Clinical and biochemical effects of omega-3 fatty acid supplementation on patients with homozygous Sickle cell disease

A thesis Submitted for the Degree of Doctor of Philosophy

By

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Dedication

In memory of my father, whose message I continue

Author`s declaration

I certify that the thesis titled: Clinical and biochemical effects of omega-3 fatty acid supplementation on patients with homozygous Sickle cell disease, submitted for the decree of : Doctor of Philosophy, has not been previously submitted for another degree in this or any other educational institution. Moreover, I confirm that the clinical research, laboratory analysis, data analysis and interpretation of the data are entirely my own work, except where otherwise acknowledged in the main text of the thesis.

Name: Ahmed Abd Almalik Daak

Signed....

Date..21-02-2012.....

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Abstract

Sickle Cell Disease (SCD) is a group of autosomal recessive genetic blood disorders due to a mutation in the sixth codon of the β goblin gene. The resultant haemoglobin S (HbS) polymerises under low oxygen tension causes rigid, sickled red blood cells. The main clinical manifestations are vaso-occlusisve painful crisis and haemolytic anaemia which lead to degenerative tissue pathology that affect major organs including the brain, eye, lung, spleen, liver, kidney, heart and bone. The pattern of the complications and survival vary considerably between patients. These clinical variations suggest, the outcome of the disease is determined by the interaction of environmental and genetic factors.

According to the classical paradigm, the pathophysiology of vaso-occlusion is primarily due to mechanical blockage of small blood vessels by the deformed sickled red cells. However, the frequency of vaso-occlusive crisis does not correlate with the number of irreversible sickled cells. In contrast, the occurrence of vaso-occlusion events and clinical severity correlate strongly with the degree of leucocyte-erythrocyte-endothelial adhesion and inflammatory state. Adhesion, aggregation, elasticity of blood cells and inflammatory response are strongly modulated by cell membrane n-3 and n-6 fatty acids. There is evidence that steady state patients with SCD have abnormal erythrocytes, platelets and mononuclear cell fatty acid composition. The abnormality is characterised by increased levels of arachidonic (AA), adrenic and osbond acids, and reduced levels of docosahexanoic (DHA) and eicosapentnoic (EPA) acids and n-3/n-6 fatty acid ratio. Hence, it is possible that amelioration of the membrane fatty acid abnormality may have beneficial clinical effects on SCD patients.

This research programme investigated whether:

- *The beneficial effects of Hydroxyurea (HU), the commonly used treatment for the disease involves modulation of blood cell membrane fatty acid abnormalities observed in sickle cell disease.* HU-treated (n=19) and untreated (n=17) Homozygous patients and healthy control subjects (n=20) were enrolled from SCD Referral Clinic, Khartoum, Sudan. Blood samples obtained from the three groups were analysed for membrane fatty acids. The data showed that HU-treated patients and healthy controls had comparable levels of docosahexaenoic (DHA) and

total n-3 fatty acids in ethanolamine and (EPG) and choline (CPG) phosphoglycerides. In contrast, the untreated group had significantly lower DHA and total n-3 compared with the controls. In addition, HU treatment selectively mobilised arachidonic acid (AA) from the inner cell membrane amino-phosphoglycerides, EPG and serine (SPG) phosphoglycerides. Hence, it appears that the clinical benefits of HU are partially mediated through modulation of blood cell membrane fatty acid composition

- *Supplementation with n-3 FA reduces the frequency of vaso-occlusive crisis through correction of the membrane fatty acid perturbation.* One hundred forty patients recruited from Sickle Cell Referral Clinical in Khartoum, Sudan, were randomised to receive high-DHA n-3 fatty acid (n=70) or placebo (n=70) capsules for one year. Treatment with n-3 fatty acids resulted in three fold increase in red cell membrane DHA and EPA, with a concomitant decrease in n-6 fatty acids particularly AA. Supplementation with the highly unsaturated and labile DHA and EPA did not induced additional oxidative stress as it was evident by the significant increase in plasma vitamin E concentration and a reduction in activity of red cell Se-glutathione peroxidise.

  Treatment with the high DHA n-3 fatty acids for one year reduced the frequency of vaso-occlusive and haemolytic crises, number of blood transfusion, and hospitalisation and absence from school due to SCD associated illnesses. None of the patients in the n-3 fatty acid group but two in the placebo group developed stroke.

  The patients treated with high-DHA n-3 fatty acids had reduced total white blood cell, integrin and lactate dehydrogenase. These findings suggest that the beneficial action of n-3 fatty acids is mediated through reduction of inflammation and adhesion.

This research programme which was based on an appreciable number of homozygous sickle cell patients clearly demonstrated that HU modulates cell membrane fatty acid abnormality, and supplementation with high-DHA n-3 fatty acids have the potential to be effective and affordable treatment option for patients with SCD. In order to test the

generalisability of the effect of n-3 on patients with SCD, a multi-centre study which involve patients from different dietary and genetic backgrounds is warranted.

Publications

Journal articles

Daak AA, Ghebremeskel K, Elbashir MI, Bakhita A, Hassan Z Crawford MA (2011). Hydroxyurea therapy mobilises arachidonic Acid from inner cell membrane aminophospholipids in patients with homozygous sickle cell disease. *J Lipids;* **2011** 718014

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Abbreviations

AA	Arachidonic acid
ACS	Acute chest syndrome
AI	Adequate intake
ALA	α-Linolenic acid (C18:2n-3)
AMDR	Acceptable macronutrient distribution range
ANOVA	One-way analysis of variance
APLT	Aminophospholipid translocase
BHT	Butylated hydroxytoluene
B-CAM/Lu	Lutheran blood-group antigen
BSA	Bovine serum albumin
CBC	Complete Blood Count
CDP- diacylglycerol	Cytidine diphospho- diacylglycerol
CD62L	L-selectin
CD62P	P-selectin
CD47	Integrine associated protein
CD11b/CD18	αMβ2 integrine
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CONSORT	Consolidated Standards of Reporting Trials
cPLA$_2$	Cytosolic phospholipase A$_2$
Cu/Zn-SOD	Cu/Zn-Superoxide Dismutase

CVD	Cardiovascular disease
cyclooxygenase-1	COX-1
cyclooxygenase-2	COX-2
CPG	Choline phosphoglycride
CYP	Cytochrome P450
DNA	Deoxyribonucleac acid
DAG	Diacylglycerol
DHA	Docosahexaenoic acid (c22:6n-3)
DHGLA	Dihomo-γ-linolenic acid
DPA	Docosapentaenoic acid (22:5n-3)
EDTA	Ethylendiaminetetraacetic acid
ECs	Endothelial cells
EFA	Essential fatty acid
EFAD	Essential fatty acid deficiency
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid (C20:5n-3)
EPG	Ethanolamine phodphoglyceride
FA	Fatty acid
FAME	Fatty acids methyl esters
Fe-CAT	Catalse
FID	Flame-ionisation detector
FSC	Forward Scatter
GalCer	Galactosylceramide
GC	Gas-Liquid Chromatography

Glycosphingolipids	GSLs
GlcCer	Glucosylceramide
GR	Glutathione reductase
GPCR	G protein-coupled receptor
Hb	Haemoglobin
HDL	High-density lipoprotein
Hb A	Haemoglobin A
HbC	Haemoglobin C
HbE	Haemoglobin E
HbF	Haemoglobin F
HbS	Haemoglobin S
HbSC	Sickle cell-haemoglobin C
HbS β^0	Sickle Cell-β^0 thalassaemia
HbS β^+	Sickle cell-β^+ thalassaemia
HbSS	Homozygous sickle cell disease
HPLC	High performance liquid chromatograph
Hp	Haptoglobin
HRP	Horseradish peroxidase enzyme
Ht	Haematocit
HU	Hydroxyurea
H_2O_2	Hydrogen peroxide
ICAM-1	Intercellular adhesion molecule-1
IL-6	Interleukin-6
IL-10	Interlukin-10

IL-1β	Interleukin-1 β
IPG	Inositol phosphoglycerides
IUPAC	International Union of Pure and Applied Chemistry
ISSFAL	International Society for the Study of Fatty Acids and Lipids
IQR	Inter-quartile range
LA	Linoleic acid (c18:2n-6)
LCPUFA	Long chain polyunsaturated fatty acid
LDL	Low density lipoprotein
LDH	Lactate dehydrogenase enzyme
LOX	Lipooxygenase
LT	Leukotriene
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobine concentration
MCV	Mean corpuscular volume
MetHb	Methaemoglobin
MUFA	Monounsaturated fatty cid
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NFκB	Nuclear favtor kappa B
n-3	Omega-3 fatty acid
n-6	Omega-6 fatty acid
n-9	Omega-9 fatty acid

OA	Oleic acid
OFN	Oxygen-free Nitrogen
OS	Oxidative stress
$O_2^{\cdot}$	Superoxide
PA	Phosphatidic acid
PBS	Phosphate buffer saline
PG	Prostaglandin
PGI2	Prostacyclin
PL	Phospholipas enzyme
PLSCR	Phospholipids scramblase
PLTs	Platelets count
PMN	Polymorphonuclear Cell
PUFA	Polyunsaturated fatty acids
Prdx	Peroxiredoxin
PPARSα	Peroxisome proliferator-activated receptor alpha
RBC	Red Blood Cell
SCD	Sickle Cell Disease
ROS	Reactive oxygen species
SPG	Serine phosphoglycerides
Rv	Resolvins
Se-GPx	Se-Glutathione Peroxidase
SD	Standard deviation
SFA	Saturated fatty acid

SMS	SM synthase
SPM	Sphingomyelin
SSC	Side scatter
TGF-β	Transforming growth factor- β
TLC	Thin-layer chromatography
TMB	3',3,5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TSP	Thrombospondin
TWBC	Total white blood cell count
TX	Thromboxane
TXA2	ThromboxaneA2
VCAM-1	Vascular cell adhesion molecule-1
VLA-4/α4β1 integrin	Very-late-activation-antigen-4

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Chapter One

Introduction

1.1 Sickle cell disease

1.1.1 Inherited haemoglobin disorders

Sickle Cell Disease (SCD) is a group of autosomal recessive genetic blood disorder due to a mutation in the sixth codon of the β goblin gene. The resultant haemoglobin S (Hbs) which polymerises under low oxygen tension causes rigid, sickled red blood cells(Serjeant 2001; Knight-Perry et al. 2009; Rees et al. 2010). The principal phenotypes are homozygous sickle cell disease (HbSS), sickle cell-haemoglobin C (HbSC), sickle cell-β^0 thalassaemia (HbS β^0), sickle cell-β^+ thalassaemia (HbS β^+), HbSO $_{Arab}$ and HbSD $_{Punjab}$ and HbS $_{Lepore\ Boston}$ SCD(Serjeant 2001; Nagel et al. 2003). The main clinical manifestations are vaso-occlusisve painful crisis and haemolytic anaemia which lead to degenerative tissue pathology that affect major organs including the brain, eye, lung, spleen, liver, kidney, heart, bone. (Ballas et al. 2010). The pattern of the complications and survival vary considerably between patients. These clinical variations suggest, the outcome of the disease is determined by the interaction of environmental and genetic factors(Driss et al. 2009).

Haemoglobin (Hb) is a heterotetramer protein that contains two polypeptide subunits related to α-globin gene subfamily and two polypeptide subunits related to β-globin gene subfamily. Globin polypeptides bind heme, which in turn allows the haemoglobin in red blood cells (RBC) to bind oxygen reversibly and transport it from the lungs to respiring tissues. The structure of human haemoglobin changes during embryonic, foetal and adult life. Adult and foetal haemoglobin have α chains combined with β (Hb A, $\alpha 2\beta 2$), δ (HbA2, $\alpha 2\ \delta 2$) or γ chains (Hb F, $\alpha 2\ \gamma\ 2$). After birth, Hb F is replaced by Hbs A (97%) and A2 (2-3.5%) over the first year of life(Weatherall and Clegg 2001; Steinberg 2009a)

HbS is caused by a single amino acid substitution of Glutamic Acid replaced by Valine at the sixth position of the β-globin chain. This is due to a single nucleotide

substitution, GAG –> GTG in codon 6 of the β-globin gene on chromosome 11 (Driss et al. 2009). The inheritance of SCD obeys the principle of Mendelian genetics (Serjeant 2001), the genetic outcome of offspring depends on whether the are parent homozygous or heterozygous for HbS. In the homozygous form of the disease (HbSS), patients inherit the gene for HbS from both parents, who are either carriers (heterozygous) or have SCD. The term sickle cell anaemia refers specifically to homozygosity for the βs allele (Rees et al. 2010).

Heterozygous Sickle cell disease results from the inheritance of HbS with a wide variety of mutations in β-globin. In haemoglobin C (HbC) the same codon 6 in β-globin that accountable for HbS mutation is changed from GAG to AAG, resulting in insertion of lysine in place of glutamic acid. In population of Africa ethnic origin, sickle cell anaemia typically account for 70% of cases of sickle cell disease, with most of the remainder having HbSC disease (HbSC) owing to the co-inheritance of βs and βc alleles(Nagel et al. 2003).

The second major type of heterozygous sickle cell disease occurs when βs is inherited with β-thalassaemia allele, causing HbS/ β-thalassaemia; this is a variable disorder dependent on the type of the β-thalassaemia mutation. Thalassemias are a group of inherited autosomal recessive hematolgical disorders that cause haemolytic anaemia because of the decreased or abscent synthesis of globin chain(Muncie and Campbell 2009). Thalassaemias are classified into α-, β-, δβ- and γδβ- depending on the affected polypeptide chains (sergeant 1994). Deletion of the entire β gene or part of δ gene causes δβ-thalassias(Henthorn et al. 1990). Deletion involving β, δ, γ genes gives rise to the rare condition γδβ-thalassaemia. B-thalassemia includes Thalassemia Major, thalassemia Intermedia and Thalassemia Minor. Subjects with thalassemia major are homozygotes or compound heterozygotes for β^{o} or β^{+} genes, subjects with thalassemia intermedia are mostly homozygotes or compound heterozgotes and subjects with thalassemia minor are mostly heterozygotes(Galanello and Origa 2010). Sickle cell β thalassemia ($HbS\beta^{o}$ or $HbS\beta^{+}$) results from co-inheritance of HbS with the β-thalassemia major ($HbS\beta^{o}$, HbA=0%) or β-thalassemia minor($HbS\beta^{+}$, HbA=20-40%) (Mukherjee et al. 2010). Disease severity decreases in the sequence HbSS ≈ HbSβo >HbSC, with $HbS\beta^{+}$ severity depending on remaining HbS percent(Dijs 2004).

Among the less frequently occurring forms of the disease are combination of HbS with other point mutations, i.e. HbD_{Punjab}, HbO_{Arab}, Hb_{lepore} and possibily HbE. In HbD_{Punjab}, glutamine replaces glutamic acids at position β 121, and in HbO_{Arab}, Lysine substitutes glutamic acid in the same location β 121 (Serjeant 2001). Hb_{lepore} is the hybrid hemoglobin (Hb) composed of two alpha-globin chains and two deltabeta hybrid chains.(McKeown et al. 2009) These variants of abnormal haemoglobin tend to co-precipitate with HbS and result in clinically significant SCD

1.1.2 Genetic modifiers of SCD

SCD is prototypical Mendelian single gene disorder, and yet, despite the apparent genetic simplicity, it has long been appreciated that patients with SCD display remarkable diversity in the clinical severity of disease(Chui and Dover 2001; Fertrin and Costa 2010). For example, in Cooperative Study of Sickle Cell Disease in the USA(Platt et al. 1991), 39% of 3578 patients with SCD had no episodes of pain but 1% had more than six per year. The complications in SCD are likely to be modulated by interactions among many genes and the environment.(Sebastiani et al. 2005; Steinberg 2005). The first study on genetic differences accounting for clinical variability in SCD reported the discovery of a site in linkage disequilibrium with the βS gene by use of restriction endonucleases(Kan and Dozy 1978). Subsequent studies characterized the presence of at least five different haplotypes of the β-like-globin gene cluster, suggesting distinct geographic origins of the same βS gene (Senegal, Benin, Bantu, Arab-Indian and Cameroon) and documented that haplotypes differed in foetal Hb (HbF) levels(Nagel and Labie 1989; Nagel and Steinberg 2001) **(Figure 1-1).** HbF (α2β2) has the ability to inhibit the polymerization of deoxygenated HbS, making its levels an important modulator of the disease(Goldberg et al. 1977; Charache 1990). Senegal and Arab-Indian haplotypes are strongly associated with higher HbF levels and hence exhibit milder clinical course(Powars et al. 1990; el-Hazmi et al. 1992). In addition, the HbF concentration in SCD which varies from 1% to 30% is inherited as a quantitative genetic trait(Rees et al. 2010). Three major loci have been identified, which account for up to 50% of this variation in SCD. Specifically, the Xmn1 polymorhism in the promoter region of the Gγ globin gene(Gilman and Huisman 1985), the HMIP locus in chromosom 6q23.3(Thein et al. 2007) and BCL11A on chromsom 2(Menzel et al. 2007).

Other primary modifier of SCD is coinheritance of α-thalassemia(de Ceulaer et al. 1983; Mukherjee et al. 1997). α-thalassaemia is present in about 30% of SCD patient(Steinberg and Embury 1986; Flint et al. 1998), it reduces the RBC mean corpuscular haemoglobin content (MCHC), deoxygenated HbS polymerization, denser dehydrated cells, irreversibly sickled cells and ultimately less haemolysis and sickling(Embury et al. 1982; Noguchi et al. 1985; Vasavda et al. 2007).

Neither HbF level nor α-globin genotype can fully explain the clinical and laboratory diversity of the sickle cell disease(Steinberg 2009b). Presently, most reported studies have examined only polymorphic genes that could potentially affect the pathogenesis of the disease and modulate the phenotype and more comprehensive genome-wide association studies (GWAS) are just beginning(Steinberg 2005). To date, a unifying theme that is emerging from the candidate gene studies is that polymorphism in genes of TGF-β/BMP pathway, a superfamily of genes modulating wound healing and angiogenesis, appear to be associated with several disease subphenotype(Bertolino et al. 2005; Steinberg 2008).

1.1.3 Environmental modifiers of the SCD

The inter-individul variation of SCD outcome not only refer to a pure genetic paradigm, but also refer to geographic, social, dietetic, traditional and socio-economical matter(Driss et al. 2009). Studies have shown a direct role of protein intake(Archer et al. 2008), water intake(Fowler et al. 2010), and temperature(Smith et al. 2009) in disease severity.

Northern Sudan, with its short rainy season, has hot daytime temperatures year round. In Khartoum, the capital, temperatures can reach 48 °C. This extreme temperature may contribute to the severity of the disease due to increased risk of dehydration. However, our own observation in Sickle Cell Referral Clinic and Khartoum hospitals, a noticeable surge of hospitalization due to SCD crises occurs during the short cold season (January and February). The possible explanation is the increased incidence of viral infection and/or the high speed of cold dry air of winter.

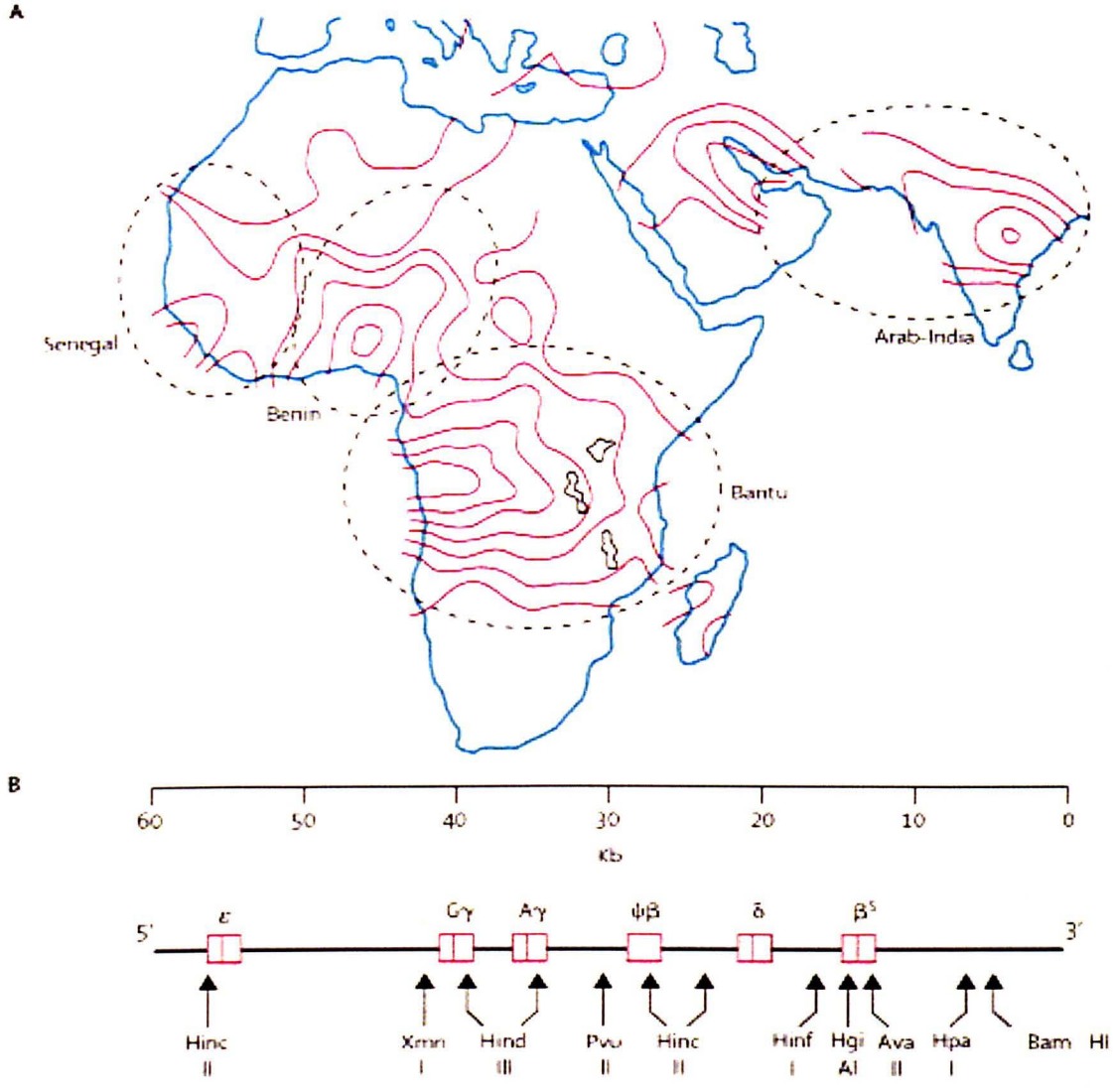


Figure 1-1Geographical distribution and schematic representation of the sickle gene
(A) Map identifies the three distinct areas in Africa and one in the Arab-India region where the sickle gene is present (dotted lines). Numbers of individuals with sickle-cell disease (red lines) in Senegal, Benin, and Bantu are higher near the coast, and falls concentrically inland. (B) The β-globin gene cluster haplotype is determined by DNA polymorphic sites (boxes) that are identified by endonuclease enzymes. With this information, haplotypes are constructed as shown. Adopted from(Stuart and Nagel 2004).

1.1.4 Epidemiology of HbS gene

1.1.4.1 Origin of the sickle cell gene

Lehmann and Huntsman, based on anthropological evidence as well as the geographical distribution of the gene within Africa hypothesised that a single gene mutation occurring in Neolithic times in the fertile Arabian Peninsula is the origin of the disease. However the studies in the DNA polymorphism (region-specific haplotypes) provided support for the hypothesis that the mutation causing HbS has occurred, and have been locally amplified, on at least two, and possibly several, separate occasions(Flint et al. 1998; Serjeant 2001). The factors that caused this amplification despite the high mortality that must have resulted in reduction of HbS gene are not fully understood(Stuart and Nagel 2004). However, substantial evidence indicates that malaria caused this amplification(Allison 1954; Aidoo et al. 2002; Rees et al. 2010). This hypothesis supported by the close geographic correlation between the frequency of HbS gene in populations and historical incidence of malaria (Flint et al. 1998)(figure 1-2) and the evidence for partial resistance of carriers to all forms of plasmodium falcuparum malaria(Williams et al. 2005b; May et al. 2007). It has been postulated the HbS resistance to malaria resulted in "balanced polymorphism", that is, a balance of deleterious and advantageous effects of a gene which allow it to remain at a relatively constant level in a population(Allison 1954; Serjeant 2001; Stuart and Nagel 2004).

The protective mechanism of HbS against malaria is yet to be elucidated. To some extend it relates to the peculiar physical or biochemical prosperities of HbAS red blood cells(Williams et al. 2005a). There is evidence that invasion, growth, and development of plasmodium falciparum parasite are all reduced in sickle red blood cells under physiological conditions in vetro(Friedman 1978; Pasvol et al. 1978), a process that may result in their premature destruction by the spleen(Shear et al. 1993). Moreover, mounting evidence suggests involvement of both innate and immune-mediated mechanisms(Marsh et al. 1989; Bayoumi et al. 1990; Abu-Zeid et al. 1992; Wellems et al. 2009).

From the primary areas of mutation the Benin haplotype spread to North Africa, Sicily, northern Greece, Albania, southern Turkey, and southwest Saudi Arabia(Serjeant

2001). The Bantu haplotype accounts for most of the disease in Kenya(Ojwang et al. 1987). The vagaries of war and slave trade have been responsible for the dissemination of HbS gene in North and South America, the Caribbean and the UK, where the predominant haplotype is Benin(Zago et al. 1992). The Arab-Indian haplotype probably originated in the Indus valley Harappa culture, and by gene flow it was distributed to Saudi Arabia, Bahrain, Kuwait and Oman. This haplotype is also linked to SCD gene in population from eastern oasis of Saudi Arabia and Adivasis tribe of India(Stuart and Nagel 2004). In Sudan, the most abundant haplotypes are the Cameroon, Benin, Bantu and Senegal haplotypes, respectively (Mohammed et al. 2006).

