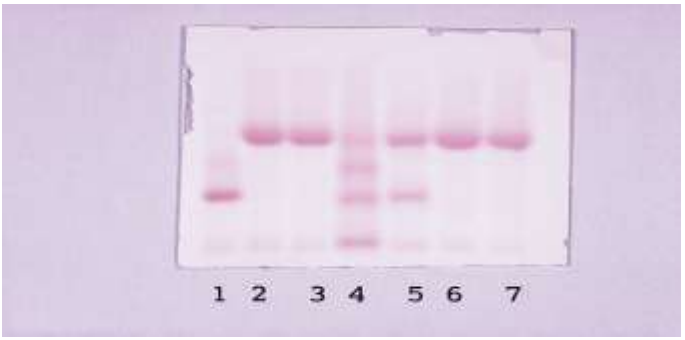

Figure (1-3): Cellulose acetate electrophoresis to separate haemoglobins A, F, S and C. Lane 4, control sample; Lanes 2, 3, 6, 7, normal; Lane 1, sickle cell anaemia; Lane 5, sickle cell trait (Howard and Hamilton 2013).

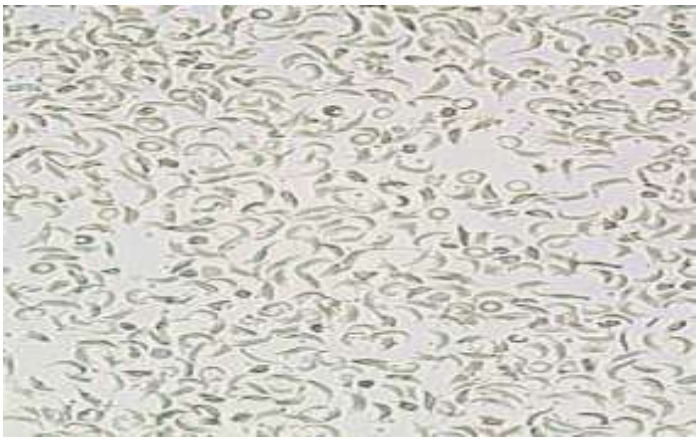

Figure (1-4): Sickle cell test under reduced oxygen tension: almost all erythrocytes appear as sickle cells in the homozygous case (Theml *et al*., 2002)**.**

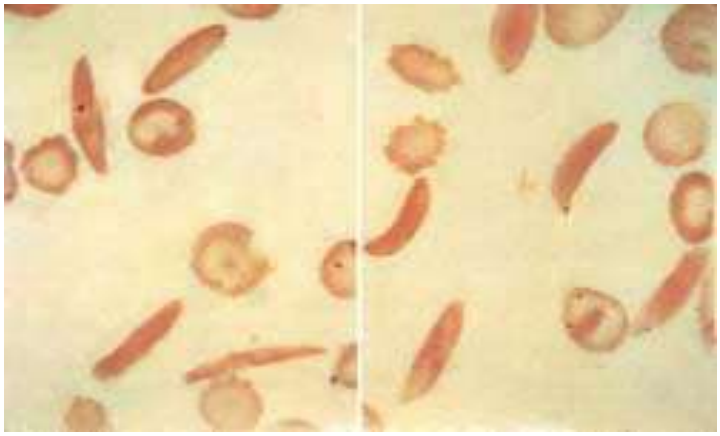

Figure (1-5): Peripheral blood film from patient with sickle cell anaemia Showing sickled erythrocyte. (Proven, 2003).

1.2.7. Sickle Cell Trait:

Sickle cell trait is a benign condition produced when a gene containing the HbS mutation is inherited along with a normal β-globin gene, and is described as HbAS (Ali *et al*., 2014). It achieved through heterozygous inheritance of Hgb S, in which an individual possesses Hb A at approximately 60% and Hgb S at approximately 40%. These individuals are hematologically normal (Hoffbrand and Moss, 2016). Individuals with sickle cell trait can suffer an excessive incidence of hematuria and a urine-concentrating defect. This is probably caused by microvascular injury in the vasa recta of the renal medulla, which has a hypoxic acidotic environment conducive to sickling even in the presence of HbA (Arceci *et al*., 2006). Individuals with sickle cell trait can develop sickle cell crisis and there is an increased incidence of sudden death in some individuals with Hb AS. This is probably related to heat exhaustion brought on by rigorous exercise in hot weather (Ali *et al., 2014*).

1.2.6.6. Treatment:

In young individuals with severe clinical diseases and a matched sibling donor, allogeneic hematopoietic cell transplantation can be curative (Schmaier and Lazarus, 2012). Hydration and pain medication are used to treat acute painful crisis. Strokes can be prevented in SCD patients by exchange RBC transfusion (Hoffbrand and Moss, 2016), which decreases the Hb S concentration to 30%. Chronic RBC transfusion is also recommended for children at high risk for Stroke (Howard and

Hamilton, 2013). Hydroxyurea, an orally – administered chemotherapeutic agent is approved for the treatment of patients with frequent painful crisis (Penkert *et al*., 2017). It has been shown to increase Hb F (Opoka *et al*., 2017). Penicillin prophylaxis should be given to the children of SCD to prevent infections (Schmaier and Lazarus, 2012).

1.2.8. Chronic inflammatory state in SCD:

Hemoglobin S polymer formation leads red blood cell membrane surface exposure of glycolipids and protein epitopes (Greer *et al*., 2014). Red and white cell adhesion to the endothelium, coupled with endothelial damage due to cell-free hemoglobin and ischemia reperfusion injury, low nitric oxide bioavailability, and activation of the coagulation cascade by glycolipids, lead to activation of the vascular endothelium(Obeagu *et al*., 2015). Endothelial activation augments NF*k*B activity and endothelin-1 production, in association with increased surface adhesion molecule expression (Zang *et al.,* 2016). Further adhesive interactions between the endothelium and red cells, leukocytes, and platelets are induced, coupled with a pancellular activation that results in an up-regulation of numerous inflammatory mediators IL-1, TNF, IL-8 and IL-6 (Obeagu *et al*., 2015). As such, a vicious circle of repeated cell activation, cellular adhesion, and inflammatory molecule production perpetuates the chronic inflammatory state that has a fundamental role in the vaso-occlusive process (Zang *et al.,* 2016).

1.2.9. Cytokines:

Proteins that are produced and secreted by many different cell types, and mediate inflammatory and immune reactions (Munker *et al*., 2007). Cytokines are principal mediators of communication between cells of the immune system (Abbas *et al*., 2015). Cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes) (Zhang and An, 2007). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action) (Zhang and An, 2007). They exhibit the attributes of pleiotropy (cytokine that induces different biological effects depending on target cell), redundancy (two or more cytokines that mediate similar functions, synergism (combined effect of two cytokines on cellular activity), antagonism

(cytokine antagonize the effects of another), and cascade induction (action of one cytokine on a target cell induces that cell to produce one or more additional cytokines) (Owen *et al.*, 2013).

1.2.9.1. CD4+ T cells and their cytokines:

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, and is responsible for many classic cell-mediated functions, including activation of cytotoxic T lymphocytes and macrophages (Siransy *et al*., 2018). The Th2 subset secretes IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, and regulates B-cell activity and differentiation (Owen *et al*., 2013). The Th17 subsets is primarily involved in recruiting leukocytes and inducing inflammation and they secrete IL-17 (Abbas *et al*., 2015). Th9 subsets secrete IL-9 which have a role in the induction and the pathogenesis of atopic disease, antiparasite immunity and immune pathological diseases of the gut (Kaplan *et al*., 2015). Regulatory T lymphocytes subset suppress immune responses and maintain self-tolerance through production of IL-10 and TGF-β, reduced ability of antigen presenting cells (APCs) to stimulate T cells and consumption of IL-2 (Sakaguchi *et al*., 2009).

