Sudan University of Sciences and Technology

College of Graduate Studies

Evaluation of Serum level of Interleukin-10 Sudanese Sickle Cell Disease Patients during Vaso-occlusive Crisis and the Steady State Conditions

تقييم مستوى المادة المصلية عند المرضى السودانيين بالانيميا المنجلية خلال حالتي الازمة الوعائية والمستقرة

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

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بِسْمِ اللَّـرَّحِيمِ
اللَّهِ الـرَّحْمَنِ

الآيـة

﴿الْـحَمْـدُ لِلَّهِ الَّـذِي أَنـزَلَ عَلَـى عَبْـدِهِ الْـكِتَـابَ وَلَـمْ يَـجْعَل لَّـه عِوَجًـا﴾

صدق الله العظيم

سورة الكهف: الآية (1)

DEDICATION

To

All Sickle cell patients

May Allah give them strength.

To

My loving parents

, brothers and sisters

And faithful friends.

Acknowledgments

Above all thanks I give my sincere thank to ALLAH .My sincere thanks to my supervisor Dr. Kawthar Abdelgaleil MohammedSalih, for her valuable guidance, kind supervision and great help.

I am also grateful to all the staff of Institute of Endemic Disease (University of Khartoum), Immunology and Molecular Biology Department, for assisting with the practical and offering their laboratory.

Abstrac

Sickle cell disease (SCD) is inherited chronic haemolytic anemias whose red blood cells have characteristic sickle shape. Cytokines are the hormonal messengers responsible for biological effects in the immune system.

SCA is associated with a pro-inflammatory state, and an enhanced inflammatory response occurs during vaso-occlusive crisis via cytokines effects.

This case-control analytical study was aimed to evaluate the IL-10 levels 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).

Sixty SCA patients age between 4-17 years 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 and (2.5ml) in EDTA container. IL-10 concentration was measured using Enzyme Linked Immunosorbent Assay (ELISA) (Biolegend's ELISA MAX^{TM}) in Institute of Endemic Diseases. Hb concentration was tested used complete blood count (CBC) device. The data was analyzed using SPSS program (Version 16) using oneway ANOVA test and independent T-test for testing difference significance, correlation test for finding potential correlation.

The result showed that means of IL-10 were (9.84± 6.4), (36.84±45.4), (14.01±16.4) in the steady state patients, VOC patients and control subjects respectively. IL-10 level was significantly elevated in VOC patients than the steady state group and control group (*P. values* were 0.811 and 0.007 respectively). There was no significant difference between IL-10 level in the steady state patients and controls (*p. value* 0.003). Hb level was significantly decreased in VOC group than the steady state group (*P. value* 0.000). There was no statistical correlation between IL-10 and Hb levels and age in the three groups (*P. values* 0.214 in steady state, 0.568 in VOC and 0.868 in controls). The difference in mean of IL-10 between males and females of the three groups was not statistically significant (*P. values* 0.413 in steady state, 0.715 in VOC, and 0.485 in controls).

The study concluded that in the studied population, IL-10 concentration may be a useful VOC prognostic marker.

مستخلص الأطروحة

انيميا الخلايا المنجليه هي انيميا وراثيه مزمنة فيها خلايا الدم الحمراء مميزه بشكل المنجل.المركبات الخلويه هي مراسيل هرمونيه مسؤله عن التأثير الحيوي على الجهاز المناعي.

انيميا الخلايا المنجليه مرتبطه بحالة إلتهابيه وتحسن الإستجابة الإلتهابية تحدث خلال أزمة إنسداد الشرايين نتيجة تأثير المركبات الخلوية. هذه الدراسة (دراسة الحالات والشواهد) هدفت لتحديد مستوى المادة الخلوية انترليوكين 10في المرضى السودانيين بالأنيميا المنجلية خلال حالتي الأزمة الوعائية و المستقرة وفي الأفراد الطبيعيين بولاية الخرطوم في الفترة من يوليو حتى ديسمبر 2018. أختير60 مريضا بالانيميا المنجلية عشوائيا , 30 منهم خلال الأزمة الوعائية و 30 خلال الحالة المستقرة تتراوح اعمارهم بين 4 – 17 عاما.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 و

(2.5مل) في أنبوبة تحتوي مضاد تجلط. قيس تركيز المركب المصلي انترليوكين 10 عن طريق فحص مقايسة الممتز المناعي المرتبط بالانزيم بمعهد الأمراض المتوطنة. حللت البيانات باستخدام برنامج الحزم الأحصائية للمجتمع (نسخة 16). بإستخدام إختبار ون واي انوفا وإختبار T المستقل لتعين الإختلاف الإحصائي و إختبار العلاقات لإيجاد العلاقات.

أظهرت النتائج الوسط الحسابي للمادة المصلية 10, 16.4±14.01, 45.4±36.84, 6.4± 9.84 في المرضى بالأنيميا المنجلية خلال الأزمة الوعائية وخلال الحالة المستقرة وفي الأفراد الطبيعيين بالترتيب.كان هنالك ارتفاع ذو دلالة احصائية في معدل انترليوكين 10 في المرضى بالأنيميا المنجلية خلال الأزمة الوعائية عند مقارنتهم بالمرضى في الحالة المستقرة والأفراد الطبيعيين(كانت القيم الاحتمالية 0.811 و 0.007 بالترتيب). بينما لا يوجد دلالة إحصائية في معدل انترليوكين 10 بين المرضى في الحالة المستقرة والأفراد الطبيعيين (كانت القيمة الاحتمالية 0.003). إنخفض معدل خضاب الدم في المرضى خلال الأزمة الوعائية مقارنة بالمرضى في الحالة المستقرة (القيمة الاحتمالية 0.000). ليس هنالك علاقة بين معدل انترليوكين 10 ومعدل خضاب الدم والعمر في المرضى خلال الأزمة الوعائية والحالة المستقرة (القيم الاحتمالية 0.214 في الحالة المستقرة ، 0.568 في حالة الأزمة الوعائية و 0.868 في الأفراد الطبيعيين) لاتوجد دلالة وصفية حسابية للمادة الخلوية انترليوكين 10 بين الرجال والنساء في الثلاث مجموعات (القيم الاحتمالية 0.413 في الحالة المستقرة، 0.715 في حالة الأزمة الوعائية و 0.485 في الأفراد الطبيعيين).

لقد توصلت الدراسة الى ان تركيز المادة الخلوية انترليوكين 10 المصلي في المجموعات المدروسة يمكن ان يستخدم كعلامة توقع دالة للأزمة الوعائية.

List of Contents

List of Tables

List of Figures

List of abbreviations

CD	Cluster of Differentiation
CD4-Th 2	Cluster of Differentiation 4T helper2
ELISA	Enzyme linked immunosorbent assay
g/dl	gram/deciliter
GMCSF	Granulocyte Monocyte Colony Stimulating Factor
Hb	Hemoglobin
HbA	Adult hemoglobin
HbS	Sickle hemoglobin
HU	Hydroxyurea
IFNγ	Interferon gamma
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-9	Interleukin-9
INF	Interferon

KD	Kilo Dalton
MHC	Major Histocompatibility Complex
N	Number
Nk cell	Natural Killer cell
NO	Nitric oxide
P. value	Probability value
Pg/ml	Pico gram/milliliter
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCD	Sickle cell disease
SCDHU	Sickle cell disease hydroxyurea
SD	Standard deviation
SPSS	Statistical package for social science
TGF-β	Transforming growth factor – beta
Th1	T helper1
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VOC	Vaso-occlusive crisis

Chapter One

Introduction and literature Review

Chapter one

Introduction

1. Introduction:

Sickle cell anemia 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 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. Repeated episodes of sickling damage the cell membrane and decreases the cell's elasticity. These cells fail to return to normal shape when normal oxygen tension is restored. 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).

Intravascular hemolysis lead to realese 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).Increased ROS and RNS levels and decreased NO levels contribute to the activation of RBCs, leukocytes, platelets and endothelial cells. This activation s leads to increased production of pro inflammatory and decrease anti-inflammatory cytokines, which gives SCA the characteristics of a chronic inflammatory disease (Greer *et al.*,2014).

Cytokines are Proteins that are produced and secreted by many different cell types, and mediate inflammatory and immune reactions well as principal mediators of communication between cells of the immune system (Abbas *et al.,* 2015).

CD4+ T cells are further divided into subsets by their function and pattern of cytokine secretion (Raghupathy , 2000).

Th1 subset secretes IL-2, IFN-γ and TNF, and responsible for many classic cell-mediated functions, including activation of cytotoxic T lymphocytes and macrophages. While 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).

Chemokines A large family of structurally homologous low–molecular-weight cytokines that stimulate leukocyte chemotaxis, regulate the migration of leukocytes from the blood to tissues by activating leukocyte integrins, and maintain the spatial organization of different subsets of lymphocytes and antigen-presenting cells within lymphoid organs(Abbas *et al.*,2015).

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine, synthesized by a wide range of cell types, including, monocytes, dendritic cells and T cells(Owen *etal*., 2013).

IL10 has roles in both innate and adaptive immunity that are manifested as either immunosuppressive or immunostimulatory effects on various cell type. Cytokines induce increased adhesion of RBC and leukocytes to the vascular endothelium, and this adhesion can cause vasoocclusion and local hypoxia IL-10 is an anti-inflammatory cytokine whose main effect is inhibition of the synthesis of various cytokines, such as TNF-α, GM-CSF, IL-1, IL-6, IL-8 and IL-12, to promote the uptake and retention of iron within reticuloendothelial system (Pitanga *et al.*,2013).

1-2-Literture Review:

1.2.1. Historical background of sickle cell anemia:

Sickle cell disease (SCD) is an inherited chronic haemolytic anemia whose clinical manifestations arise from the tendency of the hemoglobin (Hbs or sickle hemoglobin) to polymerize and deform red

the β-globing gene leading to substitution of valine for glutamic acid at position 6 of the β-globin chain (β6glu→val or βs) (Hoffbrand *et al.*,2016).

