Chapter One

1.Introduction and Literature Review

1.1.Haemoglobin

The human haemoglobin is a tetramer molecule that consists of two pairs of identical polypeptide subunit, each encoded by different family of genes. The human α-like globulins genes (δ, α1, and α2) are located on chromosome 16, and the β-like globulin genes (ε, Gγ, Aγ, δ and β) are located on chromosome 11. Interestingly, the genes are present on both chromosomes in the same order in which they are expressed during development (Weatherall and Clegg, 2001).

During fetal live, the predominant type of haemoglobin is HbF (α2γ2). During the postnatal period, Hb F is gradually replaced by Hb A (α2 β2) and Hb A2 (α2 δ2). Upon completion of the switch from Hb F to Hb A, patients with disorders of the β-globin genes start manifesting the clinical features of their diseases which commonly occurs around six months of life (Weatherall and Clegg, 2001).

1.1.1.HbS structure

HbS results from a point mutation in the gene for the beta-globin chain of adult hemoglobin. In the deoxygenated form of HbS, the beta-6 valine becomes buried in a hydrophobic pocket on an adjacent beta-globin chain, joining the molecules together to form insoluble polymers (Bunn, 1997). In sufficient concentration, these insoluble polymers give rise to the classical sickle morphology. This process causes severe damage to the red cell membrane. Sickled red cells may then aggregate and go on to cause microvascular obstruction. Also, these abnormal red cells adhere to endothelial cells (Nagel and Platt, 2001).

1.2.Sickle cell disease (SCD)

Sickle cell disease is a generic name for a group of inherited blood disorders that have two features in common; presence of sickle-shaped erythrocytes (sickle cells) in the blood, and clinical illness (disease) caused by the sickle cells (Sergeant,

2001; Modell and Darlison,2008*).*The principal phenotypes are homozygous sickle cell disease (HbSS), Hb-S/Hb-c (HbSC), sickle cell-β0 thalassaemia (HbS β0), sickle cell-β+ thalassaemia (HbSβ+), HbSOArab and HbSD Punjab and HbSLepore Boston SCD (Serjeant 2001; Nagel *et al.*, 2003). It is important to note that the sickle cell trait (HbAS) is not included in the definition of SCD because the heterozygous individuals do not have clinical illness due to the presence of sickle haemoglobin (HbS). However, under extreme low oxygen tension sickle cell trait does change from the normal biconcave disc to a half-moon (or sickle) shape (Steinberg and Rodgers, 2001).

1.2.1.Clinical manifestation and complications of SCD

The fundamental pathological processes in SCD include blood vessel occlusion, chronic haemolysis and recurrent infections due to compromised immune system (Steinberg and Rodgers, 2001; Johnsto *et al.*, 1973). Vaso-occlusion leads to ischemia and infarction, with damage and reduced function in various tissues. Sickling of red blood cells causes haemolysis and anaemia. Recurrent sepsis causes fever, dehydration, acidosis, and leukocytosis; all of which predispose to erythrocyte sickling and obstruction of blood vessels (Steinberg and Rodgers, 2001).

The organs at greatest risk for injury are those with venous sinuses where blood flow is slow and oxygen tension and pH are low (spleen and bone marrow), or those with a limited terminal arterial blood supply (eye, head of the femur and humerus). No tissue or organ is spared from hypoxic injury, which may manifest as acute (e.g. painful events, acute chest syndrome), or insidious in onset (e.g. aseptic necrosis of the hips, sickle cell retinopathy). Acute and chronic tissue injury may ultimately result in end organ damage (e.g. kidney). Particularly at young ages, Sickle cell disease affects growth (Rodgers, 1997). Stroke is one of the most serious complications of the disease. Red cell adherence to the endothelium

mediated by Von Willebrand's protein may be important in arterial occlusive stroke in sickle cell disease. Von Willebrand's protein enhances adherence of sickle red cells to endothelial cells *(*Kaul *et al*., 1993*)*. This fact concurs with the observation that high rates of blood flow through the major cerebral arteries correlates with the risk of stroke *(*Adams *et al*., 1992*;*DeBaun *et al*.,1995*)*.

1.2.2.Genetics of SCD

The sickle gene is inherited from both parents who have either sickle cell disease, or carrier of sickle genes (Ballas, 2005).

The gene defect is a known mutation of a single nucleotide (single-nucleotide polymorphism - SNP) (A to T) of the β-globin gene, which results in glutamic acid being substituted by valine at position 6. Haemoglobin S with this mutation is referred to as HbS, as opposed to the normal adult HbA. The genetic disorder is due to the mutation of a single nucleotide, from a CTC to CAC codon on the template strand, which is transcribed into a GUG codon. This is normally a benign mutation, causing no apparent effects on the secondary, tertiary, or quaternary structure of haemoglobin in conditions of normal oxygen concentration. In low oxygen concentration, the deoxy form of haemoglobin exposes a hydrophobic patch on the protein between the E and F helices. The hydrophobic residues of the valine at position 6 of the beta chain in haemoglobin are able to associate with the hydrophobic patch, causing haemoglobin S molecules to aggregate and form fibrous precipitates (Allison, 2009).

Sickle-cell gene mutation probably arose spontaneously in different geographic areas, as suggested by restriction endonuclease analysis. These variants are known as Cameroon, Senegal, Benin, Bantu and Saudi-Asian. Their clinical importance springs from the fact that some of them are associated with higher HbF levels, e.g., Senegal and Saudi-Asian variants, are known to have milder disease (Green *et al*., 1993).

1.2.3.Epidemiology of SCD

Sickle cell disease is highly prevalent in sub-Saharan Africa, India and the Middle -East (WHO, 2010). Migration of substantial populations from these areas of high prevalence to Europe and North America has dramatically increased number of patients with SCD in Western communities. It has been estimated that, 70,000 or more Americans have sickle cell disease. Three quarters of sickle-cell cases occur in Africa. A recent reported from WHO estimated that around 2% of newborns in Nigeria were affected by sickle cell anaemia giving a total of 150.000affected children born every year in Nigeria alone. Recent estimate suggest more than 230.000 affected children are born in Africa every year (0.74%of the birth in sub-Saharan Africa), which is about 80% of the global total. By comparison the estimated affected births in North America are 2600 and 2300 in Europe. The carrier frequency ranges between 10% and 40% across equatorial Africa, decreasing to 1-2% on the North African coast and <1% in South Africa. More than 2.5 million Americans have sickle cell trait (WHO, 2010).

In Sudan, two foci of the disease have been described: Western Sudan, where a prevalence rate of up to 30.4% was reported among misseria of Darfur (Vella, 1964). A survey in Kordofan reported a prevalence rate of 18% in misseria humur tribe and showed that one in every 123 children born to this tribe is in danger of having the disease (lauder and Ibrahim). The other focus in the Blue Nile area, central Sudan reported a prevalence ranging from 0-5% among the indigenous population and up to 16% was found among the immigrant tribes from Western Sudan and West Africa in the area (Ahmed and Baker, 1986) reported that sickle cell hemoglobin and beta thalassemia are the two major features responsible for haemoglobinopathies described in the capital Khartoum but prevalence is not yet reported (Travis and Susan, 2000).

1.2.4.Pathophysiology of SCD

The loss of red blood cell elasticity is central to the pathphysiology of sickle cell disease. Normal red blood cells are quite elastic, which allows the cells to deform and pass through capillaries. In sickle cell disease, low - oxygen tension promotes red blood cell sickling and a repeated episode of sickling damage the cell membrane and decreases the cells elasticity. Sickle erythrocytes are more rigid than normal red cells, and because they fail to return to normal shape when normal oxygen tension is restored; are more readily destroyed by the reticulo-endothelial system (Platt, 2000).

The classical paradigm of sickle cell pathophysiology endothelium has always considered (Hb S) polymerization and red cell sickling as the primary causative factors of the acute and chronic complications associated with disease (Hebbelm, 1991). However, emerging evidence indicates initial events in vaso-occlusion may involve complex array of factors, both polymerization dependent and polymerization independent (Embury, 2004; Kato *et al*., 2009; Frenette, 2002). The current model of vaso-occlusion in SCD views the process as a result of interaction of erythrocytes, leucocytes, platelets, plasma proteins and vascular Leucocytes indirectly enhance vaso-occlusion in SCD by activating vascular endothelium. Monocytes activate vascular endothelium by secreting the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-A) and interleukin -1b (Belcher *et al*., 2000).Activated endothelial cells increase the expression of adhesion molecules on leucocytes and erythrocytes, and facilitate adherence of circulating blood cells to the vessel wall (Schwartz *et al*., 1985).

1.3.Role of platelet in vaso-occlusion and hypercoagulability in SCD

Hypercoagulable or prothrombotic state is thought to be one of major factor that contribute to vaso-occlusion in SCD (Stuart and Setty, 2001; Ataga and Orringer, 2003). Factors underlie the hypercoagulable state in SCD include increased

thrombin generation (Francis, 1989;Tomer *et al*., 2001), decreased natural anticoagulant proteins (Wright *et al*., 1997; Westerman *et al*., 1999), increased tissue factor procoagulant activity (Key *et al*., 1998; Mohan *et al*., 2005) and increased platelet count and activity (Wun *et al*., 1997; Tomer *et al*., 2001; Blann *et al*., 2003). Steady state platelet activation manifests by elevated levels of activation markers such as surface and plasma P-selectin (CD62P) and CD40L (Wun *et al*., 1997; Inwald *et al*., 2000; Tomer *et al*.,2001). In addition, thrombospondin, platelet factor 4 and β-thromboglobulin are elevated in steady state (Tomer *et al*., 2001; Lee *et al*., 2006). Studies showed that platelet activation accelerates further during vaso-occlusive crisis (Wun *et al*.,1997; Stuart and Setty, 2001).

Several pieces of data provide evidence for the relationship between serine phosphor-glycerides externalization in RBC, decreased bio-availability of NO due to chronic haemolysis and platelet activation in SCD (Setty *et al*., 2000; Setty *et al*., 2001; Villagra *et al*., 2007).