1.1.4.2 Distribution of SCD

At least 5.2% of the world population (and over 7% of pregnant women) carry a significant haemoglobin variant. HbS account for 40% of carriers but causes over 80% of disorders because of the localised very high carrier prevalence(Modell and Darlison 2008); recent estimate suggests more than 230000 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 1300 in Europe(Modell and Darlison 2008) (**Figure1-2**).

In the Sudan, the distribution of sickle cell disease was thought to follow the historical route of the Pilgrims. Three 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(R Launder 1970). The other focus is in southern Sudan, where a sickling rate of 18% among the southern Nilotes Madi and Mandari tribes of the Equatorial region with zero prevalence among the northern Nilotes, the Dinka, Nuer and Shilok was reported(Foy et al. 1954). The third focus in the Blue Nile area showed 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 this area(Ahmed and Baker 1986). Now the disease is common in the capital (Khartoum), centre, north and east of Sudan due to the movement and intermarriage of the tribes, but the prevalence is not well reported.

Average survival is clearly strongly influenced by environmental, social and economic factors. Although almost no formal studies of survival are available in literature from African countries(Serjeant 2005), there is undoubtedly a high mortality, major determinant of which are malaria, malnutrition and infection(Serjeant 2001). It has been estimated that life expectancy among African people with sickle-cell disease is less than 20 years(Tshilolo et al. 2008). In the Cooperative Study USA, the peak incidence for death was 1-3 years(Leikin et al. 1989), and it was estimated that 85% of HbSS patients survive beyond the age of 20 years, compared to 50% 30 years ago(Scott 1970). Recent autopsy study in USA showed that the most frequent causes of death are infection (33-48%) and stroke (9.8%)(Manci et al. 2003).

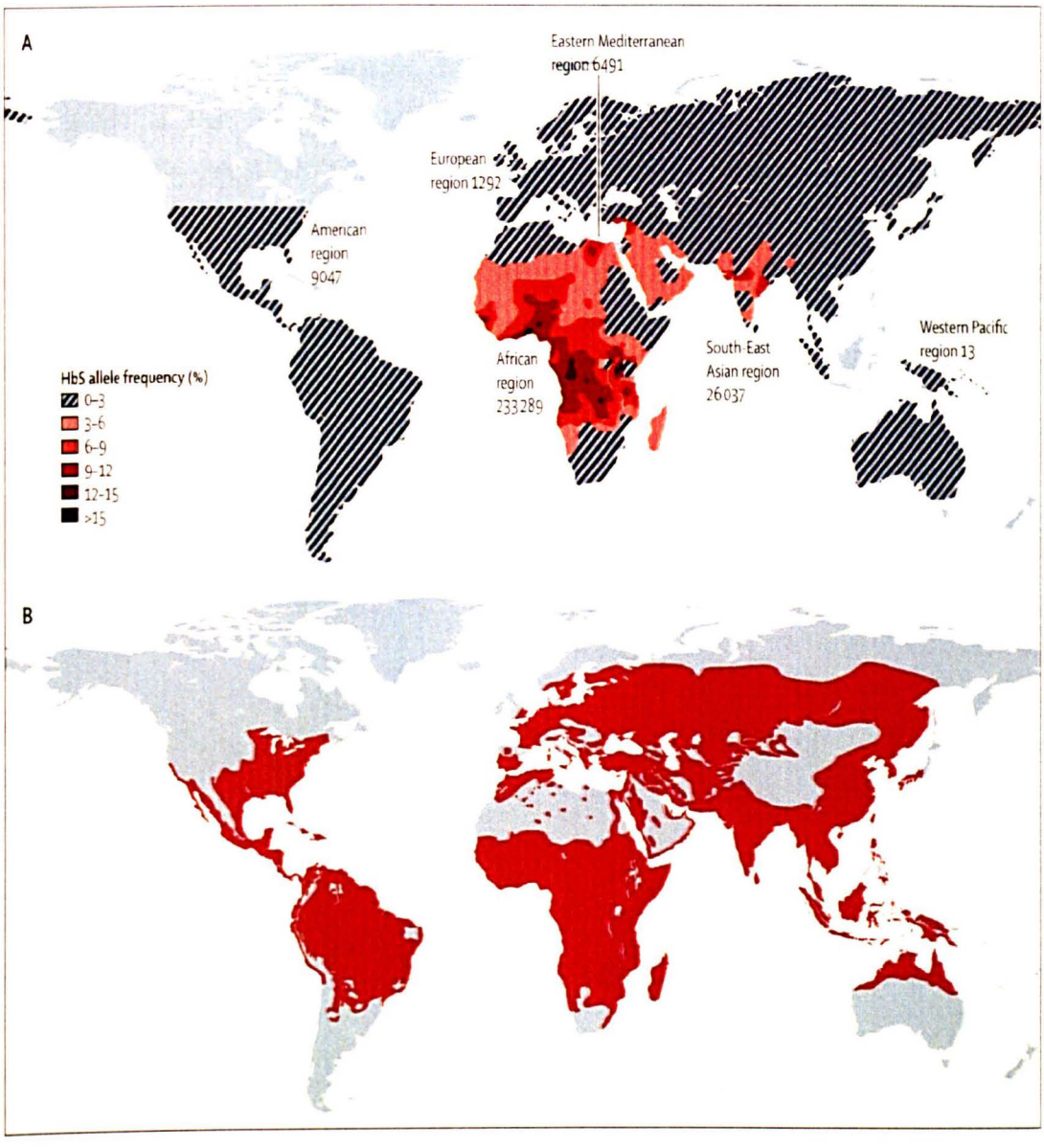


Figure 1-2Global distributions of HbS and malaria
(A) this map shows the distribution of the HbS allele. The figures indicate estimates for the combined yearly total number of individuals affected by HbSS, HbSC, and HbS/β-thalassaemia by WHO region. (B) This map shows the global distribution of malaria (red) before intervention to control malaria (adapted from(Rees et al. 2010)

1.1.5 History of SCD

Centuries before Dr Herrick first reported sickle cell disease; the people of West Africa knew the disease syndrome and gave it specific tribal names(Konotey-Ahulu 1968). The African medical literature reported this condition in the 1870's where it was known locally as ogbanjes ('children who come and go') because of the very high infant

mortality in this condition. They also knew that the disease was hereditary, with the typical presentation of cold-season rheumatism, pallor of nails, tongue, palms of hands and soles of feet, yellow coloration of the eyes and a generally low state of health(Konotey-Ahulu 1974). Herrick in 1910 was the first to describe sickle red cells in the blood of dental student from Grenada who complained of pain, dizziness and general tiredness(Herrick 2001). Using the new technique of protein electrophoresis, Pauling et al(1949) described the molecular basis of the SCD disease, the study that opened a new era of medical research(Gormley 2007).(**Tabl 1-1**).

Table 1-1 Important discoveries of the pathological and clinical features of SCD in chronological order

	Discovery	Importance
1890	Sickled erythrocytes in Grenadian dental student	First description of a disease linked to abnormal erythrocytes
1948	Pauling et al delineated the abnormal electrophoretic mobility of sickle haemoglobin	Identified pathophysiology to have molecular basis
1948	Watson(1948) noted no symptoms in infants with SCD	Beneficial effects of high concentrations of foetal haemoglobin
1951	Perutz et al(1951) describe characteristics of polymerisation of deoxygenated HbS	Primary molecular mechanism identified
1978	Kan and Dozy describe the first haplotype polymorphism associated with HbS gene	Provided insight about the genetic modifier of the disease
1980s	John et al (1984)showed the value of the penicillin in young children with SCD	Reduced mortality, role of neonatal screening
1984	Platt et al(1984) demonstrate that hydroxyurea increases HbF production in SCD	Identified potential treatment
1984	Jounson et al(1984) reported bone marrow transplant in child with sickle cell anaemia and Leukaemia	Identified potential cure
1995	Charache et al (1995) demonstrate that hydrxyurea decreases painful crises in SCD	Only-disease modifying drug identified
1998	Adams et al (1998) demonstrate reduced stroke incidence in children with abnormal transcranial dopplers who were given blood transfusion	Primary stroke prevention with fall in stroke occurance
2008	Sedgewick et al(2008) published the first genome-wide association study in SCD	Identified the role of functional motifs in HbF levels

1.1.6 Clinical manifestation of SCD

1.1.6.1 Sickle cell pain

Pain is the protean manifestation of SCD, in which episodic microvessel occlusion at on or many sites induces tissue damage accompanied by pain (nociceptive pain) and

inflammation(Stuart and Nagel 2004; Ballas 2005). It may be acute or chronic, somatic or visceral, unilateral or bilateral, localised or diffuse, moderate or severe(Ballas 1998). Typically, acute painful episodes (vaso-occlusive crisis) affect long bones and joints, with the low back being the most frequently reported site of pain(Ballas and Delengowski 1993). Other region of the body, including the scalp, face, jaw, abdomen, and pelvis, may be involved(Charache et al. 1995; Charache et al. 1996). Objective signs of a painful crisis such as fever, leukocytosis, joint effusions and tenderness occur in about 50% of patients at initial presentation(Ballas et al. 1988). Painful crisis affect virtually all patients with SCD, often beginning in late infancy and recurring throughout life(Almeida and Roberts 2005) **(Figure 1-3).** The frequency of painful crisis varies across the patients as third of the patients are asymptomatic, with small cohort (5%) accounting for a third of admission(Stuart and Nagel 2004). Platt et al calculated from hospital-based data a vaso-occlusive crises average rate of 0.8 episode per patients per year in HbSS patients(Platt et al. 1991). Brozovic et al(1987) reported that 90% of hospital admissions of patients with sickle cell disease are for the treatment of acute pain. Studies have shown that painful crises are more frequent in patients with high haemoglobin levels and high reticlucyte count(Baum et al. 1987; Platt et al. 1991). Adults who experience painful crises more often than three times per year tend to have a shorter life expectancy than those with low pain rates(Platt et al. 1991).

Chronic pain that persists for 3 or more months, may result from ongoing damage to bone and other tissues(Koshy et al. 1989; Ballas 2005; Dunlop and Bennett 2006) ; and inadequate treatment to recurrent episodes of acute pain may lead to chronic neuropathic pain that is maintained centrally by abnormal somatosensory processes and central sensitization(Ballas 2005; Niscola et al. 2009).

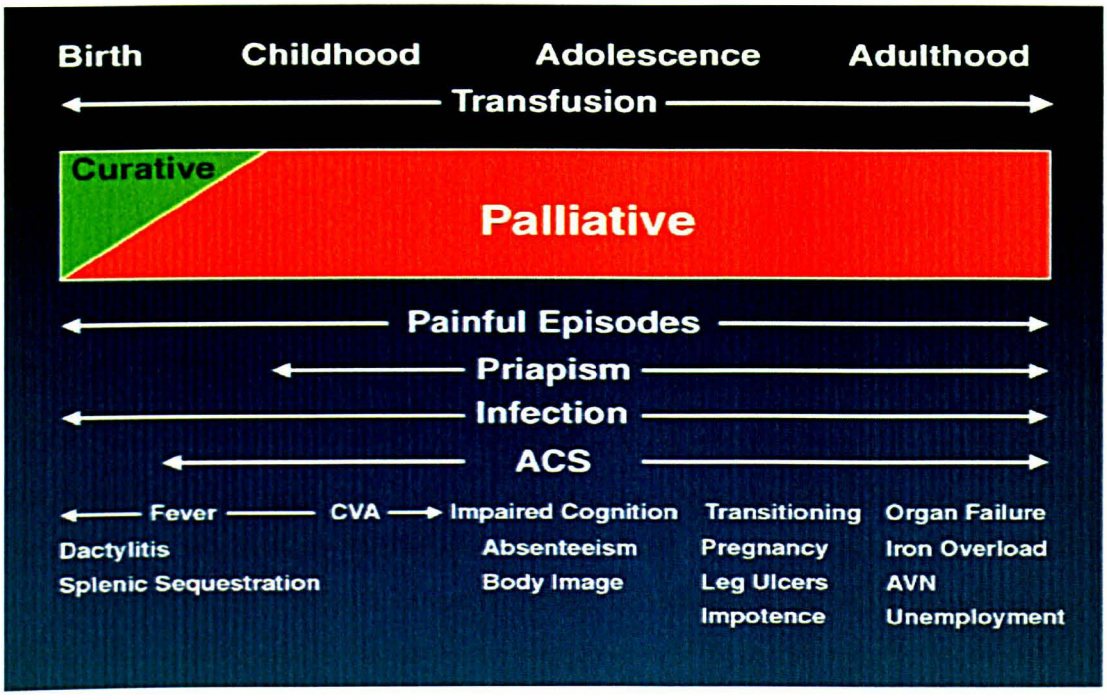


Figure 1-3 of complications of sickle cell anaemia from birth through adult life.
ACS, acute chest syndrome; AVN, avascular necrosis; CVA, cerebrovascular accident

1.1.6.2 Bone involvement in SCD

The major bone complications in SCD include vaso-oclusive crisis and osteomyelitis(Neonato et al. 2000; Almeida and Roberts 2005). While vaso-occlusion can occur in any organ, it is particularly common in the bone marrow, resulting in bone marrow infarction typically in medullary cavity or epiphyses(Lonergan et al. 2001; Kim and Miller 2002). The reasons for the vulnerability of the bone marrow to microvascular occlusion is not clear, but may be partly due to red marrow expansion (hepercellularity) leading to impaired blood flow and regional hypoxia(Smith 1996). Epiphyseal infarction has a predilection for the head of the femur (avascular necrosis), followed by the humerus, knee and small joints of the hand and feet.(Jean-Baptiste and De Ceulaer 2000; Lonergan et al. 2001). About 50% of patients with HbSS will develop epiphyseal osteonecrosis by the age of 35 years(Ware et al. 1991; Styles and Vichinsky 1996).

In infants and young children, infarction occurs in the diaphysis of small tubular bone in hands and feet causing dactylitis or "hand-foot" syndrome(Babhulkar et al. 1995). Hand-foot syndrome is common between the age 6 months and 2 years but is rare after

the age of 6 years because of the regression of red marrow in these areas with increasing age(Ejindu et al. 2007). Children often present clinically with tender and swollen hands and feet, diminished movement and fever(Stevens et al. 1981).

The effects of sickle cell anaemia on growth are thought to result from bone infarction. Epiphyseal shortening arises from vascular compromise, which causes damage to the growth plate, slowing or halting cartilage growth and leading to shortened bone(Siffert 1966). In addition, endplate depressions of vertebral bodies contribute in growth disturbance associated with SCD(Marlow et al. 1998).

Osteomyelitis and septic arthritis are serious complications of SCD and important causes of hospitalisation. The relative rate of occurrence of osteomyelitis has been estimated up to 18%, and 7% for septic arthritis (Neonato et al. 2000; Bahebeck et al. 2004). The most common cause of osteonyelitis in SCD is Salmonella especially the non-typical serotypes (salmonella typhimurium, salmonella enteriditis, salmonella choleraesuis and salmonella paratyphi B)(Piehl et al. 1993; Burnett et al. 1998). Staphylococcus aureus, the second most common causal organism, is seen in approximately 10% of cases(Ejindu et al. 2007). Gram negative organisms also implicated, and tuberculosis osteomyelitis and arthritis have been reported in SCD(Kooy et al. 1996; Burnett et al. 1998). Infection is commonly haematogenous in origin(Anand and Glatt 1994). The increased susceptibility of SCD patients to infection has been attributed to hyposplenism secondary to infarction in childhood, impaired of phagocytosis and complement dysfunction(Almeida and Roberts 2005). Moreover, infarction and necrosis of medulary bone create a good milieu for bacterial growth and spread(Ejindu et al. 2007).

1.1.6.3 Aplastic crises

Human parvovirus (HPV) B19, a common childhood infection, frequently causes transient red cell aplasia (Aplastic crises) in children with sickle cell disease (SCD)(Goldstein et al. 1987; Serjeant et al. 2001). Although most individuals spontaneously recover in a few days, the anaemia can be so sever that it causes cardiac decompensation and death(Serjeant et al. 1993; Goldstein et al. 1995).Serjeant et al (2001)reported that 70% of SCD patients seroconvert by age 20, and 67% of the infected group suffered aplastic crises.

1.1.6.4 Acute chest syndrome

Pulmonary complications represent 20 to 30% of mortality due to sickle cell and can be divided into acute and chronic events(Maitre et al. 2011). Acute chest syndrome (ACS) is a common form of acute lung injury in SCD, and second most common cause of hospital admission(Platt et al. 1994). It is defined by the development of a new alveolar pulmonary infiltrate involving at least one lung segment(Gladwin and Vichinsky 2008). The radiographic abnormality is usually accompanied by chest pain, fever, tachypnea, wheezing, or cough(Vichinsky et al. 2000). Risk factors include HbSS genotype, low HbF, high steady-state leucocyte and Hb concentration(Castro et al. 1994). Severity varies, but 13% of patients require mechanical ventilation 3% die(Vichinsky et al. 2000). Repeated episodes of ACS predispose to chronic pulmonary disease, including pulmonary hypertension (pulmonary artery systolic pressure> 35 mmHg)(Vichinsky et al. 2000; Stuart and Setty 2001a; Gladwin et al. 2004).

Acute chest syndrome is caused by a combination of pulmonary infection, fat embolism and vaso-occlusion of the pulmonary vasculature by sickled cell(Vichinsky et al. 2000) **(Figure 1-4).** The most common cause of the acute chest syndrome in children and adults is pulmonary infection by a community-acquired pathogen such as Streptococcus pneumoniae, Mycoplasma pneumonia and respiratory syncytial virus(Vichinsky et al. 2000). The pulmonary symptoms are commonly preceded by bone pain, significant reduction in Hb concentration, platelet count, increased plasma levels of free fatty acids and secretory phospholipase A2(Schuster 1994; Styles et al. 1996; Ballas et al. 2006).

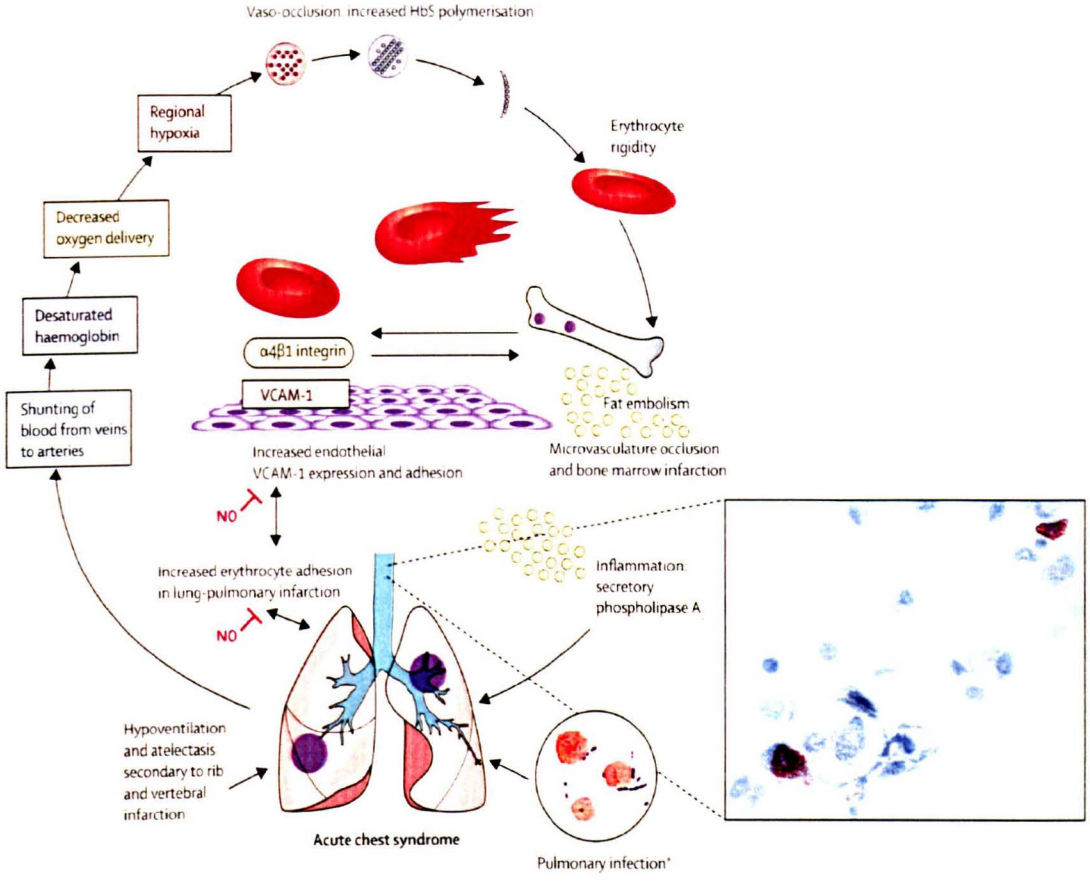


Figure 1-4 Pathophysiology of acute chest syndrome, adopted from (Rees et al. 2010).

1.1.6.5 Cardiac complications

Sickle cell patients are known to have multiple clinically significant cardiac abnormalities, primarily during adulthood(Lindsay et al. 1974; Lester et al. 1990; Ballas et al. 2010). Morphologic and physiologic changes include dilated chambers, septal hypertrophy, abnormal contractility and echocardiogram abnormalities(Covitz et al. 1995; Liem et al. 2009). These changes are thought to be due to high cardiac output, the adaptive response to reduced oxygen carrying capacity in SCD(Varat et al. 1972; Lindsay et al. 1974; Hankins et al. 2010).

1.1.6.6 Neurological complications of SCD

The developing brain is particularly vulnerable to hypoxaemia and ischaemia, to which children with SCD are frequently exposed(Hogan et al. 2006a). Neurological and cranial complications occur in at least 25% of patients with SCD(Hebbel 2005). These

complications include transient ischemic attacks, overt and silent cerebral infarction, cerebral haemorrhage, infections, moya-moya pattern, posterior reversible encephalopathy syndrome, dural venous sinus thrombosis, thickness of the diploic space, cerebral atrophy and Seizures (Liu et al. 1994; Henderson et al. 2003; Yildirim et al. 2005; Alkan et al. 2009). Cerebral infarction has been reported to be the most common cause of stroke in the first two decades of life and from fourth decade onward, whereas hemorrhagic stroke occurs more commonly in the third decade(Ohene-Frempong et al. 1998). In SCD clinical stroke with focal signs lasting more than 24 hours is 250 times more common than general paediatric population(Earley et al. 1998). SCD Patients with Low haemoglobin, high white cell count and history of previous transient ischemic event, hypertension and history of chest crisis seem to be at higher risk to devolve overt ischemic stroke. Moreover, recent studies suggest familial predisposition and epistatic polymorphisms as additional risk factors for stroke in SCD (Driscoll et al. 2003; Kirkham 2007). Besides the structural brain abnormalities, children with SCD with or without a history of overt stroke tend to have lower cognitive function(Watkins et al. 1998; Noll et al. 2001; Hogan et al. 2006a), which commonly affect attention and executive function(DeBaun et al. 1998; Hogan et al. 2006b).

Neuropathology of ischemic complications in SCD is due to narrowing and occlusion of blood vessel which results from endothelial hyperplasia, fibroblastic reaction, hyalinization and fragmentation of the internal elastic lamina, thrombi in large and small vessels, increases adhesion of blood cells and fat embolisation after bone marrow infarction (Powars et al. 1978; Rothman et al. 1986; Switzer et al. 2006). Approximately 75% of ischemic strokes are typically due to large-artery involvement. The most common subtype of overt cerebral infarction is the border-zone stroke, which occurs between the anterior cerebral artery and middle cerebral artery territories(Moran et al. 1998; Alkan et al. 2009). Recurrent stroke is generally haemorrhagic due to rupture of fragile, dilated collateral vessels from prior ischemic infarction(Ohene-Frempong et al. 1998).

1.1.6.7 Ocular and cochlear complications of SCD

Ocular manifestations are important long-term complications of SCD(Elagouz et al.; Nagpal et al. 1977). The pathological process of SCD can affect virtually every vascular bed in the eye, in its advanced stages, has the potential to cause blindness (To and Nadel 1991; Babalola and Wambebe 2005). Classification of ophthalmic manifestations in the retina can be based on the presence or absence of vascular proliferation in the eye fundus(Fadugbagbe et al.). Proliferative sickle retinopathy (PSR) is the most important causative factor of vitreous haemorrhage or retinal detachment, the severest complications of sickle cell eye disease(Nagpal et al. 1977; Moriarty et al. 1988; Kent et al. 1994). Moriarty et al (1988) have reported that incidence of visual loss in eyes with proliferative disease was 31/1000 eye-years compared with 1.4/1000 eye-years in subjects with non proliferative disease. Non-proliferative ocular manifestations of SCD include conjunctival vascular occlusions, iris atrophy, retinal pigmentary changes, retinal haemorrhages and other abnormalities of the choroid, macula, optic disc and retinal vasculature(Fadugbagbe et al.).

The prevalence of hearing impairment reported range from 0 to 66%. Different patterns and degrees of hearing loss are reported, ranging from profound bilateral losses with partial recovery over time, to mild to moderate unilateral losses— predominately in the high frequencies(Wilimas et al. 1988; Onakoya et al. 2002; Burch-Sims and Matlock 2005). The extensive vascular anatomy and the high metabolic activity requirement of the cochlear render it very vulnerable to vaso-occlusive ischaemic damage(Brown et al. 1983; Russell and Cowley 1983).

1.1.6.8 Kidney involvement in SCD

The renal features of SCD include hematuria, proteinuria, tubular disturbances, acute kidney injury and chronic kidney disease(Scheinman 2009). At early stages, the renal complications manifest as impaired urinary concentration ability, defects in urinary acidification and potassium excretion, increased phosphate reabsorption, increased creatinine secretion, glomerular hyperfilteration and proteinurea(Allon 1990; Gurkan et al. 2010). A urinary concentration defect is the most common tubular abnormality and it can present as enuresis(Devereux and Knowles 1985; Scheinman 2009). 4-21% of

adult HbSS patents suffer renal failure, and contributes to early mortality from the disease(Guasch et al. 2006).