1.2.9.2. Chemokines:

Chemokines A large family of structurally homologous low–molecular weight cytokines that stimulate leukocyte chemotaxis and maintain the spatial organization of different subsets of lymphocytes and APCs within lymphoid organs (Abbas *et al*., 2015).

1.2.9.2.1. Interleukin-8 (IL-8):

IL-8 was discovered in 1987 (Dibal *et al*., 2018). It is a pro-inflammatory chemokine that produced by many types of cells such as phagocytes, monocytes, fibroblasts, endothelial cells (Dibal *et al*., 2018), with 8.4 kD in molecular weight. It produced by processing of a precursor protein of 99 amino acids belonging to the CXC subfamily of chemokines which is characterized by two essential cysteine residues, separated by a third intervening amino acid (Qazi *et al*., 2011).There are two major forms of IL-8, that are the 72-amino acid monocyte-derived form, and the endothelial form which has five extra N-terminal amino acids. (Jundi and Greene, 2015). In many cell types, the synthesis of IL-8 is strongly stimulated by IL-1 and TNF-α (Lanaro *et al*., 2009).

Glucocorticoids, IL-4, TGF-β, inhibitors of 5-lipoxygenase, and 1.25(OH) 2 vitamin D3 inhibit the synthesis of IL8 (Qazi *et al*., 2011).

IL-8 is chemotactic for fibroblasts and stimulates deposition of fibronectin, and collagen I during wound healing (Hoffmam *et al*., 2000). IL-8 receptors are found on monocytes, T-lymphocytes, eosinophil, fibroblast and basophils as a result these cells also secrete IL-8 but their response to IL-8 is weaker than neutrophils (Qazi *et al*., 2011). Therefore, the main role of IL-8 is recruitment of neutrophils to site of tissue injury or infection (Jundi and Greene, 2015).

1.2.9.2.2. Pro-inflammatory Effects of IL-8:

IL-8 is an pro-inflammatory chemokine, released from epithelial cells following particle induced oxidative stress leading to neutrophil influx and inflammation (Palmino, 2015). Also it is a potent chemo-attractant and activator of neutrophils (Li *et al*., 2001). It not only serves as a chemotactic factor for leukocytes and fibroblasts but also stimulates fibroblast differentiation into myofibroblasts and promotes angiogenesis (Jundi and Greene, 2015).IL-8 contribute to the chronic inflammatory state that is present in various inflammatory diseases, including psoriasis, acute respiratory distress syndrome, chronic obstructive pulmonary disease, rheumatoid arthritis (Qazi *et al*., 2011) and SCD (Pitanga *et al*., 2013).

1.2.10. Relation of IL-8 with SCD:

Activation of neutrophils seen in SCA patients during VOC is believed to be mediated by IL-8 and augmented by other pro-inflammatory mediators (Alagbe *et al*., 2018). IL-8 propagates inflammation by increasing the adherence of sickle red cells to endothelium via the α4β1 integrin receptors on sickle reticulocytes (Etienne-Julan *et al*., 2004). The active process of endothelial adhesion contributes to the passive mechanical obstruction that leads to vaso-occlusion in SCA (Setty *et al*., 2008).

1.2.11. Previous studies:

In Southern Iran Keikhaei and his colleagues estimate the serum level of IL-8 in 54 SCA patients (39 VOC and 15steady state) and in 19 healthy volunteers. The result revealed that patients in VOC had highest mean levels of IL-8 than those that founded in steady state and control subjects, the mean and SD were 175±44.6, 61.39±5.02, 55.74±6.15 respectively. They compared the mean of three group and *P. values* were 0.08 (insignificant) between VOC and steady state, 0.01(significant) between VOC and control and 0.29 (insignificant) between steady state and control(Keikhaei *et al*., 2013).

Serum level of IL-8 was quantified by ELISA in Sixty adult Nigerian SCA patients (30 during bone pain crisis and 30 during steady state) and 30 hemoglobin A controls .The mean and SD were 183.92±198.58, 464±475.99 and 233.57±294.35 in control subjects, bone pain crisis patients and steady state patients respectively. Plasma IL-8 was significantly elevated in the bone pain crisis group than in the steady state group (*P. value 0.005*) and the controls (*p*.value 0.002). There was no significant difference of plasma IL-8 between steady state group and controls (*P. value* 0.438) (Alagbe *et al*., 2018)

In 2017 AL Sharif measured serum level of IL-8 in fifty asymptomatic adult sickle cell anemia Saudi patients and fifty age- and sex-matched healthy non-sickle cell disease subjects. The mean and SD were 15.32±4.25 and 11.48±3.37 in steady state SCA patients and control subjects respectively. There was significant difference between two group (*P. value* <0.05) (AL Sharif, 2017).

Duits and his colleagues analyzed the role of the potent neutrophil chemokine IL-8 by measuring serum levels in sickle cell patients during sickle cell crisis. The results were compared to non-symptomatic and healthy controls. Avaso-occlusive crisis patients showed significantly high serum IL-8 levels compared to healthy controls. Several of these patients showed extremely elevated serum IL-8 levels which were independent of the crisis inducing factor (Duits *et al*., 2009).

Interleukin-8 has been evaluated by Vicari and his colleagues in 2007 in 107 steady-state sickle cell patients and in 108 blood donors. The IL-8 levels were predominantly higher in patients than in controls (*P. value* 0.0001). (Vicari *et al*., 2007).

Lanaro and his colleagues determined plasma level of IL-8 in Brazilian SCD patients in stable health state and in control individuals. IL-8, levels were significantly (*P.*

value 0.006) higher in the plasma of SCA individuals when compared with control individuals (Lanaro *et al*., 2009).

1.2.12. Rationale:

Sickle cell anemia is one of the major types of anemia in Sudan (Ali *et al*., 2014), which characterized by red blood cells that assume an abnormal, rigid, sickle shape. Sickling decreases the cells' flexibility and results in a risk of various complications (Obeagu *et al*., 2015).

Altered inflammatory cytokine expression substantially impacts the development of Sickle cell disease VOC (Sarray *et al*., 2015). IL-8 level was tested and found to have high level which contribute to the pro inflammatory state that is present in SCD. (Cajado *et al*., 2011; Sarray *et al*., 2015).

The role of cytokine IL-8 as a VOC marker has not been well established in Sudanese SCD patients.

1.2.13. Objectives:

1.2.13.1. General objective:

-To determine serum levels of IL-8 in Sudanese sickle cell disease patients during vaso-occlusive crisis and the steady state conditions.

1.2.13.2. Specific objectives:

- To measure serum level of IL8 in sickle cell disease patients during vaso-occlusive crisis and the steady state and in normal subjects using Enzyme Linked Immunosorbent Assay (ELISA).

-To compare between serum levels of IL-8 in VOC patients , steady state patients an d normal controls.

-To correlate IL-8 level with age and gender.

Chapter Tow

Materials and Methods

Chapter Tow

Materials and Methods

2.1. Study design:

This was case control and hospital based study.

2.2. Study area, setting and duration:

Study was conducted in Al-bulk Hospital, Ahmed Gasim Hospital, Gaafar Ibn-Auf Pediatric Tertiary Hospital in Khartoum state during the period from July to November (2018).

2.3. Study population:

Study population consisted of 88 Sudanese individuals of age between 4-17years, divided into 3 groups as follows- crisis group made up of 30 sickle cell patients during crisis, Steady state group made up of 30 SCA patients during steady state and Control (Hb A) group composed of 28 Hb A individuals.