The homozygous state (HbSS or sickle cell anaemia) is the most common form of sickle cell disease, b `ut interaction of HbS with thalassaemia and certain variant haemoglobins also leads to sickling. The term 'sickle cell disease' is used to denote all entities associated with sickling of haemoglobin within red cells (Hoffbrand *et al.*,2016).

The origin of sickle cell anemia has not been identified, symptoms of the disease have been traced in one Ghanaian family back to 1670.7 Sickle cell anemia was first reported by a Chicago cardiologist, Herrick, in 1910 in a West Indian student with severe anemia. In 1917, Emmel recorded that sickling occurred in non anemic patients and in patients who were severely anemic. In 1927, Hahn sand Gillespie described the pathologic basis of the disorder and its relationship to the hemoglobin molecule. These investigators showed that sickling occurred when a solution of RBCs was deficient in oxygen and that the shape of the RBCs was reversible when that solution was oxygenated again. In 1946, Beet reported that malarial parasites were present less frequently in blood films from patients with SCD than in individuals without SCD (Keohane *et al.*,2016).

1.2.2. Prevalence:

The highest frequency of the sickle cell gene is found in sub-Saharan Africa, where each year approximately 230,000 babies are born with sickle cell disease (Hb SS), representing 0.74% of all live births occurring in this area. In contrast, approximately 2600 babies are born annually with sickle cell disease in North America and 1300 in Europe. Globally, the sickle cell gene occurs at the highest frequency in five geographic areas: sub-Saharan Africa, Arab-India, the Americas, Eurasia, and Southeast Asia (Keohane *et al.*, 2016).

1.2.4. Pathophysiology:

When Hb S is deoxygenated, it becomes polymerized and produces sickling . Subsequent hemolysis is probably a result of the extent of the red cell's capacity to sickle. The erythrocytic membrane in sickle cell anemia possesses significant membrane abnormalities, with an excessive increase in ionized calcium in the cell playing a role in the produced abnormality. Ionized calcium in the Hb SS cell is twice normal, with the most dense cells having four times the normal amount of ionized calcium (Turgeon ,2012).

Polymerization of Hb S occurs under conditions of extremely reduced oxygen and increased acidity in the blood and this polymerization produces the resultant sickling. The red cell flexibility, which is governed by the amount and alignment of this intracellular polymer, is the principal determinant of the flow of sickled red cells. Because cells that have large amounts of ordered polymer may be caught in the capillaries and venules, some cells at relatively high oxygen saturation with polymer but no deformability may have difficulty traversing the constriction of the precapillary arterioles. When the sickled cells attempt to travel through these small vessels, they become stuck and the vessels become when obstructed (Kawthalkar *et al.,* 2013).

This initiates a pattern of blood not flowing properly to the tissue and creating a lack of oxygen which causes more sickling and more deprivation of oxygen to the tissues. This process can cause intense pain. When sickled cells receive oxygen, they return to their normal shape. Repeated cycles of sickling and unsickling lead to the RBCs becoming permanently damaged . This process ends in hemolysis, which leads to anemia. In addition, repeated episodes of this type lead to the necrosis of body tissues (Turgeon, 2012).

1.2.4. Complications:

1.2.4.1. Hematologic Complications:

1.2.4.1.1.Anemia and Exacerbations:

The result of chronic hemolysis, is a typical feature of homozygous sickle cell anemia and of the compound heterozygous sickle syndromes other than sickle cell trait; in sickle cell trait, there is no anemia or hemolysis. The degree of hemolysis correlates with the number of irreversibly sickled cells, which is a function of the intracellular concentration of Hb Sand the heterogeneous distribution of fetal hemoglobin. The anemia is detectable 2 to 3 months after birth, and in any given patient the hemoglobin concentration, reticulocyte count, and magnitude of hemolysis are relatively constant. Exacerbations may be due to aplastic crises, splenic sequestration crises, hepatic sequestration crises, folic acid deficiency, iron deficiency, chronic renal disease, or bone marrow necrosis (Tefferi ,2001).

1.2.4.1.2.Aplasia:

Due to infection with B 19 parvovirus, the most common cause of transient aplasia in children with sickle cell anemia, is less common in adults. The diagnosis is made by detection of B19 parvovirus particles or of IgM anti-Bl9 antibodies. Salmonella, *Streptococcus pneumonia*, and Epstein-Barr virus infections have all been implicated in transient a plastic crises (Mehta and Hoffbrand., 2005).

1.2.4.1.3. Acute splenic Sequestration crises:

Are due to the acute intrasplenic trapping of blood, which leads to severe anemia, hypovolemia and splenomegaly. The generally accepted criteria for this diagnosis are a decrease in the steady-state hemoglobin concentration of more than 2 g/dL, evidence of a compensatory marrow response, and an acutely enlarging spleen. Attacks may be mild (resolving spontaneously) or severe (causing shock and death). Hepatic sequestration also has been described, but it is much less common (Kern, 2002).

1.2.4.2. Pulmonary Complications:

1.2.4.2.1.Acute chest syndrome (ASC)

ACS is the second most common cause of hospitalization in patients with SCD and is responsible for 25% of death. Fever, cough, chest pain and pulmonary infiltrates on chest radiography are the common presenting features and the condition may rapidly progress to life-threatening respiratory insufficiency. The most common etiologic factors are infection, pulmonary fat embolism or pulmonary infarction but it may be multi-factorial (Thachil and Quentin, 2018).

1.2.4.2.2.Management of ACS:

Supplemental oxygen should be administered to those whose oxygen saturation is less than 95%. Hydration is important but overhydration can worsen the condition. Adequate pain relief should be administered but over sedation avoided. Incentive spirometry has been shown to be beneficial in the prevention and management of ACS. Airways hyper-reactivity is treated with bronchodilators. Intravenous broad-spectrum antibiotics including a macrolide or quinolone should be administered. Top-up blood transfusion, to achieve a final hemoglobin of 10 g/dL, has been shown to be effective in children with ACS and should be administered if there is evidence of hypoxemia. In severe cases exchange transfusion may be necessary (Thachil and Quentin, 2018).

1.2.4.2.3.Chronic Pulmonary disease:

Chronic pulmonary disease is a disease of adults with SCD and affects up to 32% of patients.The true incidence is unknown as the early stages of disease are asymptomatic. The etiology is multi-factorial and includes lung damage from ACS, chronic oxygen de-saturation or sleep hypoventilation, repeated episodes of thrombo-embolism, and hemolytic anemia associated pulmonary hypertension due to reduced nitric oxide/arginine levels (Kern, 2002).

1.2.4.3. Bones and Joints:

Chronic and progressive destruction of the bones and joints may take place in the absence of clearly defined episodes of pain. The most prominent changes evolve slowly from the cumulative effect of recurrent small episodes of ischemia or infarction within the spongiosa of bone (Greer *et al.*, 2014).

1.2.4.4.Neurologie Complieations:

Stroke is a common event in children homozygous for Hgb S.In children, strokes are more frequently the result of cerebral vascular stenosis and infarction. Hemorrhage and infarct may occur together. Strokes in children who have sickle cell disease involve stenosis and occlusion of the major anterior arteries of the brain, including the carotids. The presenting symptoms of stroke can be dramatic and acute, such as coma, seizure, hemiparesis, hemianesthesia, visual field deficits, aphasia, or cranial nerve palsies. Subtle limb weakness (without pain) is often mistaken for an acute vaso-occlusive episode but can be due to stroke (Hoffbrand and moss., 2016).

1.2.4.5.Hepatobiliary and Gastrointestinal Complications:

Bilirubin gallstones can eventually be detected in most patients with chronic hemolytic anemia. Cholecystectomy may be necessary for patients with fat intolerance, presence of gallstones, and recurrent abdominal pain. Hepatomegaly and liver dysfunction may be caused by a combination of intrahepatic trapping of sickled cells, transfusion-acquired infection, or Sickle Cell Disease transfusional hemosiderosis. The combination of hemolysis, liver dysfunction, and renal tubular defects can result in very high bilirubin levels. Benign cholestasis of sickle cell disease results in severe asymptomatic hyperbilirubinemia without fever, pain, leukocytosis, or hepatic failure (Hastings *et al.*, 2012).

1.2.4.6.Genitourinary Complications:

1.2.4.6.1.Renal complications

The hypoxic, acidotic and hypertonic renal medulla favours vaso-occlusion, leading to destruction of the vasa recta and hyposthenuria in the first year of life. It presents clinically as enuresis or nocturia, and patients are susceptible to dehydration in hot weather. Haematuria as a result of papillary necrosis usually originates from the left kidney. Management is generally by bed rest and hydration, although sometimes blood transfusion and ε-aminocaproic acid are required. Some renal complications, such as hyposthenuria and haematuria, are also observed in individuals with sickle trait, as is the rare renal medullary carcinoma (Munker *et al*., 2007).