1.4.Blood cell membrane fatty acids and SCD

Adhesion, aggregation, elasticity of blood cells and inflammatory response are strongly modulated by cell membrane omega-3 and omega-6 fatty acids (Setty *et al*., 2002). This structure and function relationship of blood cells suggest sickle cell patients may have membrane lipid perturbation. Indeed, there is evidence that steady state patients with SCD have abnormal erythrocytes, platelets and mononuclear cell fatty acid composition. Pan cellular membrane lipid abnormalities, including high omega-6 low omega- 3 and an imbalance between the two fatty acid families occur in sickle cell disease (Okpala and Heanyi, 2006). These lipid abnormalities are more severe in patients with disease complications and in those with a greater degree of anaemia (Daak,2011; Daak, 2012).

1.5.Biomedical importance of n-6 arachidonic acid

The n-6 fatty acid AA is present in all biological membranes and represents up to 15%of the total fatty acids in phospholipids. Studies have shown that, low AA in maternal and cord blood is associated with low birth weight, reduced head circumference (Crawford *et al*., 1989; Leaf *et al*., 1992), intrauterine growth retardation (Vilbergsson *et al*., 1994) and impaired growth in preterm infants (Carlson *et al*., 1993). Biochemically , AA is a precursor of very active metabolites particularly prostaglandins, thromboxanes, leukotrienes and lipoxins. The eicosanoids from AA are biologically active in very small quantities and if they are formed in large amounts, they contribute to the inflammatory state, allergic reactions, particularly in susceptible people and formation of thrombus. Thus high n-6/n-3 ration may shift the physiological state to one that is prothrombotic and pro-aggregatory, with increases in blood viscosity, vasospasm, and vasoconstriction (Ferrucci *et al*., 2006; Simopoulos, 2009).

1.6.Biomedical importance of EPA and DHA n-3 fatty acids

EPA and DHA fatty acids have effects on diverse physiological processes impacting normal health and chronic diseases (Benatti *et al*., 2004). Such as the regulation of genes function and metabolism (Sampath and Ntambi,2005), cardiovascular and immune function (Cottin *et al*., 2011). Neuronal and visual development and functions (Haag, 2003). It is widely known that Omega-3 fatty acids are precursors of strong anti-inflammatory and anti-thrombotic bioactive molecules, prostaglandins, resolving and protections (Hark *et al*.,2005).

1.7.Coagulation

Coagulation (also known as clotting) is the process by which blood changes from a liquid to a gel. It potentially results in hemostasis, the cessation of blood loss from a damaged vessel, followed by repair. The mechanism of coagulation involve activation, adhesion, and aggregation of platelets along with deposition and

maturation of fibrin. Disorders of coagulation are disease states which can result in bleeding (hemorrhage or bruising) or obstructive clotting (thrombosis) (Lillicrap *et al*., 2009).

Coagulation is highly conserved throughout biology; in all mammals, coagulation involves both a cellular (platelet) and a protein (coagulation factor) component(Michelson, 2006).The system in humans has been the most extensively researched and is the best understood (Schmaier *et al*., 2011).

Coagulation begins almost instantly after an injury to the blood vessel has damaged the endothelium lining the vessel. Exposure of blood to the space under the endothelium initiates two processes: changes in platelets, and the exposure of sub-endothilial tissue factor to plasma Factor VII, which ultimately leads to fibrin formation. Platelets immediately form a plug at the site of injury; this is called primary hemostasis. Secondary hemostasis occurs simultaneously: Additional coagulation factors or clotting factors beyond Factor VII (listed below) respond in a complex cascade to form fibrin strands, which strengthen the platelet plug as seen in (figure 1.1)(Furie *et al*., 2005).

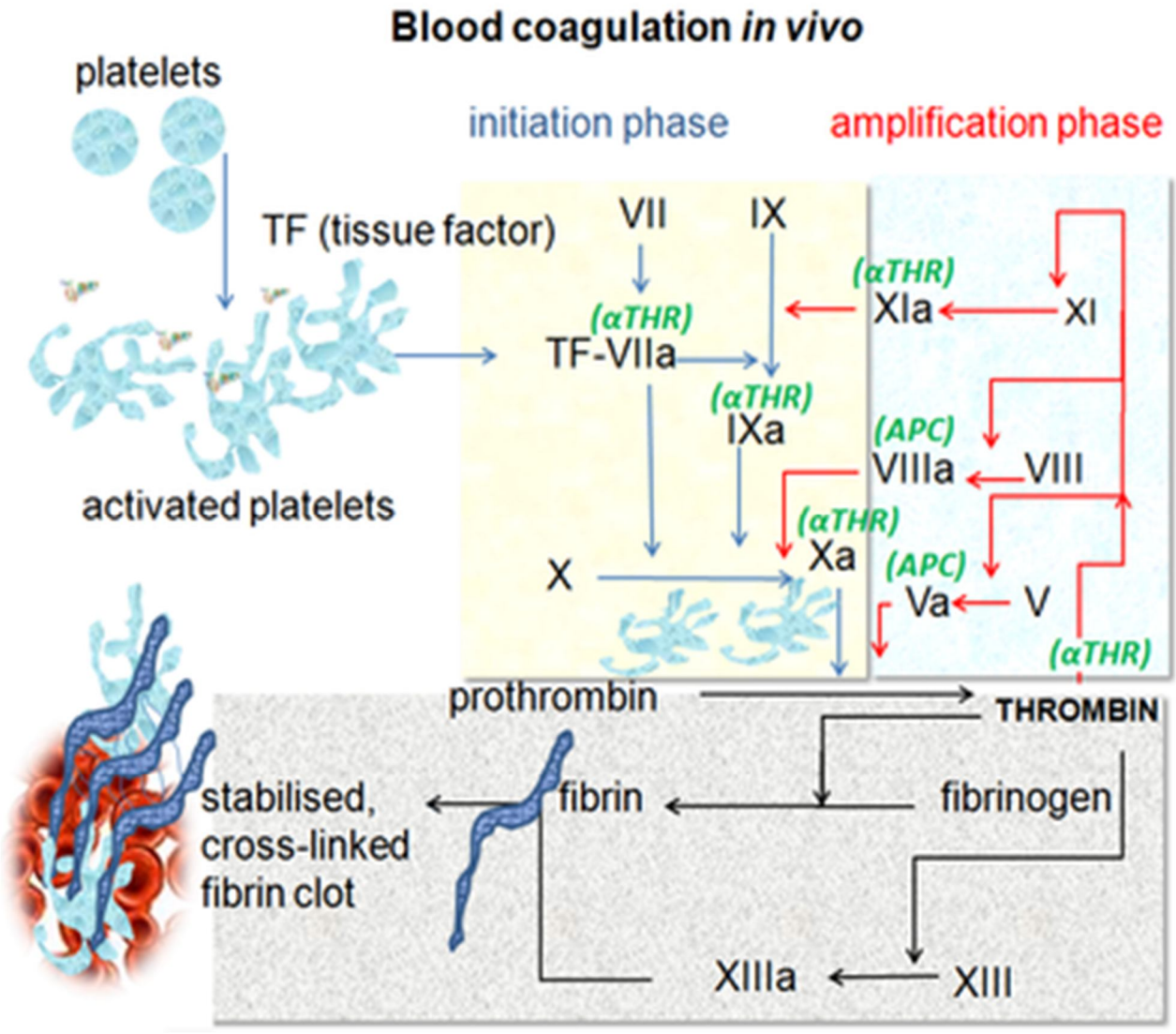

Figure1.1: Blood coagulation pathways in vivo showing the central role played by thrombin (Furie *et al*., 2005).

1.7.1.Platelet activation

When the endothelium is damaged, the normally isolated, underlying collagen is exposed to circulating platelets, which bind directly to collagen with collagen-specific glycoprotein Ia/II a surface receptors. This adhesion is strengthened further by von Willebrand factor (VWF), which is released from the endothelium and from platelets; VWF forms additional links between the platelets' glycoprotein Ib/IX/V and the collagen fibrils. This localization of platelets to the extracellular matrix promotes collagen interaction with platelet glycoprotein VI. Binding of collagen to glycoprotein VI triggers a signaling cascade that results in activation of platelet integrins. Activated integrins mediate tight binding of platelets to extracellular matrix. This process a adheres platelets to the site of injury (Key *et al*., 2009).

Activated platelets release the contents of stored granules into the blood plasma. The granules include ADP, serotonin, platelet-activating factor (PAF), VWF, platelet factor 4, and thromboxane A_2 (TXA_2), which, in turn, activate additional platelets. The granules' contents activate a G_q-linked protein receptor cascade, resulting in increased calcium concentration in the platelets' cytosol. The calcium activates protein kinase C, which, in turn, activates phospholipase A_2 (PLA_2). PLA_2 then modifies the integrin membrane glycoprotein IIb/IIIa, increasing its affinity to bind fibrinogen. The activated platelets change shape from spherical to stellate, and the fibrinogen cross-links with glycoprotein IIb/IIIa aid in aggregation of adjacent platelets (completing primary hemostasis) (Pallister *et al*., 2010).

1.7.2.Tissue factor pathway (extrinsic)

The main role of the tissue factor pathway is to generate a "thrombin burst", a process by which thrombin, the most important constituent of the coagulation cascade in terms of its feedback activation roles, is released very rapidly. FVIIa

circulates in a higher amount than any other activated coagulation factor. The process includes the following steps (Pallister *et al*., 2010).

Following damage to the blood vessel, FVII leaves the circulation and comes into contact with tissue factor (TF) expressed on tissue-factor-bearing cells (stromal fibroblasts and leukocytes), forming an activated complex (TF-FVIIa).

TF-FVIIa activates FIX and FX.

FVII is itself activated by thrombin, FXIa, FXII and FXa.

The activation of FX (to form FXa) by TF-FVIIa is almost immediately inhibited by tissue factor pathway inhibitor (TFPI).

FXa and its co-factor FVa form the prothrombinase complex, which activates prothrombin to thrombin.