Sickle cell disease (SCD) affects the kidney by acute mechanisms, as a form of the sickle crisis, and insidiously with renal medullary/papillary necrosis, with resulting tubular defects(Okpala 2006). The urinary concentration defects and haematurea are probably a consequence of RBC sickling in renal medulla combined with vascular obstruction and RBC extravasation; as it is well known that, the hypoxic, acidotic, and hyperosmolar environment of the inner medulla are strong promoters of RBC sickling, which in turn results in impairment of renal medullary blood flow, ischemia, microinfarct, and papillary necrosis(Statius van Eps LW 1997; Pham et al. 2000). Moreover, the repetitive episodes of localized ischemia and reperfusion contribute to renal damage by inciting a low-grade chronic inflammatory tissue-injuring state(Platt 2000). The mechanisms underlie proteinurea and glomerulopathy include both hyperfilteration and glomerular hypertrophy(de Jong and Statius van Eps 1985; Allon 1990). In addition, sickled cell can cause direct damage to the glomerular endothelium. Studies have also shown that, endogenous perturbation of the prostaglandin system is implicated in early development of glomerulopathy in SCD(Buckalew and Someren 1974; de Jong and Statius van Eps 1985; de Santis Feltran et al. 2002).

1.1.6.9 Acute exacerbations of anaemia

Sickle cell disorders are associated with variable degrees of anaemia depending on genotype; with the most sever disease in haemoglobin level seen in HbSS. After the first 5 years of life, the Hb concentration remains constant in the steady state over time. However, clinically significant lowering of Hb concentration below steady state values does occur episodically(Ballas et al. 2010). These episodes may result from variety of causes include hyperhaeamolysis, splenic sequestration and aplastic crisis(Manci et al. 2003; Ballas et al. 2010). Hyperhaemolyis is diagnosed when the exacerbation of anemia occurs in the absence of splenic and hepatic sequestration. Isolated hyperheamolysis in the absence of painful crisis is reffered to as haemolytic crisis(Ballas and Marcolina 2006). Intravascular haemolysis (1/3 of SCD haemolysis) and extravascular haemolysis are driven by HbS polymerisation and HbS instability respectively(Bensinger and Gillette 1974; Hebbel 2010). Recent evidence suggests that chronic intravascular

hemolysis is associated with a state of progressive vasculopathy, characterized by reduced nitric oxide (NO) bioavailability, pro-oxidant and pro-inflammatory stress , coagulopathy, pulomnary hypertension, stroke, leg ulcers and priapism (Reiter et al. 2002; Gladwin et al. 2004; Gladwin and Kato 2005; Kato et al. 2007; Morris et al. 2008). **Figure 1-5.**

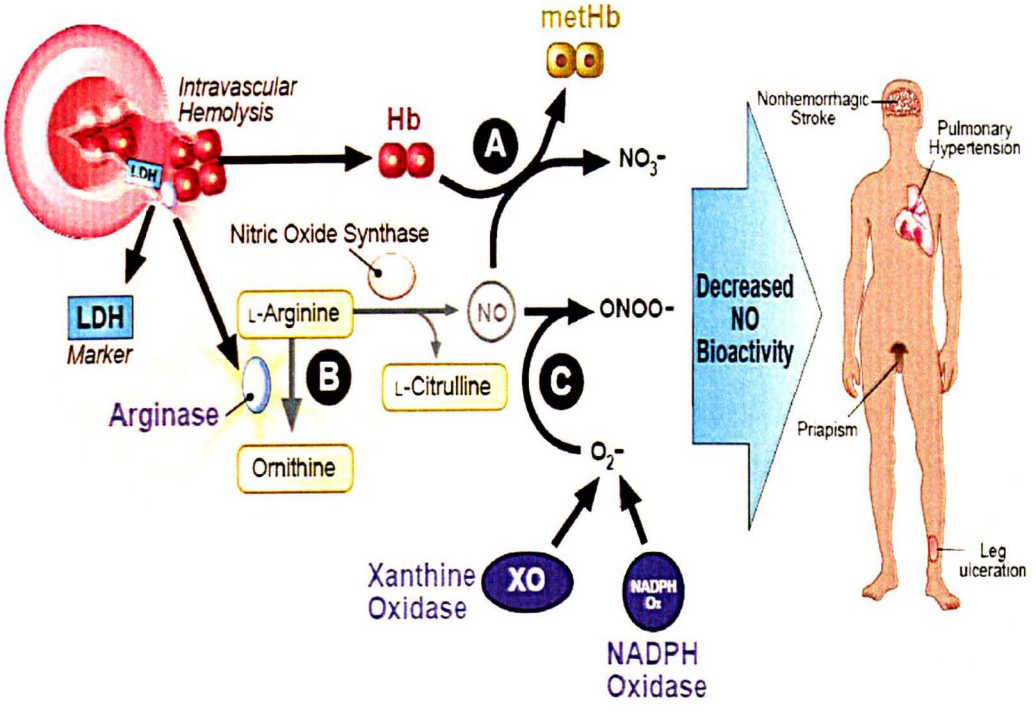


Figure 1-5 Pathophysiology of intravascular haemolysis in sickle cell disease adopted from(Kato et al. 2007).

1.1.6.10 Splenic complications

The relative hypoxic, acidic environments in the spleen coupled with slow circulation provide a uniquely hostile milieu for sickle RBC(Harrod et al. 2007). Enhanced sickling and repetitive infarctions lead to functional asplenia and ultimately splenic fibrosis and atrophy (Pearson et al. 1969; Pearson et al. 1985; Adekile et al. 2002). This functional asplenia in turn results in increased susceptibility to sepsis, particularly from encapsulated bacteria(Ballas et al. 2010). Abnormal splenic function in HbSS is common by six months of age, and affect more that 20% by one year and over 40% by two years(Serjeant 2001). The onset of functional asplenia is reflected by the

appearance of irreversibly sickle cell, anisocytosis, howell-Jolly bodies and sidrocytes(Ebert et al. 2010). Acute intrasplenic vaso-occlusion can occur, resulting in life-threatening splenic sequestration(Kinney et al. 1990; Machado et al. 2009). In HbSS patients, the life time prevalence of acute splenic sequestration has been reported to be between 7% and 30%(Emond et al. 1985; Kinney et al. 1990). The initial event occurs in toddler age group, but episodes as early as 9 weeks were reported(Pappo and Buchanan 1989; Rezende et al. 2009). Acute splenic sequestration is characterised by a tender, rapidly enlarging massive spleen due to the trapping of sickle RBC and blood constituents(Ballas et al. 2010). The haemoglobin decreases by at least 2g/dl with concomitant 20-30% increase in reticulocytes from baseline, and often moderate to severe thrompocytopenia(Singhal et al. 1995; Lonergan et al. 2001), leading rapidly to severe anaemia.

1.1.6.11 Hepatic involvement in SCD

Hepatic dysfunction is a commonly recognized complication of sickle cell disease (SCD) due to multiple factors such as intrahepatic sinusoidal sickling and congestion, bilirubin gallstones, intrahepatic cholestasis, transfusion-related viral infections or excess iron deposition (haemosiderosis)(Kakarala and Lindberg 2004; Gurkan et al. 2005). Hepatomegaly occurs in 40-80% of patients(Serjeant 2001) (Serjeant 2001), and liver infarction has been observed in 34% of autopsies (Bauer et al. 1980).Traditional liver function test may be abnormal but gneraly reflects non-hepatic pathology(Brody et al. 1975; Ebert et al. 2010). In addition to the chronic hepatic complications of SCD, there may be episodes of acute hepatic crisis and acute hepatic sequestration (AHS) (Hatton et al. 1985; Berry et al. 2007). It has been reported that 10% of hospital admission for painful crisis are due to acute hepatic crisis(Johnson et al. 1985). Patients with the uncommon AHS present with tender, progressive hepatomegaly, accentuated anaemia below baseline, reticlucytosis and hyperbilirubinemia and moderate transaminitis(Hernandez et al. 1989; Norris 2004). Because liver is not distensible as is the spleen, AHS is not usually life threatening(Sheehy 1977; Ballas et al. 2010).

1.1.7 Pathophysiology of vaso-occlustion

The two major pathophysiological processes underpinning the complications associated with SCD are vaso-occlusion with reperfusion injury and haemolytic

anaemia(Frenette 2002; Rees et al. 2010). Acute vaso-occlustion was thought to be caused by entrapment of RBC containing the rope-like fibre of deoxygenated HbS. HbS polymerises when deoxygenated, since valine which substituted glutamic acid in position six can interact hydrophobicaly with the complementary sites on adjacent globin chains(Stuart and Nagel 2004; Hebbel et al. 2009). The polymerisation of HbS is a nucleation-initiated reaction with a delay time, during which no polymer is detectable. At the end of the delay time, the critical nucleus is formed and exponential polymer formation follows(Eaton and Hofrichter 1990; Ferrone 2004). Although HbS polymerisation and red cell sickling are central to pathophysiology of the disease, emerging evidence indicates primary events in vaso-occlusion may involve a complex interplay of complex factors(Embury 2004; Kaul et al. 2009). Firestly, Studies on polymerisation kinetics have shown that, the range of the transit times of RBC in the microcirculation is short relative to the range of delay times of HbS, and consequently, most of HbS under physiological conditions fails to polymerise unless the delay times being lengthened by other factors such as inflammation and adhesion biology of sickle vascular pathobiology(Mozzarelli et al. 1987; Turhan et al. 2002; Hebbel et al. 2009). Secondly, no correlation was found between painful events and sickling. In addition, in most circumstances vaso-occlusion is triggered by polymerisation-independent antecedent events, with sickling occurring as secondary event(Billett et al. 1986; Ballas et al. 1988; Nagel 1993; Barker and Wandersee 1999). These observations led to conclusion that sickling is not necessary and sufficient or the primary initiator of vaso-occlusion(Embury 2004). The current consensus is that, the primary events crucial to vaso-occlusion include adhesion of RBC to endothelium of postcapillary venule, and leukocyte-endothelium adhesion with formation of heterocellular aggregates(Kaul et al. 1989; Turhan et al. 2002; Kaul et al. 2009), **Figure 1.6.**

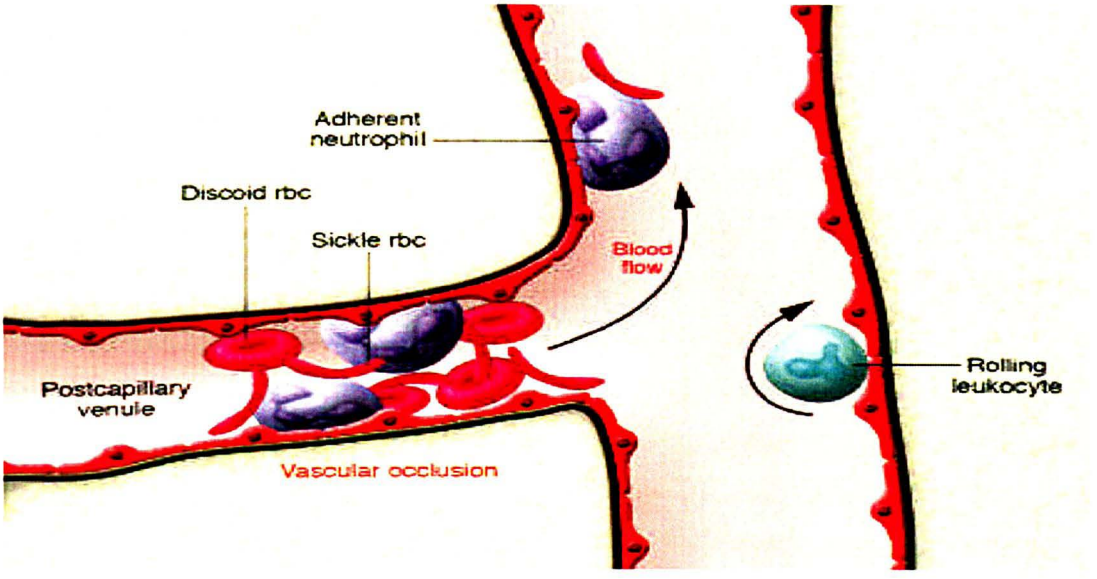


Figure 1-6 Sickle cell vasoocclusion
adopted from(Frenette and Atweh 2007)

1.1.7.1 Red cell adhesion and vaso-occlusion

Sickle red cells are abnormally adherent to endothelium(Hoover et al. 1979; Hebbel 2008). The RBC adhesion correlates inversely with the venular capillaries, and the prime sit of adhesion was shown to be the postcapillary venules(Kaul et al. 1989; Kaul and Fabry 2004; Zennadi et al. 2007). Studies have revealed that RBC adhesion act as a trigger that slows down the flow to fulfil the delay time requirement for sickling(Hebbel et al. 1980; Kaul and Fabry 2004), the finding which may explain the established positive correlation between sikcle cell adhesivity to endothelium and clinical severity of SCD. Major determinants of RBC–endothelial interactions are the RBC and endothelial cell membranes, both of which display a great variety of adhesion molecules (Hebbel 2008; Hebbel et al. 2009). In addition, interactions have been observed between sickle cell and immobilised extracellular matrix components(Stuart and Nagel 2004). The adhesion molecules commonly expressed in sick red cells include very-late-activation-antigen-4 (VLA-4/α4β1 integrin), (Joneckis et al. 1993; Brittain and Parise 2008), CD36(Gupta et al. 1999; Telen 2007), serine phosphoglycerides (SPG),(Manodori et al. 2000; Setty et al. 2002), integrine associated protein (CD47),(Brittain et al. 2001; Brittain et al. 2004), the protein that carries the Lutheran blood-group antigen (B-CAM/Lu),(El Nemer et al. 2008) and integrin receptor LW (ICAM-4),(Kaul et al. 2006; Zennadi et al. 2007).

Studies have shown different mechanisms of interaction between RBC adhesive molecule and vascular endothelium. For some mechanisms RBC adhesive molecules interact directly with endothelial molecules without participation of an intervening adhesogen, such as the interaction of α4β1 integrin with vascular cell adhesion molecule-1 (VCAM-1) on cytokine and/or hypoxia stimulated endothelial cells(Swerlick et al. 1993), and interactions between reticulocyte CD36 and endothelial cell vitronectin receptors via thrombospondin(Gupta et al. 1999; Kaul et al. 2000). On the contrary, other mechanisms involve participation of an intervening, bridging molecule such as thrombospondin (TSP) released by platelet (Sugihara et al. 1992a; Brittain et al. 1993), von Willebrand factor released by endothelial cells(Gupta et al. 1999; Kaul et al. 2000) and fibronectin(Kumar et al. 1996; Brittain et al. 2008).

1.1.7.2 White cell adhesion and vaso-occlusion

It has been known that polymorphonuclear leukocytosis correlates with severity of SCD progression(Miller et al. 2000), stroke and early death(Platt et al. 1994; Ohene-Frempong et al. 1998; Okpala 2004). This central role of white blood cell in SCD pathophysiology and vaso-occlusion is attributed to its increased propensity to adhere to endothelium and red blood cells (Frenette 2002; Frenette 2004; Canalli et al. 2008). Leukocytes adhesion to vascular endothelium is mediated by the interaction of leukocyte adhesion molecules L-selectin (CD62L), αMβ2 integrine (CD11b/CD18) and LFA-1 (CD11A/CD18) with endoendothelial adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin and P-selectin(Okpala 2002; Turhan et al. 2002; Johnson and Telen 2008). Due to fact that leukocytes are far larger, stiffer, and stickier than red cells, it has been suggested that they play a pivotal role in slowing microvasulare blood flow and, ultimately, initiation and propagation of vaso-occlusion(Chiang and Frenette 2005; Hebbel et al. 2009).

1.1.7.3 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 2001b; Ataga and Orringer 2003). Factors underlie the hypercoagulabl state in SCD include increased thrombin generation(Francis 1989; Tomer et al. 2001a), 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. 2001a; 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. 2001a). In addition, thrombospondin, platelet factor 4 and β-thromboglobulin are elevated in steady state(Tomer et al. 2001a; Lee et al. 2006). Studies showed that platelet activation accelerates further during vaso-occlusive crisis(Wun et al. 1997; Stuart and Setty 2001b). Several pieces of data provide evidence for the relationship between serine phosphoglycerides externalisation 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).

1.1.8 Management of SCD

1.1.8.1 General measures

The care of patients with SCD has undergone important advances in recent years. The institution of newborn screening programme in many countries, paved the way for early identifications of the disease and application of the comprehensive care programme (Steinberg 1999; Hankins and Aygun 2009). Early in life, when the risk of infection is highest, simple and cost-effective measures such as counselling the parents about the prophylactic therapy, the detection of enlarging spleen, the dangers of fever and increasing pallor and periodic visits to the physician can positively influence the clinical outcome and may be life-saving(Vichinsky et al. 1988; Rahimy et al. 2003; Frempong and Pearson 2007).

1.1.8.2 Prophylactic therapy

The mortality rate due to streptococcus pneumoniae pneumonia, sepsis and meningistis was historically very high prior to the age of 6 years in children with SCD(Ramakrishnan et al. 2010). This high mortality rate has been lowered tremendously by three manoeuvres. Firstly, immediate initiation of prophylactic penicillin after diagnostic screening and continued until 5 years old(Gaston et al. 1986),

and secondly, introduction of immunisation programme with heptavalent pneumococcal-conjucated vaccine at 2,4,6 and 12 months of age(Aliyu et al. 2006). The third is immunisation with 23-valent pneumococcal polysaccharide vaccine at 2 and 5 years of age(AAP 2000). In addition, 1 mg of folate daily provided as a supplement to meet the increased metabolic requirement for folate in SCS is frequently administered(Wang 1999).

1.1.8.3 Supportive therapy

The supportive management of pain includes hydration, pain relief, blood transfusion and psychosocial support(Aliyu et al. 2006; Verduzco and Nathan 2009; Edwards and Edwards 2010; Tanabe et al. 2010).

1.1.8.3.1 Pharmacologic management of pain

Nonopioids, opioids and co-analgesics are the three major classes of compounds commonly used for pharmacologic management of pain in SCD(Stinson and Naser 2003; Ballas 2005; Dunlop and Bennett 2006). Unlike opioids, nonopioids have a "ceiling effect", a dose above which no additive analgesic effects is attainable(Beaver 1988). Co-analgesics are heterogeneous compounds that potentiate the analgesic effect of opioids, ameliorate their side effects and have their own mild analgesic effect(Ballas 2005). They include antihistamines, antidepressant, benzodiazpines and anti-convulsant.

Acute painful episodes of mild or moderate severity are usually treated at home using a combination of nonpharmacologic and nonopioids analgesics(Field et al. 2009). Sever acute sickle painful episodes are usually treated in hospitals using parenteral analgesics(Benjamin et al. 2000).

Management of chronic sickle pain entails multidisciplinary approach. Chronic pain due to avascular necrosis and leg ulceration necessitate input of wound care centre, orthopaedics, physical therapy, rehabilitation and rheumatology(Ballas 2005; Dowsett 2005; Marti-Carvajal et al. 2009). Neuropathic pain management in SCD involves anticonvulsant, specially Gabapentin that is general used in this complication(Ballas 2005; Wilkie et al. 2010).

1.1.8.3.2 Blood transfusions

Blood transfusions are administered in SCD to increase oxygen carrying capacity, replace rigid, sickle-shaped red blood cells with normal, deformable cells, and restore blood flow(Vichinsky et al. 1995; Adams et al. 1998). Transfusions may be indicated for a variety of reasons in patients with SCD, including acute or episodic symptoms or long-term management(Inati 2009). Simple transfusions are usually indicated for symptomatic anaemia, acute neurologic event, acute chest syndrome, acute splenic or hepatic sequestration and acute multiorgan failure(Vichinsky 2001a; Lottenberg and Hassell 2005); Whereas chronic simple transfusion is generally indicated for prevention of recurrent stroke, recurrent acute chest syndrome and pulmonary hypertension(Adams et al. 1998; Vichinsky 2001a; Lottenberg and Hassell 2005). Exchange transfusions are recommended for severe acute chest syndrome, acute multiorgan failure and acute neurologic event(Inati 2009). Despite the beneficial effects of blood transfusions in SCD, it is limited by myriad complications which include auto and allo-immunisation, anaphylaxis, acute lung injury, delayed haemolytic transfusion reaction (Norol et al. 1994; Vichinsky 2001b; Wanko and Telen 2005; Raphael et al. 2008; de Montalembert et al. 2011), enhanced blood viscosity and risk of central nervous system infarction(Ohene-Frempong et al. 1998), infections and iron overload(Vichinsky 2001b; Vichinsky et al. 2011). Although iron chelation has been shown to reduce iron stores(Olivieri et al. 1995; Inati et al. 2011), and reverse congestive cardiomyopathy resulting from iron overload(Rahko et al. 1986), improvements do occur only in 50% of patients due to poor compliance and high cost(Delea et al. 2007; Payne et al. 2007; Vichinsky et al. 2008).

1.1.8.4 Curative treatment and strategies

1.1.8.4.1 Haemopoietic cell transplantation

A matched allogeneic Haemopoietic cell transplantation (HSCT) is the only curative treatment for SCD(Johnson et al. 1984; Pinto and Roberts 2008). Most groups reported event-free survival rates of around 80–90%(Bernaudin et al. 2007; Panepinto et al. 2007). However, the HSCT is restricted by the availability of matched related donors and the potential serious complications(Fitzhugh et al. 2008; Pinto and Roberts 2008). Other emerging alternative options are unrelated donor marrow HSCT(La Nasa et al.

2005) or cord blood transplantation from an unrelated or related donor, sometimes in conjunction with preimplantation genetic diagnosis(Qureshi et al. 2005; Kuliev et al. 2011).

1.1.8.4.2 Gene Therapy

The strategy for gene therapy for SCD relies on production of an anti-polymerization globin in a cell producing a full complement of globin chains(Perumbeti and Malik 2010). Lentiviral-mediated gene transfer can correct haematological defects and organ damage in mice with SCD(Pawliuk et al. 2001; Levasseur et al. 2003). Nevertheless, to date no randomised controlled trials of gene therapy have been reported, hence no objective conclusions about gene therapy are established(Olowoyeye and Okwundu 2010).

1.1.8.5 Sickle cell Disease-modifying drugs

1.1.8.5.1 HbF-inducing drugs

The potential therapeutic effect of HbF modulation has been derived from the observations that infants with SCD have few symptoms until their HbF levels fall(Watson 1948), and patients with Hereditary Persistence of Foetal Haemoglobin have a milder clinical course(Perrine et al. 1978). Moreover, studies have shown that HbF levels predict the frequency of vaso-occlusive crises, acute chest syndrome, osteonecrosis and death(Castro et al. 1994; Platt et al. 1994). Different drugs have the ability to increase HbF production, using different mechanisms(Bank 2006; Fathallah and Atweh 2006). However, only hydroxyurea (HU) is commonly used for treatment of adult and children with SCD(Hankins and Aygun 2009).

1.1.8.5.1.1 Hydroxyurea

Hydroxyurea (HU) is the commonly used, effective therapy for adults, children and infants with severe sickle cell disease (Hoppe et al. 2000; Stuart and Nagel 2004; Segal et al. 2008; Strouse et al. 2008a). HU clinical effects, mechanism of action and side effects are discussed in the relevant section, chapter 3, section 3.1.

1.1.8.5.1.2 De-methylating agents

decitabine and its analogue 5-azacytidine are cytidine surrogate(Hankins and Aygun 2009). They incorporate in the DNA and form covalent bonds with DNA methyltransferase, leading to depletion of this enzyme and ultimately DNA hypomethylation(Creusot et al. 1982). The induction of HbF is attributed to trigger effect on γ-globin gene promoter due to drug-induced hypomethylation(Charache et al. 1983; DeSimone et al. 1983). The initial reports about efficacy of 5-azacytidine in SCD were very encouraging. However concern about its carcinogenic effects halted further investigation(Carr et al. 1984). There have been no clinical studies of decitabine in children with SCD(Trompeter and Roberts 2009). In adults with SCD small studies of intravenous or subcutaneous decitabine show that it increases HbF and total Hb, as well as reducing red cell adhesion and endothelial damage with no apparent tumorigenic risks(Koshy et al. 2000; DeSimone et al. 2002; Sauntharajah et al. 2003). The long-term clinical effects, long-term side effects, effects in children and non-toxic oral formulations have not yet been investigate(Hankins and Aygun 2009; Trompeter and Roberts 2009).

1.1.8.5.1.3 Butyrate derivatives

Short chain fatty acid butyrate inhibits histone deacetylase and promotes elevated levels of core histone acetylation, affecting chromatine structure and transcription rate of γ-globin genes and HbF level(McCaffrey et al. 1997; Davie 2003). Studies of intermittent, but not continuous, intravenous infusion of arginine butyrate or pulsed-dosing with oral phenylbutyrate show sustained induction of HbF in most adult patients with SCD(Atweh et al. 1999; Hines et al. 2008). Moreover, it has been reported that butyrate has beneficial effect in treatment of intractable leg ulcer(Sher and Olivieri 1994). The disadvantages of butyrate centre on its short half-life, requiring continuous IV dosage through a central line or very high oral dose (40 tablets per day) and the unpalatable nature of the treatment(Collins et al. 1995).

1.1.8.5.1.4 Erythropoietin

In vitro and in vivo experiments have demonstrated recombinant human erythropoietin's ability to raise the concentration of HbF(Al-Khatti et al. 1987; Stamatoyannopoulos et al. 1987). Moreover, SCD patients have some degree of erythropoietin deficiency(Saraf et al. 2011). The studies investigated potential effects of

erythropoietin on HbF levels in patients with SCD in combination with HU showed mixed results(Goldberg et al. 1990; el-Hazmi et al. 1995). However, emerging data suggest potential role to erythropoietin in improving patient tolerance to hydroxyurea dose escalation and prevention of HU-induced erythroid toxicity(Little et al. 2006; Hankins and Aygun 2009).