2.4. Inclusion criteria:

Patients were considered in sickle cell crisis if they complain from signs and symptoms of crisis and necessitating hospital admission. Stable health state in SCA patients who did not have pain or any other crisis. Control group was age and sex matched apparently health subjects.

5.5. Exclusion criteria:

All sickle cell patients with situations that affect cytokine level including physiological factors such as (Pregnancy, smoking and alcohol consumption) and other diseases such as (autoimmune diseases, infectious disease, allergy, hypersensitivities, cancer, heart failure and Parkinson disease) were excluded.

5.6. Sample size:

A total of 88 subjects were enrolled in this study. 60 samples were collected from sickle cell patients (30 during crisis and 30 during steady state) and 28 samples were collected from healthy volunteer.

2.7. Data collection:

Samples were collected randomly.Questionnaire was used to collect demographic, clinical and laboratory data and it was full filled by us.

2.8. Sampling:

Three ml of venous blood was collected from patients and control in a plain container, and allowed to clot at room temperature. Then the samples were centrifuged and serum was separated in a sterile container and stored at -20 °C until analysis. Serum levels of IL-8 were measured using ELISA (Biolegend's ELISA MAX^{TM}).

2.9. Principles and procedures:

2.9.1. Principle of the ELISA:

BioLegend's ELISA MAX™ Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human IL-8 specific monoclonal antibody is irst coated on a 96-well plate. Standards and samples are added to the wells, and IL-8 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-8 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB Substrate Solution, producing a blue color in proportion to the concentration of IL-8 present in the sample. Finally, the Stop Solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate reader (biolegend, USA, 2013).

2.10.1.1. ELISA procedure:

100 µL of diluted Capture Antibody solution was added to all wells of a 96-well plate; plate sealed and incubated overnight (16-18 hrs) between 2°C and 8°C.Then plate washed 4 times with at least 300 µL Wash Buffer per well and residual buffer was blotted by firmly tapping plate upside down on absorbent paper**.** To block non-specific binding and reduce background, 200 µL 1X Assay Diluent was added per well, plate sealed and incubated at RT for 1 hour with shaking at 200 rpm on a plate shaker.Plate washed 4 times with Wash Buffer. 50 µL of Matrix Diluent was added to the standard wells. 50 µL of Assay Diluent A was added to the sample wells. 50 µL/well of standards were added to the standard wells. 50 µL/well of serum samples were added to the sample wells. Plate sealed and incubated at RT for 2 hours with shaking. Plate washed 4 times with Wash Buffer. 100 µL of diluted Detection Antibody solution was added to each well, plate sealed and incubated at RT for 1 hour with shaking. Plate washed 4 times with Wash Buffer.100 µL of diluted Avidin-HRP solution was added to each well, plate sealed and incubated at RT for 30 minutes with shaking. Plate washed 5 times with Wash Buffer. For the final wash, wells were

soaked in Wash Buffer for 30 seconds to 1 minute for each wash. To minimize background.100 µL of TMB Substrate Solution C was added and incubated in the dark for 15 minutes. Positive wells were turned blue in color. Reaction was stopped by addition of 100 µL of Stop Solution to each well. Positive wells were turned from blue to yellow. Absorbance was read at 450 nm within 30 minute. For results calculation the standard curve was plotted on log-log axis graph paper with analyte concentration on the x-axis and absorbance on the y-axis. A best fit line was drawn through the standard points. To determine the unknown analyte concentrations in the samples, the absorbance value of the unknown on the y-axis was found and a horizontal line was drawn to the standard curve. At the point of intersection, vertical line was drawn to the x-axis and the corresponding analyte concentration was read.

2.9.1.2. ELISA Washer principle:

First the wash solution is pump from the wash bottle, the solution is dispense to the cuvette by short pins, and then the wash liquid is aspirate from the cuvette by the long pins, at the end the waste liquid was pumped into the waste bottle by the vacuum pump (www.diasource.be).

2.9.1.3. 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)

2.10. Ethical Consideration:

Permission to carry out the study was obtained from the college of Medical Laboratory Science. Permission of hospital manger was taken before beginning. Every sample was collected after verbal approval by the parents of volunteer.

2.11. Statistical analysis:

Data was analyzed by using statistical package for social sciences (SPSS) programme (version 16.0) using one way ANOVA test and independent T-test for testing difference significance, correlation test to find out correlations. The probability value ≤ 0.05 was considered significant.

Chapter Three

Results

Chapter Three

Results

3. Results:

Eighty eight subjects of age between four to seventeen years were enrolled in this study divided into steady state group, vaso-occlusive crisis group and control group. Steady state group composed of thirty SCD patients with mean of age 9±3, 40 % were males and 60% were females. VOC group consised of thirty SCD patients with mean of age 8±4, 47% were males and 53% were females. Twenty tow were healthy subjects comprised the control group with mean of age 10±3, 61% were males and 39% were females. Among VOC patients twenty one of them were having musculoskeletal pain, four having hemolytic crisis, four having acute chest syndrome and one has retinopathy. IL-8 was measured using ELISA and Hb level was obtained from records.

Results were analyzed using Statistical Package of Social Science (SPSS) (version 16.0). Mean and SD of Hb (g/dL) were 7.3±1.2, 5.9±1.4 in the steady state patients and VOC patients respectively, *P. value* was 0.000. IL-8(pg/ml) mean and SD were 92.78±32.17, 148.45±165.72, 90.48±24.94 in the steady state patients, VOC patients and healthy subjects respectively. In multiple comparisons of IL-8 levels in three groups, *P. values* were 0.033 between the steady state and VOC groups, 0.930 between steady state and control groups and 0.029 between VOC and control groups. IL-8 mean of males and females of the steady state group were 99.58 and 82.57 respectively, *P. value* was 0.116. In VOC group mean of IL-8 were 120.91 in males and 179.92 in females ،*P .value* was 0.37. IL-8 mean of males and females of control group were 93.77 and 88.35 respectively, *P. value* was 0.584. In correlation of IL-8 level with age in three group *P. values* were 0.961 in steady group, 0.524 in VOC group and 0.266 in control group.

Table (3-1): Mean ± SD of age and gender distribution among the study groups:

Study group	Mean ± SD of age	Male	Female
Steady state group (N = 30)	9±3	N= 18 40%	N=12 60%
VOC group (N = 30)	8±4	N=16 47%	N=14 53%
Control group (N = 28)	10±3	N=11 61%	N=17 39%
Total		45	43

Table (3-2): Comparison of IL-8 level between steady-state group and vaso-occlusion crisis (VOC) group:

Subject	Steady state group (N = 30)	VOC group (N = 30)
mean±SD of IL-8	92.78 ± 32.17	148.45±165.72
P. value	0.033	

Table (3-3): Comparison of IL-8 level between steady-state group and control group:

Subject	Steady state group (N = 30)	Control group (N = 30)
mean±SD of IL-8	92.78 ± 32.17	90.48 ± 24.94
P. value	0.930	

Table (3-4): Comparison of IL-8 level between VOC group and control group:

Subject	VOC group (N = 30)	Control group (N = 28)
mean±SD of IL-8	148.45±165.72	90.48 ± 24.94
P. value	0.029	