1.2.4.6.2.Priapism

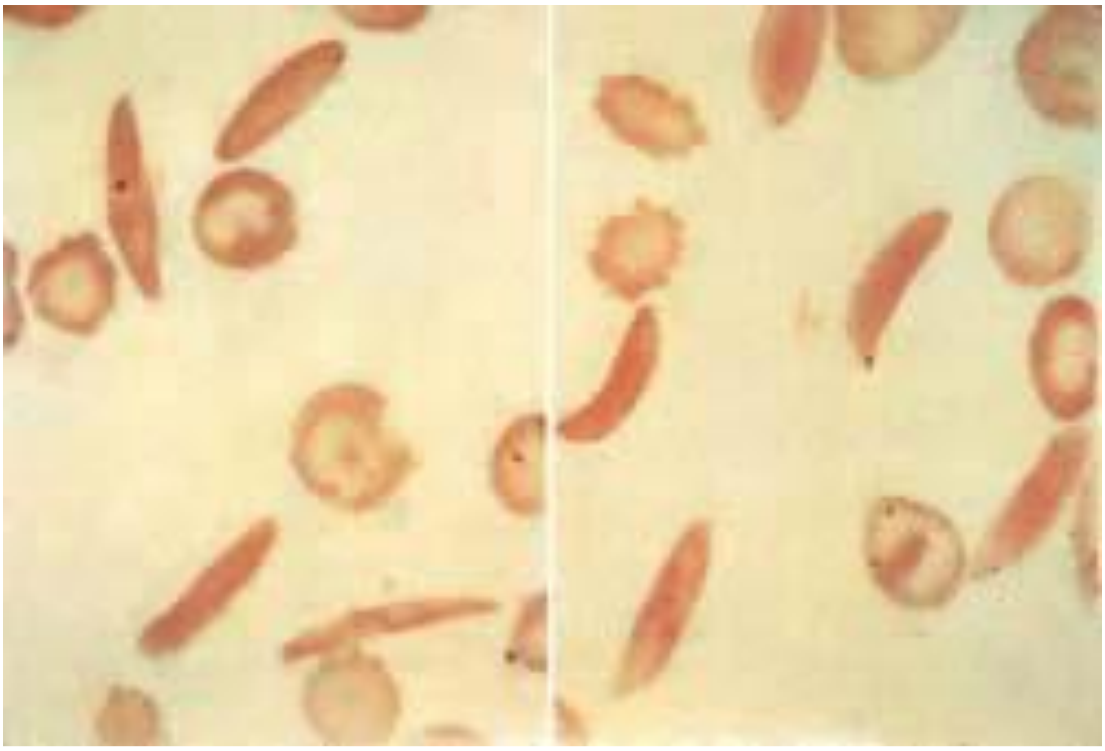
Priapism is caused by vasoocclusion leading to obstruction of venous drainage from the penis. It typically affects the *corpora cavernosa* alone, resulting in a hard penis with a soft glans. Episodes can be brief (stuttering) or prolonged, when they last for longer than 3 hours (Munker *et al.*, 2007).

1.2.4.7.Ophthalmic Complieations:

The eye is particularly sensitive to hypoxia. Vaso-occlusion of retinal vessels and hypoxia of the retina cause permanent retinal damage. Blood in the anterior chamber of the eye (hyphema) becomes rapidly deoxygenated and permanently sickled, obstructing the outflow from the aqueous humor. The accumulation of aqueous humor in the anterior chamber increases intraocular pressure leading to decreased blood flow to the retina until the perfusion pressure of the globe is reached. This leads to sudden vascular stasis and blindness (Hastings *et al.*, 2012).

1.2.5.Diagnosis

Sickle cell anaemia should be suspected in any patient of an appropriate racial group with a haemolytic anaemia. It can be confirmed by a sickle cell test, although this does not distinguish between heterozygotes and homozygotes. A definitive diagnosis requires haemoglobin electrophoresis and the demonstration of the sickle cell trait in both parents. (Provan, 2003)



(Figure 1-2) Peripheral blood film from patient with sickle cell anaemia showing sickled erythrocytes (Provan, 2003)

1.2.6.Mangment of sickle cell anemia:

1.2.6.1.Agents that elevate HbF levels:

It has been recognised for some time that HbF levels ameliorate b thalassaemia and sickle cell disease. HbF reduces HbS polymerisation and hence sickling. HbF level of >10% reduces episodes of aseptic necrosis; levels >20% HbF are associated with fewer painful crises (Provan *et al.*, 2004).

1.2.6.2.Hydroxyurea

Hydroxyurea was shown to reduce the frequency of pain crises, acute chest syndrome, and the need for blood transfusions. The mechanisms of action of hydroxyurea include increased production of HbF and an alteration of the adhesive properties of leukocytes (Munker *et al.*,2007).

1.2.6.3.Erythropoietin, 5-azacytidine:

Short chain fatty acids and Gene therapy are not widely used and still under trail, gene therapy potentially curative but experimental. Globin gene transfer has been attempted with variable results. Expression of exogenous gene has been at levels too low to be of benefit (Provan *et al*., 2004).

1.2.6.4. Bone marrow transplantation:

Sibling donor transplants for sickle cell disease have been carried out in a number of centers. Since the mortality from sickle cell disease has dropped over recent years from 15% to 71%, and with the advent of hydroxyurea therapy, there is a less compelling argument for BMT in sickle cell disease (Kawthalkar *et al.,* (2013): Kern *et al.,* 2002).

1.2.7. Prevention and Management:

Premarital, antenatal, and neonatal screening programs have been established in some high income countries. The development of cheap and reliable point-of care diagnostic tests with high sensitivity and specificity could hugely facilitate screening for sickle cell disease in lower-income countries (Piel *et al*,. 2017).

Clinical outcomes have gradually improved over the years, mostly as a result of developments in supportive care and treatment with hydroxyurea. Relatively few interventions have a strong evidence base, but those that do include penicillin prophylaxis in children, primary stroke prevention with the use of transcranial Doppler screening and blood transfusion, regular blood transfusions to prevent the progression of silent cerebral infarction, and hydroxyurea to prevent acute pain and the acute chest syndrome as well as primary stroke and with growing evidence of the safety and efficacy of hydroxyurea in both adults and children. (Ciesla,. 2012).

Alternative sources of donor cells is used although allogeneic stem-cell donation may be superseded by gene therapy and gene editing approaches .A recent case report describing the use of a self-inactivating lenti viral vector to inhibit HbS polymerization as a proof of concept of complete clinical remission with correction of hemolysis and biologic hallmarks of the disease certainly reflects the fast pace of current developments in gene therapy for sickle cell disease. A better understanding of genetic modifiers is essential for advances in gene therapy and drug development (Piel *et al.,* 2017).

1.2.8.Cytokines:

This group of soluble molecules plays an extremely important role in clinical immunology. They are secreted by macrophages only and may act as stimulatory or inhibitory signals between cells. Cytokines that initiate chemotaxis of leucocytes are called chemokines (Zabriskie ,2009).

1.2.8.1.Cytokines Role and Nomenclature:

Cytokines Stimulate growth and differentiation of lymphocyte ,activates immune cells to eliminate microbes & Ag, stimulate hematopoiesis and used in medicine as therapeutic agent. It nomenclature is according to producing cell is divided into: monokines produced by macrophage/monocyte, lymphokines produced by lymphocyte, Interleukins produced by leucocytes and act on other leucocytes eg IL-1 & IL-2 & IL-3…..and Biologic response modifier which used clinically to increase or reduce immunity (Hawas ,2016).

1.2.8.2.Classification of cytokines:

Cytokines are classified into mediators and regulators of innate immunity which are produced mainly by macrophages, and NK cells (Tumor necrosis factor TNF-α, IL-1, IL-12 **,** Interferon IFN-α and IFN-) and mediators and regulators of adaptive immunity which are produced by T lymphocytes (IL-2, IL-4, IL-5, IFN-γ)and cytokines that stimulators of hematopoiesis such as Graulocyte-monocytes colony stimulating factor GM-CSF, 7/IL-3 and IL-7) (Horton-Szar *et al,*. 2012).

1.2.8.3.Cytokine Profiles of Th1 and Th2 Subsets

Th1is induced byIFN-γandIL-12 to produce IFN-γ, IL-2, TNF and LT that function in cell-mediated immune response while Th2 **is** induced by IL-4 to produce IL-4, IL-5, IL-10 and IL-13 to Function in humoral immune response (Hawas ,2016).

1.2.8.4.Properties of the major cytokines produced by CD4 helper T lymphocytes:

Cytokines are produced transiently in response to antigen and usually acts on same cell that produces the cytokine (autocrine) or nearby cells (paracrine) and each cytokine has multiple biologic actions(Pleiotropism) Multiple cytokines may share the same or similar biologic activities (Redundancy) (Abbas *et al.,* 2015).

(Table 1-2-1) Some important characteristics of the major cytokines of innate immunity are listed (Abbas *et al.*, 2015).

Cytokines	Principal cell source(s)	Principal cellular targets and biologic effects
Tumer necrosis factor (TNF)	Macrophage, T cells	Endothelial cells: activation (inflammation, coagulation) Neutrophils: activation Hypothalamus: fever Liver: synthesis of acute phase proteins Muscle, fat: catabolism (cachexia) Many cell types: apoptosis
Interleukin (IL-1)	Macrophage, endothelial cell, some epithelial cells	Endothelial cells: activation (inflammation, coagulation) Hypothalamus: fever Liver. synthesis of acute phase proteins
Chemokines	Macrophage, endothelial cell, T lymphocytes, fibroblasts, platlets	Leukocytes: chemotaxis, activation
Interleukin-12 (IL-12)	Macrophage, dendritic cells	NK cells and T cells: IFN-r. synthesis, increased cytolytic activity T cells: T H1 differentiation
Interferon-*y* (IFN-*y*)	NK cells, Tlymphocytes	Activation of macrophages Stimulation of some antibody responses
Type 1 IFNs (IFN-*a*,IFN-*B*)	IFN-*a*:T lymphocytes IFN-*B*:Fibroblastes	All cells: antiviral state, increased class I MHC expression NK cells: activation
lnterleukin-10 (IL-10)	Macrophages, T cells (mainly T H2)	Macrophages: inhibition of IL-12 production, reduced expression of costimulators and class 11 MHC molecules
lnterleukin-6 (IL-6)	Macrophages, endothelial cells, Tcells	Liver: synthesis of acute phase proteins B cells: proliferation of antibody-producing Cells
lnterleukin-15 (IL-15)	Macrophages, others	NK cells: proliferation T cells: proliferation
lnterleukin-18 (IL-18)	Macrophages, others	NK cells and T cells: IFN-'Y synthesis

1.2.9.Interlukin-10(IL-10):

Interleukin 10 (IL-10) cytokine is required for regulating immune functions by promoting the widespread suppression of immune responses through its pleiotropic effects. The autocrine/ paracrine capabilities of IL-10 by direct binding to leukocytes and resultant containment of immune responses is considered the primary function of this cytokine. IL-10 secretion from CD4+CD25 regulatory cells (Tregs), macrophages and other leukocytes followed by subsequent binding to IL-10 receptors on macrophages and dendritic cells (DCs) has been linked to reduced antigen presentation and increased T-cell anergy (Bijjiga and Martino,. 2013).