Thrombin then activates other components of the coagulation cascade, including FV and FVIII (which activates FXI, which, in turn, activates FIX), and activates and releases FVIII from being bound to VWF.

FVIIIa is the co-factor of FIXa, and together they form the "tenase" complex, which activates FX; and so the cycle continues. ("Tenase" is a contraction of "ten" and the suffix "-ase" used for enzymes.)

1.7.3.Contact activation pathway (intrinsic)

The contact activation pathway begins with formation of the primary complex on collagen by high-molecular-weight kininogen (HMWK), prekallikrein, and FXII (Hageman factor). Prekallikrein is converted to kallikrein and FXII becomes FXIIa. FXIIa converts FXI into FXIa. Factor XIa activates FIX, which with its co-factor FVIIIa form the tenase complex, which activates FX to FXa. The minor role that the contact activation pathway has in initiating clot formation can be illustrated by the fact that patients with severe deficiencies of FXII, HMWK, and prekallikrein do not have a bleeding disorder. Instead, contact activation system seems to be more involved in inflammation (Pallister *et al*., 2010).

1.7.4.Final common pathway

The division of coagulation in two pathways is mainly artificial, it originates from laboratory tests in which clotting times were measured after the clotting was initiated by glass (intrinsic pathway) or by thromboplastin (a mix of tissue factor and phospholipids). In fact thrombin is present from the very beginning, already when platelets are making the plug. Thrombin has a large array of functions, not only the conversion of fibrinogen to fibrin, the building block of a hemostatic plug. In addition, it is the most important platelet activator and on top of that it activates Factors VIII and V and their inhibitor proteinC (in the presence of thrombomodulin), and it activates Factor XIII, which forms covalent bonds that crosslink the fibrin polymers that form from activated monomers (Pallister *et al.*, 2010).

Following activation by the contact factor or tissue factor pathways, the coagulation cascade is maintained in a prothrombotic state by the continued activation of FVIII and FIX to form the tenase complex, until it is down-regulated by the anticoagulant pathways (Pallister *et al.*,2010)(Figure 1.2).

1.7.5.Coagulation cascade cofactors

Various substances are required for the proper functioning of the coagulation cascade:

Calcium and phospholipid (a platelet membrane constituent) are required for the tenase and prothrombinase complexes to function. Calcium mediates the binding of the complexes via the terminal gamma-carboxy residues on FXa and FIXa to the phospholipid surfaces expressed by platelets, as well as procoagulant microparticles or microvesicles shed from them. Calcium is also required at other points in the coagulation cascade.

Vitamin K is an essential factor to a hepatic gamma-glutamyl carboxylase that adds a carboxyl group to glutamic acid residues on factors II, VII, IX and X, as

well as Protein S, Protein C and Protein Z. In adding the gamma-carboxyl group to glutamate residues on the immature clotting factors Vitamin K is itself oxidized. Another enzyme, Vitamin K epoxide reductase, (VKORC) reduces vitamin K back to its active form. Vitamin K epoxide reductase is pharmacologically important as a target of anticoagulant drugs warfarin and related coumarins such as acenocoumarol, phenprocoumon, and dicumarol. These drugs create a deficiency of reduced vitamin K by blocking VKORC, thereby inhibiting maturation of clotting factors. Vitamin K deficiency from other causes (e.g., in mal absorption) or impaired vitamin K metabolism in disease (e.g., in liver failure) lead to the formation of PIVKAs (proteins formed in vitamin K absence) which are partially or totally non-gamma carboxylated, affecting the coagulation factors' ability to bind to phospholipid (Pallister *et al.*, 2010).

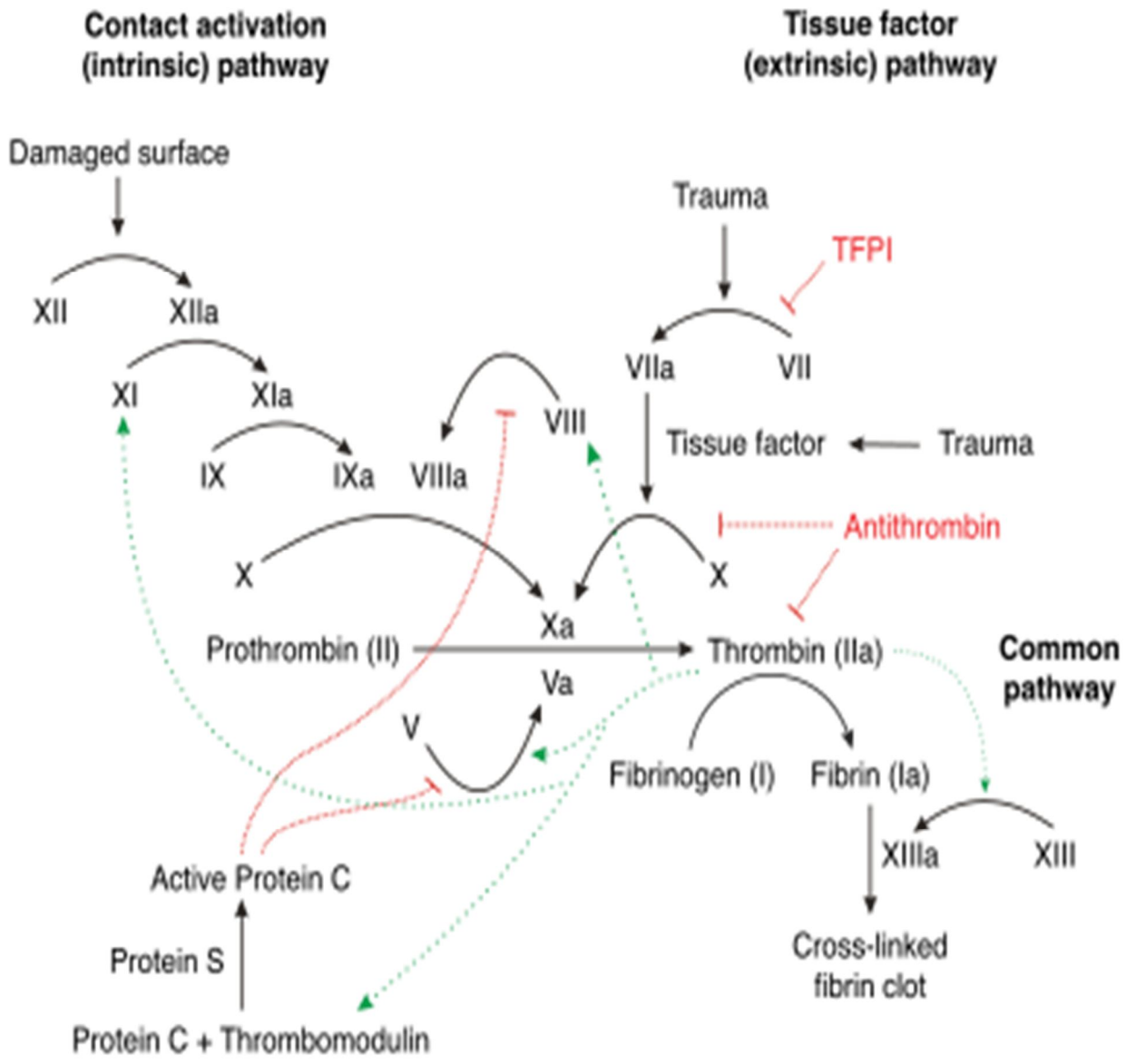

Figure1.2:Coagulation with arrows for negative and positive feedback (Pallister *et al.*,2010).

1.7.6.Protein S

Protein S (also known as S-Protein) is a vitamin-dependent plasma glycoprotein synthesized in the endothelium. In the circulation, Protein S exists in two forms: a free form and a complex form bound to complement protein C4b-binding protein (C4BP). In humans, protein S is encoded by the PROS1gene (Lundwall *et al*., 1986; Long *et al*., 1988).

The characterized function of Protein S is its role in the anti-coagulation pathway, where it functions as a cofactor to Protein C in the inactivation of Factors Va and VIIIa. Only the free form has cofactor activity (Castoldi *et al*., 2008).

Protein S can bind to negatively charged phospholipids via the carboxylated GLA domain. This property allows Protein S to function in the removal of cells which are undergoing apoptosis. Apoptosis is a form of cell death that is used by the body to remove unwanted or damaged cells from tissues. Cells which are apoptotic (i.e. in the process of apoptosis) no longer actively manage the distribution of phospholipids in their outer membrane and hence begin to display negatively charged phospholipids, such as phosphatidyl serine, on the cell surface. In healthy cells, an ATP (Adenosine triphosphate)-dependent enzyme removes these from the outer leaflet of the cell membrane. These negatively charged phospholipids are recognized by phagocytes such as macrophages. Protein S can bind to the negatively charged phospholipids and function as a bridging molecule between the apoptotic cell and the phagocyte. The bridging property of Protein S enhances the phagocytosis of the apoptotic cell, allowing it to be removed 'cleanly' without any symptoms of tissue damage such as inflammation occurring.

Protein S also binds to the nascent complement complex C5,6,7 and prevents this complex from inserting into a membrane. This function prevents the inappropriate activation of the complement system, which would cause uncontrolled systemic inflammation. In fact, Protein S was first discovered in 1977 in this role and it is

named after the membrane site that it occupies in the complex (Eckhard *et al*., 1977).

1.7.7.Protein C

Protein C, also known as auto prothrombin IIA and blood coagulation factor XIV (Mather *et al*., 1996; Hall *et al*., 1999). is a zymogen, the activated form of which plays an important role in regulating anticoagulation, inflammation, cell death, and maintaining the permeability of blood vessel walls in humans and other animals. Activated protein C (APC) performs these operations primarily by proteolytically inactivating proteins Factor Va and Factor VIIIa. APC is classified as a serine protease as it contains a residue of serine in its active site (Nicolaes *et al*., 2003).In humans, protein C is encoded by the *PROC* gene, which is found on chromosome 2 (Foster *et al*., 1985).