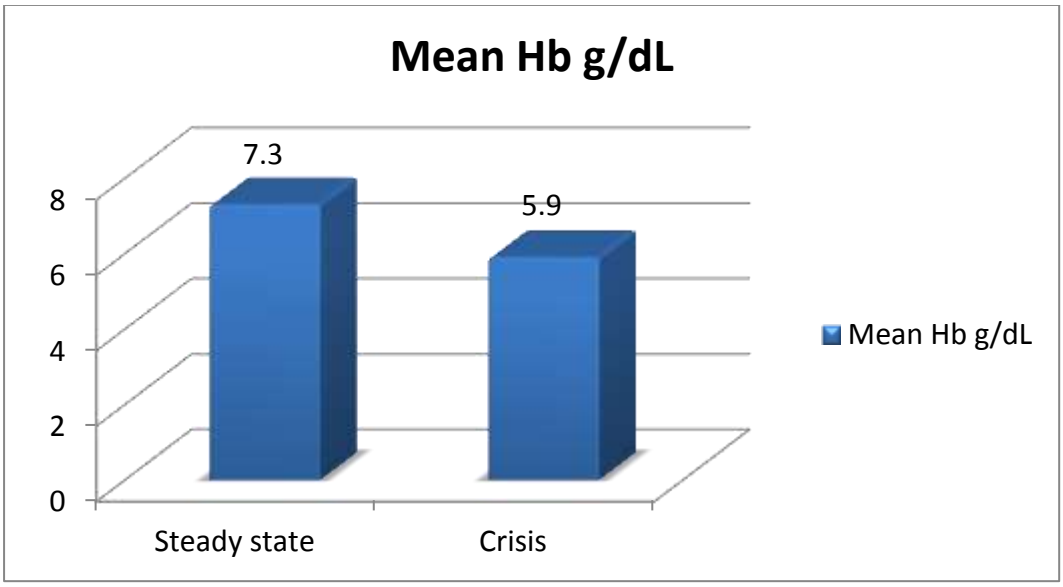

Figure (3-1): Mean of Hb in the SCA patients during the steady state and during VOC. Mean of Hb was higher in the steady state patients than VOC patients. The difference was significant (*P. value* 0.000).

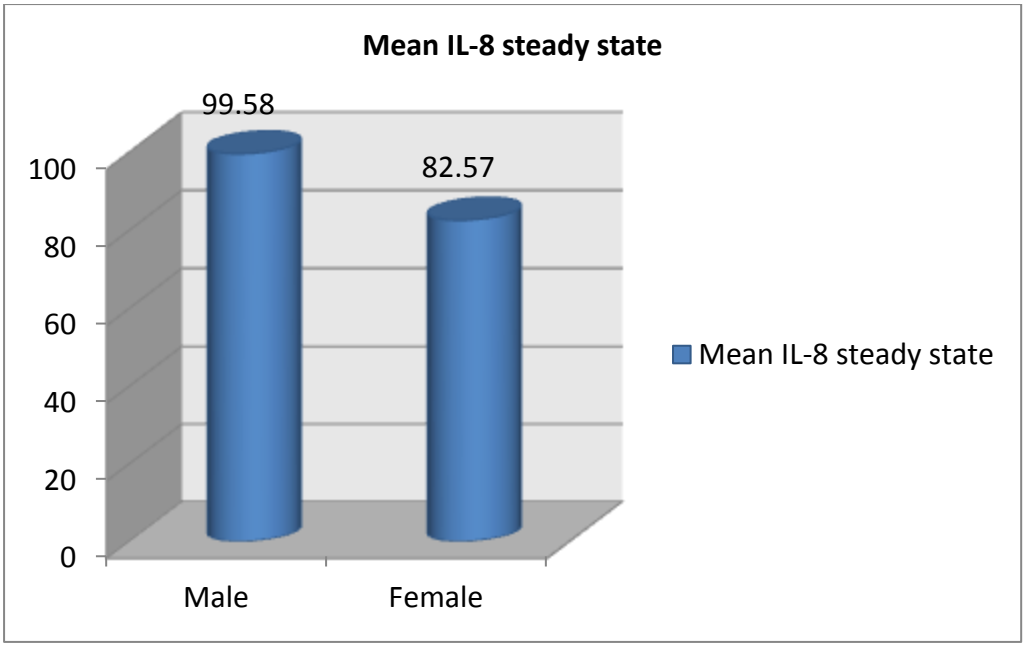

Figure (3-2): Mean of IL-8 in males and females of the steady state group. Mean of IL-8 in males was higher than female's mean but the difference was insignificant (*P. value* 0.116).

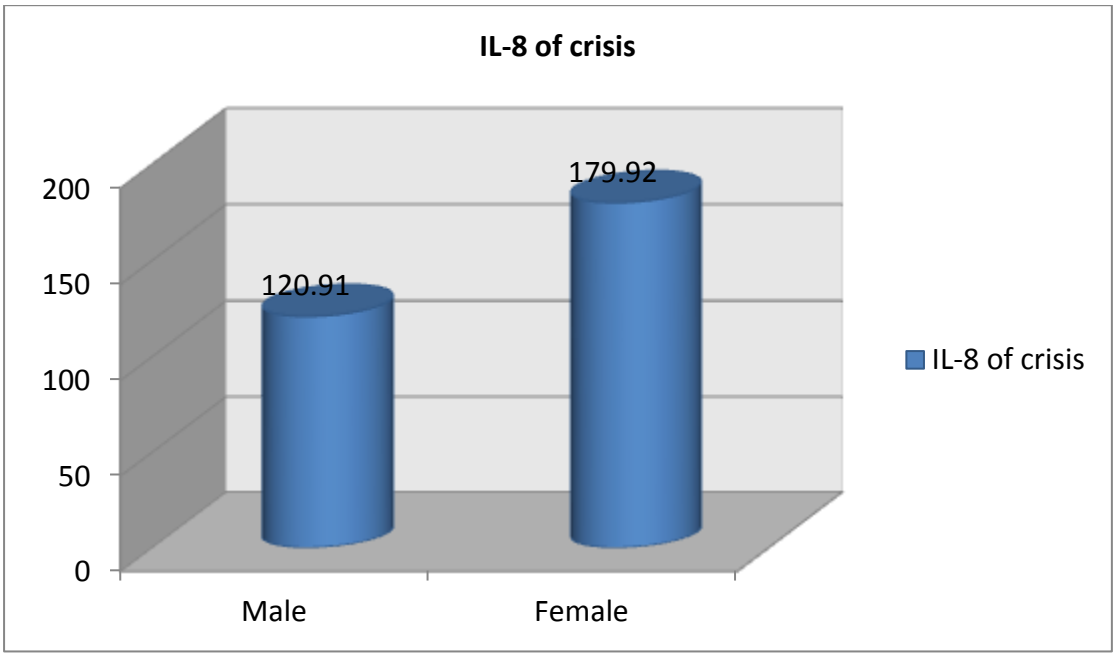

Figure (3-3): Mean of IL-8 in males and females of the VOC group. Mean of IL-8 in females was higher than male's mean but the difference was insignificant (*P. value* 0.37).