IL-10 also functions to minimize the development of Th1 responses by decreasing Th1 related cytokines (IL-12 and IFN-γ) and encouraging Th2 responses by increasing levels of Th2 related cytokines (IL-4, IL-5 & IL-13) (Bijjiga and Martino,. 2013).

1.2.10.Relation between IL-10 and SCD:

IL-10 is an anti-inflammatory cytokine whose main effect is inhibition of the synthesis of various cytokines, such as TNF-*α*, GM-CSF, IL-1, IL-6, IL-8 and IL-12, to promote the uptake and retention of iron within mono- cytes and the reticuloendothelial system. IL-10 also inhibits the proliferation of TH1 cells, decreasing cytolytic function and the secretion of TH1 cytokines and facilitating the development of a TH2 response (Hoffman *et al.*, 2000).

There are conflicting reports on the role of IL-10 in SCA patients. One study demonstrated that patients undergoing HU therapy had high levels of IL-10, but the mechanism was not described (lanaro *et al.,* 2006). In 2009, a Sudanese study of children with malarial infection demonstrated that asymptomatic children with sickle cell trait had significantly lower levels of IL-10 than HbAA children with severe malaria. However, these children had higher IL-10 levels than HbAA children with mild malaria. These results suggest that IL-10 has a protective effect against the occurrence of severe malaria in patients with sickle cell trait. A study of inflammation and iron- overloading observed higher levels of IL-10 and lower levels of non-transferrin bound iron in SCA patients than in thalassemia patients, confirming the contribution of this cytokine to the regulation cellular iron status . Thus, abnormal production of these anti-inflammatory cytokines can

affect both cell-mediated and humoral immune responses and increase the risk of morbidity in sickle cell patients (Hoffman *et al.*,2000).

IL-10 levels are inversely correlated with disease incidence and severity and reduced level of IL-10 contributes to inflammatory response in VOC (Sarray and Wassim., 2015).

1.2.11. Previous studies:

- A study was conducted in Manama , Bahrain on a147 sickle cell patients in vasoocclusive crisis(VOC) and 63 pain free sickle cell patients (steady state) , IL-10 levels were measured using ELISA kites , IL-10 levels were significantly low in VOC compared to steady state group and in contrast high levels of IL-10 in steady state compared to VOC group. Correlation analysis demonstrated significant association between reduced IL-10 and duration of VOC, frequency, type, severity and need for hydroxyurea treatment (Sarray and Wassim.,2015).

-In Brazil a study was conducted on 50 SCD in steady state and 33 healthy controls to evaluate the level of IL-10 and to study the effect of hydroxyurea. IL-10 plasma levels showed no significant diferance between the IL-10 levels in SCD steady state group and control group. IL-10 levels were higher in SCD patients receiving HU therapy than SCD patients not receiving HU therapy (Lanaro *et al.*,2009).

-Other study in Brazil aimed to evaluate possible immunologic relationship between sickle cell anemia (SCD) and periodontal inflammation and its impact on serum cytokines. 25 Brazilian children of African descent were involved, 10 SCD patients and 15 non SCD controls. Intergroup analysis had shown high IL-10 levels in SCD group only. Within the limit of the study it was concluded that SCD increases IL-10 levels regardless of the presence of the periodontal inflammations (Veiga *et al.*,2013).

-Astudy was conducted to evaluate the effect of HU therapy on cytokines levels in sickle cell patients. IL-10 plasma levels in healthy controls, SCD patients in steady state and SCD patients on HU therapy (SCDHU) was determined using ELISA assay. Detectable levels of IL-10 (>3.8pg/ml)were found in 3(15%)of 20 SCD patients and 1(11%)of 9controls , whilst 7(50%)of 14 SCD on HU therapy demonstrated detectable IL-10 levels. HU therapy appear to have augmenting effect on IL-10 (Lanaro *et al.*,2006).

-A study done in Brazil aimed to determine the association between IL-10 and TNF-a levels and the course of sickle cell disease. The study was conducted on 25 sickle cell patients in steady state and 26 healthy controls. IL-10 levels were reduced in poly transfusion and in Acute chest syndrome (Cavalcante ,2016).

- Bourantas and his colleagues did astudy in Greece aimed to identify a possible acute phase response during the steady state of sickle cell disease. IL-10 was measured in 21 steady state patients and was not detected in any of them (Bourantas *et al.*,1998).

-I did not find any published studies about IL10 level in sickle cell disease in Sudanese patients.

1.3. Rationale:

Sickle cell anemia 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).Sickle cell anemia is one of the major types of anemia in Sudan and SCA has serious complications such as Stroke , Acute chest syndrome, Pulmonary hypertension ,Organ damage , Blindness, leg ulcer, gall stone and priapism, (Ali *et al*., 2014). IL10 is a key anti-inflammatory cytokine which comes from activated immune cell, (Obeagu *et al*., 2015). It plays an important role in the control of immune response in view of role of IL10 as an anti-inflammatory cytokine in sickle cell disease it was tested and found to have reduced level which contribute to the pro inflammatory state that is present in SCD, also reduced level of IL10 is an early indicator of osteomyelitis complication in sickle cell disease patients, (Cajado *etal*., 2011; Sarray and Wassim.,2015).

1.4. Objectives:

1.4.1. General objective:

-To evaluate serum levels of IL10 in Sudanese sickle cell disease patients during vasoocclusive crises and the steady state condition.

1.4.2. Specific objectives:

_To measure serum level of IL10 in only patients during vasooclusive crises and the steady state and in normal subjects using Enzyme-linked Immunosorbent Assays (ELISA) Kits (Biolegend's ELISA MAX^{TM}).

_To compare levels of serum IL-10 between SCD patients and healthy control.

_ To study possible correlation between IL-10 level and other factors (age, gender and Hb).

Chapter Two

Materials & Methods

Chapter Two

Materials and Methods

2.1. Study design:

This is descriptive case control hospital based study.

2.2. Study Area and duration:

The study was conducted in Al-bulk Hospital, Ahmed Gasim Hospital and Dr. 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 4-17 years, divided into 3 groups as follows- crisis group made up of 30 sickle cell patients during crisis, Steady state (steady) group made up of 30 SCA steady state patients, and Control (Hb A) group composed of 28 HbA individuals.

2.4. Inclusion criteria*:*

Known diagnosed patients in both sickle cell crises if they complain from bone and joint pain, multiple sites of pain, or other type of crises necessitating hospital admission and stable health state SCD patient who do not have any other crisis. Control group was age and sex matched apparently healthy volunteers.

2.5. Exclusion criteria:

All non-sickle cell patients and sickle cell patients with other disease that affect cytokines levels such as autoimmune diseases, heart failure, allergy, infectious disease, hypersensitivity, cancer, parkinson disease and paget disease of bone were excluded .

2.6. Sample size:

Total of 88 subjects and classified into (60) sickle cell patients (30) during crises and (30) during steady state and (28) healthy volunteer.

2.7. Sample collection and prepration:

Three ml of Vinous blood was collected in a plain container and allowed to clot at room temperature. Then samples were centrifuged and serum was separated in a sterile

container and stored at -20c until analysis. Serum levels of IL-10 was measured using ELISA kits (Biolegend's ELISA MAX^{TM}).

2.8. Data collection:

Samples were collected randomly.

Structive questionnaire was used and included demographic, clinical and laboratory data.

2.9.Principle and Procedure:

2.9.1.Principle of test:

BioLegend's ELISA MAX™ Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human IL-10 specific rat monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-10 binds to the immobilized capture antibody. Next, a biotinylated rat monoclonal anti-human IL-10 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- 10 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. (www.biolegend.com).

2.9.2.Procedure of the test:

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

-One day prior to running the ELISA, we diluted Capture Antibody in 1X Coating Buffer A as described in Reagent Preparation. We addwd 100 µL of this Capture Antibody solution to all wells of a 96-well plate provided in the set. We Sealed the plate and incubated it overnight (16-18 hrs) between 2°C and 8°C.

-We brought 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.

-We washed the plate 4 times with at 300 µL Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper.

- To block non-specific binding and reduce background, we added 200 µL 1X Assay Diluent A per well.

-We Sealed plate and incubated it at RT for 1 hour with shaking on a plate shaker (e.g. 500 rpm with a 0.3 cm circular orbit). All subsequent incubation with shaking were performed similarly.

-We washed plate 4 times with Wash Buffer.

- We Added 100 µL/well of standards or samples to the appropriate wells.

- We Sealed the plate and incubated it at RT for 2 hours with shaking.

-We washed plate 4 times with Wash Buffer.

-We added 100 µL of diluted Detection Antibody solution to each well, sealed the plate and incubated at RT for 1 hour with shaking.

-We wash plate 4 times with Wash Buffer.

-We added 100 µL of diluted Avidin-HRP solution to each well, sealed the plate and incubate at RT for 30 minutes with shaking.

-We Washed the plate 5 times with Wash Buffer. For this final wash, we soaked wells in Wash Buffer for 30 seconds to 1 minute for each wash to minimize background.

-We added 100 µL of freshly mixed TMB Substrate Solution and incubated it in the dark for 30 minutes.

-We stop reaction by adding 100 µL of Stop Solution to each well.

-We read absorbance at 450 nm within 15 minutes.