The zymogenic form of protein C is a vitamin K-dependent glycoproteinthat circulates in blood plasma. Its structure is that of a two-chain polypeptide consisting of a light chain and a heavy chain connected by a disulfide bond (Foster *et al*., 1985). The protein C zymogen is activated when it binds to thrombin, another protein heavily involved in coagulation, and protein C's activation is greatly promoted by the presence of thrombomodulin and endothelial protein C receptors (EPCRs). Because of EPCR's role, activated protein C is found primarily near endothelial cells (i.e., those that make up the walls of blood vessels), and it is these cells and leukocytes (white blood cells) that APC affects (Nicolaes *et al*., 2003; Mosnier *et al*., 2007). Because of the crucial role that protein C plays as an anticoagulant, those with deficiencies in protein C, or some kind of resistance to APC, suffer from a significantly increased risk of forming dangerous blood clots (thrombosis).

Research into the clinical use of activated protein C also known as drotrecoginalfa-activated (branded Xigris) has been surrounded by controversy. The manufacturer

Eli Lilly and Company ran an aggressive marketing campaign to promote its use in people with severe sepsis and septic shock including the sponsoring of the 2004 Surviving Sepsis Campaign Guidelines (Eichacker *et al*., 2006).In (2011)Cochrane review however found that its use cannot be recommended as it does not improve survival (and increases bleeding risk). (Carvajal *et al*., 2011).

1.7.8.D-dimer

D-dimer (or D dimer) is a fibrin degradation product (or FDP), a small protein fragment present in the blood after a blood clot is degraded by fibrinolysis. It is so named because it contains two cross linked D fragments of the fibrin protein as shown in figure 1.3 (Adam *et al*., 2009).

D-dimer concentration may be determined by a blood test to help diagnose thrombosis. Since its introduction in the 1990s, it has become an important test performed in patients with suspected thrombotic disorders. While a negative result practically rules out thrombosis, a positive result can indicate thrombosis but does not rule out other potential causes. Its main use, therefore, is to exclude thromboembolic disease where the probability is low. In addition, it is used in the diagnosis of the blood disorder disseminated intravascular coagulation (Adam *et al*., 2009).

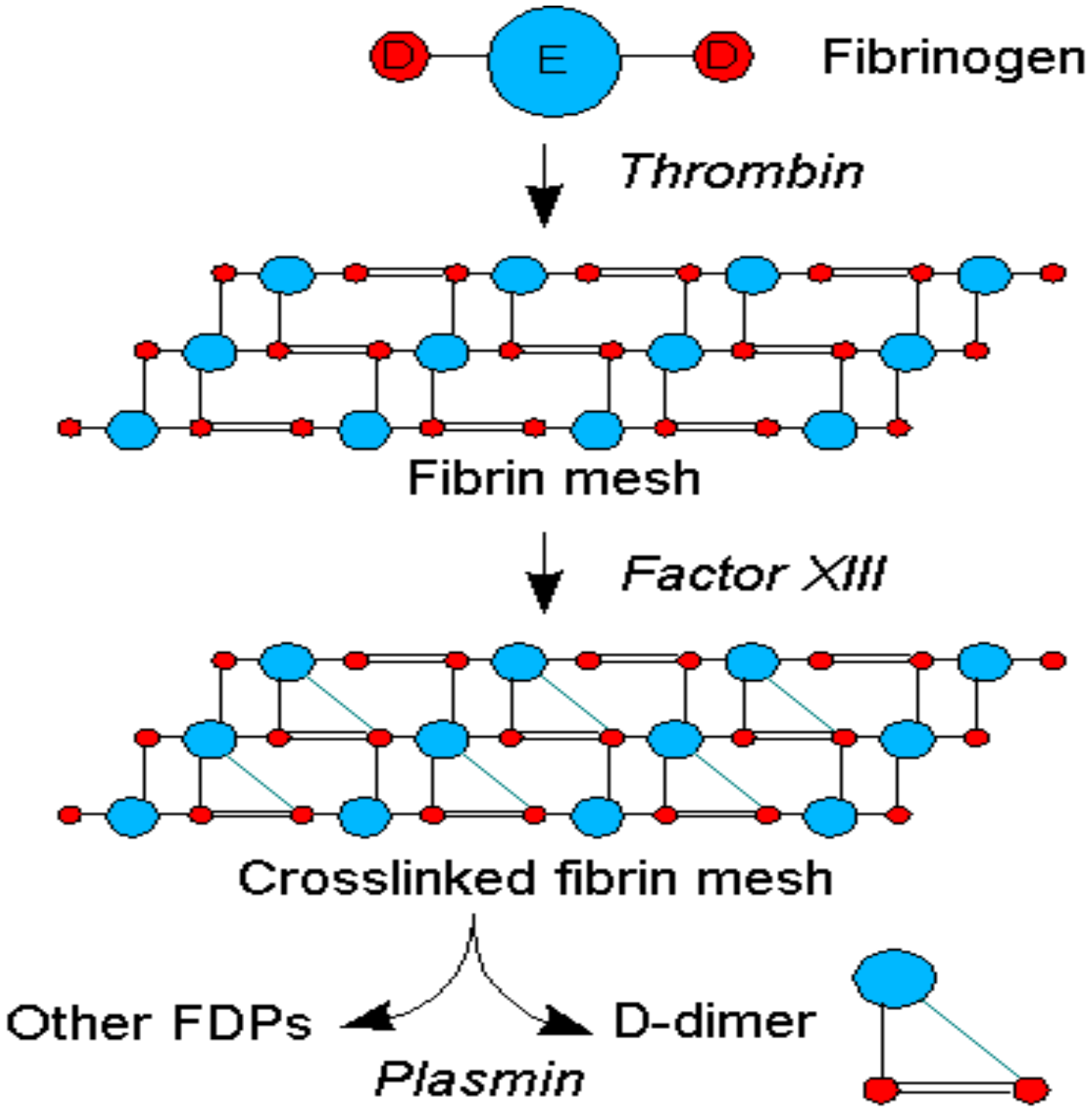

Figure 1.3: Principles of D-dimer testing (Adam *et al.*, 2009).

1.7.8.1.Principles of D-dimer testing

Coagulation, the formation of a blood clot or thrombus, occurs when the proteins of the coagulation cascade are activated, either by contact with damaged blood vessel wall (intrinsic pathway) or by activation of factor VII by tissue activating factors. Both pathways lead to the generation of thrombin, an enzyme that turns the soluble blood protein fibrinogen into fibrin, which aggregates into proteofibrils. Another thrombin-generated enzyme, factor XIII, then cross links the fibrin proteofibrils at the D fragment site, leading to the formation of an insoluble gel which serves as a scaffold for blood clot formation (Adam *et al*., 2009).

The circulating enzyme plasmin, the main enzyme of fibrinolysis, cleaves the fibrin gel in a number of places. The resultant fragments, "high molecular weight polymers", are digested several times more by plasmin to lead to intermediate and then to small polymers (fibrin degradation products or FDPs). The cross-link between two D fragments remains intact, however, and these are exposed on the surface when the fibrin fragments are sufficiently digested. The typical D-dimer containing fragment contains two D domains and one E domain of the original fibrinogen molecule (Adam *et al*., 2009).

D-dimers are not normally present in human blood plasma, except when the coagulation system has been activated, for instance because of the presence of thrombosis or disseminated intravascular coagulation. The D-dimer assay depends on the binding of a monoclonal antibody to a particular epitome on the D-dimer fragment. Several detection kits are commercially available; all of them rely on a different monoclonal antibody against D-dimer. For some of these, the area of the D-dimer to which the antibody binds is known. The binding of the antibody is then measured quantitatively by one of various laboratory methods (Adam *et al*., 2009).

1.8.Rationale

Randomized placebo control studies have proved that omega-3 fatty acid is effective treatment for patients with SCD (Daak *et al*., 2012). However, the mechanism of action (s) is not well elucidated. Importantly, in a pilot study investigated the effect of omega-3 fatty acids on co-agulation factors some improvements were reported. However, the sample size was small (n=10) and the duration of the time of supplementation was short (Tomer *et al*., 2001). It is widely known that prothrombotic state is one of the major patho-physiologyical factors of the disease, and omeg-3 fatty acids are known precursors of potent anti-inflammatory and anti-thrombotic bioactive factors. Hence, it is plausible to postulate that the observed beneficial effect of omega-3 fatty supplementation in SCD might be partially a reflection of amelioration of SCD associated hyper-coagulable state.

1.9.Objectives

1.9.1.General objective

The aim of this study was to assess the effect of long term supplementation with omega-3 fatty acids on the coagulation parameters and blood counts in patients with homozygous sickle cell disease.

1.9.2.Specific objectives

1. To measure the coagulation parameters (PT, PTT, D- Dimer, Protein C, and Protein S) inomega-3 supplemented and non-supplemented SCD patients.
2. To measure the blood counts (Hb, HCT, RBCs, WBCs, MCV, MCH, MCHC) in omega-3 supplemented and non-supplemented SCD patients.
3. To compare blood counts and coagulation parameters between HbSS-omega-3 treated, HbSS-omega-3 untreated, HSS-Hydroxyurea treated and HbAA-healthy controls.

1.10.Alternative Hypothesis

Long term supplementation with omega-3 fatty acid has effect on:

a) Coagulation parameters.
b) Blood counts.

1.11.Null hypothesis

Long term supplementation with omega-3 fatty acid has no effect on:

a) Coagulation parameters.
b) Blood counts.

Chapter Two

2.Materials and methods

2.1.Study design

This study was a comparative, analytical case control study.

2.2.Study population and area

This study included a total of 156 participants, including homozygous sickle cell disease patients (HbSS); omega-3supplemented (n=44); non-supplemented controls(n=52);sickle cell disease patients HbSS treated with hydroxurea (8); and healthy volunteers as controls (n=52); the patients were from a SCD referral clinic to the Biochemistry Department, Faculty of Medicine, University of Khartoum, Khartoum State, Sudan.