1.1.8.5.2 Vasodilators

1.1.8.5.2.1 Inhaled nitric oxide

Nitric oxide (NO) is the critical effector of endothelial-dependent vasodilatation and exerts pleiotropic effects on vascular and circulating blood cells, including the inhibition of platelet aggregation, down-regulation of cellular adhesion molecules, and modulation of ischemia-reperfusion injury, all pathways adversely affected duringVOC(Ignarro et al. 1987; De Caterina et al. 1995; Villagra et al. 2007; Akinsheye and Klings 2010). Preclinical studies in transgenic mouse models have consistently demonstrated effects on inhibition of Gardos channels; reduction in red cell density; improved perfusion; and reductions in lung injury, microvascular vaso-occlusion, and mortality(Martinez-Ruiz et al. 2001; de Franceschi et al. 2003; Dasgupta et al. 2006). Early single-centre and multi-centre clinical trials suggested decreased pain severity and reduced opioid analgesic use, with trends toward reductions in length of hospitalization(Weiner et al. 2003; Head et al. 2010). However, a recent well designed multi-centre, double-blind, placebo controlled, randomized clinical trial in SCD patients found no difference in painful crisis between Inhaled nitric oxide versus inhaled nitrogen placebo(Gladwin et al. 2011).

1.1.8.5.2.2 Arginine

L-arginine is deficient in adults with steady state Hb SS disease, and decreases to even lower levels during a vaso-occlusive episodes(Enwonwu et al. 1990; Lopez et al. 2003). In children with SCD, arginine decreases during vaso-occlusive crisis. L-arginine is the precursor to NO(Morris et al. 2000a; Morris et al. 2000b). Oral arginine supplement has been showed to increase NO levels during vaso-occlusive crisis and reduce pulmonary hypertention(Morris et al. 2000a; Morris et al. 2003; Morris 2006). Based on these promising results clinical trial has been conducted but no significant change was found in any laboratory outcome including arginine level(Hankins and Aygun 2009).

1.1.8.5.2.3 Sildenafil

Sildenafil is an oral phophodiesterase-5 inhibitor. In tissues that express phosphodiesterase-5, such as the lungs and corpora cavernosa, sildenafil amplifies NO signaling by inhibiting hydrolysis of cGMP, the mediator of NO signal transduction(Atz and Wessel 1999; Corbin and Francis 1999). Machado et al have reported improvements in pulmonary hypertension and exercise capacity in patients with SCD after treatment with sildenafil(Machado et al. 2005) . In addition, studies have shown encouraging therapeutic effects on priapism(Bialecki and Bridges 2002; Burnett et al. 2006). However, the observations that long term treatment with sildenafil is associated with frequency of vaso-occlusive crises may limit its use as treatment for SCD(Lane and Deveras 2011).

1.1.8.5.2.4 Statins

Independent of their cholesterol-lowering, emerging data indicate that many of statins clinical effects are largely conferred through modulation of NO, via inhibition of Ras/Rho proteins and direct upregulation of endothelial nitric oxide synthase(Hernandez-Perera et al. 1998; Laufs et al. 1998; Kano et al. 1999; Laufs 2003). Subsequent studies have shown that statins down-regulate inflammation, adhesion and thrombosis(Diomede et al. 2001). Data from transgenic mouse models indicate that statins attenuate endothelial activation in SCD(Solovey et al. 2004). Preliminary data from pilot studies in SCD patients showed increase in NO levels and improvement in measures of NO-dependent vascular dysfunction and inflammatory markers after treatment with simvastatin(Hoppe et al. 2011). These findings suggest a potential therapeutic role for SCD has to be yet verified.

1.1.8.5.3 Antioxidant Therapy

Oral L-glutamine has been shown to significantly increase NAD redox potential and NADH concentration in sickle RBCs, thereby reducing oxidative damage(Niihara et al. 1998). Moreover, supplementation with L-glutamine resulted in improvement of sickle RBC adhesion(Niihara et al. 2005). Other possible anti-oxidant strategy is repletion of over consumed tocopherol (vitamin E)(Browne et al. 1998).

1.1.8.5.4 Drugs for prevention of red cell dehydration

HbS concentration directly depends upon cellular hydration status(Hankins and Aygun 2009). Therefore, dehydrated RBCs have higher intracellular HbS concentration and enhance propensity of RBC to sickle(Eaton and Hofrichter 1987). Inhibition of sickle cell dehydration has been demonstrated to reduce dense cell formation and improve anaemia in patients with SCD(Ataga et al. 2008b). Major pathways in cell membrane involve in red cell dehydration include calcium-activated potassium efflux channel (Gardos channel)(Canessa 1991), KCl co-transporter(Lauf and Adragna 2000) and the Na^+ pump(Joiner et al. 1986). Abnormal cation transport is implicated in formation of dehydrated dense sickle cell and irreversible sickle cells(Bookchin and Lew 2002; Lew and Bookchin 2005). Hence, inhibition of any of these pathways can potentially prevent RBC dehydration and prevent polymerization of HbS(De Franceschi et al. 1994; Brugnara 1995; Brugnara 2003).

1.1.8.5.4.1 Senicapoc (ICA-17043)

Senicapoc (ICA-17043) is an ion-channel blocker that selectively blocks potassium efflux through the Gardos channel in human RBCs(McNaughton-Smith et al. 2008). Placebo controlled Clinical trails investigated the efficacy and safety of senicapoc consistently reported increased haematocrit, haemoglobin, and decreased numbers of both dense erythrocytes and reticulocyte in active group(Ataga et al. 2008b; Ataga et al. 2011). However, no significant improvement in the rate of sickle cell painful crises was observed in patients treated with senicapoc compared to those on placebo(Ataga et al. 2011).

1.1.8.5.4.2 Magnesium Pidolate

Magnesium (Mg^{++}) is an important regulator of cellular cation transporters such as the KCl co-transporter, Ca^{++} and K^+ channels(De Franceschi et al. 1996). Increased intracellular Mg^{++} inhibits K^+ efflux from the sickle erythrocyte and consequently prevents RBC dehydration(Brugnara and Tosteson 1987). Patients with sickle cell anaemia have decreased magnesium levels in plasma and erythrocytesand increased urinary excretion of magnesium(Olukoga et al. 1990; Olukoga et al. 1993). Experimental studies in transgenic sickle mice have shown that magnesium supplementation can significantly reduce KCl co-transport activity, decrease MCHC,

red cell density, and reticulocyte count, when compared to mice receiving a low magnesium diet(De Franceschi et al. 1996). Administration of magnesium supplementation to adult patients with SCD has been associated with minimal toxicity and possibly a reduced frequency of vaso-occlusion(De Franceschi et al. 1997; De Franceschi et al. 2000). A Phase II clinical trial is underway to investigate the role of magnesium pidolate in SCD (http://clinicaltrials.gov/ NCT00040456).

1.1.8.5.5 Anticoagulants and Antiplatelet Agents

Multiple studies of anticoagulants and antiplatelet agents have been conducted in SCD patients with varying results(Ataga and Key 2007). Mautsi et al(2002) have found that unfractionated heparin decreases sickle cell adhesion to endothelium under static conditions. In a randomized double-blind trial of low molecular weight heparin, tinzaparin, showed a significant reduction in the overall duration of painful crisis, number of days with the most severe pain scores, and duration of hospitalization(Qari et al. 2007).

1.1.8.5.6 Anti-inflammatory and antiadhesion treatment

1.1.8.5.6.1 Sulfasalazine

The inflammatory state of sickle cell disease and the presence of a chronic vasculopathy with increased blood cell-endothelium interaction argue for application of anti-inflammatory therapeutic approaches(Hebbel et al. 2004). Nuclear factor-kappa B (NFkB) is a transcription factor that activates expression of endothelial adhesion molecules(Ataga 2009). In transgenic mice, inhibition of NFkB significantly reduced leukocyte adhesion and improved microvascular blood flow(Kaul et al. 2004). Solovey et al(2001) have reported similar results from few numbers of patients with SCD.

1.1.8.5.6.2 Steroids

Steroids have been reported to reduce the duration of severe pain episodes, as well as the severity of acute chest syndrome in children and adolescents, although there appears to be a substantial risk for rebound painful events(Griffin et al. 1994; Bernini et

al. 1998; Strouse et al. 2008b). In addition, treatment with corticosteroids may also be associated with an increased risk of hemorrhagic stroke in children with SCD(Strouse et al. 2006).

1.1.8.5.6.3 Integrin and selectin inhibition

Phase 1 trials of eptifibatide α2β3 Integrin antagonist have shown decreases in platelet activation and inflammatory markers with increase in vasodilation parameters in patients with sickle cell anemia(Lee et al. 2007). Blocking of integrin binding on the ICAM-4 receptor by synthetic peptides may also have some promise in sickle cell adhesion therapy(Kaul et al. 2006). In addition, experimental data showed improved microcirculatory blood flow and survival after intervention with pan-selectin inhibitors(Chang et al. 2010).

1.2 Cell membrane and SCD

It has been increasingly recognised that cell membrane defects in sickle cell has a significant influences on pathophysiology and clinical severity of the disease(Hebbel 1991). The major membrane abnormalities observed in SCD are compromised membrane transport defects(Gibson and Ellory 2002), dysfunctional lipid bilayer(Kuypers 2007; Barber et al. 2009), perturbation of fatty acids composition of membrane phospholipids(Connor et al. 1997; Ren et al. 2006) and enhanced lipid peroxidation due to iron deposition in cell membrane(Repka and Hebbel 1991; Sugihara et al. 1992b; Browne et al. 1998).

Dysfunctional lipid bilayer in sickle cell membrane is characterised by externalisation of serine phosphoglycerides (SPG)(Middelkoop et al. 1988; Kuypers et al. 1996). The SPG externalisation in SCD is found to be due to abnormal activities of aminophospholipid translocase (APLT) and phospholipids scramblase (PLSCR), the enzymes responsible for maintenance of phospholipids asymmetry across cell membrane(de Jong et al. 2001; Kuypers and de Jong 2004; Barber et al. 2009). It has been proposed that external SPG contributes to thrombogenesis(Atichartakarn et al. 2002), enhanced adhesion with other blood cells and endothelium(Schwartz et al. 1985b; Manodori et al. 2000; Setty et al. 2002), shortened RBC lifespan in SCD (Bratosin

et al. 2001; Lang et al. 2005), providing a cellular substrate for secretory phospholipase A2(Fourcade et al. 1995; Neidlinger et al. 2006), and ultimately, vaso-occlusion and chronic haemolysis(Boas et al. 1998).

Besides red blood cell, membrane phospholipids fatty acids perturbation has also been observed on platelets and mononuclear cell fatty acid composition(Ren et al. 2005b). The abnormality is characterised by high omega-6, low omega-3 and an imbalance between the two fatty acid families (Connor et al. 1997; Manodori et al. 2000; Ren et al. 2005b; Ren et al. 2006). The n-3 and n-6 LCPUFA are vital structural and functional components of cell and sub-cellular component; Studies have shown that the balance between these two fatty acids families influences blood cell adhesion, aggregation, blood coagulation, cell deformability and inflammatory response (Mills et al. 1993; Nishiyama et al. 2000; Saito and Kubo 2003; Mukherjee et al. 2004). Hence, It has been postulated that an imbalance in membrane n-6/n-3 LCPUFA is the antecedent of the loss of membrane asymmetry, blood cell adhesion and aggregation and vaso-occlusion in SCD(Ren et al. 2005a).

1.2.1 Cell membrane composition

All cells are delimited by membranes, which confer them spatial identity and define the boundary between intracellular and extracellular space. These membranes are composed of lipids, proteins and carbohydrates(Simons and Sampaio 2011). However, the proportions of these components vary wildly between cells types, as well as between the same cells of different animal species. Generally speaking, lipids and proteins are at the ratio of 30% and 70% respectively (Cooper and Hausman 2007). Human red blood cell contains approximately 52% (w/w) proteins, 40% lipids, and 8% carbohydrates(Steck 1974). The corresponding proportions in liver are 54%, 36% and 8% and those in myelin membranes are 20%, 75% and 5%, respectively(Guidotti 1972).

1.2.2 Membrane structure and organisation

The basic structure of all cell membranes is the lipid bilayer, the oldest still valid molecular model of cellular structures(Gorter and Grendel 1925). Mammalian cell membranes lipids are glycerophospholipids, sphingolipids and cholesterol(Simons and Sampaio 2011). The fluid mosaic membrane model proved to be a very useful hypothesis in explaining many phenomena taking place in biological membranes,

specially the distribution of molecular components in the membrane(Vereb et al. 2003). Membranes had been considered by the Singer-Nicolson fluid mosaic model as "a two dimensional oriented solution of integral proteins freely diffuse in the viscous phospholipid bilayer"(Singer and Nicolson 1972; Jacobson et al. 1995). The phospholipids bilayer is composed of two layers of phosphoglycerides that have their hydrophobic fatty acyl groups pointed toward each other and the polar hydrophilic phospholipid head groups oriented outwards to the extracellular and cytoplasmic surface(Yeagle 1989), figure **1-7.** The four major phospholipids species present in the membrane choline phosphoglycride (CPG, PC),ethanolamine phodphoglyceride (EPG, PE), Serine phosphoglyceride (SPG, PS), and sphingomyelin (SPM) are distributed asymmetrically across the bilayer leaflet resulting in an enrichment of choline-phospholipid (CPG and SPM) in the outer leaflet and of amino-phospholipid (EPG and SPG) in the cytoplasmic leaflet(Schwartz et al. 1985a).

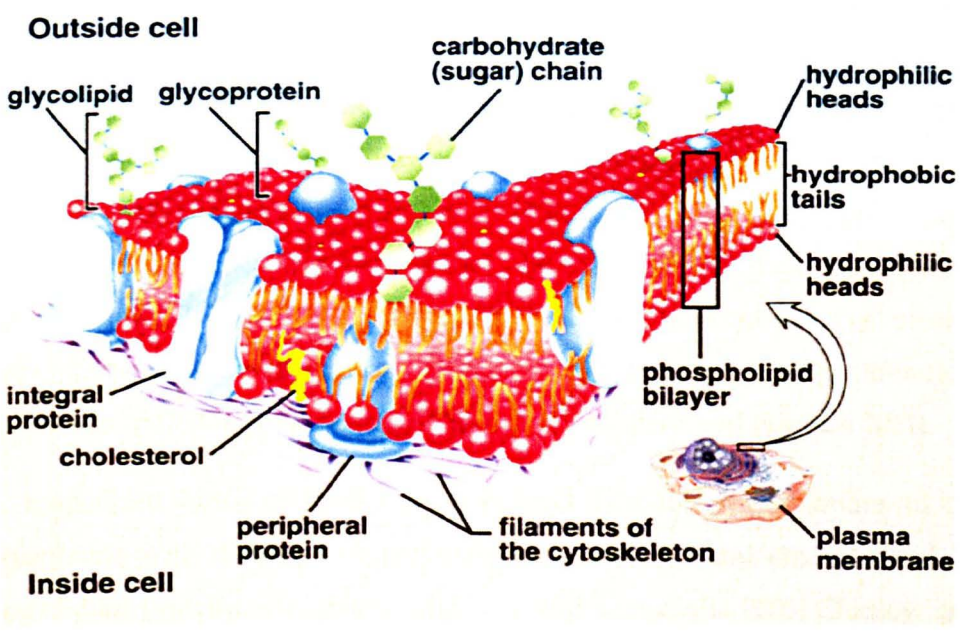


Figure 1-7 Fluid-mosaic model
Adopted from facstaff.bloomu.edu/gdavis/Cell

1.2.2.1 Rafts and caveolae

Experimental evidence accumulated over the last three decades indicated that the lateral motion of membrane components was not free after all as predicted by the fluid mosaic model, but constrained by various mechanisms such as direct and indirect interactions with cytoskeleton elements(Marguet et al. 2006). In additions, a heterogeneous membrane regions (microdomains) do exist(Lingwood and Simons 2010). Thus, the straightforward application of the S-N model as a frame of events is impossible without introducing a new model that incorporate the microdomains concept and non-random distribution of a significant number of cell-surface molecules(Vereb et al. 2003; Lingwood et al. 2009). Microdomains are sub-compartments that have specific lipid and protein composition(Kurzchalia and Parton 1999; Mukherjee and Maxfield 2000). Two important microdomains have been identified; lipid rafts and subset of rafts known as caveolae(Maxfield 2002), **Figure 1-8**.

Lipid rafts were firstly isolated as membrane structures insoluble in non-ionic detergents(Brown and London 2000b). Membrane rafts are defined as dynamic nanoscale sterol, sphingolipid-enriched, ordered assemblies of specific proteins(Lingwood and Simons 2010; Simons and Gerl 2010). Among the proteins that occur in rafts are phosphoglyceride-anchored proteins and proteins involved in cell signalling(Lingwood and Simons 2010; Stuermer 2010). Studies have shown that, these highly dynamic raft domains are essential in signalling processes and also form sorting platforms for targeted protein traffic(Patel et al. 2008; Patra 2008). Lipid rafts are also involved in protein endocytosis that occurs via caveolae or flotillin-dependent pathway(Lajoie and Nabi 2007; Lajoie and Nabi 2010). Non-constitutive protein components of rafts fluctuate dramatically in cancer with impacts on cell proliferation, signalling, protein trafficking, adhesion and apoptosis(Staubach and Hanisch 2011).

Caveolae, specialized forms of lipid rafts, are small tube-like invaginations of the plasma membrane with diameter of approximately 60-80nm, and characterized by specific scaffolding proteins, the caveolins(Le Lay and Kurzchalia 2005; Chidlow and Sessa 2010; Bastiani and Parton 2011). The lipid composition of caveolae (cholesterol, sphingomyelin, glycosphingolipids and saturated fatty acids) is similar to that of "lipid rafts(Stan 2005). Caveolae occur at different densities in different cell types, being most prominent in vascular endothelial cells (ECs), adipocytes, fibroblasts, and epithelial

cells(Chidlow and Sessa 2010). Mounting evidence indicates that caveolae function as cell signalling platforms and regulate the kinetics of vesicle transport, making them both versatile and highly integrated into cellular physiology(Quest et al. 2004; Pike 2005). In terms of EC function, caveolae are important regulators of vascular tone through modulation of endothelial nitric oxide synthase (eNOS) activity(Sowa et al. 2001; Bernatchez et al. 2005). Caveolin-1 has also been shown to regulate important inflammatory signalling mediators including cyclooxygenase-2 (COX-2)(Engelman et al. 1998; Chen et al. 2010). In-vitro and animal studies show that rafts and caveolae are potentially modifiable by diet particularly n-3 fatty acids, and potentially influencing a wide range of biological processes, including immune function, neuronal signaling, cancer cell growth and insulin resistance in metabolic disorders(Garattini 2007; Siddiqui et al. 2007; Yaqoob and Shaikh 2010).

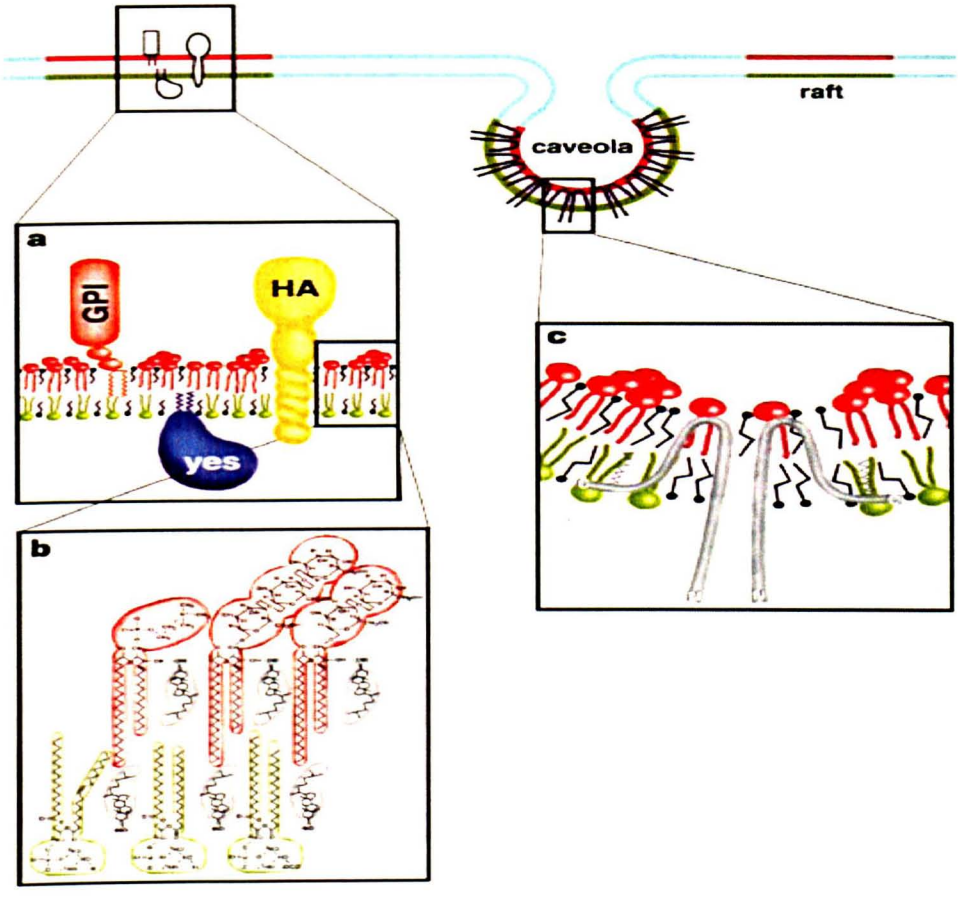


Figure 1-8 Organisation of lipid rafts and caveolae membranes
(Simons and Ikonen 1997)

a. Rafts contain specific proteins attached to the exoplasmic leaflet of the bilayer (i.e. Glycosylphosphatidylinositol (GPI) anchored, the Src-family kinase Yes, the influenza virus protein haemagglutinin (HA))

b. Rafts lipid organization: sphingomyelin and glycosphingolipids (red) enriched in the exoplasmic leaflet and glycerolipids (for example phosphatidylserine and phosphatidylethanolamine; green) in the cytoplasmic leaflet. Cholesterol (grey) is present in both leaflets and fills the space under the head groups of sphingolipids or extends the fatty acyl chain in the apposing leaflet.

c. Caveolae are formed by self-associating caveolin molecules making a hairpin loop in the membrane.

1.2.3 Membrane lipids

At the time the fluid mosaic model was formulated, evidence was become available that membrane's lipids are not as considered before biologically inert compounds, simply form a barrier between the inner and external environment of cells(Spector and Yorek 1985). It becomes increasingly evident that membrane lipids, independently and in concert with proteins, play a crucial role in the regulation of cell functions(Williamson et al. 2003; Lee 2005), including cellular metabolism(Di Paolo and De Camilli 2006; Worgall 2008), signal transduction(Wymann and Schneiter 2008), regulation of gene expression(Sampath and Ntambi 2004; Sampath and Ntambi 2005) and major determinant of cell membrane fluidity and permeability(van Meer et al. 2008).

The major structural lipids in mammals membranes are the glycerophospholipids, sphingolipids and sterols, specifically cholesterol, are the main component of animal cell membrane lipids(van Meer et al. 2008; Mannock et al. 2010). In human red blood cells the content of total lipids is approximately 5.0×10^{-10} mg per red cell. In which phospholipids are about 60%, neutral lipids (chiefly free cholesterol) 30% and the rest is glycolipids(Yawata 2003).

1.2.3.1 Membrane cholesterol

Cholesterol is a major and essential lipid component of the plasma membranes of the cells of higher animals, and is also found in lower concentrations in certain intracellular membranes in vesicular communication with the plasma membrane(Liscum and Munn 1999; Mannock et al. 2010). It typically accounts for 20-25% of the lipid molecules in plasma membrane (Ikonen 2008), but it can increase up to 50% in red blood cells(Rog et al. 2009). Structurally, cholesterol is a steroid built from four linked hydrocarbon rings attached to hydrocarbon tail at one end, and hydrophilic hydroxyl group to the other end, **Figure 1-9**. Cholesterol affects cellular processes by interacting both with other membrane lipids as well as with specific proteins. Because of the rigid sterol backbone, cholesterol is preferentially positioned in close proximity to saturated hydrocarbon chains of neighbouring lipids, hence resulting in increased lateral ordering of membrane lipids, and consequently affects the biophysical properties of the membrane, by decreasing fluidity and reducing the permeability of

polar molecules(Simons and Vaz 2004; Ikonen 2008). Moreover, sterol-induced alterations in membrane biophysical properties and direct interaction with proteins could affect the cell membrane protein functions and cell signalling(Lee 2004; Olkkonen et al. 2006; Raychaudhuri and Prinz 2010). Interestingly, There is evidence patients with SCD do have high concentration of cholesterol in RBC membrane, However the underpinning cause (s) and implications of this is not well elucidated(Muskiet and Muskiet 1984; Sasaki et al. 1986)

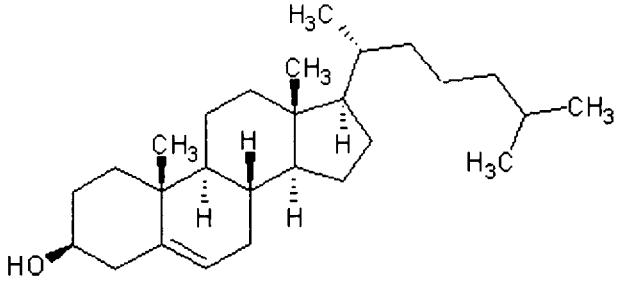


Figure 1-9 Cholesterol structure
Adopted from http://www.ganfyd.org

1.2.3.2 Spingolipid

Sphingolipids are vital cell components, mainly residing in the external layer of the plasma membrane. The lipid moiety of sphingolipids is named ceramide. Ceramide is constituted by a long chain amino alcohol, 2-amino-1,3-dihydroxy-octadec-4-ene, sphingosine, connected to a fatty acid by an amide linkage(Karlsson 1970; Merrill 2002), **Figure 1-10.** The major sphingolipids in mammalian cells are sphingomyelins (SMs) and the glycosphingolipids (GSLs)(Feizi 1985; Sonnino et al. 2006).