*****Optimal substrate incubation time depends on laboratory conditions and the optical linear ranges of ELISA plate readers. (www.biolegend.com)

2.9.3. Hb Estimation:

Blood is diluted in Drabkins solution which consist of potassium cyanide and potassium ferricyanide. Potassium ferricyanide converts Hb to methemoglobin. Further potassium cyanide converts methemoglobin to cyanmethemoglobin (HiCN). The absorbance of solution is measured in spectrophotometer at a 540 wavelength.

2.10. Ethical Consideration:

This study was approved by College of Medical Laboratory Science and ethical committees, permission from hospital manger was taken before beginning. Every sample was collected after verbal approval by the volunteers parents. Provide privacy and confidentially for every participant.

2.11. Data Analysis:

Statistical analysis was performed using statistical package for social science {SPSS} (version 16.0) using Mean ± SD one way ANOVA test and independent T test for testing difference significance, correlation test to find out correlation and frequencies to obtain mean and stander deviation. Probability value (PV) less than or equal 0.05 was considered statistically significant.

Chapter Three

Results

Chapter Three

Results

3. Results

Eighty eight subjects age between 4 to17 years old were recruited to this study with mean age and STD (9±3) and male: female was (52:48). The study subjects were clinically classified into 30 SCD patients in steady state with mean of age (9±3), and male: female was (60:40), 30 SCD patients during VOC with mean of age (8±4), males: female was (53:47) and 28 healthy subjects with mean of age (10±3), male: female was (39.2:61.7) (Table3-1). 21 of VOC patients were having musculoskeletal pain, 4 were having hemolytic crisis, 4 having acute chest syndrome and only one had retinopathy (Table3-1).

IL-10 was measured using ELISA and Hb levels were estimated Drabkins method. Results were statistically analyzed using Statistical Package of Social Science (SPSS) (version 16.0).

The mean and STD of IL-10 (3.59±39, 2.56±28 and 6.07±90) in VOC patients during musculoskeletal pain crisis, hemolytic crisis and ACS crisis respectively, *P value* were (0.383, 0.209 and 0.016) respectively (Table 3-1). Mean± STD of Hb (g/dl) were 7.3±1.2, 5.9±1.4 in steady state and VOC patients respectively, *P value* was 0.000 (Figure 3-1). IL-10 (pg/dl) mean and SD were 9.8428± 6.4, 36.8459±45.4, 14.0160±16.4 in the steady state, VOC patients and healthy subjects respectively.

In correlation between IL-10 levels and demographic date age and gender *P values* were 0.409 and 0.514 respectively (Table 3-1). IL-10 mean of males and females of steady state group were 9.1 and 2.5 respectively, *P value* 0.413 (Figure3-3). IL-10 mean of males and females of VOC patients were 4.3 and 2.5 respectively, *P value* 0.715 (Figure3-3). IL-10 mean of males and females of control group were 15.4 and 13.1 respectively, *P value* 0.485 (Figure3-3).

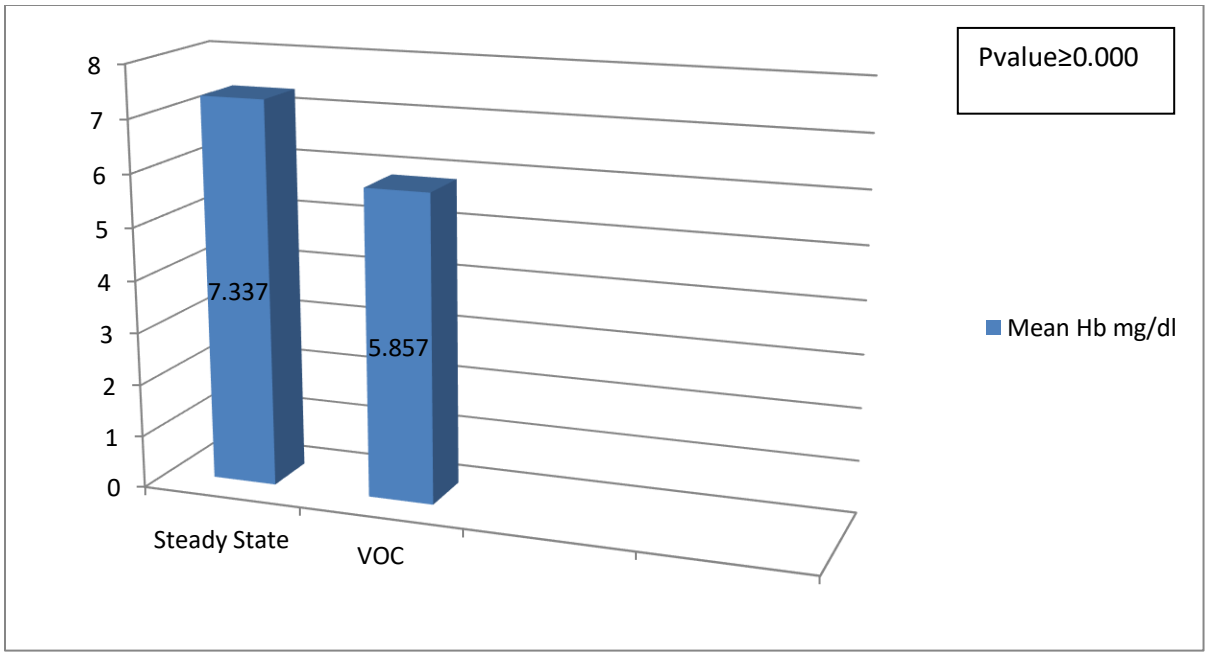
In correlation of IL-10 levels with age in three group P values were 0.214 in steady state group, 0.568 in VOC patients and 0.868 in control group (Figures 3-4, 3-5 and 3-6).

In correlation between IL-10 levels and clinical remarks of SCD patients during different VOC, musculoskeletal pain crises, hemolytic crisis and acute chest syndrome *p values* were (0.383 and 0.209 and 0.016) respectively (Figures 3-7, 3-8 and 3-9).

In multiple comparisons of IL-10 levels between the three groups, *P values* were 0.003 between steady state group and VOC group, 0.811 between steady state and control group and 0.007 between control group and VOC group (Table 3-4). In correlation between IL-10 levels in the steady state group and VOC group *P values* were 0.587 and 0.381 respectively (Table 3-4).

Table (3-1): Mean ± SD of age, gender distribution and demographic, clinical data and study subjects among study groups.

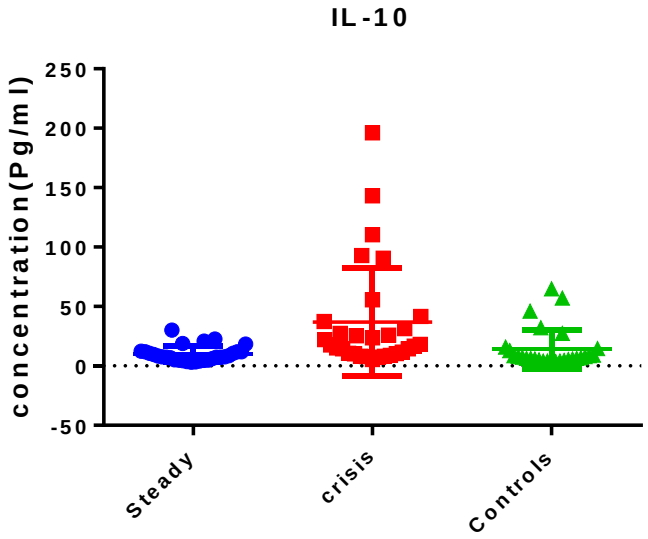
Study groups	**Steady state group** (N = 30)	**VOC group** (N = 30)	**Control group** (N = 28)	***P value***
Mean and SD age 9±3	9±3	8±4	10±3	0.409
Gender				
Male				
N 45	18	16	11	
% 52%	60%	54%	39.2%	0.514
Female				
N 43	12	14	17	
% 48%	40%	46%	61.7%	
Clinical Remarks				
Musculoskeletal pain	**(N = 0)**	**(N = 21)**	**(N = 0)**	
Mean IL-10		3.59±39		0.383
Hemolytic crisis	**(N = 0)**	**(N = 4)**	**(N = 0)**	
Mean IL-10		2.56±28		
Acute chest syndrome	**(N = 0)**	**(N = 4)**	**(N = 0)**	0.209
Mean IL-10		6.074±90		
Retinopathy	**(N = 0)**	**(N = 1)**	**(N = 0)**	0.016
Mean IL-10		7.25		



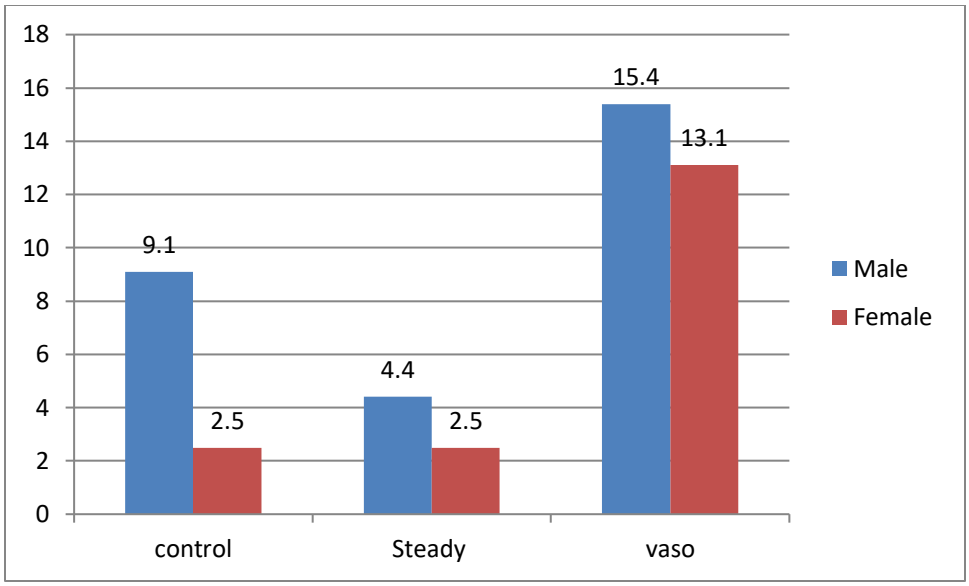
(Figure 3-1) Mean Hb concentration in SCA patients during steady state and during vaso-occlusive crisis (VOC).