The patients on omega-3 fatty acids in this study (n=44) were part of the active arm of the randomized control trial (RCT) carried by the main supervisor, and received a daily dosage of one, two, three or four capsules according to the patient's age (< 5), (5-10) , (11-16) and (>17) years, respectively. The omega-3 capsule contains 277.8 mg docosahexaenoic acid (DHA) and 39.0 mg eicosapentnoic acid (EPA) and (1.5 mg) of Vitamin E to prevent fatty acids peroxidation. The omega-3 fatty acid group in this study received the same dosage of omega-3 fatty acids for two years after the end of the RCT. The hydroxyurea groups were treated daily with a dose of 20 mg per kilogram body weight orally for one year. All of the HbSS patients were on regular folate supplement before and during the study period. The healthy control subjects did not receive folate or any other nutritional supplement, they were selected conveniently. The study patients and controls were matched by age and gender.

2.3.Inclusion criteria

Only steady state patients (HbSS) and healthy controls (HbAA) were enrolled in this study."Steady state" is defined as being free from acute painful crisis or other

medical condition for at least one month prior to the study. The healthy controls were mostly siblings of the patients to ensure matching in ethnic and socioeconomic status. Hemoglobin phenotypes were confirmed using cellulose acetate electrophoresis at pH 8.5.

2.4.Exclusion criteria

Patients in sickle cell crisis or with other chronic disease, blood transfused during last two months, patients on anti-coagulant treatment and pregnant women were excluded from the study. Healthy people receiving vitamin, folate or any other nutritional supplement were excluded from the study.

2.5.Sample size calculation and sampling technique

Sample size was calculated according to the following equation:

The equation ($n = (Z\alpha/2 + Z\beta)\ 2\ *2*\sigma2 / d2$) was employed to calculate the sample size, and the assumption that the mean difference of D-dimer between the omega-3 treated and untreated patients 1µg/ml compared to untreated. The population variance was assumed to equal to 2µg/ml. To detect the assumed mean difference with 95% power at a 5% significance level, minimum of 43 patients is required in each arm.

2.6.Data collection tools

A pre-designed, researcher- administered questionnaire was used to collect the demographic and clinical data (Appendix One).

2.7.Blood sample collection

Following disinfection by alcohol swab of anti-cubic area, five ml of venous blood were took from each patient; 2.5ml were collected in tri-sodium citrate anticoagulant container to obtained plasma for (PT , APTT , D- Dimer , protein C and protein S) and another 2.5ml were collected in EDTA anticoagulant container for blood count.

2.8.Biochemical measurements

-Blood counts were determined by automated hematology cell counter (Sysmex Kx-21N, Japan).

-Prothrombin time (PT) and activated partial thromboplastin time (APTT) were estimated by an automatic coagulometer (DiaMed-CD2, Switzerland).

-Protein C and Protein S were estimated by enzyme linked immune-sorbent assay **(**ELISA**)**(ASYS model Expert Plus, Type G020150, Austria).

-D-Dimer was estimated by a fluorescence immunoassay (Boditech Med Inc. IChroma reader, Version 2014-04-18).

2.9.Methodology

2.9.1.Complete blood count

Sysmex is an automated hematology cell counter that is based on the size and shape of the cells (electrical resistance principle) which depends on the fact that blood cells are non-conductive to electricity, so when they pass through an electrical field they will increase the electrical impedance (resistance).

The counter has two chambers; one for red blood cell and platelet counting and sizing, while the other chamber is for WBCs counting and sizing according to their size of particles they can be discriminated into cell types or populations.

2.9.2.The coagulometer

The automatic coagulometer (DiaMed-CD2) is an instrument for the determination of the main parameters used in the plasma coagulation methods.

Theory and principle

The coagulometer has an optical measurement system which detects a sudden variation in optical density when a clot is formed. The chronometer and the stirring system are activated by a sudden change of the time measurement when the sample is added to the reagent and stop the measurement time at the moment that clot is formed. The continuous mixing guarantees a perfect homogeniziation and makes

the measurement possible of low concentrations of fibrinogen by grouping the fibrin filaments in the center of the optical pass. The system has programmable security time during which variations in optical density, when the reagent and the plasma are still in the homogenization phase, cannot activate the detection cell.

2.9.2.1.Prothrombin time (PT)

Principle

The test measures the clotting time of plasma in the presence and optimal concentration of tissue extract (thromboplastin) and indicates the overall efficiency of the extrinsic clotting system.

Although originally thought to measure prothrombin, the test is now known to depend on reactions with factors V, V11, and X and on the fibrinogen concentration of the plasma

Reagents

- Platelets poor plasma (ppp) from patient and control (Obtained by centrifugation of citrate anticoagulant blood on 3000rpm for 15 min)
- Commercially prepared thromboplastin (containing tissue factor and phospholipids).
- Calcium chloride ($CaCl_2$) as starter for activation of PT.

Procedure

1. The PT reagent was incubated to 37C^o for 10 minutes to maintain the suspension of the reagent by magnetic stirring.
2. 0.1 ml of test or control plasma was pipette into a test cuvette.
3. Incubated at 37C^o for 1 min.
4. 0.2 ml of the pre-incubated PT reagent was rapidly added, simultaneously the timer was started.
5. Clotting time was recorded in seconds.

Expression of results

The results were expressed as the mean of the duplicate readings in seconds or as the ratio of the mean patient's plasma time to the mean normal control plasma time or as an international normalized ratio.

Normal values

The normal value of PT is between (11- 16) Seconds.

2.9.2.2.Activate partial thromboplastin time (APTT)

Principle

The test measures the clotting time of plasma after activation of contact factor without addition of tissue thromboplastin, and indicates the overall efficiency of intrinsic and common pathway.

Reagents

- Platelets poor plasma (PPP) from patient and control. (Obtained by centrifugation of citrate anticoagulant blood on 3000rpm for 15 min).
- Kaolin cephaline (phospholipids) reagent for APTT.
- Calcium chloride ($CaCl_2$) as starter for activation of APTT.

Procedure

1- The calcium chloride was incubated to 37Cº for 10 min.

2- 0.1ml of test or control plasma was pipette into a test cuvette and then Incubated at 37Cº for 1 to 2 min.

3- 0.1ml of the APTT reagent was added to the cuvette containing the plasma and maintained the suspension of the reagent by magnetic stirring. Then incubated mixture at 37Cº for 3 min.

4- 0.1ml of the pre- incubated calcium chloride was rapidly added and simultaneously the timer starts.

5- The clotting time was record in seconds.

Normal values

The normal values for APTT ranged between (26 – 40)Sec.

2.9.3.Protein C and S were measured by Elisa

Principle

The protein C or S is a sandwich ELISA using micro plates coated with a capture antibody specific for human protein C or S. 1: 5diluted patient plasma is incubated in the wells allowing protein C or S present in the plasma to bind to the antibody. The unbound fraction is removed by washing. Afterwards anti-human protein C or S detection antibody conjugated to horseradish peroxidase (conjugate) is incubated and reacts with the antigen-antibody complex on the micro well surface. Following incubation, unbound conjugate is washed off. Addition of TMB-substrate generates an enzymatic colorimetric (blue) reaction, which is stopped by diluted acid (color changes to yellow). The rate of color formation from the chromogen is measured in optical density units with a spectrophotometer at 450 nm. Using a curve prepared from the reference plasma provided with the kit, the protein C or S antigen relative percent concentration in patient plasma can determined.

Reagents

- Platelets poor plasma (PPP) from patient and control (Collected with 3.8 % sodium citrate as an anticoagulant centrifuge samples on 3000 rpm for 15 min).
- Sample buffer.
- Wash buffer.
- Reference plasma.
- Control N.
- Control D.
- Conjugate.
- TMB substrate sample buffer.
- Stop solution.

- Microtiter plate.

Procedure

- 0.1ml of each patient diluted plasma in to the designated micro wells.
- 0.1ml of each working dilution of the reference plasma and the diluted controls were added in to the designated wells. Then the plate was incubated for 30 minutes at (25Cº).
- Plate was washed 3× with 0.3 ml washing buffer (diluted 1:50).
- 0.1ml conjugate in to each well.
- Incubated for 30 minutes at (25Cº).
- Plate was washed 3× with 0.3 ml washing buffer (diluted 1:50).
- 0.1ml TMB substrate in to each well.
- Incubated for 30 minutes at (25Cº), protected from intense light.
- 0.1ml stop solution into each well, using the same order as pipetting the substrate.
- Incubated 5 min.
- Plate agitated carefully for 5 sec.
- Absorbance read at 450 nm (optionally 450-620nm) within 30minutes.

Normal values

A normal value of protein C is between (70% -140 %).

A normal value of protein S is between (60% -150 %).

2.9.4.IChroma reader for estimation of D-Dimer

Ichroma D-dimer along with the Ichroma reader is a fluorescence immunoassay that quantifies the total D-dimer concentration in plasma. The test is used as an aid in the post therapeutic evaluation of thromboembolic disease patients.

Principle

The test uses the sandwich immune detection method. Such that the detection antibody in buffer binds to D-dimer in the plasma sample and antigen-antibody

complexes are captured by antibodies that have been immunobilized on the test strip as sample mixture migrates through nitrocellulose matrix. The more D-dimer antigen in the plasma, the more antigen-antibody complexes is accumulated on the test strip. Signal intensity of fluorescence on detection antibody reflects amount of antigen captured and is processed by Ichroma reader to show D-dimer concentration in the specimen. The working range of Ichroma D-dimer test is 50 - 10.000ng/m

Reagents

Platelets poor plasma (PPP) from patient and control (Collected with 3.8 % sodium citrate as an anticoagulant centrifuge samples on 3000rpm for 15 min).

Sealed test cartridges.

ID chip.

Package inserts.

Detection buffer tubes.

Procedure

1- 0.01ml of serum/plasma/control sample was transferred using a transfer pipette to a tube containing the detection buffer.

2- The lid of the detection buffer tube was closed and the sample was mixed thoroughly by shaking it 10times. The sample mixture was used immediately.

3- 0.075 ml of a sample mixture was pipette out and dispensed it into the sample well on the test cartridge.

4- The sample was Left to load in test cartridge at room temperature for 12 min.