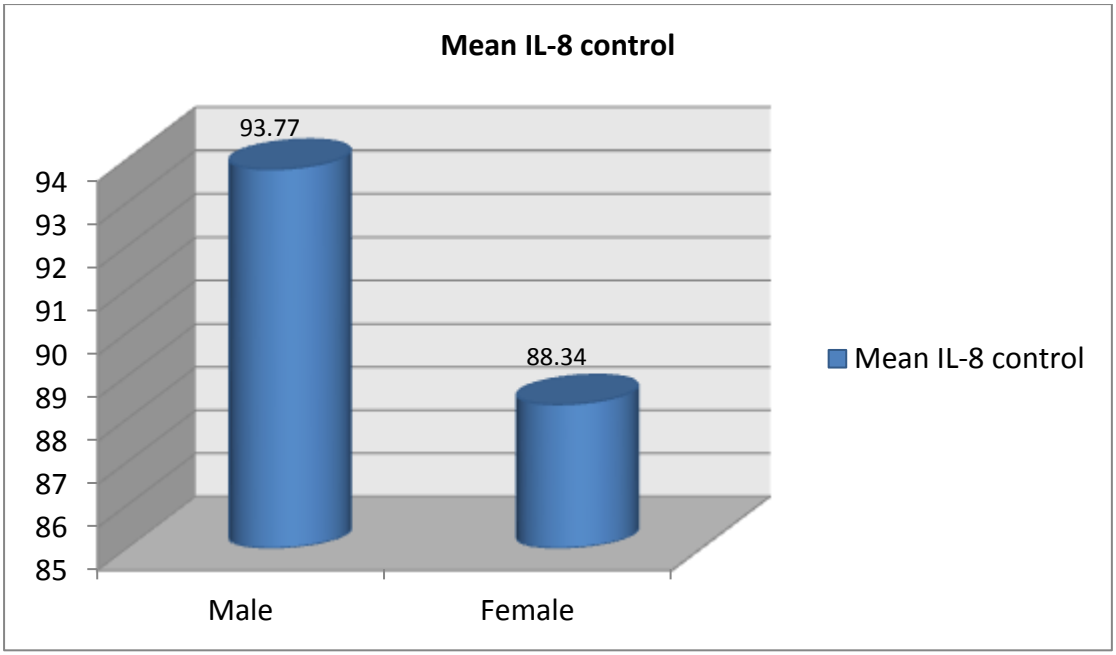

Figure (3-4): Mean of IL-8 in males and females of the control group. Mean of IL-8 in males was higher than female's mean but the difference was insignificant (*P. value* 0.584).

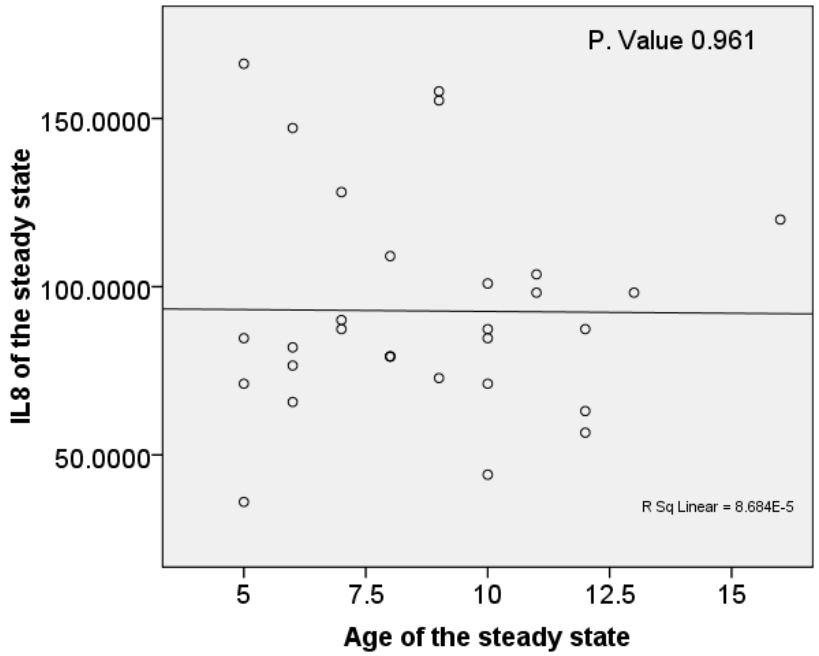

Figure (3-5): Correlation between IL-8 level and age of SCA patients during the steady state. There was no correlation between IL-8 level and age of the steady state patients (*P. value* 0.961).

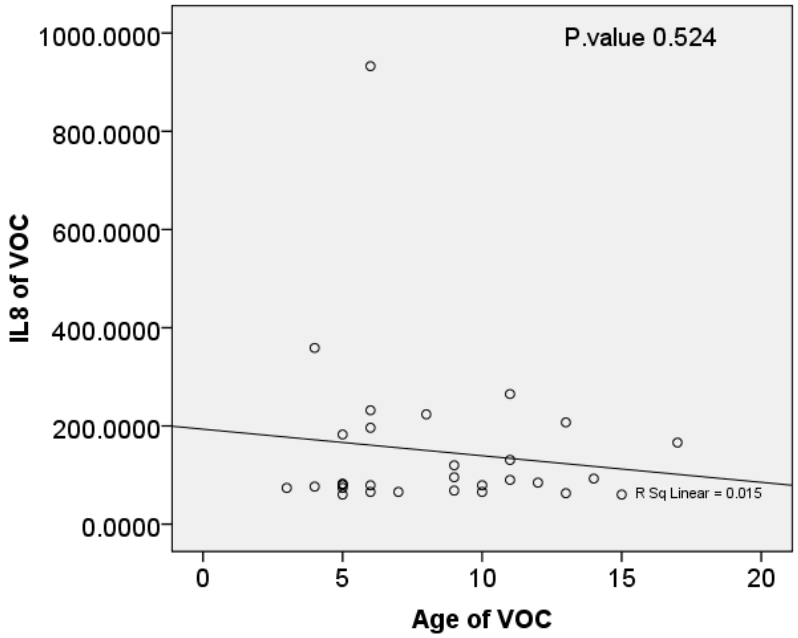

Figure (3-6): Correlation between IL-8 level and age of SCA patients during VOC. There was no correlation between IL-8 level and age of SCA patients during VOC (*P. value* 0.524).

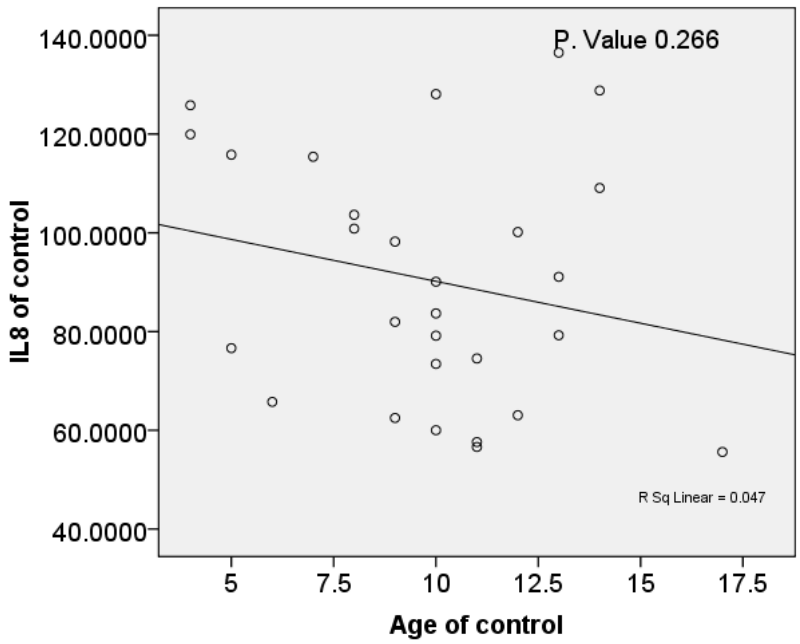

Figure (3-7): Correlation between IL-8 level and age of control subjects. There was no correlation between IL-8 level and age of control subjects (*P. value* 0.266).

Chapter four

Discussion, Conclusions and Recommendations

Chapter four

Discussion, Conclusions and Recommendations

4.1. Discussion:

SCA is associated with pro-inflammatory state, and an enhanced inflammatory response occurs during vaso-occlusive crisis.IL-8 propagates inflammation by increasing the adherence of sickle red cells to endothelium via the α4β1 integrin receptors on sickle reticulocytes (Etienne-Julan *et al*., 2004).