(Table3-2): **Mean ± SD of IL-10 in Sickle Cell Disease patients in VOC and SCD steady state and control group.**

Variables	steady state group	VOC group	IL-10 in control group
Number	30	30	28
Mean	9.84±6.4	36.84±45.4	14.01±16.4



(Figure 3-2) IL-10 concentrations comparison between SCD in crisis, SCD in steady state and control group.



(Figure 3-3) Mean IL-10 in male and female among study group.

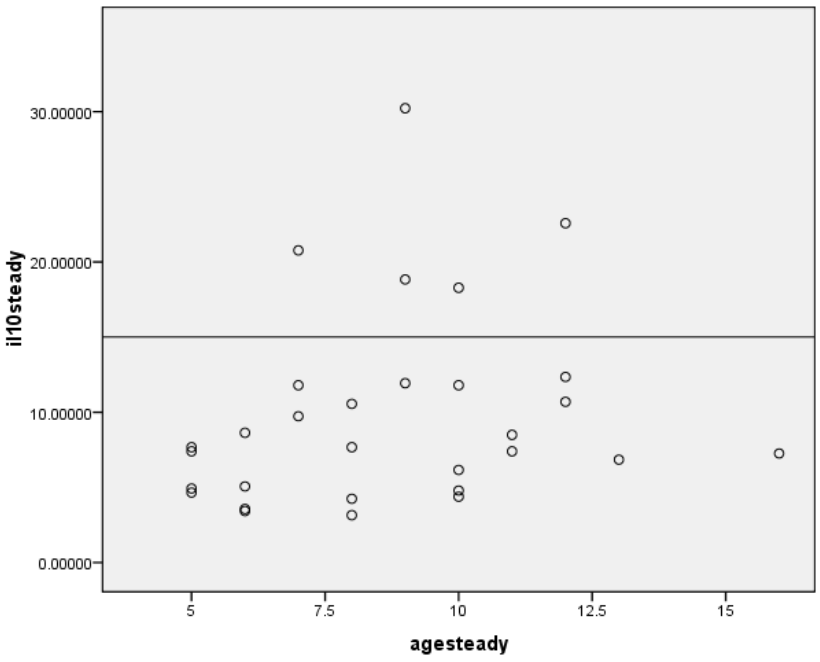


Figure (3-4): Correlation between IL-10 levels and age of SCA during steady state.

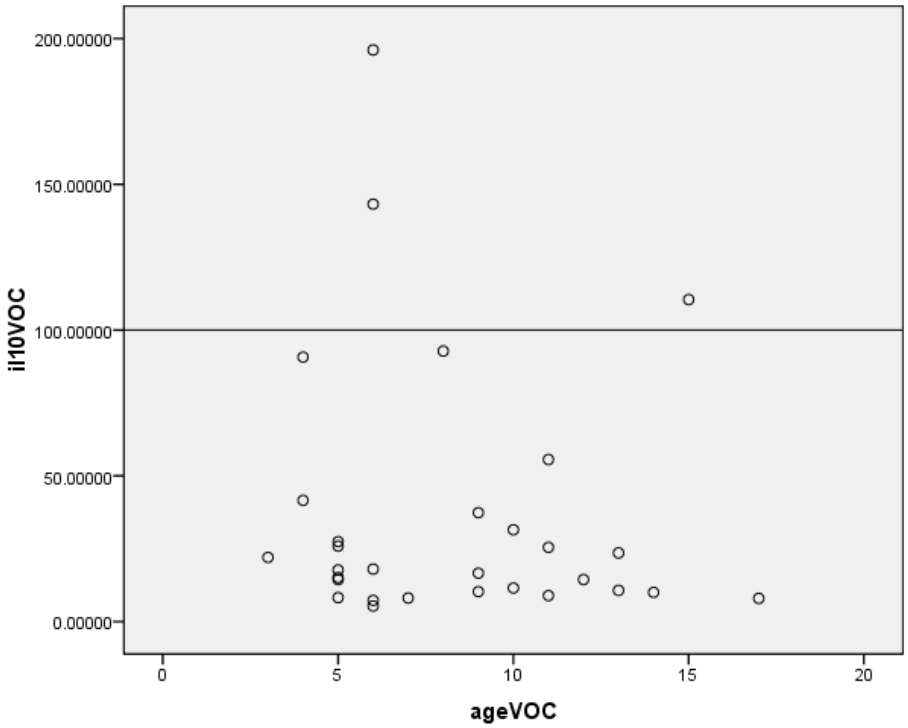


Figure (3-5): Correlation between IL-10 levels and age of SCA during VOC.

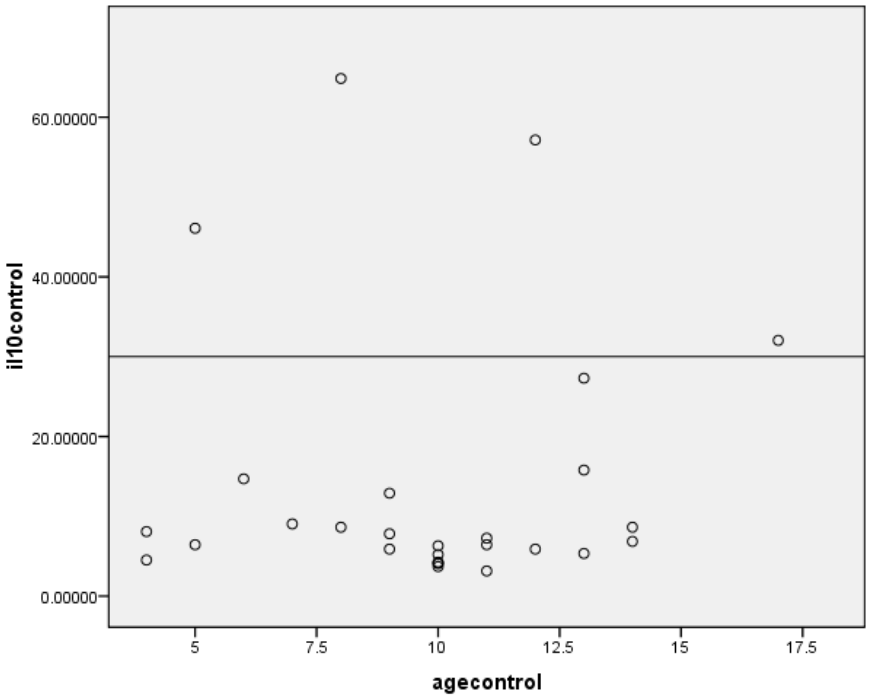


Figure (3-6):Correlation between IL-10 levels and age of Healthy control subjects.

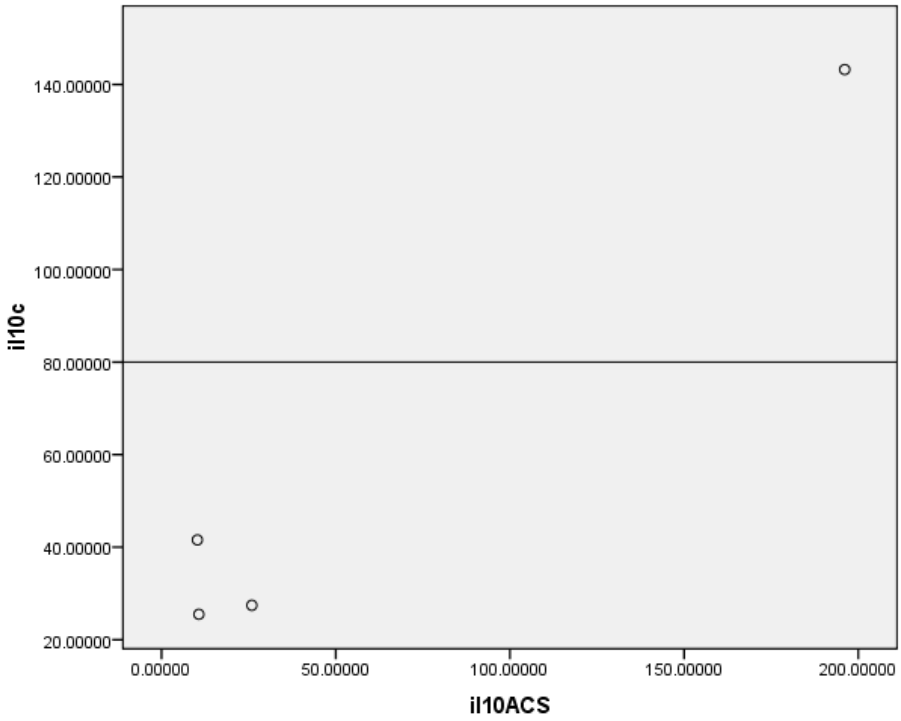


Figure (3-7): Correlation between IL-10 levels in SCD patients during ACS and SCA during VOC in general.