5- For scanning inserted in to the test cartridge holder of the Ichroma reader. Proper orientation of the test cartridge was ensured before pushing it all the way inside the test cartridge holder. An arrow has been marked on the test cartridge especially for this purpose.

6- Select button was pressed on the Ichroma reader to start the scanning process.
7- Ichroma Reader started scanning the sample-loaded test cartridge immediately.
8- The test result was read on the display screen of the Ichroma reader.

Normal value

Up to 500ng/ml

2.10.Data analysis and presentation

The data were expressed as mean ± sd or median and interquartile rage (IQR) as pertinent. The groups were compared for blood counts, coagulation parameters, anticoagulant proteins and D-dimer levels by using one-way analysis of variance (ANOVA).When statistical differences were indicated, Bonferroni pair wise multiple comparison post-hoc tests were performed. The statistical significance was assumed at a “p” value of less than 0.05. The data were analyzed with SPSS for Windows, Version 23 (SPSS Ltd., Surrey, UK).

2.11.Ethical consideration

This study was a part of Sickle Cell Disease Project, Department of Medical Biochemistry, Faculty of Medicine, and University of Khartoum. The Research Ethics Committee of the Faculty of Medicine, University of Khartoum approved the study and REC of Sudan University for Sciences approved the study. All participants or their guardians provided informed written consent.

Chapter Three

3.Results

3.1.Demographic data

This study included a total of 156 participants. Out of them, the number of sickle cell disease HbSS omega-3 treated subjects cases (44), sickle cell disease HbSS hydroxurea treated cases (8), sickle cell disease HbSS untreated patients (52) and healthy HbAA controls (52) (Table 3.1).

Gender distribution of sickle cell disease HbSS omega-3 treated patients in (M/F) ratio was (1/1) cases, sickle cell disease HbSS hydroxurea treated was (3/1) cases, sickle cell disease HbSS untreated was (1.2/1) cases and healthy HbAA controls was (1.2/1) (Table 3.1).

The mean of age in the sickle cell disease HbSS omega-3 treated, sickle cell disease HbSS untreated, sickle cell disease HbSS hydroxyurea treated, and healthy HBAA, was 9.8 ±2.9,10.8 ± 4.0,17.4 ± 0.9, and 11.3 ± 4.0, respectively (Table 3.1).

3.2.Comparison of coagulation parameters in study groups (Tables 3-2).

Omega-3supplemented group had significant decrease compared with non-supplemented group, in plasma PT (17.2±1.8 versus 31.3±11.1 sec, P< 0.001), plasma PTT (38.3±4.2 versus 57.0±11.3sec, P< 0.001), INR (1.2±0.14 versus 2.3±0.9, P< 0.001), and PLTs count (489.5±121.1versus 533.6±98.7×10^3/µL, P= 0.031). However, there was no statistically significant reduced between omega-3 treated group compared with healthy controls in plasma PT (17.2±1.8 versus 14.5±0.7 sec, P= 0.05), plasma PTT (38.3±4.2 versus 37.0±3.8 sec, P=0.388), INR (1.2±0.14 versus 1.01±0.05, P= 0.071) and PLTs count(489.5±121.1versus 330.3±72.5×10^3/µL, P< 0.001).

The hydroxyurea treated group had significant decrease compared with non-supplemented group in plasma PT (18.5±1.8 versus 31.3±11.1 sec, P< 0.001),

plasma PTT(42.8±2.7 versus 57.0±11.3 sec, P< 0.001), INR (1.3±0.12 versus 2.3±0.9, P< 0.001) and PLTs count(414.5±109.9 versus 533.6±98×10^3/µL, P= 0.002). Nevertheless, there was no statistically significant reduced between the hydroxyurea treated group and healthy controls in plasma PT (18.2±1.8 versus 14.5±0.7 sec, P=0.139), plasma PTT (42.8±2.7 versus 37.0±3.8 sec, P= 0.05), INR (1.3±0.12 versus 1.01±0.05, P=0.182) and PLTs count (414.5±109.9versus 330.3±72.5×10^3/µL, P=0.026).(Table 3-2).

3.3.Comparison of blood count in study groups (Tables 3-3).

omega-3 supplemented group and non-supplemented had insignificantly different levels in Hb(7.56±1.62 versus 7.59±1.03g/l, P=0.999),HCT (22.9±4.7 versus 22.9±3.0%, P=0.941),RBCs count (2.8±0.7 versus 2.7± 2.7±0.5 x 10^6/µl, P=0.510),WBCs count (12.4±4.3versus13.3±3.4×10^3/µL,P=0.094),MCV(84.3±8.6 versus 86.5±7.1fl, P=0.197), MCH (27.9±3.4versus 28.6±3.0pg, P=0.244) and MCHC (33.3±2.0 versus 33.1±1.4 g/dl, P=0.567),but a significant difference was found when omega-3 supplemented group compared with healthy controls in Hb (7.56±1.62 versus 12.60±1.09 g/l, P< 0.001), HCT (22.9±4.7 versus 38.0±3.5 %, P< 0.001),RBCs count (2.8±0.7 versus 4.6±0.5×10^6/µL, P< 0.001)and WBCs count (12.4±4.3 versus 6.3±1.3×10^3/µL, P<0.001). Moreover, there was no statistically significant difference between the two groups for MCV (84.3±8.6 versus 82.3±4.7 fl, P=0.202),MCH (27.9±3.4 versus 27.6±1.9 pg, P=0.580) and MCHC (33.3±2.0 versus 33.5±1.5 g/dl, P= 0.627).

There were no significant differences between hydroxyurea treated groups when compared with non-supplemented group in Hb(7.95±1.03 versus 7.59±1.03 g/l, P=0.478), HCT (23.8±2.8 versus 22.9±3.0%, P=0.533),RBCs count(2.8±0.6 versus 2.7±0.5×10^6/µL, P=0.729),WBCs count(13.6±3.7 versus 13.3±3.4×10^3/µL, P=0.980),MCV(87.8±11.6versus86.5±7.fl,P=0.535),MCH(29.5±4.4versus 28.6±3.0pg, P=0.403) and MCHC (33.4±0.9 versus 33.1±1.4g/dl, P=0.618), were

encountered. However, there a significant difference when hydroxyurea treated groups was compared with healthy controls in Hb(7.95±1.03 versus 12.60±1.09g/l, P< 0.0001),HCT (23.8±2.8 versus 38.0±3.5 %, P< 0.001), RBCs count (2.8±0.6 versus 4.6±0.5,$\times 10^6$/µL p< 0.001) and WBCs count (13.6±3.7 versus 6.3±1.3$\times 10^3$/µL, P< 0.001), Furthermore, there was no statistically significant difference when hydroxyurea treated compared with healthy controls in MCV (87.8±11.6 versus 82.3±4.7fl, P=0.046),MCH (27.5±4.4 versus 27.6±1.9pg, P=0.079) and MCHC (33.4±0.9 versus 33.5±1.5 g/dl, P=0.942).(Table 3.3).

3.4.Comparison of D-dimer level in study groups (Figure 3.1).

The healthy control subjects had a lower level of plasma D-dimer concentration than the omega-3 fatty acid treated, HU-treated and un-treated patients, (p<0.001). The n-3 fatty acid treated group compared with the HU treated (Median =1.14 (IQR=0.74) µg/mL versus median= 2.33 (IQR=3.17) µg/mL, (p<0.001)) and untreated (median =1.14 (IQR=0.74) µg/mL versus median= 1.75(IQR=1.16) µg/mL, (p<0.001)). Omega-3 treated patients had a lower plasma D-dimer level. Patients treated with HU had a higher levels of plasma D-dimer compared to HbSS untreated, (P=0.002).(Figure 3-1).

3.5.Comparison of Protein-S level in study groups (Figure 3.2).

Plasma protein S concentration was lower in the treated (n-3 and HU) and untreated patients than in the healthy controls (median =139.5 (IQR=28.0) µg/mL, (p<0.001)). There was no difference in protein S level between the untreated (median =42.5 (IQR=18.0) µg/mL), omega-3 treated (median =45.5 (IQR=14.0) µg/mL) and HU-treated patients (median =40.5 (IQR=11) µg/mL), (p=0.394). (Figure3.2).

3.6.Comparison of Protein-C level in study groups (Figure 3.3).

Plasma protein C concentration was lower in the treated (omega-3 and HU) and untreated patients than in the healthy controls (median =90.5 (IQR=20.0) µg/mL, ($p<0.001$)). The omega-3 treated (median =60.5 (IQR=19.0) µg/mL) and HU-treated (median =59.5 (IQR=13) µg/mL) patients ($p=0.229$), treated and the un treated (median =60.0(IQR=23.0) µg/mL) patients had comparable concentration of protein C, ($p=0.367$).(Figure3.3).