In the present study serum level of IL-8 was measured in 60 SCA patients (30 during VOC and 30 during steady state) and in 28 apparently health subjects. Steady state patients had higher Hb level compared with VOC patients and the difference was significant (*P.value* 0.000). This result was supported by Siransy *et al* who demonstrated that significantly elevated Hb level in the steady state patients versus VOC (Siransy *et al*., 2018). Higher levels of IL-8 in vaso-occlusive crisis patients compared to the steady-state patients, with statistically significant differences (*P.value*s were 0.033 between the steady state and VOC group). This finding was supported by Keikhaeil *et al*, and Alagbe *et al* who showed significantly elevated concentration of IL-8 in crisis versus the steady state SCA patients (Keikhaeil *et al*., 2013 and Alagbe *et al*., 2018). Also results revealed that VOC patients had higher IL-8 level than control subjects with statistically significant difference (*P. value* was 0.029), this result consistent with findings of Keikhaeil *et al* Alagbe *et al* and Duits *et al* (Keikhaeil *et al*., 2013; Alagbe *et al*., 2018 and Duits *et al*., 2009). Our results also demonstrated that no statistically significant difference in IL-8 level between the steady state patients and control subjects (*P. value* 0.930), Keikhaeil *et al*, Alagbe *et al*, Al-Sharif and Lanaro *et al* agree with us in this finding (Keikhaeil *et al*., 2013; Alagbe *et al*., 2018; AL Sharif, 2017 and Lanaro *et* ., 2009). In the comparison of mean of IL-8 between males and females in the three groups study revealed that males of the steady state and control groups had higher levels than those of females but the differences were statistically insignificant (*P. values* were 0.116, 0.584 respectively), while females of VOC group had higher mean of IL-8 than those of males with statistically insignificant difference (*P. value* was 0.37).Study revealed that no statistical correlations between IL-8 levels and age in steady state group (*P. value* was 0.961), VOC group (*P. value* was 0.524) and control group (*P. value* was 0.266).

4.2. Conclusions:

High serum level of IL-8 in SCD patients during VOC compared with SCD patients during steady state and control subjects with statistically significant differences (*p. values* 0.033 and 0.029 respectively).

IL-8 can be considered useful marker for vaso-occlusive crisis.

There was no correlation between IL-8 level and age and gender.

4.3. Recommendations:

- Serum IL-8 level in adult Sudanese sickle cell anemia should be determined.

- Effect of hydroxyurea on serum IL-8 level in Sudanese SCA patients should be determined.

- Regular measurement of serum IL-8 level to access crisis in SCD patients .

- Large sample size should be used.

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Appendices

Appendix (1)

Sudan University of Sciences and Technology

Evaluation of Interleukin-8 Level in Sudanese Sickle Cell Disease Patients during Vaso-occlusive Crisis and the Steady State Condition

تقييم مستوى المادة الخلوية انترليوكين 8 المصلي عند المرضى السودانيين بالانيميا المنجلية خلال حالة الازمة الوعائية والحالة المستقرة

_Date: / /2018

_ID Number: ……..

_Age: …….Years

_Gender:

Male :{ } Female: { }

-Clinical remarks:……
pain…….

Acute chest syndrome……

Other ……If yes specify…….

Date: …… Signature: ……

Appendix (2)

استمارة موافقة مشاركة
تقييم مستوى المادة الخلوية انترليوكين 8 المصلي عند المرضى السودانيين بالانيميا المنجلية خلال حالتي الازمة الوعائية و المستقرة

أنيميا الخلايا المنجليه هي أحد أنواع فقر الدم وهي تصيب كريات الدم الحمراء وهي من أشهر أمراض الدم الوراثيه الإنحلاليه التى تسبب تكسر كريات الدم الحمراء. من مضاعفات المرض السكتة الدماغية ،متلازمة الصدر الحاده، إرتفاع الضغط الدموي وتضرر الاعضاء.

هل توافق على المشاركة في البحث العلمي عن مرضى أنيميا الخلايا المنجليه ، التابع لجامعة السودان للعلوم والتكنولوجيا كلية علوم المختبرات الطبيه ، وأنت على علم تام بمحتوى البحث ، مشاركه عن طريق التبرع بعينه من دمك، مشاركه 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:.....

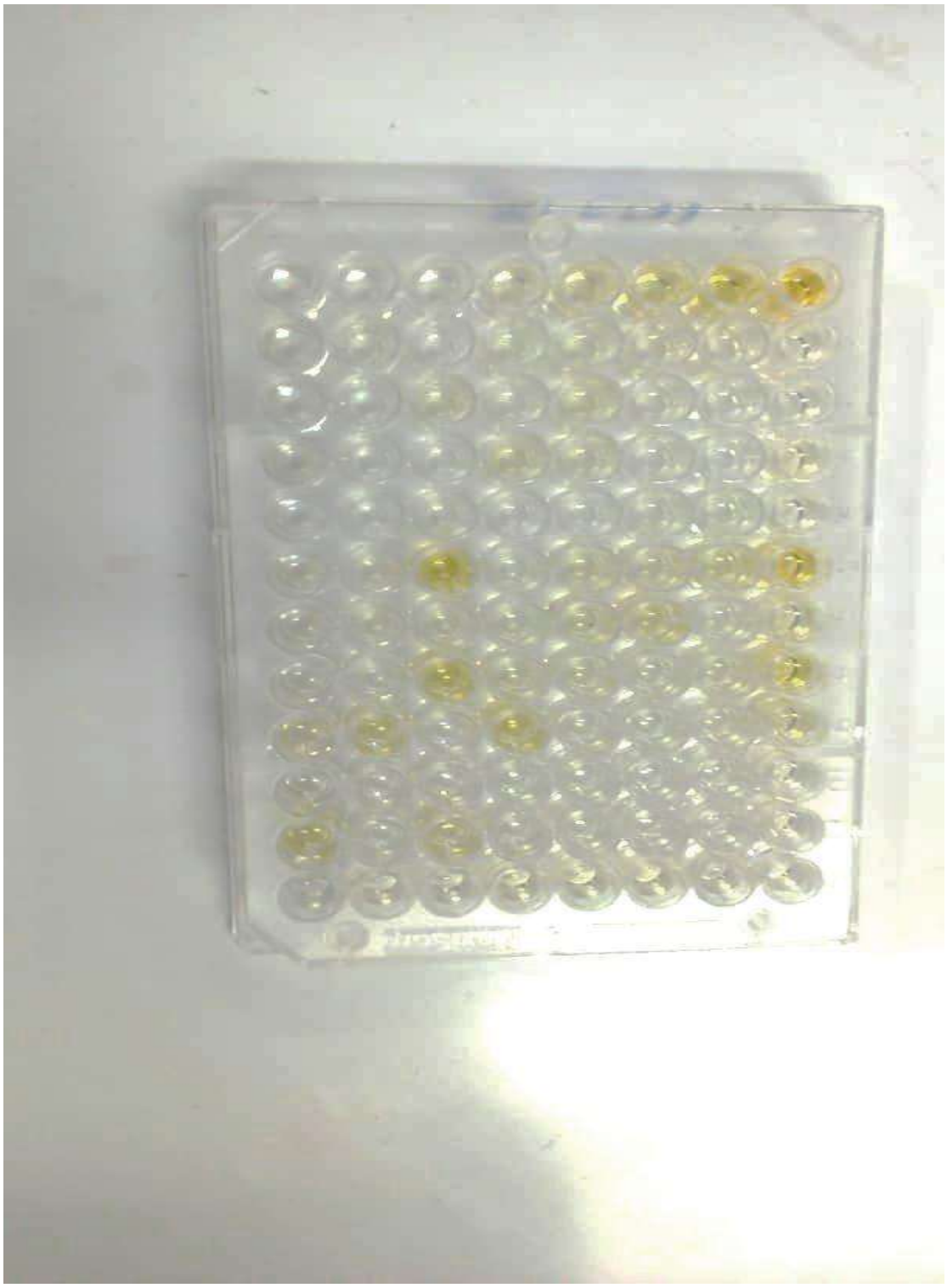
رقم تلفون الباحث.....