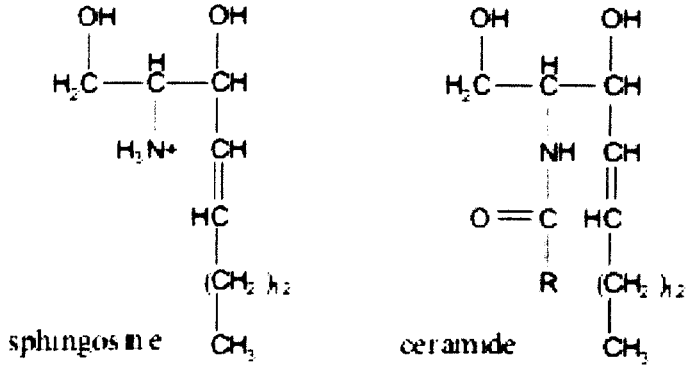


Figure 1-10 structure of sphingosine and ceramide
Adopted from www.rpi.edu/dept/bcbp/molbiochem

1.2.3.3 Glycosphingolipid

Glycosphingolipids (GSLs) contain mono-, di- or oligosaccharides based on glucosylceramide (GlcCer) and sometimes galactosylceramide (GalCer)(van Meer and Lisman 2002). The major GSLs in animal cell membranes are cerbrosides and gangliosides(Hakomori 2003). Cerbrosides also called monoglycosylceramides (**Figure 1-11 b**), have ceramide linked with amide bond to glucose (glucocerbroside) or glactose (glactocerbrosides). Cerbrosides are particulary occur in neuronal tissues(Tan and Chen 2003), and they appear to be involved in the regulation of specific aspects of neuronal proliferation, differentiation, survival and apoptosis(Buccoliero and Futerman 2003). In gangliosided (**Figure 1-11a**), the carbohydrate moiety is oligosaccharide which carries residues of sialic acid or neuraminic acid, the trivial names used for the derivatives of 5-amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid(Cantu et al. 2011). Besides its structural importance, glycosphingolipids are involved in the regulation of signalling through the growth factor and adhesion receptors(Odintsova et al. 2006; Sonnino and Prinetti 2010).

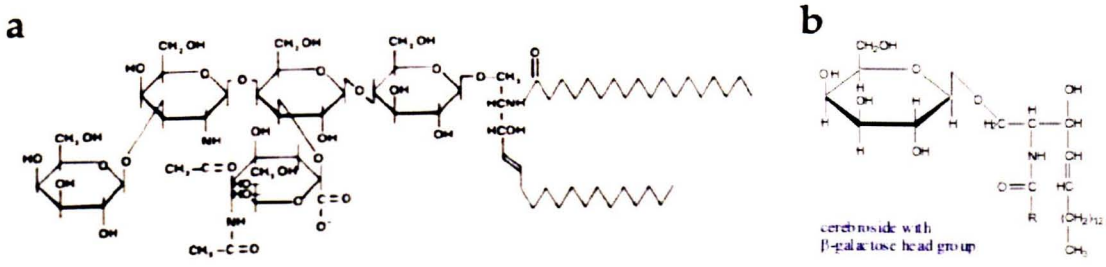


Figure 1-11 the structure of glycosphingolipids
a) One of gangliosides b) Cerebroside. Adopted from http:www.cyberlipid.org/glycolip

1.2.3.4 Sphingomyelin

Sphingomyelins (SMs) constitute the most common sphingolipids in mammalian membranes(Talbott et al. 2000). These molecules result from the addition of a polar head group, phosphorylcholine or phophorylethanolamine to ceramides, **Figure 1-12**. The acyle group is generally saturated (16:0, 18:0, 22:0, 24:0) or monosaturated (18:1, 20:1, 22:1, 24:1) fatty acids(Karlsson et al. 1998; Byrdwell and Perry 2007). Sphingomyelin (SPM, ceramide-1- phosphorylcholine) is the most abundant sphingomyelins that present mainly in the outer cell membrane(Ramstedt and Slotte 2002; Daleke 2008). Tissues in which SPM is the predominant phospholipid include the sheath of nerve cell axons, myelin (25%), erythrocytes (18%) and lenses (10 to 15%) of the phospholipids content (Calhoun and Shipley 1979; Borchman et al. 1994). SPM synthesis occurs in the lumen of the Golgi as well as on the cell membrane(Huitema et al. 2004). The synthesis process which is catalysed by SM synthase (SMS), involves the transfer of phosphocholine from phosphatidylcholine onto ceramide, yielding diacylglycerol in the process (Villani et al. 2008; Gault et al. 2010). Because SMS activity directly regulates the level of ceramide and diacylglycerol, it has also been proposed to play an essential role in regulating cellular functions(Gault et al. 2010).

Sphingomyelin high packing density and affinity for sterols help provide a rigid barrier to the extracellular environment and play a role in the formation of lipid rafts, signal transduction and membrane trafficking(Holthuis et al. 2001; Ohanian and Ohanian 2001; Tafesse et al. 2007).

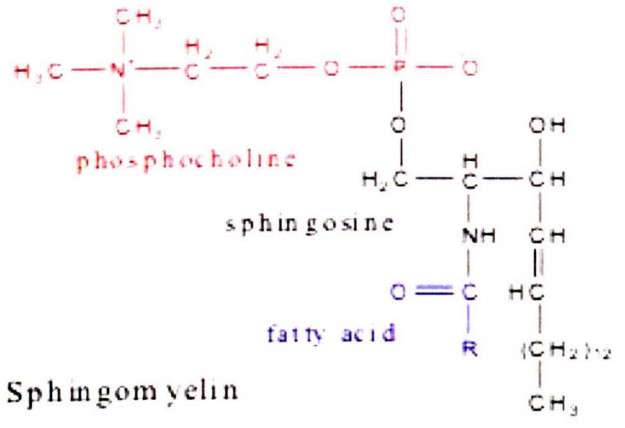


Figure 1-12 the structure of sphingomyelin and ceramide
Adopted from www.rpi.edu/dept/bcbp/molbiochem

1.2.3.5 Phosphoglyceride

1.2.3.5.1 Structure and function

Phosphoglycerides are the most abundant cell membrane lipids in virtually all mammalian membranes. Phosphoglycerides consist of a glycerol core binding to a phosphate group (hydrophilic head) and two fatty acids (hydrophobic tail), **Figure 1-13**. According to structure of the head group, Phosphoglycerides are classified into choline (CPG), ethanolamine (EPG), serine (SPG), and inositol phosphoglycerides (IPG), phosphatidic acid (PA), phosphatidyglycerol (PG) and cardiolipin, **Figure 1-13.** Choline phosphoglyceride accounts for >50% of the phospholipids in most eukaryotic membranes(van Meer et al. 2008). While the hydrocarbon chain at the sn2 position is always ester-linked to the glycerol moiety, that in the sn1 position can be linked via an ester, ether or a vinylether bon. The length of the alkyl chain typically varies from 14 to 24 carbons and the number of double bonds from 0 to 6. The alkyl chain in the sn1 position is typically saturated or monounsaturated, while that in the sn2 position is often polyunsaturated(Hermansson et al. 2011). Because of the large number of different alkyl chain combinations, each phospholipid class in turn consists of numerous molecular species, i.e. molecules that have the same head group but differ in respect of the acyl chains(Han and Gross 2005; Taguchi and Ishikawa 2010).

Besides serving as key structural components of membranes, phosphoglyceride many have other functions particularly in signalling transduction(Fernandis and Wenk 2007; Meyer zu Heringdorf and Jakobs 2007; Guan and Wenk 2008; van Meer et al. 2008).

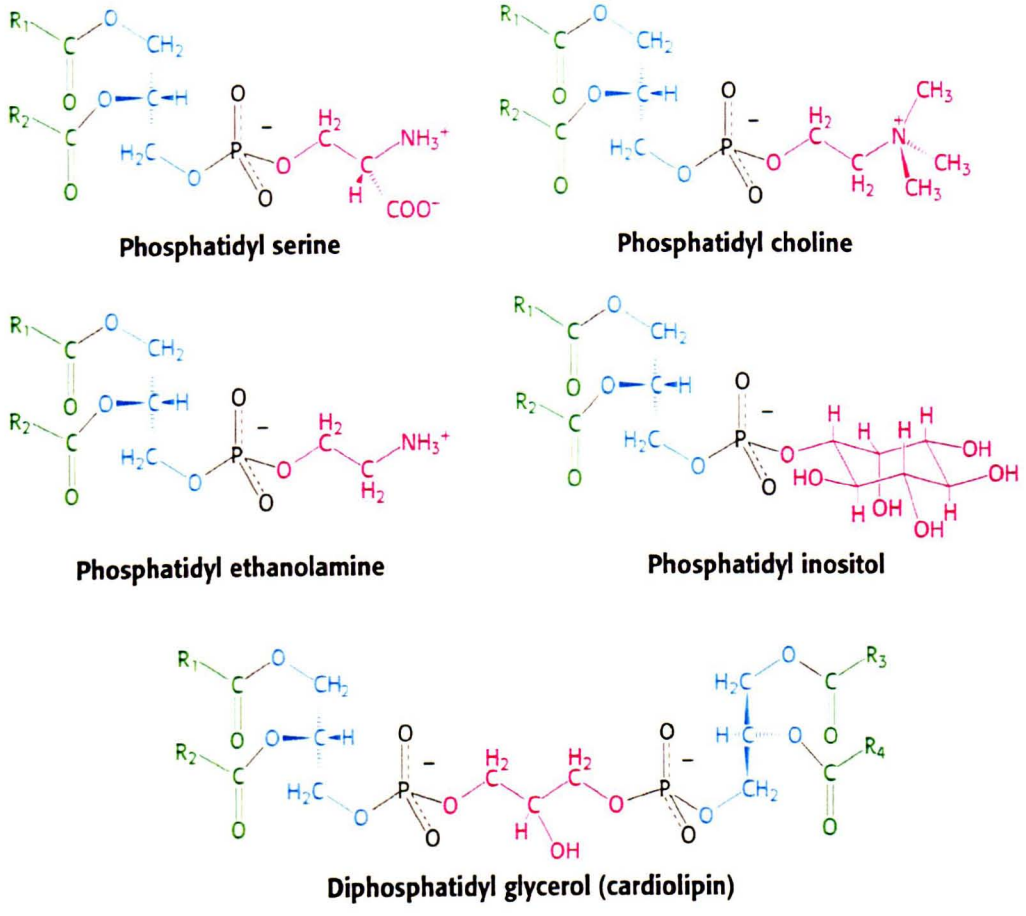


Figure 1-13 Phosphoglyceride structure
(Adopted from www.Biochem.uthscsa.edu)

1.2.3.5.2 Phosphoglyceride synthesis

Phosphatidic acid (PA) is a key building block of all the mammalian phospholipids. PA is mainly synthesised by acylation of glycerol-3phosphate by the action of glycerol-3-phosphate acyltransferase. The product 1-acyl-glycerol-3-phosphat (lyso Phosphatidic

acid) is subsequently acylated by another acyltransferase enzyme, **Figure 1-14**. The donor of the fatty acyl chains of phosphatidic acid is acyl-CoA(Das et al. 1992; Vance and Vance 2004). A small amount of PA is also formed from dihydroxyacetone phosphate (DHAP). The pathway involves acylation of DHAP to 1-acyl DHAP which in turn reduced and acylated to produce lyso Phosphatidic acid(Dircks and Sul 1997; Chakraborty et al. 1999). Notably, beside de novo biosynthesis, PA can also be generated via phosphorylation of diacylglycerol (DAG) by a diacylglycerol kinase(Cai et al. 2009; Topham and Epand 2009).

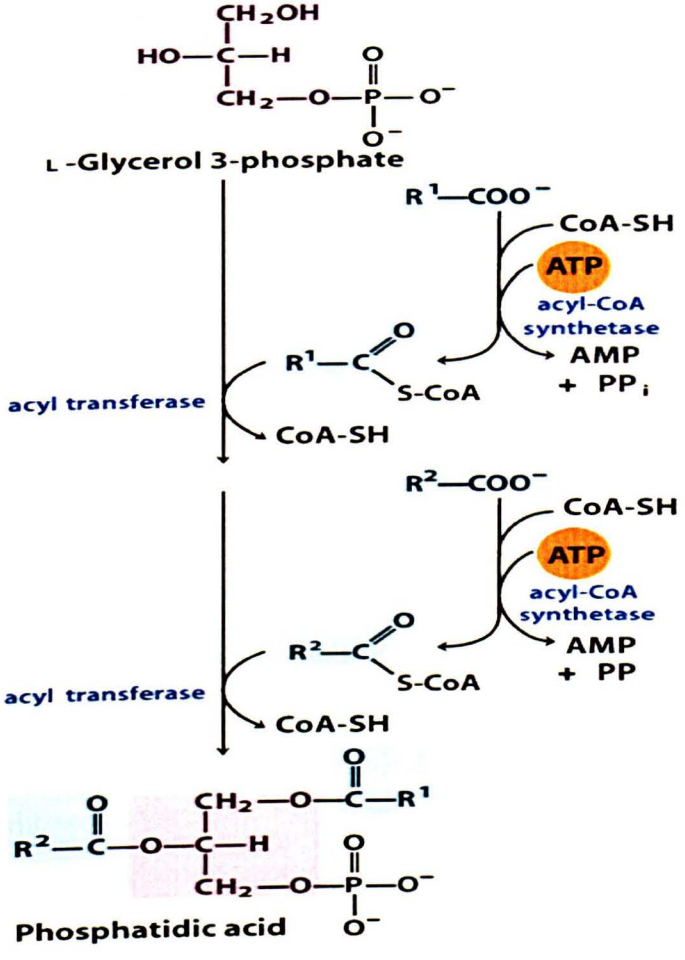


Figure 1-14 De novo synthesis of phosphatidic acid
(Adopted from www.med.ufl.edu/.../PL%20&%20cholesterol%20biosynthesis)

The biosynthesis of CPG, EPG, and SPG requires a source of diacylglycerol (DAG) that is generated through the hydrolysis of phosphatidic acid(Shindou and Shimizu 2009).

The enzyme catalyzing this reaction is phosphatidic acid phosphatase. This phosphatase can be regulated by reversible translocation from a less active form in the cytosol to a more active form in endoplasmic reticulum (ER) membranes(Hermansson et al. 2011). A second fate of phosphatidic acid is its conversion to cytidine diphospho-diacylglycerol (CDP-diacylglycerol) which is used for the synthesis of phosphatidylinositol (IPG), phosphatidylglycerol (PG), and diphosphatidylglycerol (cardiolipin)(Heacock and Agranoff 1997). Mammalian CDP-diacylglycerol synthase, which converts phosphatidic acid into CDP-diacylglycerol, is present in both mitochondrial and microsomal fractions, although 90%–95% of the activity resides in microsomes(Vance and Vance 2004).

All mammalian cells and tissues synthesize CPG via the cytidine 5'-phosphate (CDP)-choline (Kennedy) pathway, but hepatocytes can produce this phospholipid also by sequential methylation of phosphatidylethanolamine. Synthesis of CPG via the CDP-choline pathway involves three steps. First, choline is phosphorylated by choline kinase to phosphocholine(Wu and Vance 2010). Second, CTP:phosphocholine cytidylyltransferase catalyzes the condensation of phosphocholine and CTP thus producing CDP-choline(Choy et al. 1980; Sugimoto et al. 2008). Finally, phosphocholine is transferred from CDP-choline to diacylglycerol by CDP-choline:diacylglycerol cholinephosphotransferase thus forming CPG(McMaster and Bell 1997).

Mammalian cells synthesize EPG via CDP-ethanolamine pathway and decarboxylation of SPG(Arthur and Page 1991). The synthesis of EPG via CDP-ethanolamine (Kennedy) pathway is analogous to that of CPG(Hermansson et al. 2011). The main route of the synthesis of SPG is by base-exchange from CPG or EPG by one of the SPG synthases. Phosphatidylserine synthase 1 replaces the head group of CPG with L-serine and phosphatidylserine synthase 2 catalyses the analogous reaction with EPG(Vance 2008). IPG is synthesised by the condensation of myo-inositol and CDP-DAG by CDP-diacylglycerol: inositol-3-phosphatidyl transferase(Antonsson 1997). A similar reaction between glycerol-3-phosphate and CDP-diacylglycerol produces PG(Ohtsuka et al. 1993), which contributes in formation of cardiolipin by condensation with CDP-diacylglycerol(Houtkooper and Vaz 2008), **Figure 1-15.**

After their synthesis de novo many phospholipid species undergo acyl chain remodelling (Lands' Cycle), a process which involves phospholipase A and acyl transferase or transacylase(MacDonald and Sprecher 1991; Lands 2000). Phosphoglyceride remodelling is thought to be responsible for establishing and maintaining the specific molecular species composition of the various Phosphoglyceride classes(Shindou et al. 2009).

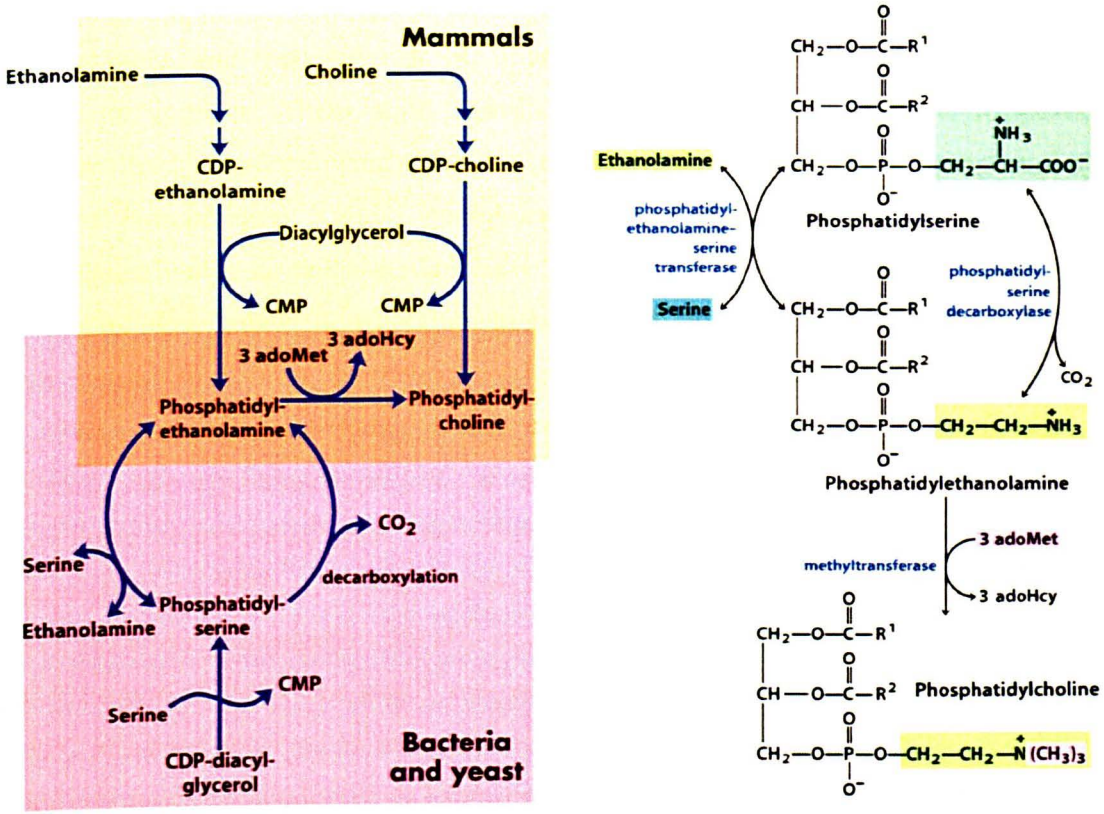


Figure 1-15 Summary of Pathways to Phosphatidylcholine and Phosphatidyethanolamine synthesis (www.wlu.ca/.../02-05_Biosynthesis_of_Membrane_Lipids)

1.2.4 Membrane phospholipids asymmetry

The plasma membrane is characterized by a strict lipid asymmetry in which CPG and sphingomyelin (SPM) are mainly localized in the extracellular leaflet, whereas EPG and SPG are exclusively present in the cytoplasmic(inner) leaflet(Contreras et al. 2010).

Other minor phospholipids, such as phosphatidic acid (PA) and IPG, are also enriched on the cytofacial (inner) side of the membrane(Daleke 2003). The distribution of glycosylsphingolipids favors the external leaflet of the plasma membrane(Kolter et al. 2002). In red blood cell membrane, approximately 80% of SPM and CPG are located in the outer cell membrane. In contrary, 80% of EPG and 100% of SPG occur in the inner leaflet of the cell membrane(Bretscher 1972; Schwartz et al. 1985a).

The asymmetric distribution of phospholipids in the plasma membrane may be the result of the selective trafficking of phospholipids from endoplasmic reticulum (ER) and Golgi apparatus, or regulation of lipid transporting proteins(Daleke 2003). Establishment and maintenance of lipid asymmetry involves several different membrane proteins. These lipid translocators include ATP-dependent flippases (catalyze lipid transfer towards the inward) and floppases (catalyze lipid transfer outward monolayer) and energy-independent bi-directional transporters scramblases(Pomorski and Menon 2006; Devaux et al. 2008), **Figure 1-16**. The scramblase flips aminophospholipids in particular PS from the plasma membrane inner to outer monolayer upon increased level of calcium in the cytosol(Williamson et al. 1992). Although asymmetry is the rule for normal cells, loss of asymmetry, especially the appearance of SPG at the cell surface is associated with many physiologic phenomena(Zwaal and Schroit 1997), such as thrombosis(Zwaal 1978; Heemskerk et al. 2002), cells phagocytosis and apoptosis(Martin et al. 1995; Fadok et al. 2001). Moreover, tumorigenic cells also express relatively large amounts of outer-leaflet SPG(Utsugi et al. 1991). The loss of red cell membrane asymmetry and externalisation of SPG has been observed in many diseases associated with co-agulopathy including sickle cell disease(Manodori et al. 2000), stroke and diabetes(Wilson et al. 1993).

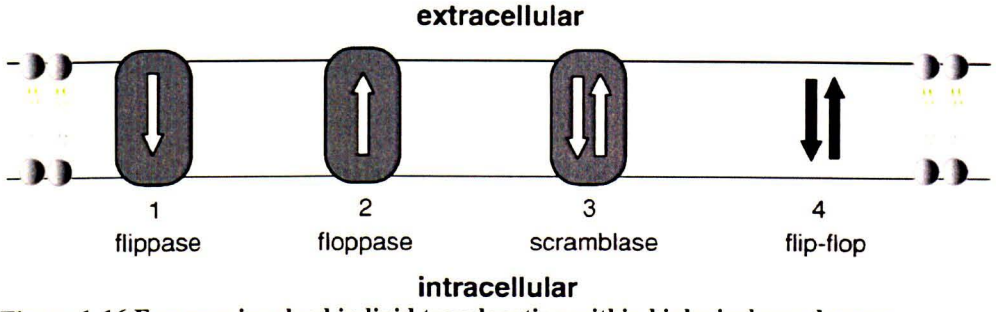


Figure 1-16 Enzymes involved in lipid translocation within biological membranes

(Adopted from(Devaux et al. 2008)

1.2.5 Fatty acid

Fatty acids are carboxylic acids with long aliphatic tail, whose length may vary from 4 to 30 carbons. However, most of the fatty acids found in plasma membrane have 16 to 24 evenly numbered carbon atoms. The predominant fatty acids are straight chain, can be saturated (SFA) or contain carbon-carbon double bonds with an even number of carbon atoms. Based on the number of double bonds, fatty acids are further classified into monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). Fatty acids are the major lipid building block of complex lipids, and therefore are the one of the most fundamental categories of biological lipids.

1.2.5.1 Nomenclature

There are several systems of nomenclature in use for fatty acids. Trivial or common names are non-systematic historical names are the most frequent naming system used in literature. In the International Union of Pure and Applied Chemistry (IUPAC) system of nomenclature, the carbons in fatty acids are numbered consequently from the end of the chain, the carbon of the carboxyl group being considered as number 1. By convention, a specific bond in a chain is identified by the lower number of the two carbons that it joins. The double bond configuration is labelled Z/E notation (Z for *cis* and E for *trans*), where appropriate. In the cis conformation, the two hydrogen atoms are situated on the same side of the double bond, whereas in the *trans*, the two hydrogen atoms sit on each side of the double bond.

Another system of nomenclature in use for unsaturated fatty acids is the delta system. This system is similar to that of IUPAC; the only difference is the Greek delta symbol

(Δ) is used to indicate the position of double bond counting from the carboxylic carbon. Whereas, the Greek symbol omega (ω), or letter n denote the position of the first double bond from the methyl end. The delta symbol is preceded by a cis- or trans- prefix indicating the configuration of the double bond.

1.2.5.2 Saturated fatty acids

Saturated fatty acis contain only single carbon-carbon bonds (no double bonds), **Figure 1-17.** Most of the SFAs occurring in nature have unbranched and even number of carbon atoms. They have general formula R-COOH, in which R group is a straight chain hydrocarbon of the form CH3(CH2)X. SFA are the least reactive chemically and therefore, they are more stable. The melting point of SFA increases with chain length. Decanoic and longer chain fatty acids are solid at room temperature.

The saturated fatty acids are further classified into three subgroups, short, medium and long. Short chain fatty acids contain less than eight carbons. Butyric (4:0), and caproic (6:0) are the most important member of this group and they occur mainly in milk fat. The second subgroup, medium chain fatty acids are fatty acids with carbon atom from 8-14. Capric (10:0), lauric (12:0) and myristic (14:0) are members of this group. The long chain fatty acids are fatty acids with 16 carbons or more. Plamitic (16:0) and stearic (18:0) are the most important fatty acid of the group. Plmitic acid is the most widely occurring SFA. Saturated fatty acids longer than stearic, arachidic (20:0), behenic (22:0) and lingceric (24:0) are minor dietary components(Ratnayake 2008).