Table 3.1:Demographic characteristics of the patients and healthy controls

		HbSS-omega-3 treated group	**HbSS-omega-3 untreated group**	**HbSS-HU treated group**	**HbAA-Healthy control group**
Number of patients (*n*)		44	52	8	52
	Male	22 (50%)	28 (53.8%)	6 (75%)	28 (53.8%)
	Female	22 (50%)	24 (46.2%)	2 (25%)	24 (46.2%)
Age years, (mean± SD)		9.8 ± 2.9	10.8 ± 4.0	17.4± 0.9	11.3± 4.0

Table 3.2:Comparison of coagulation parameters in the study groups

	HbSS-omega-3 treated (Mean±SD)	**HbSS- untreated (Mean±SD)**	**HbSS-HU treated (Mean±SD)**	**HbAA- Healthy controls (Mean±SD)**
PT (sec)	$17.2\pm1.8^{\dagger\dagger\dagger}$	$31.3\pm11.1^{+++}$	18.2 ± 1.8^{xxx}	14.5±0.7
PTT (sec)	$38.3\pm4.2^{\dagger\dagger\dagger}$	$57.0\pm11.3^{+++}$	42.8 ± 2.7^{xxx}	37.0±3.8
PLT	$489.5\pm121.1^{\dagger\dagger\dagger}$	$533.6\pm98.7^{+++}$	414.5 ± 109.9^{xxx}	330.3±72.5
INR	$1.2\pm0.14^{\dagger\dagger\dagger}$	$2.3\pm0.90^{+++}$	1.3 ± 0.12^{xxx}	1.01±0.05

*No significant difference was found between HbSS-HU treated versus HbSS-omega-3 treated

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HbSS-omega-3 treated versus HbSS- untreated: †p<0.05, ††p<0.01,†††p<0.001

HbSS-omega-3 treated versus healthy controls: *p<0.05, **p<0.01, ***p<0.001

HbSS-untreated versus healthy controls: $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$

HbSS-HU treated versus HbSS-omega-3untreated:$^{x}p<0.05$,$^{xx}p<0.01$,$^{xxx}p<0.001$

Table 3.3:Comparison of blood count in the study groups

	HbSS-omega-3 treated (Mean±SD)	**HbSS-untreated (Mean±SD)**	**HbSS-HU treated (Mean±SD)**	**HbAA-Healthy controls (Mean±SD)**
Hb (g/l)	7.56±1.62	$7.59\pm1.03^{+++}$	7.95±1.03	12.6±1.09***
HCT (%)	22.9±4.7	$22.9\pm3.0^{+++}$	23.8±2.8	38.0±3.5***
RBC	2.8±0.7	$2.7\pm0.5^{+++}$	2.8±0.6	4.6±0.5***
MCV (fl)	84.3±8.6	86.5±7.1	87.8±11.6	82.3±4.7
MCH (pg)	27.9±3.4	28.6±3.0	29.5±4.4	27.6±1.9
MCHC (g/dl)	33.3±2.0	33.1±1.4	33.4±0.9	33.5±1.5
WBC	12.4±4.3	$13.3\pm3.4^{+++}$	13.6±3.7	6.3±1.3***

*No significant difference was found between HbSS-HU treated versus HbSS-omega-3 treated

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HbSS-omega-3 treated versus HbSS- untreated: †p<0.05, ††p<0.01, †††p<0.001

HbSS-omega-3 treated versus healthy controls: *p<0.05, **p<0.01, ***p<0.001

HbSS-untreated versus healthy controls: +p<0.05, ++p<0.01, +++p<0.001

HbSS-HU treated versus HbSS-untreated: ^{x}p<0.05, ^{xx}p<0.01, ^{xxx}p<0.001

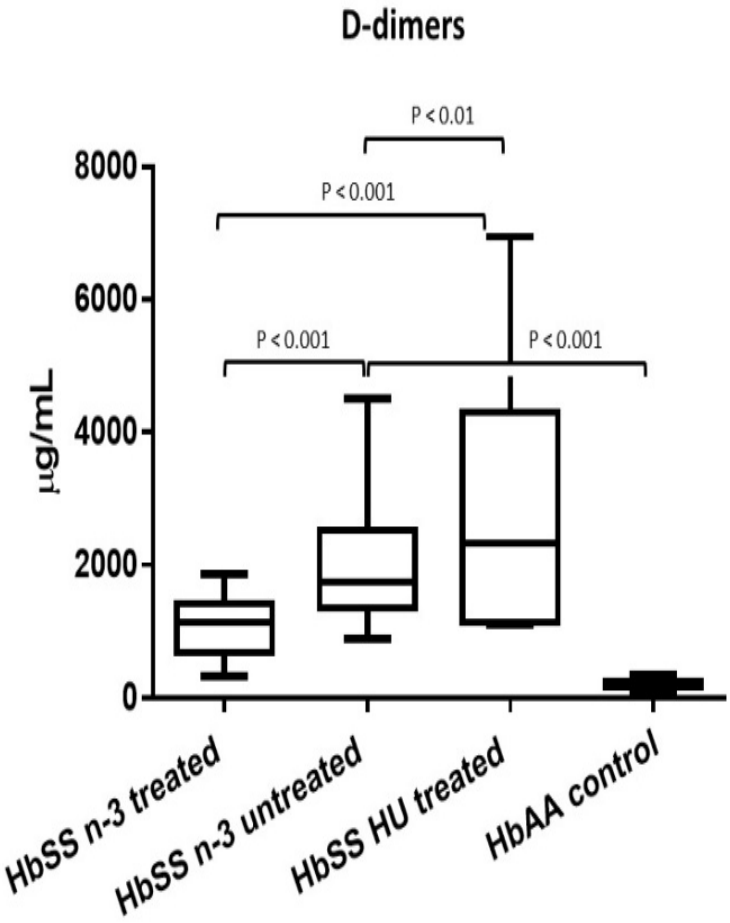

Figure 3.1:Comparison of D-dimer level in the study groups.

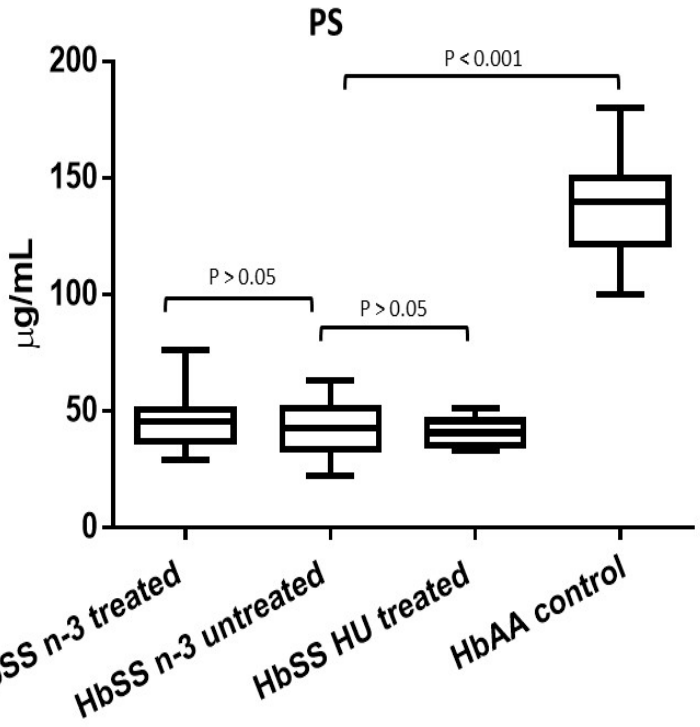

Figure3.2:Comparison of Protein-S level in the study groups.

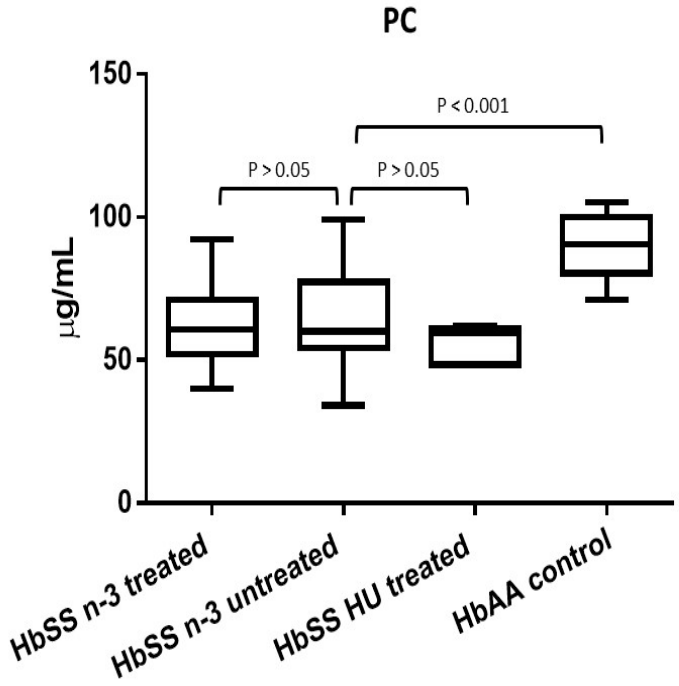

Figure3.3:Comparison of Protein-C level in the study groups.

Chapter Four

4.Discussion, conclusion and recommendation

4.1Discussion

Hydroxyurea (HU), which is a cytotoxic, antimetabolic and antineoplastic agent, is the only disease-modifying therapy approved for sickle cell disease (McGann and Ware, 2011). Hydroxyurea has been shown to be partially effective in reducing the frequency of vaso-occlusive events; but, there is no evidence that it prevents organ damage (Wang *et al.*, 2011). One of the factors which restricts HU usage is that it undergoes renal clearance and hence there is a need for careful dose adjustment and close monitoring of myelotoxicity in individuals with renal impairment (Yan *et al.*, 2005). This vital requirement is hardly possible to undertake in most middle and low income countries where SCD is highly prevalent because of lack of functional facilities and expertise. Therefore, there is a need for safe, effective and easily manageable treatment(s) for children and adult patients with sickle cell disease. Clinical trials have provided evidence that n-3 fatty acids are effective in reducing frequency and severity of vaso-occlusive episodes, severe anemia, blood transfusion rate, markers of inflammation and oxidative stress (Daak *et al.*, 2012; Okpala *et al* .,2011).

This is the first case control study in Sudan to have performed the effect of supplementation with omega-3 fatty acids for at least two years on coagulation parameters in patients with homozygous sickle cell disease, the cases subject (HbSSomega-3treated and hydroxyurea treated) and controls subjects (HbSS untreated and HbAA healthy volunteers). Cases and controls were broadly matched for age and gender. It was interestingly found that the coagulation profile (PT, PTT, INR and PLT) were significantly decreased in the omega-3 treated group when compared with untreated group. Nonetheless, the same parameters were insignificantly reduced in the omega-3 treated group when compared with healthy

controls. Also there was significant decrease in (PT, PTT, INR and PLT) among hydroxyurea treated group when compared with untreated. Moreover, the same parameters were insignificantly reduced among hydroxyurea treated group when compared with healthy controls. Similar findings have been reported in American (Raffini *et al*., 2006), Nigerian children(Chinawa *et al*., 2013) and Jamaican adults (Wright *et al*., 1997) with sickle cell disease. In contrast, another study comprising of 17 subjects did not find a difference in mean PT between SCD and healthy children (Bayazit and Kilinc, 2001). In the latter study it is possible that the difference between the groups might have been obscured by the small sample size.