Appendix (3)

ELISA Reader

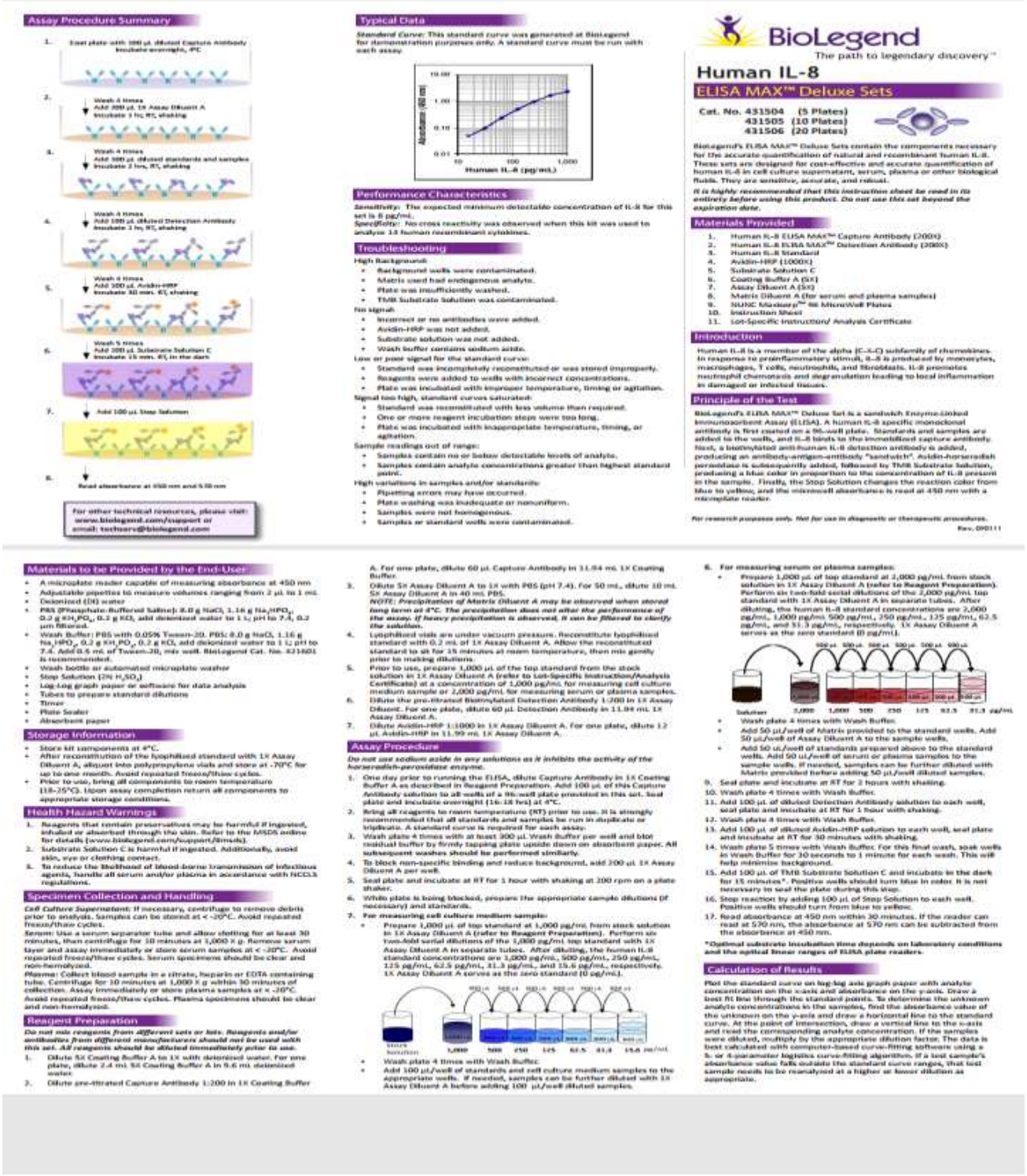
ELISA Washer

Appendix (4)



ELISA micro plate

Appendix (5)



Assay Procedure Summary

1. Coat plate with 100 µL diluted Capture Antibody. Incubate overnight, 4°C.
2. Wash 4 times. Add 300 µL 1X Assay Diluent A. Incubate 1 hr, RT, shaking.
3. Wash 4 times. Add 100 µL diluted standards and samples. Incubate 2 hrs, RT, shaking.
4. Wash 4 times. Add 100 µL diluted Detection Antibody. Incubate 1 hr, RT, shaking.
5. Wash 4 times. Add 100 µL Avidin-HRP. Incubate 30 min, RT, shaking.
6. Wash 5 times. Add 100 µL Substrate Solution C. Incubate 15 min, RT, in the dark.
7. Add 100 µL Stop Solution.
8. Read absorbance at 450 nm and 570 nm.

For other technical resources, please visit: www.biolegend.com/support or email: techserv@biolegend.com

Typical Data

Standard Curve: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.

Performance Characteristics

Sensitivity: The expected minimum detectable concentration of IL-8 for this set is 8 pg/mL.
Specificity: No cross reactivity was observed when this kit was used to analyze 13 human recombinant cytokines.

Troubleshooting

High Background:
- Background wells were contaminated.
- Matrix used had endogenous analyte.
- Plate was insufficiently washed.
- TMB Substrate Solution was contaminated.

No signal:
- Incorrect or no antibodies were added.
- Avidin-HRP was not added.
- Substrate solution was not added.
- Wash buffer contains sodium azide.

Low or poor signal for the standard curve:
- Standard was incompletely reconstituted or was stored improperly.
- Reagents were added to wells with incorrect concentrations.
- Plate was incubated with improper temperature, timing or agitation.

Signal too high, standard curves saturated:
- Standard was reconstituted with less volume than required.
- One or more reagent incubation steps were too long.
- Plate was incubated with inappropriate temperature, timing, or agitation.

Sample readings out of range:
- Samples contain no or below detectable levels of analyte.
- Samples contain analyte concentrations greater than highest standard point.

High variations in samples and/or standards:
- Pipetting errors may have occurred.
- Plate washing was inadequate or nonuniform.
- Samples were not homogenous.
- Samples or standard wells were contaminated.

BioLegend
The path to legendary discovery™

Human IL-8
ELISA MAX™ Deluxe Sets

Cat. No. 431504 (5 Plates)
431505 (10 Plates)
431506 (20 Plates)

BioLegend's ELISA MAX™ Deluxe Sets contain the components necessary for the accurate quantification of natural and recombinant human IL-8. These sets are designed for cost-effective and accurate quantification of human IL-8 in cell culture supernatant, serum, plasma or other biological fluids. They are sensitive, accurate, and robust.

It is highly recommended that this instruction sheet be read in its entirety before using this product. Do not use this set beyond the expiration date.

Materials Provided

1. Human IL-8 ELISA MAX™ Capture Antibody (200X)
2. Human IL-8 ELISA MAX™ Detection Antibody (200X)
3. Human IL-8 Standard
4. Avidin-HRP (1000X)
5. Substrate Solution C
6. Coating Buffer A (5X)
7. Assay Diluent A (5X)
8. Matrix Diluent A (for serum and plasma samples)
9. NUNC Maxisorp™ 96 MicroWell Plates
10. Instruction Sheet
11. Lot-Specific Instruction/ Analysis Certificate

Introduction

Human IL-8 is a member of the alpha (C–X–C) subfamily of chemokines. In response to proinflammatory stimuli, IL-8 is produced by monocytes, macrophages, T cells, neutrophils, and fibroblasts. IL-8 promotes neutrophil chemotaxis and degranulation leading to local inflammation in damaged or infected tissues.