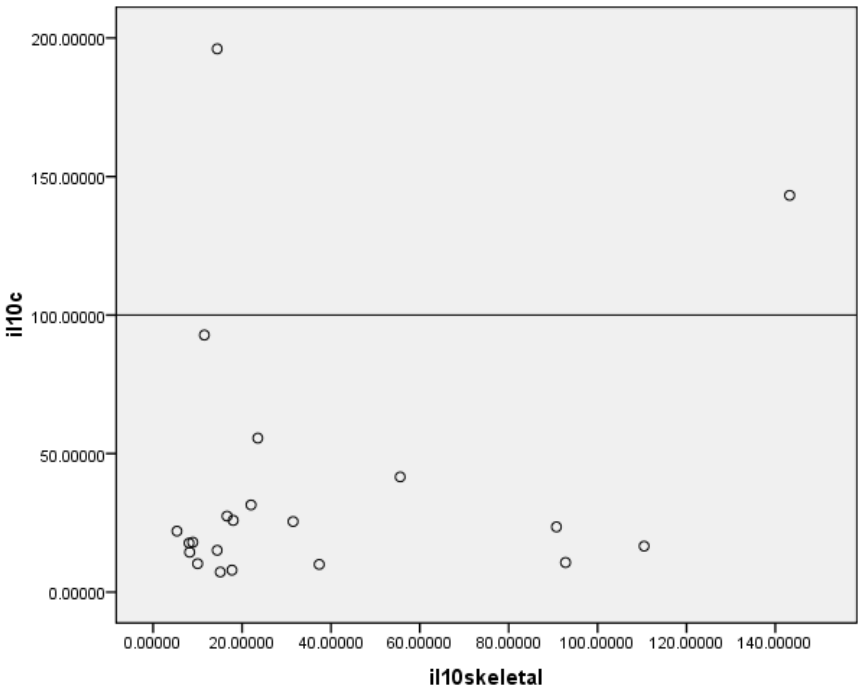


Figure (3-8): Correlation between IL-10 levels in SCD patients during musculoskeletal pain crisis and SCA during VOC in general.

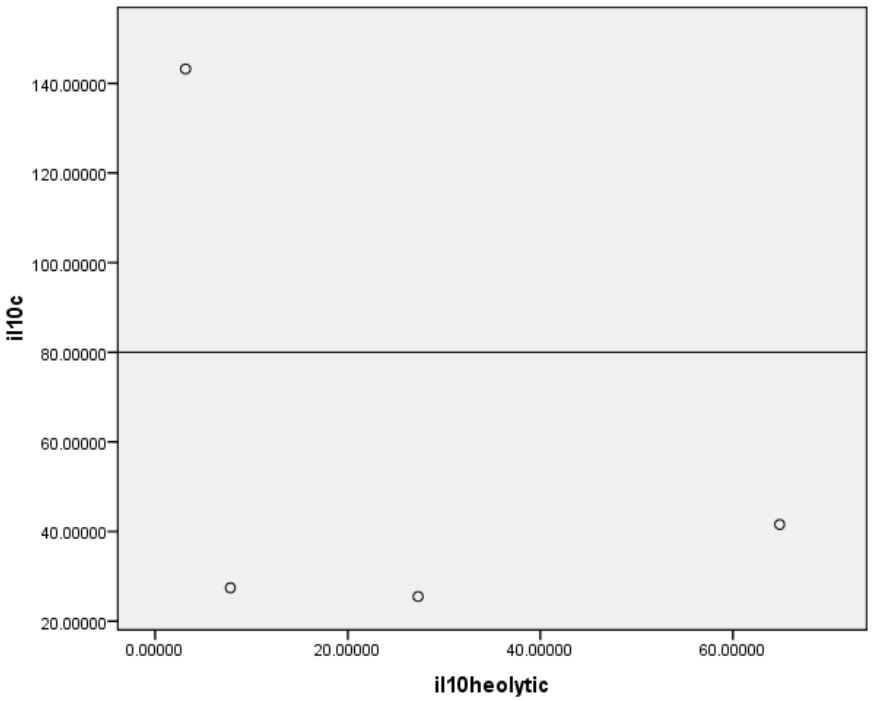


Figure (3-9): Correlation between IL-10 levels in SCD patients during hemolytic crisis and SCA during VOC in general.

(Table 3-4) *P value* and difference significance among study groups

	Significance	Degree of significance(by stars)	*P value*
Steady vs. VOC	Yes	**	0.003
Steady vs. Controls	No	0	0.811
VOC vs. Controls	Yes	**	0.007

Chapter One

Introduction and literature Review

Chapter four

Discussion, Conclusion and Recommendation

4.1.Discussion:

Mean± STD of IL-10 was higher in SCD patients during ACS crisis than SCD patients during musculoskeletal pain crisis and hemolytic crisis.

In correlation between IL-10 levels and age and gender, there was no clinically significant difference in IL-10 levels in different age groups and between male and female, *P values* were 0.409 and 0.514 respectively.

IL-10 levels were higher in male than female in steady state patients, VOC patients and control group but the difference was insignificant *P values were* (0.413, 0.715 and 0.485) respectively.

In correlation between IL-10 levels and clinical remarks of SCD patients during different VOC, musculoskeletal pain crises and hemolytic crisis there was no correlation (0.383 and 0.209) respectively, where there was positive correlation between IL-10 levels and acute chest syndrome *p value* (0.016).

Steady state patients had higher Hb levels than those in VOC and the difference was significant (*p value* = 0.000). These findings are supported by Conclaves *et al* and Siransy *et al* who demonstrated that significantly elevated Hb levels in steady state patients compared to VOC patients (Conclaves *et al., l2001;* Siransy *et al*., 2018).

IL-10 levels were found to be elevated SCD patient during VOC compared to both SCD patients during steady state and the healthy control group *P values* 0.003 and 0.007 respectively. This study result are in contrast to the result obtained from the study of Sarray *et al and Cavalcante* which showed significantly low levels of IL-10 in VOC subjects (Sarray *et al.*, 2015;Cavalcante, 2016).

IL-10 levels are higher in control group than SCD patients in steady state, but the difference was insignificant (P value 0.811), possibly due to two extreme values (64.85741 and 57.14748) and the two extreme values may be to un know underlying disease because the control are apparently healthy individuals and are not tested in this

study for other diseases that might affect IL-10 concentrations. This result consists with lanaro *et al* study in which IL-10 levels were not significantly different between the steady state group and the healthy control group (lanaro *et al*., 2009). In Veiga et al and Lanaro etal studies it was found that IL-10 levels were elevated in SCD patients in general (Veiga , *et al*., 2013; Lanaro *et al.*, 2006).

Data from studies have been conflicting, possibly as a result of the different study populations under investigation and the use of different methods for the detection of IL-10 in individuals with SCD. The current availability of more sensitive detection tools, however, probably explains the discrepancy between newer and older researches.

This study result maybe due to the anti-inflammatory role of IL-10 , which contribute to infections in SCD patients by reducing the production of pro-inflammatory cytokines and chemokines.

4.2. Conclusion

- IL10 levels are high in sickle cell patients during VOC than sickle cell patients in steady state and control group, and IL-10 levels are not significantly different between sickle cell in steady state and control group.
- There was no correlation between IL-10 levels and other risk factors, age, gender and Hb, but Hb levels were higher in SCD patients in steady state compared to SCD patients in VOC.

4.3. Recommendations:

-More studies with large sample size should be conducted to evaluate IL-10 levels in SCD patients.

-IL-10 levels should be measured and the effect of hydroxyurea should be determined in Sudanese SCD patients.

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Appendixes

Appendix (1)

Questionnaire

Sudan University of Sciences and Technology

College of Graduate studies

Evaluation of Interleukin-10 Level in Sudanese Sickle Cell Disease Patients During Vaso-occlusive Crisis and the Steady State Condition

تقييم مستوى المادة الخلوية المصلي 10عند المرضى السودانيين بالانيميا المنجلية في حالتي الازمة الوعائية و المستقرة

_Date: / /2018

_ID. Number:……..

_Age: …….Years

_Gender:

Male:{ } Female: { }

-Clinical remarks:
Fever……

pain
……

Acute chest
syndrome…….

Others…… If you specify
…….……

Laboratory Results

HB\b: …….

IL 10 result: ……

Appendix (2)

استمارة موافقة مشارك

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 وسوف تكون هذه المعلومات لغرض البحث العلمي فقط , وتتمتع بالسريه الكامله.

اسم المشارك والتوقيع:

.....

رقم تلفون المشارك.....

اسم الباحث والتوقيع:.....

رقم تلفون الباحث.....