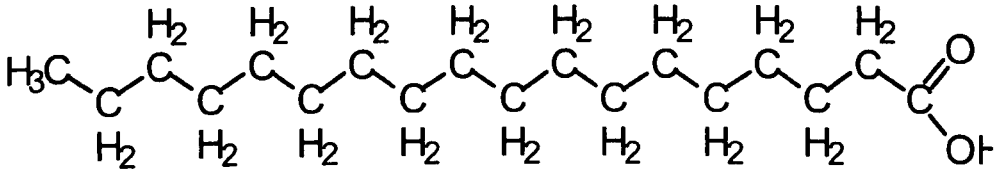
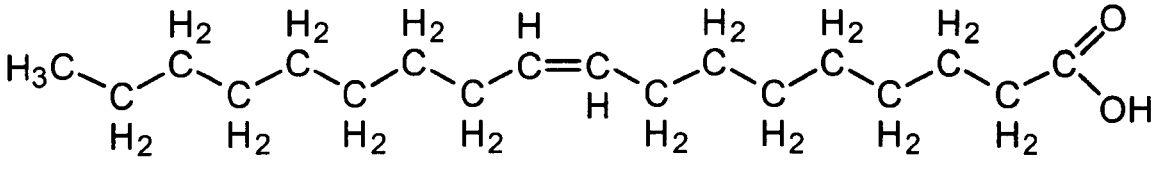


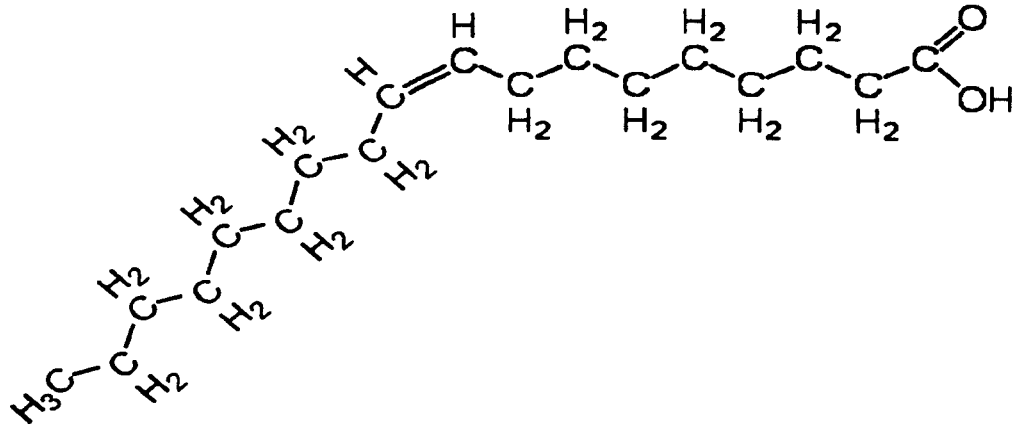
Figure 1-17 Saturated fatty acid (stearic acid).
(Adopted from www.anslab.iastate.edu)

1.2.5.3 Monounsaturated fatty acids

The monounsaturated fatty acids contain a single carbon-carbon double bond. The presence of a single double bond in the structure allows two possible conformations; the *cis* and the *trans*, **Figure 1-18.** The *cis* configuration results in a non-straight structure, which confers more fluidity to membranes as compared to the *trans* conformation. *Trans* fatty acids are in fact straight chains with properties similar to saturated fatty acids, and they increase membrane rigidity. The fatty acids present in animal cell membranes have cis configurations(Shantha and Napolitano 1992). The double bond is most likely located at Δ9 position. Oleic acid (*cis*-9-octadecenoic) is the commonest cis-MUFA and it is also the most widely distributed of all natural lipids, **Figure 1-18.**



***trans*-Oleic acid**



***cis*-Oleic acid**

Figure 1-18 Trans and cis configurations of a monounsaturated fatty acid
(Adopted from www.anslab.iastate.edu)

1.2.5.4 Polyunsaturated fatty acids (PUFA)

Polyunsaturated fatty acids have two or more double bonds. The double bonds can be uninterrupted (allenic), conjugated one-methylene-interrupted or polymethylene-interrupted. Most animal cells have one-methylene-interrupted double bonds. The presence of several double bonds lowers their melting point compared to saturated fatty acids of the same length, and produces extremely flexible molecules. Therefore, high "liquidity" of cell membranes is usually associated with the presence of increased number of unsaturated fatty acids. The natural PUFA with methylene interrupted double bonds and with all cis configuration can be divided into 12 different families ranging from double bonds located from the n-1 position to n-12 position counted from the terminal carbon of the fatty acid chain(Gunstone 1999). The most important families, in term of extent of occurrence and human health and nutrition are n-3, n-6 and n-9 families, **Figure 1-19.** All of which are metabolized using the same group of enzymes. Besides their vital role as integral part of the cell membranes, PUFAs serve as the major constituents of plasma lipoprotein particles phospholipids, triglycerides, and cholesterol esters(Le et al. 2009).

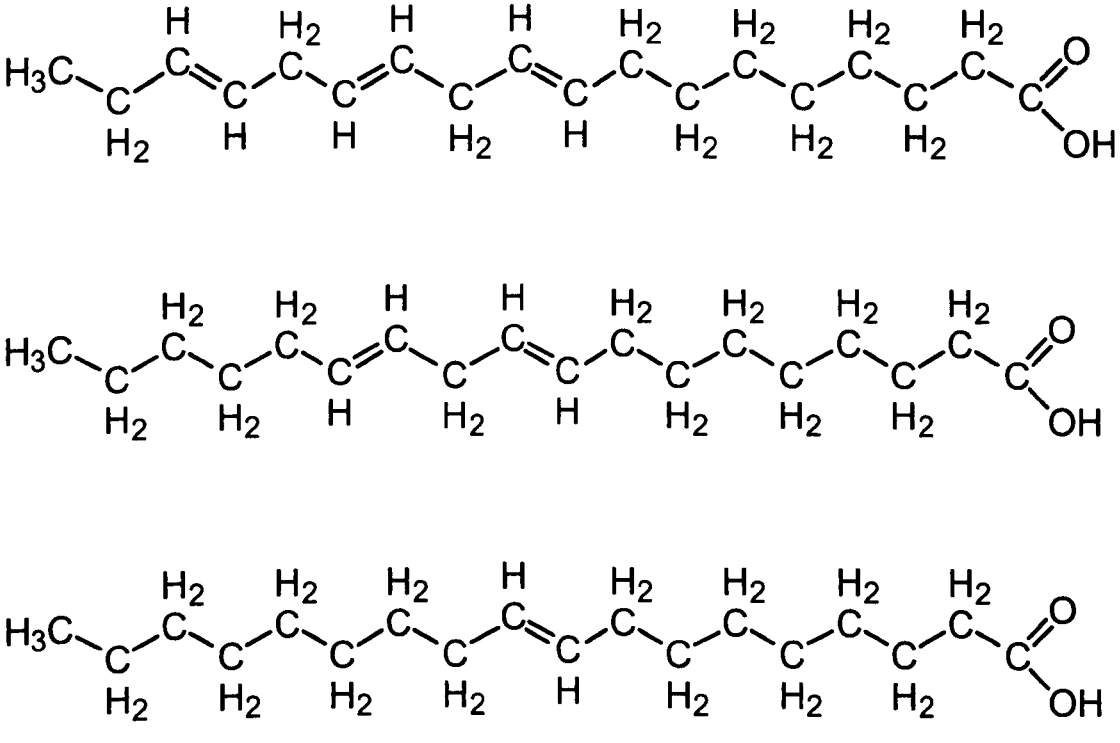


Figure 1-19 Strucure of polyunsaturated fatty acids a-n-3, b- n-6, c- n-9 fatty acid
(Adopted from www.anslab.iastate.edu)

1.2.5.5 Essential fatty acids (EFA)

An 'essential' nutrient is one that is needed for normal development and function of mammalian cells throughout the life cycle(Cunnane 2003). The term EFA was first coined by Burr and Burr in 1929 when these FAs were used to reverse the symptoms developed in young rodents fed with a fat-free diet. The fat-free diets resulted in dermatitis, kidney lesion, infertility, premature death and impaired growth. These symptoms were reversed by the introduction of linoleic (LA) and α-linolenic (ALA) acids in the diets(Burr and Burr 1973). However, ALA was not as effective as LA in curing the condition. In human, the importance of LA was not realised until 1958 when Arild Hansen and colleagues demonstrated for the first time that LA- deficient milk is associated with skin symptoms and growth retardation(Hansen et al. 1958). In contrast to LA, ALA essentiality was not established in human until Holman et al reported that ALA deficiency causes neurological and visual impairment and these lesions are treatable by supplementation with ALA(Holman et al. 1982). ALA deficiency symptoms ere also observed other mammals. In monkeys, ALA deficiency reported to cause scaly dermatitis, alopecia and visual impairment. In rats, visual impairments, learning difficulties, lower reproduction, impaired lactation and increased prenatal mortality were also reported. However, there are no dose-response studies in animals or humans investigated the ability of ALA, EPA, or DHA to reverse n-3 FA deficiency.

In humans, the essentiality of dietary intake of polyunsaturated fatty acids (PUFAs) is increasingly recognised as it proved that the body is unable to synthesize LA and ALA and the de novo synthesis rat of EPA and DHA don not meet the metabolic needs of the body(Gomez Candela et al. 2011). It is know accepted that the clinical manifestations of LA and ALA are due to the deficiency of their respective longer chain metabolites, such as Dihomo-γ-linolenic (DHGLA), arachidonic acid (AA), eicosapentanoic (EPA) and docosahexaenoic (DHA) acids. Hence, AA, EPA, and DHA are considered conditional EFAs because their production may be inadequate in certain conditions and necessitate exogenous supplementation(Le et al. 2009)

1.2.5.6 Endogenous synthesis of LCPUFAs

Among these three families of PUFAs, only OA, a parent n- 9 FA, can be synthesized by mammals from simple carbon precursors. Both the parent n-3 and n-6 FAs must be

acquired from the diet, as mammals cannot insert double bonds at position-3 and position-6 to produce ALA and LA, respectively, because the lack of Δ12 and Δ15 desaturases(Pudelkewicz et al. 1968). ALA and LA can be further desaturated and elongated to varying degrees depending on the animal species, Δ6 and Δ5 desaturases and elongases enzymes activity and tissue location(Innis 1991; Wang et al. 2005; Zheng et al. 2005), **Figure 1-20.** The changes in expression levels of the desaturase and the elongase genes are thought to involve transcription factors such as peroxisome proliferator-activated receptor alpha (PPARα)(Keller et al. 1993). There is evidence to suggest that the activity of desaturase genes is related to the prevailing concentrations of PUFAs, and these genes are upregulated in response to low dietary PUFAs and suppressed at high PUFA/LCPUFA levels(Igarashi et al. 2007; Tu et al. 2010).

The pathway leading to the biosynthesis of DHA from docosapentaenoic acid (DPA, 22:5n-3) has only recently been deciphered in mammals. Until 1991, it was postulated that a Δ4 desaturase catalyzes the conversion of DPA to DHA in the microsome. However, radiolabeling studies and lack of evidence of Δ4 presence in mammalian cells paved the way for a new model for DHA synthesis (Sprecher pathway)(Voss et al. 1991; Sprecher et al. 1995). DPA is elongated to C24:5n-3, which is then desaturated by a Δ6 desaturase to generate C24:6n-3 in the microsome. C24:6n-3 is then thought to be transported to the peroxisomes, wherein it undergoes β-oxidation (retroconversion) to generate DHA. The Sprecher pathway has been accepted as a working model, though the exact enzymes or specific mechanism involved in retroconversion are not fully delineated(Pereira et al. 2003; Benatti et al. 2004).

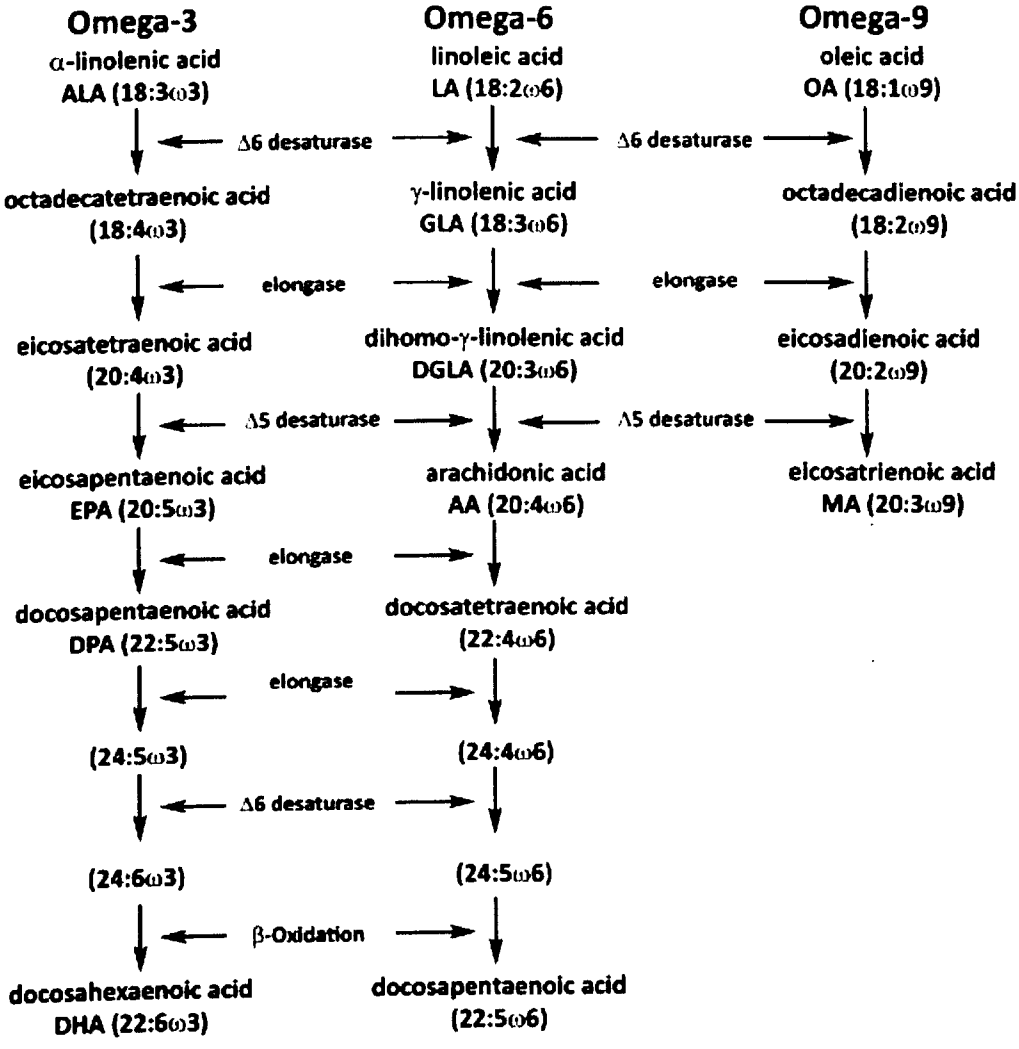


Figure 1-20 Pathway of metabolism and synthesis of n-3, n-6, and n-9 LCPUFAs Adopted from(Le et al. 2009).

1.2.5.7 Clinical and Biochemical markers of essential fatty acid deficiency

Essential fatty acid deficiency (EFAD) typically occurs when less than 1-2% of total calories are provided from EFAs(Holman 1960). In humans, biochemical changes consistent with EFAD can occur in as little as a few days in infants. However, Clinical symptoms of EFAD may take 4-6 weeks to appear in older patients. Clinical signs of EFAD include growth retardation, hair loss, infertility, coagulopathies, dry and scaly skin(Alfin-Slater and Aftergood 1968). The biochemical indicator of EFAD is increased oliec acid (OA) conversion to mead (MA, 20:3n-9) and di-homo mead (22:3n-9) acids(Siguel et al. 1987). This metabolic switch is seen as a compensatory mechanism to

maintain the number of double bonds in FAs incorporated in cell membranes(Le et al. 2009). Desaturase enzymes display differential activity in the following order of preference: n-3>n-6>n-9. As a result, conversion of OA to MA (n-9), only occurs when there are low dietary levels of both ALA and LA. Hence, a mead and arachidonic acid ratio of less than 0.2 is equated with linoleic acid sufficiency(Mascioli et al. 1996). Likewise, an insufficient intake of DHA, if n-6 fatty acids are not limiting, leads to decrease in DHA, and concomitant increase in n-6 docosapentaenoic acid (22:5n-6) synthesised from n-6 docosatetraenoic acid (22:4n-6). Therefore, the ratios of 22:5n-6/22:4n-6 and DHA/22:5n-6 are regarded as reliable DHA markers of status.

1.2.5.8 Dietary requirements of LPUFA

Because LA is abundant in the human diet, the amount of AA available almost always exceeds the level needed to maintain the metabolic needs. n-6 fatty acids abundantly present in liquid vegetable oils, including soybean, corn, safflower oil and cotton seed oils. It was only upon the introduction of parenteral nutrition that LA and AA became more common. In rodents, AA alone has been shown to be as effective as LA in preventing symptoms of essential fatty acids deficiency (EFAD)(Thomasson 1962; Hansen et al. 1986). These studies demonstrate that AA can replace LA as the sole source of dietary omega-6 FAs(Le et al. 2009).

Unlike n-6 fatty acids, n-3 fatty acids do not occur in large amounts in plants food and western human diet. Moreover, ALA conversion process to EPA and DHA in humans is not efficient as only 5-10% are converted to EPA, and a mere 2-5% to DHA(Arterburn et al. 2006). N-3 fatty acids are abundantly present in fish and shellfish. In fact, fish-oil supplements typically contain 30–50% of n-3 FAs(Russo 2009). The current adequate intake (AI) for ALA issued by the Institute of Medicine of the National Academies, USA (2005) is 1.6g/day for men 19–47 years and 1.1 g/day for women 19–47 years. The acceptable macronutrient distribution range (AMDR) for ALA is 0.6–1.2% of energy. The lower boundary of the range meets the AI for ALA. Approximately 10% of the AMDR for ALA can be consumed as EPA and/or DHA. The dietary guidelines (2005) also note that consumption of approximately two servings of fish per week (approximately 224 g total) may reduce the risk of mortality from

coronary heart disease. However, since the physiological potency of EPA and DHA is greater than ALA and there has been a substantive increase in the evidence base about the health benefits of omega-3 fatty acids(Mozaffarian and Rimm 2006), the recommendations of International Society for the Study of Fatty Acids and Lipids (ISSFAL) for minimum DHA and EPA intake is 500 mg/day(ISSFAL 2004).

The traditional diet of northern Sudanese population is characterised by high carbohydrate and low fat intake, particularly n-3 fatty acids due to paucity of marine food and absence of dietary supplementation culture. Nyuar et al (2010) have recently reported that the breast milk of Sudanese mothers contains the lowest level of DHA compared to 106 studies conducted in several countries all over the world(Brenna et al. 2007).

Another important and more controversial aspect in LCPUFA dietary intake is the relationship between the n-6 and n-3 intake. There is a controversial point of view regarding the n-6/n-3 fatty acids ration role in health and disease(Crawford 2000; Griffin 2008; Simopoulos 2009). Nevertheless, it can be stated that an adequate intake of both FAs, n-3 and n-6 fatty acids, is essential for good health, though it is not clear whether the ratio between them is of crucial importance(Willett 2007).

1.2.5.9 Biomedical importance of n-6 and n-3 LCPUFAs

1.2.5.9.1 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). Biochemical, 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 proaggregatory, with increases in blood viscosity, vasospasm, and vasoconstriction(Ferrucci et al. 2006; Simopoulos 2009).

1.2.5.9.2 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).

The adult brain contains approximately 50%-60% of its dry weight as lipid and approximately 35% of these lipids are LCPUFAs particulate DHA. In (1976) Crawford et al compared thirty different mammalian species and found that, despite a wide variation in the fatty acid composition of the livers, that of brain tissue was remarkably constant, with DHA making up 25% of grey matter phospholipid fatty acids. In the photoreceptor outer segments, DHA constitutes 60% of total fatty acids(Jeffrey et al. 2001). The functional importance of DHA in eye may be related to the interaction of the photoactive protein rhodopsin with DHA containing phospholipids. Moreover, Phospholipid bilayers rich in DHA have a high fluidity and enhanced rates of fusion and permeability which are characteristics vital to the normal functioning of photoreceptor cells(Gordon and Bazan 1990; Giusto et al. 2000).Martinez and colleague (2000) provided a compelling evidence about the fundamental role of DHA in brain by showing that supplementing patients with Zellweger syndrome (Generalized peroxisomal disorders) with DHA improves myelination, vision, liver function, muscle tone, and social contact.

Evidence from animal studies suggests that DHA deficiency can decrease brain DHA levels, increase the n-6/ n-3 ratio in nerve membranes and result in poor functioning(Auestad and Innis 2000). In addition, studies have shown enhanced visual development and cognitive performance due to increased intakes of docosahexaenoic acid (DHA)(Moriguchi et al. 2000; Neuringer 2000; Helland et al. 2003). However, conclusive evidence in human subjects is lacking(Transler et al.; Ruxton et al. 2005).

The cardioprotective effects of DHA and EPA fatty acids supplementation have been extensively studied since firstly highlighted by Dyerberg and Bang(Dyerberg and Bang 1979). It is now widely accepted that DHA and EPA supplementation decreases the risk of cardiovascular diseases (CVD)(von Schacky and Harris 2007), such as fatal Coronary heart disease (CHD)(Hu and Willett 2002) and stroke(He et al. 2004). These cardioprotective effects of DHA and EPA have been attributed to their anti-inflammatory EPA-derived eicosanoids(Calder 2008a), anti-arrhythmic and anti-aggregatory effects, as well as their beneficial effects on vascular and endothelial functions. In addition, both EPA and DHA are precursors of a novel class of lipid mediators, known as E-series resolvins (Rv) from EPA and D-series Rv and neuroprotectin D1 from DHA, which are involved in the resolution of inflammation(Serhan and Chiang 2008), figure 1-24. Anti-thrombotic properties of fish oils were initially attributed to EPA due to its competition with AA in the cyclooxygenase (COX) and lipooxygenase (LOX) pathways(Nomura et al. 2003). However, animal human studies suggest that DHA is a more potent anti-aggregatory agent than EPA at high doses(Adan et al. 1999; Woodman et al. 2003; Cottin et al. 2011). Consistent with the anti-inflammatory effects of DHA and EPA, several clinical studies have shown that n-3 fatty acid supplementation had beneficial effects in atopic disease(Calder 2003), and conditions characterised by chronic inflammatory state such as arthritis(Calder and Zurier 2001; Sales et al. 2008), psoriasis(Mayser et al. 2002; Zulfakar et al. 2007) and inflammatory bowel disease(Calder 2008a). Interestingly, and may be of more importance in regard to sickle cell disease, there is a preliminary evidence that genetic disorder cystic fibrosis which associated with chronic inflammatory state is responsive to n-3 fatty acids supplementation(Keen et al. 2010).

Although the role of individual fatty acids in human cancer risk has hitherto been poorly investigated, epidemiological and experimental data linked a high dietary intake of n-6 PUFA, especially in association with a low intake of n-3 PUFA, to increased risk for cancer of the breast, colon, and possibly prostate. n-6 PUFA enhance tumorigenesis and metastasis in experimental animals by several mechanisms, whereas n-3 PUFA can inhibit the growth of initiated cancer cells (Benatti et al. 2004; Gleissman et al. 2010).

1.2.5.10 Eicosanoid

Eicosanoids are 20-carbon derivatives of essential fatty acids group of biologically active compounds that play major roles in human physiology in both health and disease. Eicosanoids include prostanoids (prostaglandins, thromboxanes, prostacyclin), leukotrienes and lipoxins(Cook 2005; Miller 2006; Wang and Dubois 2010). The first step in synthesis of eicosanoids from its fatty acids precursors (AA, EPA, DHGLA) is cleavage of these fatty acids from sn-2 position of cell membrane phospholipids the by the action of phosphoslipases under effect of specific stimuli(Russo 2009). The Eicosanoids exert their biological effects in an autocrine or paracrine manner (autocoids) by binding to their cognate cell surface receptors which belong to the G protein-coupled receptor (GPCR) family, or bind nuclear receptors such as peroxisome proliferator-activated receptors (PPARs)(Wang et al. 2004; Wang and Dubois 2010). The eicosanoides contribute significantly to the regulation of inflammation(Dobrian et al. 2011), renal function(Imig 2006; Harris 2008), vascular function(Feletou et al. 2011), insulin secretion (Luo and Wang 2011) and reproduction(Olson and Ammann 2007; Roldan and Shi 2007).

1.2.5.10.1 Phospolipases

Phospholipases (PL) are a group of enzymes that catabolise membrane phosphoglycerides. They are classified into phospholipase A1 (PLA_1), A_2 (PLA_2), B (PLB), C (PLC) and D (PLD), according to the bond they cleave(Exton 1994; Murakami and Kudo 2002; Fukami et al. 2010), **figure 1-21**. Pospholipase A_1 and A_2 catalyse the hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate free fatty acids and lysophospholipids. Whereas, phospholipases B hydrolyze both acyl groups, resulting in only minimal accumulation of lysophospholipid product. Hence, these enzymes often also have lysophospholipase activity, removing the remaining acyl moiety on lysophospholipids(Ghannoum 2000). Phospholipase C enzymes are phosphodiesterases that cleave the glycerophosphate bond(Kohler et al. 2006). Thirteen PLC isozymes have been identified, of these the phosphoinositide-specific isoform has been recognised to have a key rol in cell signalling. It hydrolyzes phosphatidylinositol 4,5-bisphosphate into two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol (DAG). 1,4,5-trisphosphate triggers the release of calcium from intracellular stores, and DAG mediates the activation of protein kinase (Fukami et al.