Principle of the Test

BioLegend's ELISA MAX™ Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human IL-8 specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-8 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-8 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB Substrate Solution, producing a blue color in proportion to the concentration of IL-8 present in the sample. Finally, the Stop Solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate reader.

For research purposes only. Not for use in diagnostic or therapeutic procedures.

Rev. 090111

Materials to be Provided by the End-User

- A microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Deionized (DI) water
- PBS (Phosphate Buffered Saline): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1 L; pH to 7.4, 0.2 µm filtered.
- Wash Buffer: PBS with 0.05% Tween-20. PBS: 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1 L; pH to 7.4. Add 0.5 mL of Tween-20, mix well. BioLegend Cat. No. 421401 is recommended.
- Wash bottle or automated microplate washer
- Stop Solution (2N H_2SO_4)
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Sealer
- Absorbent paper

Storage Information

- Store kit components at 4°C.
- After reconstitution of the lyophilized standard with 1X Assay Diluent A, aliquot into polypropylene vials and store at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
- Prior to use, bring all components to room temperature (18–25°C). Upon assay completion return all components to appropriate storage conditions.

Health Hazard Warnings

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details (www.biolegend.com/support/#msds).
2. Substrate Solution C is harmful if ingested. Additionally, avoid skin, eye or clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum and/or plasma in accordance with NCCLS regulations.

Specimen Collection and Handling

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at < -20°C. Avoid repeated freeze/thaw cycles.
Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 X g. Remove serum layer and assay immediately or store serum samples at < -20°C. Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.
Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 X g within 30 minutes of collection. Assay immediately or store plasma samples at < -20°C. Avoid repeated freeze/thaw cycles. Plasma specimens should be clear and non-hemolyzed.

Reagent Preparation

Do not mix reagents from different sets or lots. Reagents and/or antibodies from different manufacturers should not be used with this set. All reagents should be diluted immediately prior to use.

1. Dilute 5X Coating Buffer A to 1X with deionized water. For one plate, dilute 2.4 mL 5X Coating Buffer A in 9.6 mL deionized water.
2. Dilute pre-titrated Capture Antibody 1:200 in 1X Coating Buffer A. For one plate, dilute 60 µL Capture Antibody in 11.94 mL 1X Coating Buffer.
3. Dilute 5X Assay Diluent A to 1X with PBS (pH 7.4). For 50 mL, dilute 10 mL 5X Assay Diluent A in 40 mL PBS.
   NOTE: Precipitation of Matrix Diluent A may be observed when stored long term at 4°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.
4. Lyophilized vials are under vacuum pressure. Reconstitute lyophilized standard with 0.2 mL of 1X Assay Diluent A. Allow the reconstituted standard to sit for 15 minutes at room temperature, then mix gently prior to making dilutions.
5. Prior to use, prepare 1,000 µL of the top standard from the stock solution in 1X Assay Diluent A (refer to Lot-Specific Instruction/Analysis Certificate) at a concentration of 1,000 pg/mL for measuring cell culture medium sample or 2,000 pg/mL for measuring serum or plasma samples.
6. Dilute the pre-titrated Biotinylated Detection Antibody 1:200 in 1X Assay Diluent. For one plate, dilute 60 µL Detection Antibody in 11.94 mL 1X Assay Diluent A.
7. Dilute Avidin-HRP 1:1000 in 1X Assay Diluent A. For one plate, dilute 12 µL Avidin-HRP in 11.99 mL 1X Assay Diluent A.

Assay Procedure

Do not use sodium azide in any solutions as it inhibits the activity of the horseradish-peroxidase enzyme.

1. One day prior to running the ELISA, dilute Capture Antibody in 1X Coating Buffer A as described in Reagent Preparation. Add 100 µL of this Capture Antibody solution to all wells of a 96-well plate provided in the set. Seal plate and incubate overnight (16-18 hrs) at 4°C.
2. Bring all reagents to room temperature (RT) prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
3. Wash plate 4 times with at least 300 µL Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes should be performed similarly.
4. To block non-specific binding and reduce background, add 200 µL 1X Assay Diluent A per well.
5. Seal plate and incubate at RT for 1 hour with shaking at 200 rpm on a plate shaker.
6. While plate is being blocked, prepare the appropriate sample dilutions (if necessary) and standards.
7. **For measuring cell culture medium samples:**
   - Prepare 1,000 µL of top standard at 1,000 pg/mL from stock solution in 1X Assay Diluent A (refer to Reagent Preparation). Perform six two-fold serial dilutions of the 1,000 pg/mL top standard with 1X Assay Diluent A in separate tubes. After diluting, the human IL-8 standard concentrations are 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, and 15.6 pg/mL, respectively. 1X Assay Diluent A serves as the zero standard (0 pg/mL).

   - Wash plate 4 times with Wash Buffer.
   - Add 100 µL/well of standards and cell culture medium samples to the appropriate wells. If needed, samples can be further diluted with 1X Assay Diluent A before adding 100 µL/well diluted samples.
8. **For measuring serum or plasma samples:**
   - Prepare 1,000 µL of top standard at 2,000 pg/mL from stock solution in 1X Assay Diluent A (refer to Reagent Preparation). Perform six two-fold serial dilutions of the 2,000 pg/mL top standard with 1X Assay Diluent A in separate tubes. After diluting, the human IL-8 standard concentrations are 2,000 pg/mL, 1,000 pg/mL 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, and 31.3 pg/mL, respectively. 1X Assay Diluent A serves as the zero standard (0 pg/mL).

   - Wash plate 4 times with Wash Buffer.
   - Add 50 µL/well of Matrix provided to the standard wells. Add 50 µL/well of Assay Diluent A to the sample wells.
   - Add 50 uL/well of standards prepared above to the standard wells. Add 50 uL/well of serum or plasma samples to the sample wells. If needed, samples can be further diluted with Matrix provided before adding 50 µL/well diluted samples.
9. Seal plate and incubate at RT for 2 hours with shaking.
10. Wash plate 4 times with Wash Buffer.
11. Add 100 µL of diluted Detection Antibody solution to each well, seal plate and incubate at RT for 1 hour with shaking.
12. Wash plate 4 times with Wash Buffer.
13. Add 100 µL of diluted Avidin-HRP solution to each well, seal plate and incubate at RT for 30 minutes with shaking.
14. Wash plate 5 times with Wash Buffer. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
15. Add 100 µL of TMB Substrate Solution C and incubate in the dark for 15 minutes*. Positive wells should turn blue in color. It is not necessary to seal the plate during this step.
16. Stop reaction by adding 100 µL of Stop Solution to each well. Positive wells should turn from blue to yellow.
17. Read absorbance at 450 nm within 30 minutes. If the reader can read at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

*Optimal substrate incubation time depends on laboratory conditions and the optimal linear ranges of ELISA plate readers.

Calculation of Results

Plot the standard curve on log-log axis graph paper with analyte concentration on the x-axis and absorbance on the y-axis. Draw a best fit line through the standard points. To determine the unknown analyte concentrations in the samples, find the absorbance value of the unknown on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the corresponding analyte concentration. If the samples were diluted, multiply by the appropriate dilution factor. The data is best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If a test sample's absorbance value falls outside the standard curve range, that test sample needs to be reanalyzed at a higher or lower dilution as appropriate.