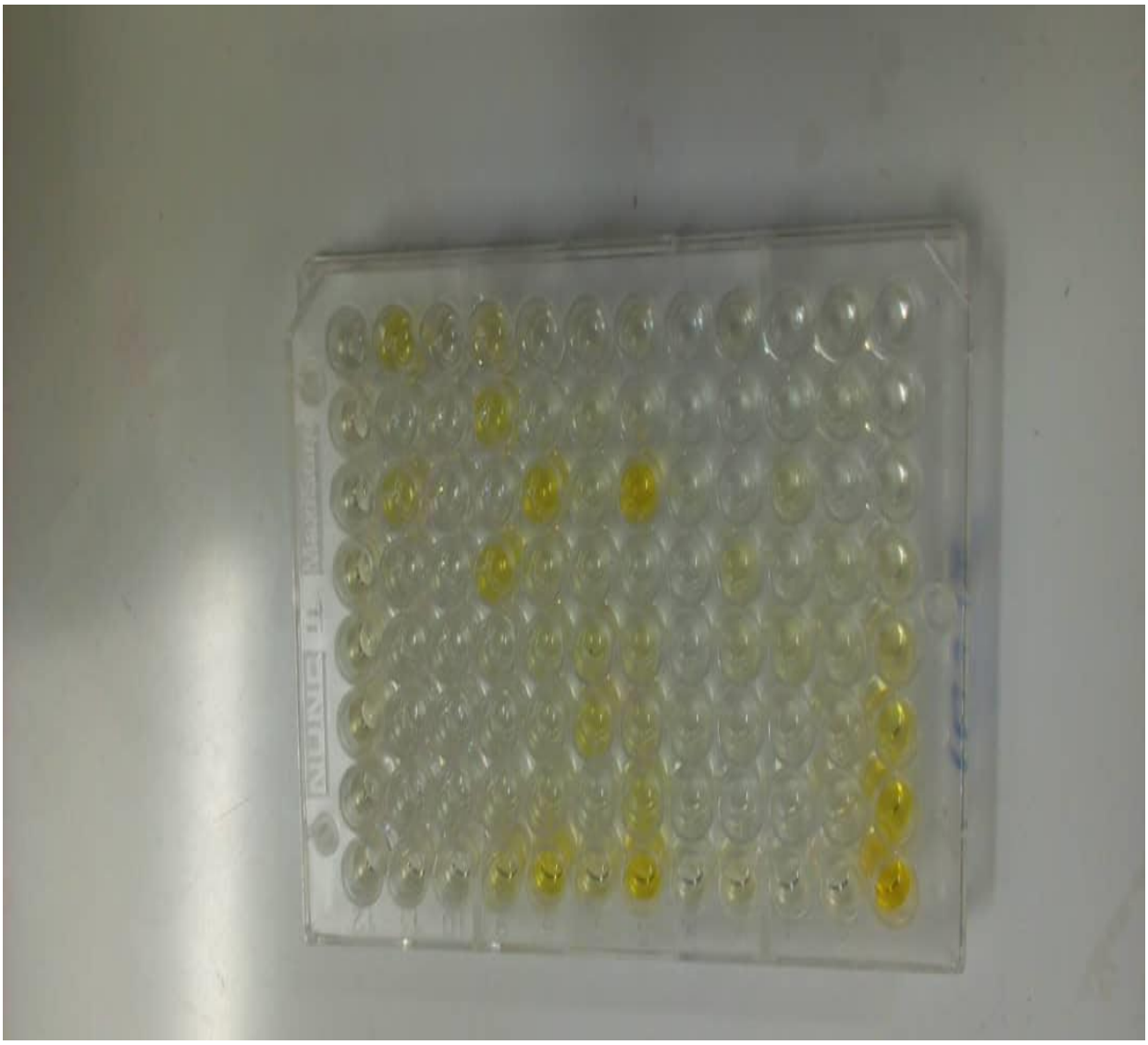
Appendex(3)

ELISA washer device.

Appendex (4)

ELISA Reader device

Appendix (5)



ELISA micro plate.

Appendix (6)

BioLegend

The path to legendary discovery™

Human IL-10

ELISA MAX™ Standard Sets

Cat. No. 430601 (5 plates)
430602 (10 plates)
430603 (20 plates)

BioLegend's ELISA MAX™ Standard Sets contain the capture and detection antibodies, recombinant protein standard, and Avidin-HRP required for the accurate quantification of natural and recombinant human IL-10. These sets are cost-effective and designed for experienced ELISA users. Optimization of reagent concentrations and assay conditions may be required.

It is highly recommended that the instruction sheet be read in its entirety before using this product. Use the recommended assay protocol, microwell plates, buffers, diluent, and substrate solution to obtain desired assay results. Do not use this set beyond the expiration date.

Materials Provided

1. Human IL-10 ELISA MAX™ Capture Antibody (200X)
2. Human IL-10 ELISAMAX™ Detection Antibody (200X)
3. Human IL-10 Standard
4. Avidin-HRP (1000X)
5. Lot-Specific Certificate of Analysis/ELISA MAX™ Standard Set Protocol

Introduction

Human IL-10 was originally described as Cytokine Synthesis Inhibitory Factor (CSIF) due to its ability to inhibit cytokine production by T_H1 clones. IL-10 is expressed in activated $CD8^+$ and $CD4^+$ T cells, activated monocytes, mast cells, and Ly-1 B cells. IL-10 shares over 80% sequence homology with the Epstein-Barr virus protein BCRFI. The functions of IL-10 include inhibition of macrophage-mediated cytokine synthesis and suppression of the delayed type hypersensitivity response.

Principle of the Test

BioLegend's ELISA MAX™ Standard Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human IL-10 specific rat monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-10 binds to the immobilized capture antibody. Next, a biotinylated rat monoclonal anti-human IL-10 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-10 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.

Materials to be Provided by the End-User

- Microwell plates: BioLegend Cat. No. 423501 is recommended.
- Plate Sealers: BioLegend Cat. No. 423601 is recommended.
- Phosphate-Buffered Saline (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 1.0 L, pH to 7.4.
- Coating Buffer: 8.4 g $NaHCO_3$, 3.56 g Na_2CO_3, add deionized water to 1.0 L, pH to 9.5 (BioLegend Cat. No. 421701 is recommended).
- Assay Diluent: 10% Fetal Bovine Serum or 1% BSA in PBS (BioLegend Cat. No. 421203 is recommended).
- Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
- TMB Substrate Solution: BioLegend Cat. No. 421101 is recommended.
- Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4
- 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
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Absorbent paper

Storage Information

- Store kit components between 2°C and 8°C.
- After reconstitution of the lyophilized standard with Assay Diluent, aliquot into polypropylene vials and store at -70°C. Do not repeatedly freeze/thaw the recombinant protein standard as loss of activity may occur.
- Prior to use, bring all components to room temperature (18°C-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/msds).
2. TMB substrate solution 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.

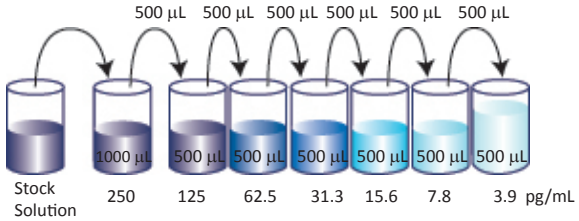
Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 X *g*. Remove serum layer and assay immediately or store serum samples at < -20°C.
Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.

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 and Sample Preparation

Do not mix reagents from different sets or lots. Avidin-HRP, Human IL-10 Standard, and/or antibodies from different manufacturers should not be used with this set. All reagents should be diluted immediately prior to use.

1. Dilute the pre-titrated Capture Antibody 1:200 in Coating Buffer. For one plate, dilute 60 µL Capture Antibody in 11.94 mL Coating Buffer.
2. Reconstitute the lyophilized standard with 0.2 mL of Assay Diluent, re-cap vial, and mix well. Allow the reconstituted standard to sit for 15 minutes at room temperature, then invert/vortex to mix.
3. Prior to use, prepare 1,000 µL of the top standard at a concentration of 250 pg/mL from stock solution in Assay Diluent (**refer to Lot-Specific Certificate of Analysis/ELISA MAX™ Standard Set Protocol**). Perform six two-fold serial dilutions of the 250 pg/mL top standard with Assay Diluent in separate tubes. After diluting, the human IL-10 standard concentrations are 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL 7.8 pg/mL, and 3.9 pg/mL, respectively. Assay Diluent serves as the zero standard (0 pg/mL).



4. Dilute the pre-titrated Biotinylated Detection Antibody 1:200 in Assay Diluent. For one plate, dilute 60 µL Detection Antibody in 11.94 mL Assay Diluent.
5. Dilute Avidin-HRP 1:1000 in Assay Diluent. For one plate, dilute 12 µL Avidin-HRP in 11.99 mL Assay Diluent.
6. Prepare all other reagents required for the assay including TMB Substrate Solution. Refer to reagent description in the section **"Materials to be Provided by the End-User"**.
7. Samples: For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. If dilutions are necessary, samples should be diluted in the corresponding cell culture medium.
   For other sample types, such as serum and plasma, optimization of reagent concentrations and assay conditions may be required.

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 Coating Buffer. 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 between 2°C and 8°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 Assay Diluent per well.
5. Seal plate and incubate at RT for 1 hour with shaking on a plate shaker(e.g. 500 rpm **with a 0.3 cm circular orbit). All subsequent incu**bation with shaking should be performed similarly.

6. While plate is being blocked, prepare standard dilutions and appropriate sample dilutions (if necessary).
7. Wash plate 4 times with Wash Buffer.
8. Add 100 µL/well of standard dilutions and samples to the appropriate wells. If needed, samples can be further diluted with Assay Diluent before adding 100 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 and incubate **in the dark for 30 minutes or until the desired color develops*.** 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 15
18. 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 optical linear ranges of ELISA plate readers.**

Assay Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, incubate overnight between 2°C and 8°C.

Day 2

1. Wash plate 4 times
2. Add 200 µL Assay Diluent to block, incubate at room temperature for 1 hour with shaking
3. Wash plate 4 times
4. Add diluted standards and samples to the appropriate wells, incubate at room temperature for 2 hours with shaking
5. Wash plate 4 times
6. Add 100 µL diluted Detection Antibody solution to each well, incubate at room temperature for 1 hour with shaking
7. Wash plate 4 times
8. Add 100 µL diluted Avidin-HRP solution to each well, incubate at room temperature for 30 minutes with shaking
9. Wash plate 5 times, soaking for 30 seconds to 1 minute per wash
10. Add 100 µL of TMB Substrate Solution to each well, incubate in the dark for 30 minutes or until the desired color develops
11. Add 100 µL Stop Solution to each well
12. Read absorbance at 450 nm and 570 nm

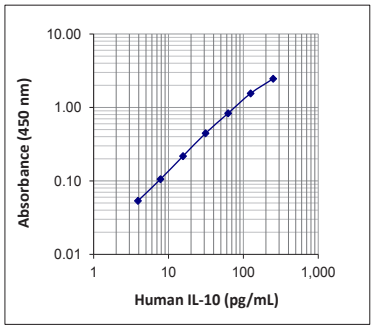
For more information about BioLegend ELISA MAX™ Sets and LEGEND MAX™ ELISA Kits with precoated plates, visit **www.biolegend.com.**

Calculation of Results

Plot the standard curve on log-log axis graph paper with cytokine concentration on the x-axis and absorbance on the y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine 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 cytokine 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 ranges, that test sample needs to be reanalyzed at a higher or lower dilution as appropriate.

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

Specificity: No cross reactivity was observed when this kit was used to analyze multiple human, mouse and rat recombinant proteins.

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 contained 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 inappropriate 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, Inc.

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