2010). Phospholipase D enzymes remove the base group of phospholipids and generate the membrane lipid phosphatidic acid (PA). Phosphatidic acid is a negatively charged phospholipid which is thought to facilitate membrane vesicle fusion and fission. In addition, PA can be converted to other signalling lipids, specifically lyso-PA, by phospholipase A_2, or diacylglycerol (DAG), by lipid phosphatases(Peng and Frohman 2011).

To date, more than 30 enzymes that possess PLA_2 or related activity have been identified in mammals, based on their structures, catalytic mechanisms and localizations, these enzymes have been subdivided into secretory PLA_2 ($sPLA_2$) family, cytosolic PLA_2 ($cPLA_2$) family, Ca^{2+}-independent PLA_2 ($iPLA_2$), The platelet-activating factor (PAF) acetylhydrolase (PAF-AH) family and lysosomal PLA_2s and adipose-specific PLA_2 (AdPLA) family(Murakami et al. 2011).

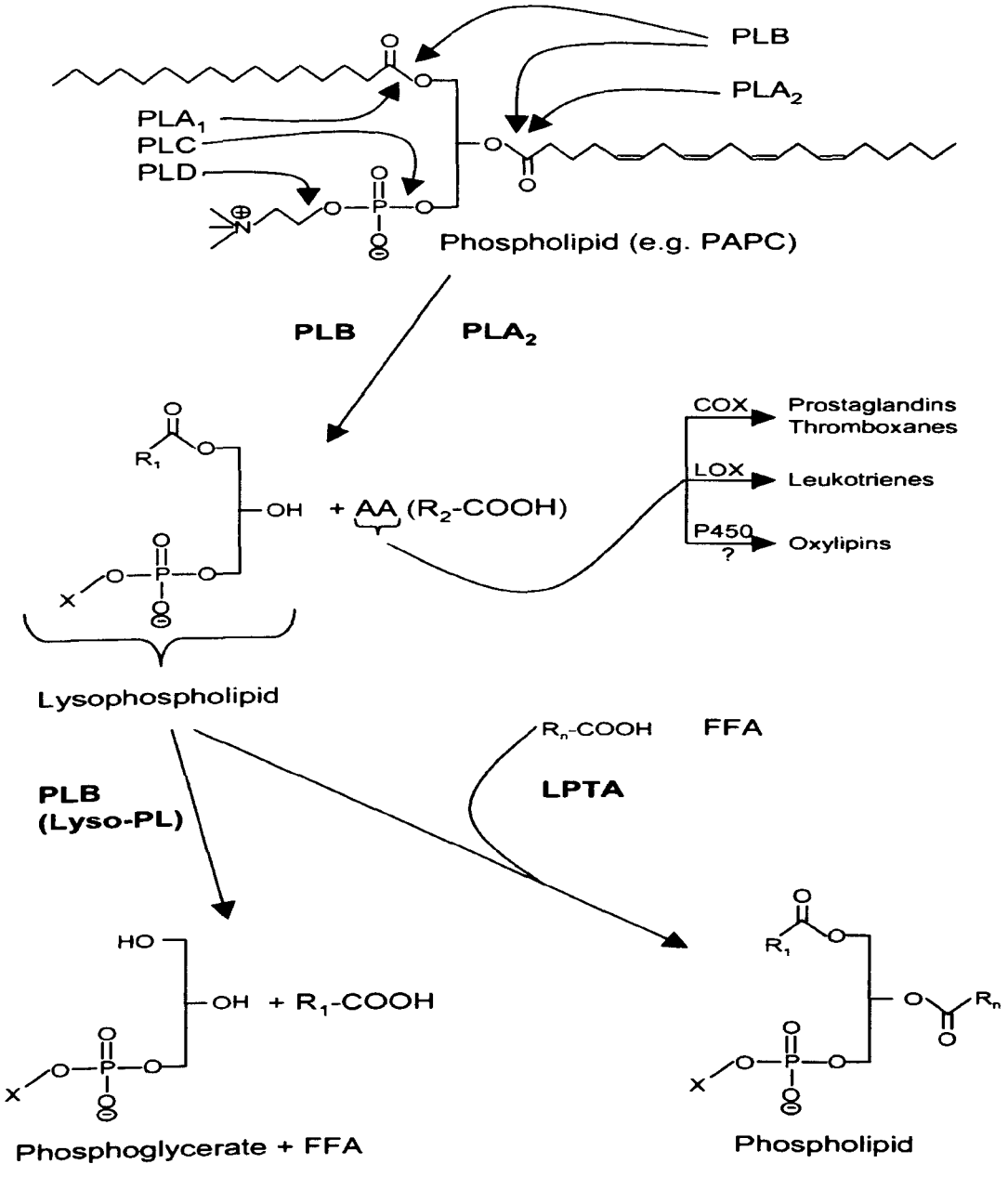


Figure 1-21 The positions of hydrolytic activities of and Mechanism of action of phospholipases (A, B, C, D),**(Adopted from (Kohler et al. 2006))**

The secretory PLA_2 ($sPLA_2$) are stored in cytosolic granules or synthesised on stimulation, secreted extracellularly, and have no selectivity for specific fatty acid(Jaross et al. 2002). The levels of $sPLA_2$ in serum or exudative fluids are well correlated with the severity of inflammatory conditions such as rheumatoid arthritis, Crohn's disease and sepsis, as well as cardiovascular diseases such as brain and

myocardial infarction(Menschikowski et al. 2006). In SCD serum $sPLA_2$ level is elevated threefold in steady state SCD patients compared to healthy controles(Styles et al. 2000). Moreover, $sPLA_2$ level is strong predictor of acute chest syndrome(Ballas et al. 2006).

The cytosolic ($cPLA_2$) isoforms (IVA. IVB, IVC) are primarily located in the cytosol. Group IVA (cPLA2α), the most extensively studied isoform in the $cPLA_2$ family, is widely expressed in mammalian cells and mediates the production of functionally diverse lipid products in response to extracellular stimuli(Ghosh et al. 2006). This enzyme has PLA_2 and lysophospholipase activities and is the only PLA_2 that shows specificity for phospholipid substrates containing AA(Murakami et al. 2011). Hence, its activation is important for regulating various physiological and pathological processes involve the eicosanoids. In contrast to $sPLA_2$s those require mM-levels of Ca^{2+} and iPLA2s and PAF-AHs that do not require Ca^{2+}, cPLA2α is activated by μM-levels of Ca^{2+} concentration(Kita et al. 2006).

1.2.5.10.2 Eicosanoid biochemistry

Non-esterified AA, DHGLA and EPA released from the sn-2 position of the membrane phospholipids by the action of phospholipases are substrates for modification by three major pathways, including cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP), into biologically active eicosanoids(Jump 2002; Arnold et al. 2011), **Figure 1-22.**

There are two isoforms of COX, COX-1 and COX-2. COX-1 is constitutively expressed in most cells, whereas COX-2 is inducible by inflammatory or stimulatory events in tissues and responds to specific inducers(Vane et al. 1998; Cheng and Harris 2004). The major COX-derived eicosanoids are prostaglandins (PGs) and thromboxane (TX). The metabolism of AA by COX-1 or COX-2 generates the 2-series prostaglandins which include PGD2, PGE2, PGI2, PGF2α and thromboxane A2 (TXA2)(Luo and Wang 2011). The action of these PGs and TXA2 is mediated through the binding of these products into their membrane-bound receptors, including DP, EPs (EP1 to EP4), IP, FP, and TP receptors(Robertson 1998; Hao and Breyer 2007b). Although derived from the same parent molecule, PGI2 (prostacyclin) is a potent vasodilator and an inhibitor of platelet aggregation, whereas TXA2 is a potent vasoconstrictor and a pro-aggregatory

substance(Kawabe et al. 2010). Therefore, the balance between PGI2 and TXA2 in the circulation is important for cardiovascular homeostasis.

Lipoxygenases (LOX) catalyse the reactions that convert AA to 4-series leukotrienes (LTA4, LTB4, LTC4, LTD4, and LTE4), hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), and hydroxyoctadecadienoic acids (HODEs)(Dobrian et al. 2011). The human LOX enzymes include 5-LOX (which produces (LTs), 12-LOX and and 15-LOX(Kuhn et al. 2002; Mochizuki and Kwon 2008). Of circulating cells, LTs are mainly synthesized by eosinophils, neutrophils, monocytes or macrophages, and mast cells(Camara et al. 2009). A substantial body of evidence indicates that 5-LOX products exert proinflammatory effects by increasing the production of proinflammatory cytokines; also, 5- LOX products act as chemotaxins to recruit inflammatory cells in the blood vessels(Poeckel and Funk 2010). Hence, 5-LOX products are considered to be detrimental factors in pathological conditions, including cardiovascular and renal diseases(Hao and Breyer 2007a; Mochizuki and Kwon 2008). In steady state SCD, consistent with high levels of $_{S}PLA_2$ and high cell membrane AA, LTB4 was found significantly high compared to healthy controls(Setty and Stuart 2002). In addition, studies have shown that LTB4 stimulates sickle erythrocyte adhesion, the effect which can be attenuated by anti-ILB4 treatment(Haynes and Obiako 2002; Knight-Perry et al. 2009).

AA is also a precursor of CYP-derived eicosanoids (HETEs)(Luo and Wang 2011), and have been shown to exert potent biological effects on regulation of cardiovascular and renal function. Notably, 20-HETE is a vasoconstrictor in the renal and cerebral microcirculation(Imig and Hammock 2009; Yousif et al. 2009).

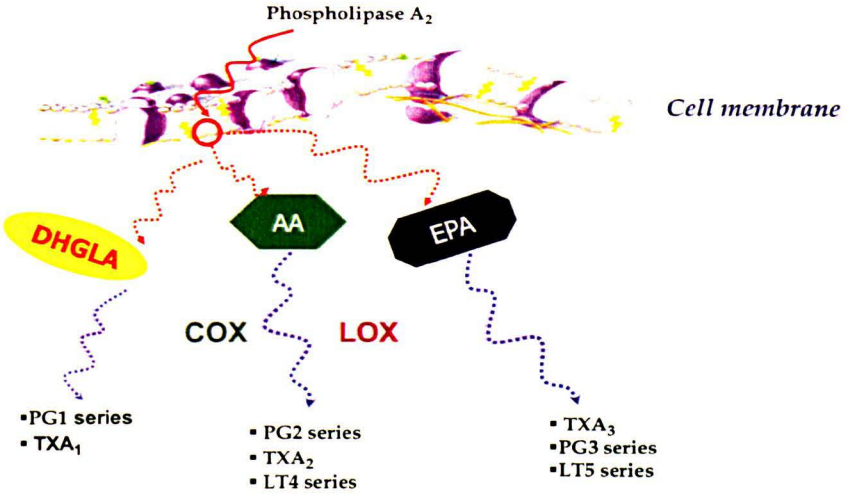


Figure 1-22 Biosynthesis of eicosanoids.
LT, leukotriene; PG, prostaglandin; TX, thromboxane; COX, cycooxygenase; LOX, lipooxygenase.

AA, DHGLA EPA compete at all steps of prostanoid biosynthesis resulting in specific alterations in metabolite production and action(Wada et al. 2007). When catalysed by cyclooxygenases, DHGLA and EPA produce 1- and 3-series prostanoids (prostaglandins, prostacyclins and thromboxanes), respectively. Moreover, EPA is converted to 5-series leukotriense by the action of the 5-lipoxygenases. In contrast to AA LTB4, LTB5 from EPA a metabolite at least 30 times less potent than LTB4(Wu et al. 1996). However, recent studies reveal that the anti-inflammatory effects of EPA and DHA are not only due to an exchange of the classical AA derived pro-inflammatory eicosanoids for their less potent n-3 counterparts. EPA and DHA are the precursors of novel lipid mediators resolvins, protectins and maresin, which have highly potent anti-inflammatory and pro-resolution properties and may play an essential role in the protection against various inflammatory diseases(Serhan 2009; Levy 2010; Serhan et al. 2011). In addition, EPA and DHA also interfere with cytochrome P450 (CYP) pathway. The same CYP isoforms that metabolize AA accept EPA and DHA as efficient

alternative substrates(Konkel and Schunck 2011). These interactions may have important physiological implications and provide novel insight into the mechanisms of the vascular and cardioprotective effects of DHA and EPA(Arnold et al. 2011), **Figure 1-23.**

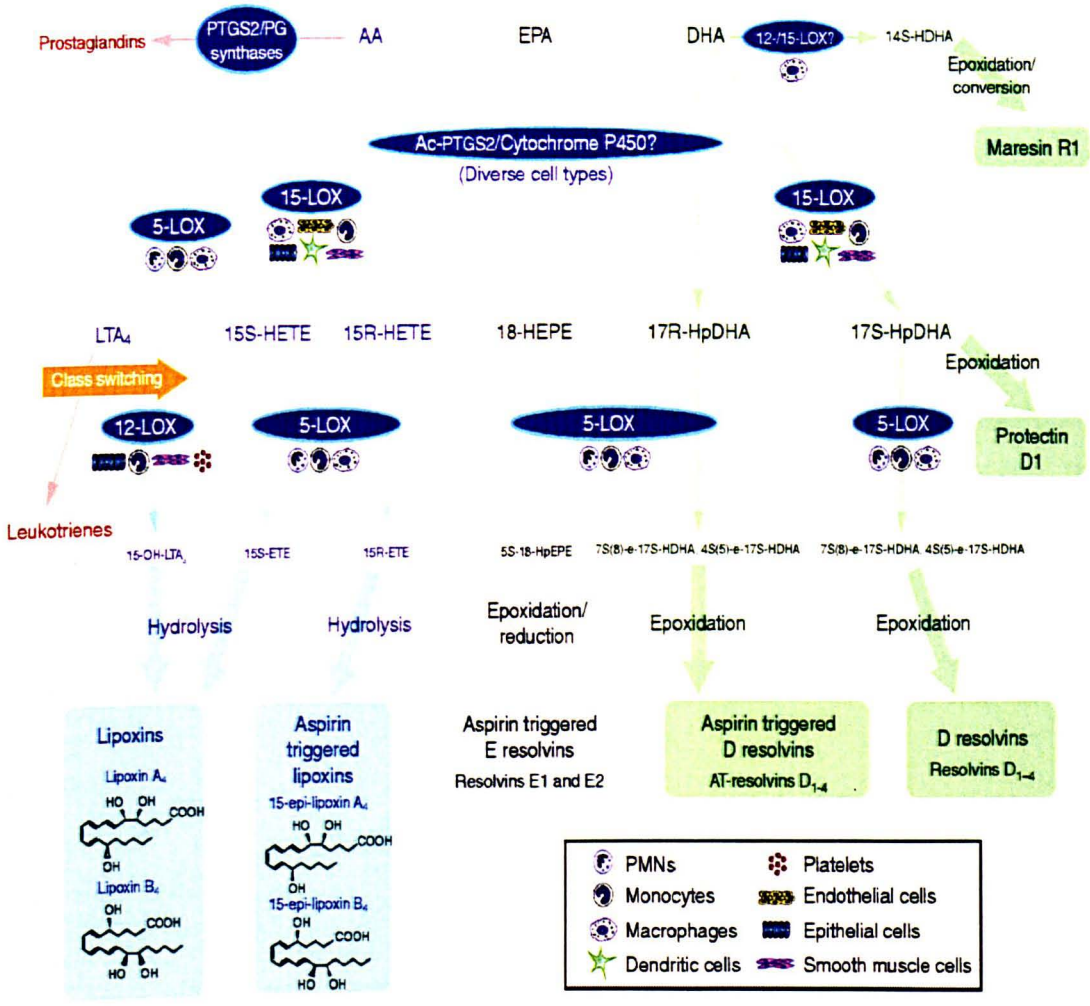


Figure 1-23 pathways of lekotriens, resolvines, protectines and maresin Adopted from (Hutchinson et al. 2011)

1.2.5.11 DHA and EPA supplementation, and membrane responsiveness

Despite the rapid turnover of membrane lipids, changes in the fatty acid composition of the diet can have varying effects on the fatty acid composition of membrane lipids.

Studies have shown that variation in dietary composition of saturated and monosaturated fatty acids cause a very little change in percent saturation of membrane lipids(Hulbert et al. 2005). In contrast, membrane lipids are very responsive to variation to dietary composition of LCPUFA. The greatest sensitivity to changes in dietary fatty acids was for n-3 PUFA and n-6/n-3 fatty acids ratio. Membrane fatty acid composition varies from a 6% change in cerebral myelin up to a 75% change in cerebral synaptosomes in response to a 100% change in dietary n-3 PUFA(Srinivasarao et al. 1997). Cao et al(2006) have reported 300% increase in red blood DHA and EPA composition in subjects supplemented with 1296 mg EPA and 864 mg DHA. Similarly, 10 fold increase in EPA concentration of mononuclear cell membrane phospholipids have been observed four weeks after supplementation(Hocker et al. 1990). Studies using multiple doses of fish oil show that the incorporation of these fatty acids in immune cells occurs in a manner that is highly correlated with the amount of the fatty acid consumed(Healy et al. 2000; Rees et al. 2006). Typically the increase in content of n-3 PUFAs occurs at the expense of n-6 PUFAs, especially arachidonic acid. There is evidence, changing the cell membrane EPA and DHA composition modulate the eicosanoids and resolvine production, expression of adhesive molecules and the overall inflammatory state(Calder 2008b).

1.3 Overview and Aims

For a long time, it was thought; vaso-occlusive crisis in SCD is caused by a mechanical blockage of small blood vessels by rigidly distorted (sickled) red blood cells. However, there is no relationship between number of irreversible sickle cells and vaso-occlusive events. Moreover, steady state SCD patients have high levels of inflamatory markers and enhanced expression of adhesive molecules in red cells, neutrophils and lymphocytes. These observations led to a new paradigm that excessive tendency of red cells (sickled and non-sickled) to adhere to vascular endothelium and activation of platelets and leukocytes are the primary causative factors of vasoocclusion. Adhesion, aggregation, elasticity of blood cells and inflammatory response are strongly modulated by long chain polyunsaturated fatty. There is evidence that SCD patients in steady state have abnormal erythrocytes, platelets and mononuclear cell fatty acids composition. The abnormality is characterised by high omega-6 arachidonic (AA), adrenic and osbond acids, and low omega-3 docosahexanoic acid (DHA) and eicosapentnoic acid (EPA). Hence, it is conceivable that cell membrane fatty acid composition may have a role in pathophysiology of vaso-occlusion. In this study we have investigated whether the current treatment of SCD (hydroxurea) or supplementation with DHA and EPA ameliorate the perturbation in fatty acids composition of blood cell membrane, and the effect of n-3 fatty acid supplementation on clinical severity of the disease and overall life quality of the patients.

1.3.1 Hypothesis

1- N-3 fatty acid Supplementation of patients with homozygous Sickle Cell Disease (HbSS) will not have effect on:

Cell membrane fatty acid composition, clinical course of the disease, haematological manifestations, blood cell adhesion, Markers of haemolysis, inflammation and oxidative stress

2-Hydroxyurea, the chemotherapic agent used for the treatment of sickle cell disease, does not alter fatty acid composition of red blood cell membrane

1.3.2 Specific Aims

1-In n-3 Fatty acid (DHA & EPA) supplemented and un-supplemented HbSS patients:

- The red blood cell membrane fatty acid composition at Zero-time and one year after supplementation, frequency and severity of vaso-occlusive crisis, number of hospital admission and types and number of new complications will be collected through two-arm, randomised, placebo-controlled, double blinded clinical trial.
- Full blood count (reticulocytes, leukocytes, platelets, total haemoglobin (Hb), foetal Haemoglobin (Hb F), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV)) and concentration of other pertinent blood parameters (Lactate dehydrogenase, haptoglobin) will be determined.
- Expression of TNF-α and IL-10 cytokines and leukocytes adhesion molecules (αMβ integrin, L-selectin) will be assessed.
- Plasma anti-oxidant vitamins concentration and anti-oxidant enzymes activities (Cu/Zn Superoxide dismutase, Se-glutathione peroxidase) will be assayed

2-In hydroxyurea treated and untreated HbSS patients and healthy controls fatty acid composition of red blood will be assessed

Chapter Two

Methods

2.1 Patients recruitment and determination of haemoglobin phenotype

Steady-state HbSS patients aged 2-24 years who were in regular follow-up at SCD referral clinic, Abnaoaf Pediatrics Hospital, Khartoum, Sudan were enrolled for the studies. Steady state was defined by absence of evidence of fever, infection or crisis for at least four weeks before the study. The phenotypic characteristic was confirmed by cellulose acetate electrophoresis at pH 8.5 using membranes from Lablnkoop Benelux, Germany (Cat. No 11200-57-145-N). The studies design and patients' characteristics will be discussed on the appropriate section.

2.2 Blood collection and processing

Whole blood, 10 ml, was taken from patients with homozygeous sickle cell and healthy controls in a tube containing the anticoagulant, ethylendiaminetetraacetic acid (EDTA) (K2 EDITA (spray-dried), BD Company). In sub-group of patients 3 ml of blood was taken in a tube containg gold hemogard (Vacutainer tube serum separator, BD) for serum preparation.

2.2.1 Determination of haemoglobine concentration and the pertinent blood parameters

Haemoglobine concentration (Hb), haematocit (Ht), Mean corpuscular volume (MCV), mean corpuscular haemoglobine (MCH), mean corpuscular haemoglobine concentration (MCHC), total white blood cell count (TWBC), platelets count (PLTs) total red blood cell count were obtained by using automated haematology analyzer Sysmex KX-21N (Sysmex Corporation, Kobe, Japan).

2.2.2 Preparation of plasma, serum and red blood cells

The whole blood collected in EDTA-containing tube was fractionated into RBC and plasma by cold centrifugation at 3000 rpm (g) for 15 min. The top plasma layer was carefully siphoned off and transferred into another tube. The lower red cell layer was washed three times with physiological saline (0.85% NaCl) and centrifuged at 3000 rpm (g) to remove traces of plasma and buffy coat. The resulting plasma and RBC pellet were stored at -80 °C until analysis.

The whole blood collected in serum separator tube was fractionated into RBC and plasma by cold centrifugation at 3000 rpm (g) for 15 min. the tope layer (serum) was separated from the other blood component gold hemogard gel. The serum was used immediately to re-suspend mononuclear cell, granulocyte and platelet of the respective participant.

2.2.3 Preparation of mononuclear cells, granulocytes, platelets

In subgroup of the recruited subjects, the plasma, red blood cell, monocytes, granulocytes and platelet were separated through a sequence of centrifugation steps using histopaque-1077 (density 1.077 g/m) and histopaque-1119(density 1.119 g/m) (Sigma-Aldrich, UK) . Firstly, 3 ml of histopaque-1119 was added to a 50 ml tube followed by 3ml of histopaque-1077 to form two distinct phases. Consequently, 5ml of the collected whole blood was layered carefully over the histopaque and centrifuged at 700 xg for 50 minutes at 22 °C (room temperature), **Figure 2-1**. Separation of plasma, mononuclear cells/platelets, granulocytes and red blood cells was obtained. The top layer (plasma) was transferred to cryotubes and stored at -80 °C. The mononuclear cell/ platelet fraction was transferred to a new tube and re-suspended in 10 ml 1X PBS (Sigma-Aldrich, UK) and centrifuged at 250 x g for 10 min at 22 °C. The supernatant (platelet) was transferred to a new 15ml tube, whereas the remaining pellet was re-suspended with 10 ml 1X PBS and washed twice by centrifugation at 250 x g for 10 minutes. Mononuclear cells, granulocytes and platelets were washed twice with 10 ml 1X PBS at 2000 x g for 5 min, resuspended in 450 µl serum (described on section 2.2.2) and 50µl Dimethyl sulfoxide (DMSO), and stored at -80 °C until the analysis. The red blood cell was processed as described on section 2.2.2.

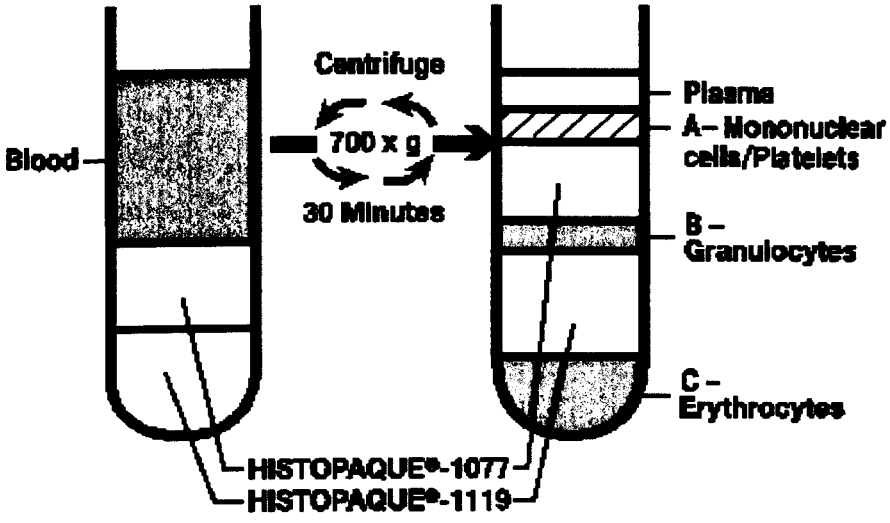


Figure 2-1 Blood cell separation

2.3 Fatty acid analysis

2.3.1 Lipid extraction

Total lipids were extracted by the method of Folch et al(1957) by homogenising of processed RBC as described in section (2.2.2) in 2:1 v/v chloroform/methanol containing 0.01% (w/v) butylated hydroxytoluene (BHT), under nitrogen. The ratio of the samples to extracting solvent mixture was 1:90 in all analysis. The extraction procedure used is as following. 0.5 ml of RBC carefully transferred to a 100 ml extraction tube containing 15 ml methanol and shake gently to prevent clumping. Subsequently, 30 ml of chloroform + BHT was added to RBC- methanol mixture, vortexed thoroughly and flushed with oxygen-free nitrogen for one minute and capped instantly. Each sample extracted for 24 hours at 4 °C. The chemical and reagent used and their sources are detailed in **appendix 2**.