The mechanism behind the prolongation of PT in children with SCD is not fully understood. It is suggested that impaired liver function (Raffini *et al.*, 2006) and depletion of coagulation factors (De Franceschi *et al*., 2011) play a role in the prolongation process. However, it is worth pointing out that a relationship between an abnormal liver function and coagulation prolongation is yet to be unequivocally established.

In contrast to the findings of the current study, a high intake n-3 fatty acid did not have a significant effect on PT and APTT in adult patients with sickle cell disease (Tomer *et al*., 2001). The subjects in the latter study were adults of different ethnic background with normal coagulation parameter values at baseline. In addition, the n-3 fatty acid composition of the oil/supplement used in the two studies was different from the high DHA capsules given to the children in the current study. The observed normalization of coagulation parameters in treated SCD patients could be the result of increased availability of the coagulation factors and the possible concomitant reduction in coagulation activation that could surpass its potential detrimental hypo-coagulant effect (Vanschoonbeek *et al*., 2004; Nieuwenhuys *et al*., 2001). HU treatment had a similar effect as n-3 fatty acids on

PT and APTT suggesting that children with abnormal coagulation profile responsive to either therapy.

In blood counts (Hb, HCT, RBC, MCV, MCH, MCHC, WBC) no significant differences found between omega-3 treated and untreated, and also similar results when compared with healthy controls in (MCV, MCH, MCHC) on the other hand(Hb, HCT, RBC, WBC) were significantly reduced in omega-3 treated group and untreated group compared with healthy control. The same result was observed between hydroxyurea treated group when compared with healthy controls.

There is evidence indicates that HU mediates its beneficial effects in SCD, partially, by lowering leukocyte, reticulocyte and platelet counts (Ballas *et al*., 1999). In the current study, HU treatment reduced platelet count significantly but had no noticeable effect on WBCs. This unexpected effect of HU on WBC among Sudanese children with SCD might be a reflection of the fact that HU is being administered to the most severely ill children with SCD (Tripathi *et al*., 2011), or a response peculiar to Sudanese SCD patients that warrant further research.

D-dimer was significantly reduced in the omega-3 treated group when compared with untreated group, but also still increased when compared with healthy controls. D-dimer was significantly reduced in hydroxyurea treated group when compared with omega-3 untreated, but still increased when compared with healthy controls.

The high levels of D-dimer in patients in the current study confirm that hypercoagulability state is one of the major elements of pathophysiology of the disease (Hebbel *et al*., 2009; Ataga *et al*., 2012). Previous studies have reported that n-3 fatty acid intake is inversely associated with the level of fibrinogen, factor VIII and von Willebrand factor (VWF) (Shahar *et al*.,1993) and D-dimer (Tomer *et al*., 2001). Consistent with the latter study, the current investigation demonstrates high DHA omega-3 fatty acid, but not HU, treatment reduces plasma D-dimer concentration in patients with SCD. The decrease of D-dimer by n-3 fatty acids has

implications for clinical management of patients because plasma D-dimer level is associated with a history of stroke in SCD (Ataga *et al*., 2012).

This study did not attempt to elucidate the mechanism through which n-3 fatty acids, particularly DHA and EPA, mediate their anti-coagulant effect. Nevertheless, it is well established that some of the metabolites of these fatty acid are antithrombotic, antiaggregatory, anti infalammrory and vasodilatory. EPA by competing with arachidonic acid (AA) for cyclooxygenase and lipooxygenase enzymes (Nomura *et al*., 2003). inhibits the synthesis of the prothrombotic proaggregatory, pro-inflammatory and vasoconstrictor metabolites of AA. Recent animal and human studies suggest that DHA is more potent anti-aggregatory agent than EPA at high doses (Cottin *et al*., 2011; Adan *et al*., 1999). Interestingly, studies in SCD have demonstrated that endothelial tissue factor expression is specifically dependent upon the nuclear factor-kappa B (NFκB) component of blood mononuclear cells (Kollander *et al*., 2010). It has been shown that treatment with high DHA n-3 fatty acid was associated with down regulation of NFκB gene expression in mononuclear cell and amelioration of SCD-associated chronic inflammatory state (Daak *et al*., 2015;Kalish *et al*.,2015). Hence, it is justifiable to attribute the observed improvements in the patient's hypercoagulable state after high DHA intervention to its suppressive effect on NFκB gene expression and partial resolution of the chronic inflammatory state (Esmon, 2005).

No statistically significant difference was found in protein-S in omega-3 treated group when compared with untreated group but it was found significantly reduced in (treated and untreated group) when compared with healthy controls. Also, there was no statistically significant difference in protein-S in hydroxyurea treated group when compared with HbSS untreated group but it was found significantly reduced when compared with healthy controls.

Moreover, there was no statistically significant difference in protein-C in omega-3 treated group when compared with untreated group but it was found significantly reduced in (treated and untreated group) when compared with healthy controls. Furthermore, there was no statistically significant difference in protein-C in hydroxyurea treated group when compared with HbSS untreated group but it was found significantly reduced when compared with healthy controls.

The low level steady-state proteins-C and S in the Sudanese children with SCD, agrees with previous findings (Wright *et al*., 1997;Piccin *et al*., 2015), underscores that a down regulation of anti-coagulant pathway in SCD results in activation of both the inflammatory and coagulant pathways (Hebbel *et al*., 2009). Protein C, activated by thrombin in the presence of protein S, inhibits the clotting ability of factor V and VIII (Marlar *et al*., 1982). The underlying cause of the natural anti-coagulant deficiency is yet to be elucidated. However, it is speculated that this deficiency is due to either known SCD hemostatic abnormalities or hepatic dysfunction (Bayazit and Kilinc, 2001). An earlier study reported a decrease in proteins-C in children with sickle cell disease treated with hydroxyurea. Koc et al study comprised of 11 children, 5 of them were homozygous SCD (Koc *et al*., 2003). The discrepancy of HU effect on proteins-C and S in the current study and the previous study could be due to the fact reported by Koc *et al* who was relatively underpowered to detect the true HU effect on natural anti-coagulant system (Koc *et al*., 2003).

Despite the observed improvement in coagulation parameters and hypercoagulable state, treatment with n-3 fatty acids did not result in a significant change on the level of the natural anti-coagulant proteins-C and S. These findings may indicate that the liver role on low natural anticoagulant in SCD might outweigh the effect of over-consumption due to SCD-associated hypercoagulable state.

The current study did not assess liver function in order to have a better understanding of the observed abnormalities of coagulation system and the responses to HU and n-3 treatments. In addition, the effect of n-3 and HU treatments on markers of thrombin generation such as prothrombin fragment F1, 2 and thrombin-anti-thrombin complexes was not investigated. Due to the fact that only patients above 10 years old and those of very severe clinical course are treated with HU in Sudan, we did not manage to recruit sample size of patients on HU matched by age and gender. These significant limitations will be addressed in future studies.

4.2.Conclusion

- (PT,PTT,INR and PLT) were significantly reduced in omega-3 treated and hydroxyurea treated patients compared with untreated HbSS patients. Nonetheless, the same parameters were not significantly different in the omega-3 and hydroxyurea treated groups when compared with healthy controls.

- (Hb, HCT, RBC, WBC) were significantly reduced in omega-3 treated, hydroxyurea treated and untreated HbSS patients compared with healthy control.

- D-dimer was significantly reduced in omega-3 treated and hydroxyurea treated group compared with untreated HbSS patients but still increased compared with healthy controls.

- Protein-C and Protein-S were significantly reduced in omega-3 treated and hydroxyurea treated patients compared with healthy controls.

4.3.Recommendations

This study recommended that

1- Omega-3 fatty acids might be safe an effective treatment for patients with homozygous sickle cell disease.

2- Regulator monitoring of coagulation parameters could be a good clinical practice that may help prevent hyper-coagulopathy complications on patients with the disease.

3- Further studies are warranted to investigate the role of omega-3 to prevent hyper-coagulopathy related complications.

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Appendix One

Questionnaire

Sudan University of Science and Technology

College of Graduate Studies

Effects of Long Term Supplement with Omega-3 Fatty Acids on Some Coagulation Parameters and Blood Counts in Patients with Sickle Cell Disease

Name…….....

Code No……...

Sex: Male ( ) Female ( ) …….

Age……. ……

Tribe…….....

Residence…….

Phone No…….

On omega-3 fatty acids supplementation Yes ( ) No ( ) ……

Family history (No of siblings): None ( )Yes ( ) ……...

Investigation

1- Full blood count(Hb ….. HCT…RBCs count ……WBCs count …. PLT count … MCV ….. MCH …. MCHC) …….

2- PT…….

3- APTT……...

4- D-Dimer ……..

5- Protein C …….

6- Protein S …….

Appendix Two

Informed consent

موافقه مستنيرة

هذه دعوة منى الباحث / شيخ عووضه القاسم فضل الحاج

للمشاركة فى برنامج بحثى هدفه تقييم

تأثير العلاج الطويل المدى بالأحماضالدهنيةغير المشبعة (اومقا-٣)على تخثر الدم لدى السودانيين المصابين بداء الأنيمياالمنجلية.

اذا رغبتم فى انجاحهذا البرنامج فانى أسنقوم ب :-

- أخذ عينة من الدم لقياس مستويات تأثير العلاج الطويل المدى بالأحماضالدهنيةغير المشبعة (اومقا-٣)على تخثر الدم.
- ملء أستمارة بمعلومات تخصكم لها علاقه بموضوع البحث.
- ونفيدكم بأن المعلومات التي تخصكم ستكون سرية.
- ومشاركتكم فى البرنامج تسعدنا وتساعد في انجاح اهداف البحث، ولكم كامل الحرية في المشاركة أو عدم المشاركة .

التاريخ .....

اسم المشارك او من ينوب عنه وتوقيعه .....

توقيع الباحث .....