2.3.2 Partitioning-Separation of lipid and non-lipid components

The tube containing the extracted samples were taken from the refrigerator and kept at room temperature in dim light for 30 minutes to equilibrate, then filtered into 100 ml separating funnel (Whatman International Ltd. England). The extraction tube and filter paper were washed twice with total of 15 ml chloroform/methanol 2:1 v/v + BHT. Afterwards, the filter paper was removed, and 25%v/v of 0.85% NaCl was added to the separating funnel to ensure complete partitioning, flushed thoroughly by bubbling

nitrogen gas through the liquid. Finally, the funnels were closed tightly and stored overnight at 4 °C.

2.3.3 Collection of lipids

The separating funnels were taken out the refrigerator and kept at room temperature in dim light for 30 minutes. Subsequently, the lower organic layer obtained after partitioning was drained into 250 ml round bottomed flasks and the solvent removed under reduced pressure in a water bath at 37 °C, using a rotary evaporator (vacuum pump V-700, Buchi, Switzerland). To remove any trace of water, the lipid residue was re-dissolved twice in 2 ml of methanol and dried. After complete drying, the whole lipid extract were removed from the round bottomed flasks to a 10 ml vial by washing them three times with chloroform/methanol 2:1 v/v + 0.01 % BHT. The samples were reduced to one ml under a stream of OFN at 370C and stored at -20 °C until applied to thin-layer chromatography (TLC) as described in the next section.

2.3.4 Thin-layer chromatography (TLC)

- **Principle-** Thin-layer chromatography (TLC) is an indispensable tool for lipid analysis and separation of complex lipid classes. Separation occurs in stationary phase is due to polarity differences of the analytes(Fuchs et al. 2011). The stationary phase is normally a thin layer of finely ground silica gel adhered to glass, aluminium or plastic plate. The resolving efficiency of a stationary phase is dependent on particle size of the silica gel powder, pore diameter and thickness of silica layer on the plate. In normal phase TLC, the stationary phase (normally silica gel) is polar and the mobile phase is quite apolar. The mobile phase mixture is composed of two or more solvent with different polarities and elutropic values, which is the ability of the solvent to displace lipid component from a stationary phase. Solvent, such as chloroform, methanol and water, which have high polarity and elutropic value, are suitable for resolving phospholipids.

- **TLC plates preparation-** 20X20 cm thin-layer chromatography (TLC) plates pre-coated with 0.25 mm thick silica gel were used for the analysis (Merck Kga, Germany). The silica was scored from top to bottom 0.5 cm in from each side, and from left to right about 3 cm from the top. Then, the plates were activated

at 100 °C before use in order to dry off any moisture from the plate. After one hour, the plates were removed from the oven and kept in a desiccator to cool.

- **Equilibration of developing chamber-** The solvent mixture chloroform/methanol/methylamine (65:30:15, v/v/v) containing 0.01% was used as mobile phase. 200 ml of the freshly prepared solvent was placed in a glass tank (10x21x21) lined with filter paper and closed instantly, to help the solvent saturate the atmosphere within the tank. The tanks were kept close for 30 minutes before use.

- **Application of samples on TLC plates-** The activated plates were gently placed on a surface lined with filter paper, in a room with a dimmed light. A line was lightly drawn with a soft pencil 2 cm from the bottom edge of the plate, just above the tope surface of the mobile phase. The samples were completely dried under a stream of oxygen-free nitrogen (OFN) and the total lipid extract re-dissolved in 1 ml of chloroform/methanol 2:1 v/v + 0.01 % BHT. The total lipids extract was applied as a narrow band along the bottom pencil line using a capillary tube. A maximum of three samples were applied on each plate.

- **Development and visualization of TLC plates-** The plates were put quickly into the equilibrated tank and developed for 90 minutes. Next, the plates were removed from the tank, dried and sprayed with 0.1% solution of 2,7-dichlorofluorescein in methanol, and dried again under stream of cold air in a darkened fume cupboard. Subsequently, the different phospholipids bands were visualised under UV light (533 nm) and the boundaries of the bands were carefully demarcated with pencil. The phospholipids classes were identified by comparison with authentic standards, (figure 2-2). CPG, EPG, SM and SPG phospholipids bands were scraped onto filter paper using a blunt spatula and transferred to their respective nethylation tube.

2.3.5 Preparation of fatty acids methyl esters

Transmethylation- Freshly made methylating reagent (15% acetyle chloride in dry methanol), 4 ml, was added to each nethylation tube, flushed with N_2, tightly capped and incubated at 70 °C for three hours. Each tube was hourly checked for leakage of

methylation reagent and vortexed. In case of leakage, methylation reagent was added to maintain the original volume.

- **Extraction of fatty acids methyl esters (FAMEs)** - After oven incubation, the tubes were cooled down at room temperature in dimmed light cupboard. To each tube, 4 ml of 5% NaCl plus 2 ml of petroleum ether were added and mixed vigorously. Emulsions resulted from vortexing were cleared by adding few drops of methanol. The upper petroleum ether layer containing the FAMEs was transferred into a new tube and its acidity was neutralised by addition of 2% potassium bicarbonate. Afterwards, 2 ml of petroleum ether were added to the original tubes, the tubes were shaken and the upper petroleum ether was transferred to the same tube containing 2% potassium bicarbonate. The tubes were vortexed and the upper layer was transferred to a new tube containing 100 mg of dried granular sodium sulphate, to remove any moisture possibly comes from the aqueous solution of potassium bicarbonate. Finally, the moisture-free FAMEs solution was transferred into 3 ml vial and dried down at 37 °C under OFN and re-dissolved in 1ml heptane (containing 0.01% BHT). The samples were flushed under nitrogen and store at -20 °C until analysed by gas chromatography.

2.3.6 Analysis of fatty acids methyl esters by Gas-Liquid Chromatography (GC)

Principal- Like all chromatographic techniques, gas chromatograph separate mixture of volatile derivatives of analytes by taking advantage of their deferential distribution between the stationary and mobile phases. The distinctive feature of GC is the use of an inert gas as a mobile phase, and a layer of immobilised liquid film coated on an inert solid support of a long tube (column) as stationary phase(Willett 1987). All gas chromatograph must have gas supply with controlled flow rate, a column contained with a thermostatted oven, sample injection system and sensitive detector to determine and record the concentration of separated constituent at the end of the column, **Figure 2-2.**

The commonly used GC columns are packed and capillary column. The capillary columns are high purity fused silica tubes, usually 25 to 60 meters I length and 0.1 to

0.5 millimetres in internal diameters. Unlike packed column, capillary columns do not contain a packing but have their stationary phase supported on their inner walls in one of two ways. The simplest version is Wall Coated Open (WCOT), in which the liquid stationary phase is a thin film over the smooth, inner surface of the capillary. The other type called Support Coated Open Tubular columns (SCOT) has the liquid stationary phase deposited onto a thin layer of small support particles on the inner wall of the column. The absence of packing in the centre of the bore of capillary columns makes them much more permeable to gas flow than packed column, and hence can be longer without using extremes of gas pressure, and better resolution of the separated mixture components(Baugh 1993). The efficiency of separation of analytes in GC is mainly dependant on the column length, column's internal diameter and film thickness, oven temperature and the carrier gas flow.

The mobile phase (the carrier gas) is inert and highly diffusive or low resistant to mass transfer. The most frequently gas used in UK is nitrogen, followed by helium, hydrogen and carbon dioxide. The choice of the gas depends on safety, cost and most importantly compatibility with the detector. While the nitrogen provide the highest chromatographic efficacy in accordance to Van Deemter Plot, the optimum velocity of hydrogen is four times greater than the nitrogen, the characteristic that renders it more preferable over the nitrogen and helium, especially after the wide availability of cheap and low capacity hydrogen generator which minimised the hazard associated to hydrogen use. Besides its use as a carrier gas, hydrogen is used in GC as a fuel gas for flame-ionisation detectors (FIDs).

The main detection systems used in GC are flame-ionization detectors (FIDs), thermal conductivity detector (TCD) and electron capture detector (ECD). The FID detectors are very stable and highly sensitive to most organic compounds including fatty acids methyl esters (FAMEs). The FID detector consists of a flame ionisation tip and a collector plate. The column effluent is mixed with hydrogen and passed through a jet into a chamber through which air is passed, and hydrogen is ignited to produce continuous flame. As compound from the column enter the flam, they undergo combustion and a very few proportion (0.001%) of the carbon atoms will be ionized.

An electrode collects these ions and the resulting electrical current is amplified to provide the chromatographic signal(Bartle 1993).

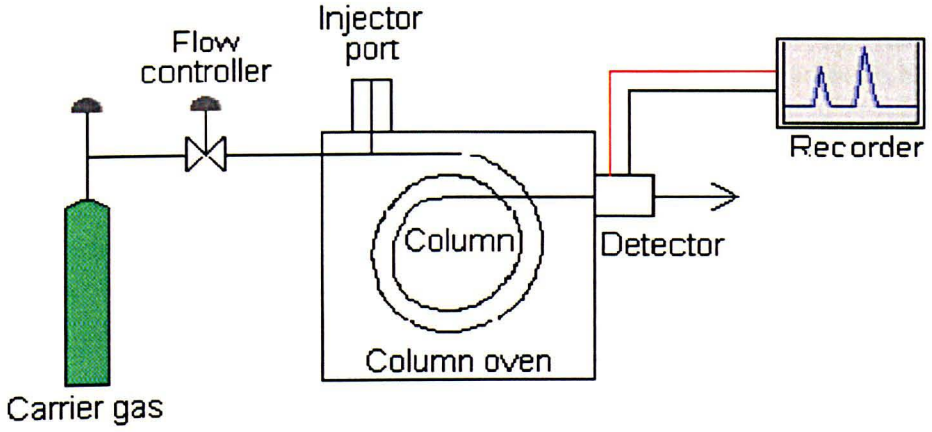


Figure 2-2 Block diagram of a gas chromatograph

- **Analysis of fatty acids methyl esters-** FAMEs were separated by a capillary gas chromatograph (HRGC MEGA 2 series, Fisons Instruments, Italy), fitted with a BPX-70 capillary column (60 m x 0.32 mm ID, 0.25μ film, SGE Europe Ltd (UK). Hydrogen was used as a carrier gas at 2 ml/min, and the injector, oven and detector temperatures were 250, 230 and 280°C, respectively. Specifications regarding gas chromatography instrumentation are listed in **Appendix 1**.

Quality certified fatty acid methyl ester standard mixture (Supelco® 37 Component FAME Mix. U47885-U, Sigma-Aldrich, Dorset, UK), and GC-MS authenticated fatty acid methyl esters prepared from lipid extract of vegetable seed oils which contain alpha-linolenic, gamma-linolenic and stearidonic acids, and from bovine brain L-A-phosphatidylethanolamine Type 1 (Sigma-Aldrich, Dorset, UK) were used to identify the fatty acids. Peak areas were computed with EZChrom chromatography data system (Scientific Software, Inc, San Ramon, CA). The area of each peak was expressed as percentage of total identified peaks (**Appendices 3&8&9**).

2.4 Analysis of plasma lipids

Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol) and triglyceride concentrations were analysed by the use of Ace Alera Clinical Chemistry System (Alfa Wassermann Diagnostic Technologies B.V. Pompmolenlaan 24, 3447 GK Woerden, The Netherlands). Low-density lipoprotein cholesterol (LDL-cholesterol) was computed with the equation:

{LDL-Cholesterol = Total Cholesterol − (HDL-Cholesterol + Triglyceride/5)}. Equation 2-1

2.5 Determination of plasma retinol and α-tocopherol

2.5.1 Extraction

Plasma sample (prepared as described in section 2.2.2) , 500 µl, was deproteinised with 500 µl of ethanol (HPLC-grad, Sigma-Aldrich Co. UK), the resulted mixture was extracted with 1 ml hexane (HPLC-grad, Sigma-Aldrich Co. UK). Both solvents (ethanol and hexane) contained 0.01% of the antioxidant butylated hydroxytoluene (BHT). The extract was vortexed thoroughly for 5 minutes and centrifuged at 2000 rpm. The upper organic layer containing the vitamins was transferred to another new glass tube, and the remaining precipitate was extracted twice with 1ml hexane described above. The collected organic extract (3ml of hexane) was evaporated to dryness under nitrogen and subsequently dissolved in 100 µl of 98% HPLC-grade methanol. All the extraction steps were conducted in a dimmed light.

2.5.2 Separation and identification

The retinol, α-tocopherol and β-carotene in the plasma extract were separated and identified with use of an Agilent 1100 series high performance liquid chromatograph (HPLC), connected to autosampler and diod-array detector (Agilent Technologies Deutchland GmbH, Waldbronn, Germany). A reverse phase C18 column (5µ, 250x 4.6 mm, Thermo Scientific) was used (**Apprenix 1**). 98% HPLC-grade methanol and 2% HPLC-grade water were used for the mobile phase at a flow rate of 1ml/min, and the column temperature was maintained at 20 °C. Retinol and α-tocopherol were detected at their respective maximum absorption wavelength 325 nm and 292 nm. Based on authentic standards, the retention times were 4.5 for the retinol and 8.4 for α-tocopherol.

2.5.3 Quantification

Standard stock solutions of retinol (650 ug/dl) and α-tocopherol (30 mg/dl) were prepared by dissolving the obtained standards from Sigma-Aldrich Co Ltd, UK in HPLC-grade methanol. The concentrations of stock solutions of the retinol and α-tocopherol were confirmed by measuring the absorbance at wave length 325 and292.8 respectively (**Equation 2-2, 2-3**), using Lamda 35 UV/Vis spectrophotometer (PerkinElmer Ltd., UK). Working standards with concentration ranges of 219.3-1.7 µg/ml for retinol and 10.2-0.08 for α-tocoherol were prepared from the stock solution. Linear regression lines of concentrations (**Figure 2-3, Figure 2-4**) were obtained by plotting the concentrations versus HPLC peak areas. The concentrations of the vitamins in the sample were calculated from their respective standand regression line equations, (Figure 2-4, Figure 2-5) The coefficient variations of the reproducibility of the assay were 1.5% for retinol and 3.95 for α-tocoherol. The computer software, Agilent ChemStation (Agilent Technologies Deutschland GmbH, Waldborn, Germany) was employed to compute peak areas and the concentrations of the vitamins.

Retnol µg/ml = (Absorbance/1780)*10 Equation 2-2

α-tocoherol mg/ml = (Absorbance/75.8)*10 Equation 2-3

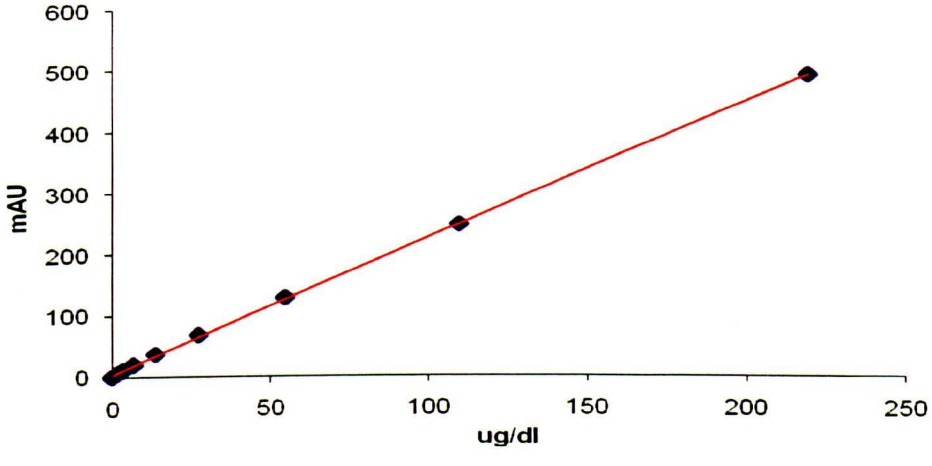


Figure 2-3 Standard curve of retinol

Equation of the regression line- retinol µg/ml= (Peak area Mau) - 3.44)/2.23

Correlation coefficient(r) =0.99, coefficient of determination (r^2) = 0.98.

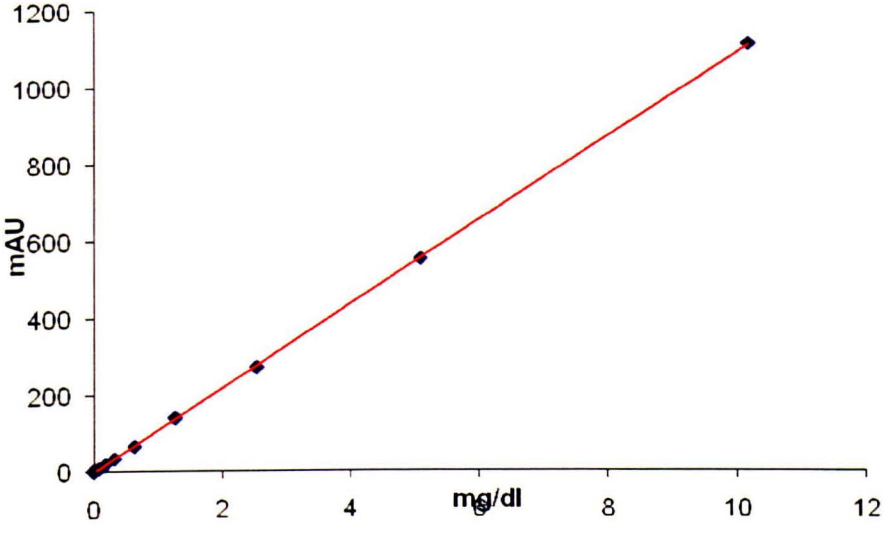


Figure 2-4 Standard curve of α-tocopherol
Equation of the regression line- α-tocoherol mg/ml= (Peak area (mAU)-+2.5)/109.6 Correlation coefficient(r) =0.99, , coefficient of determination (r^2) = 0.98.

2.6 Determination of anti-oxidants enzymes activities

2.6.1 Preparation of red cell haemolysates

50µl red cell as prepared in 2.2.2, was added to 200µl ice-cold deionised distilled water (4/1, v/v), thoroughly vortexed and centrifuged at 10,000xg for 15 minutes at 4°C. The supernatant (haemolysate) was collected and stored at -80°C until needed. Haemolysates were used to analys the activities of superoxide dismutase, glutathione peroxidase and catalase enzymes.

2.6.2 Determination of Cu/Zn-Superoxide Dismutase (Cu/Zn-SOD) activity

Principle- The activity of CU/Zn-SOD in erythrocytes was assayed based on the method of Woolliams et al (1983) using a kit (catalog No. 706002) from Cayman (Cayman Chemical Company, 1180 East Ellsworth Road, Ann Arbor, Michigan 48108, USA). The kit utilizes a water soluble tetrazolium salt (WST-1) for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The oxidised tetrazolium salt forms a highly water soluble formazan dye (WST-1 formazan) that

absorbs light at the wavelength of 440-460. The formation of WST-1 formazan is inhibited by CU/Zn-SOD, which competitively converts the generated superoxide radicals to hydrogen peroxide (H_2O_2) and oxgen (O_2). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical (**Figure. 2-5**).

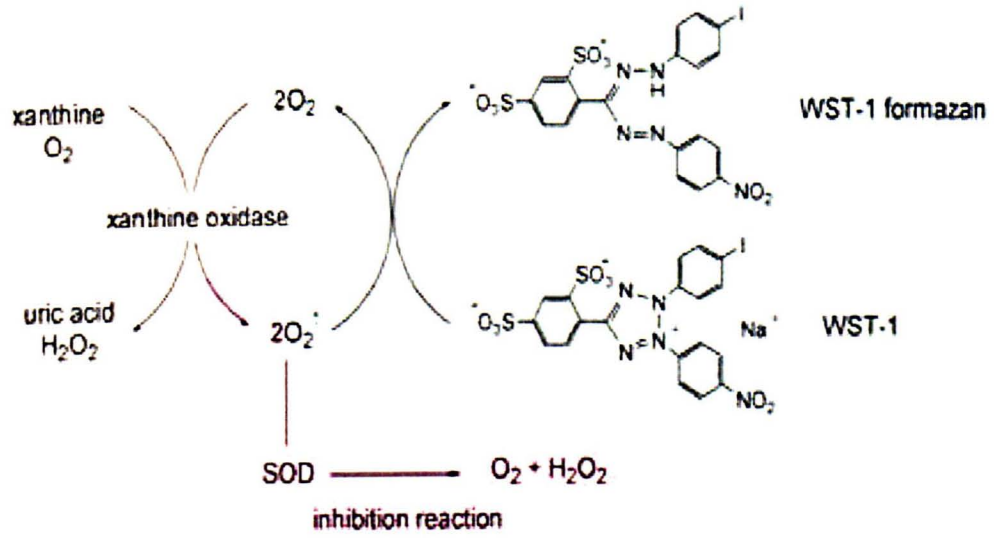


Figure 2-5 Scheme of the Superoxide Dismutase Assay

2.6.2.1 Preparation of Cu/Zn-SOD reagents and working standards

Assay buffer- The final assay buffer (50 mM Tris-HCl, PH 8.0, containing 0.1 mM diethylenetriaminpentaacetic acid (DTPA) and 0.1 mM hypoxanthine) was prepared by diluting 3 ml assay buffer concentrate (10X) (Cayman Chemical Company, UK) with 27ml HPLC-grade water.

Sample buffer- sample buffer (50 mM Tris-HCl, PH 8.0) was prepared by diluting 2ml sample buffer concentrate (10X) (Cayman Chemical Company, UK) with 18ml HPLC-grade water.

Radical detector- Radical detector (tetrazolium sat) was prepared by diluting 50 µl of the supplied solution (Cayman Chemical Company, US) with 19.5 ml of diluted assay

buffer. The final solution was kept well protected from light and used within two hours after preparation.

Xanthine oxidase- Xanthine oxidase reagent was prepared by diluting 50 µl of supplied enzyme (Cayman Chemical Company, US) with 1.95 ml of diluted sample buffer. The enzyme solution was kept on ice and used within one hour after preparation.

Working standards- Cu/Zn-SOD working standards (Std A- Std G) were Prepared as described in the kit obtained from Cayman (Cat NO. 706006), Cayman Chemical Company, US). 20 µl of supplied Cu/Zn SOD standard was diluted with 1.98 sample buffer (diluted) to obtain the SOD stock solution. The working standards (STD A, B, C, D, G), their concentration ranges from 0-0.25 U/ml, were prepared by serial dilution of stock standard with sample buffer.

Sample preparation- An aliquot (5 µl) of red blood lysate which was prepared as described in 2.5.1, was diluted with 495 µl sample buffer in order to obtain final Cu/Zn-SOD activity within the dynamic range of the kit (0.025-0.25 U/ml). The diluted red blood cell lysate was assayed for Cu/Zn SOD activity.

Standard curve of Superoxide dismutase- The change of absorbance due to inhibition of formation of formazan dye by the Cu/Zn SOD working standards in a reaction mixture containing 10 µl of standard (STD A- STD G), 200 µl of the diluted radical detector was determined. The reaction was initiated by 20 µl of diluted xanthine oxidase. After 20 minutes of incubation at room temperature, the absorbance (Abs) was read at 450 nm by plate reader (Fluor Star Omega, BMG Labtech).

The average absorbance from triplicates of each standard was calculated. The linearized rate (LR) was obtained by dividing standard A's absorbance by itself and all other standard (STD B- STD G), **equation 2-4.**

LR for Std B= Abs Std A/Abs Std B (Equation-2-4).

The linearized Cu/Zn-SOD standard rate was plotted (**Fig 2-6**) as a function of final Cu/Zn-SOD activity (U/ml) in accordance to value determined in table 2-1. The

equation obtained from linear regression of the standard cure was employed to calculate the samples final Cu/Zn-SOD activity.

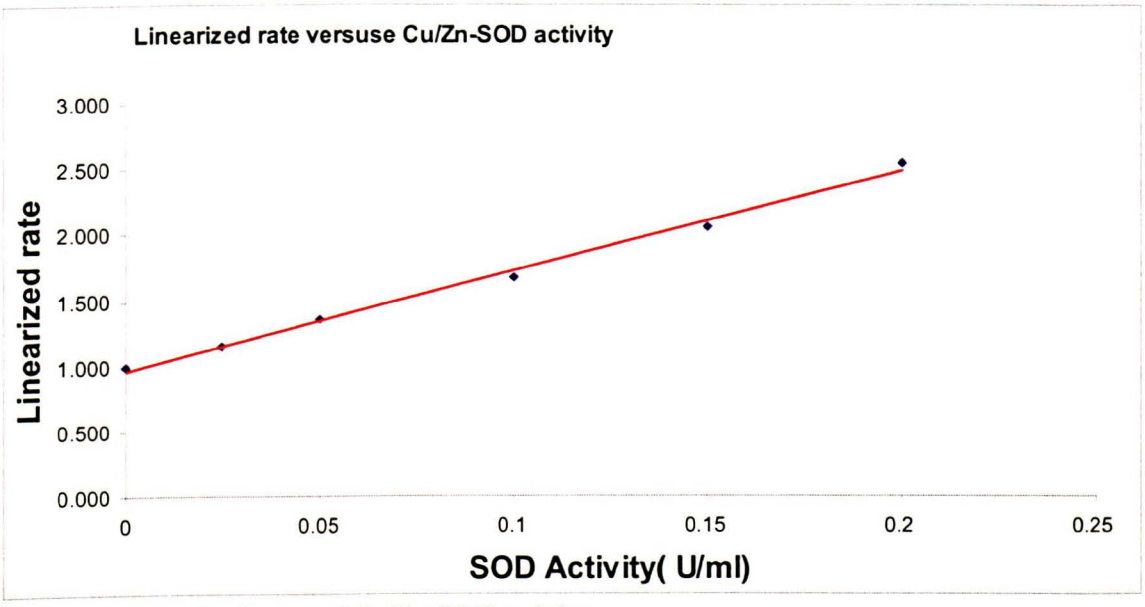


Figure 2-6 Standard curve of Cu/Zn-SOD activity.
Equation of the regression line: Cu/Zn-SOD activity (U/ml) = (Linearized rate- 0.9678) /7.6065
Correlation coefficient(r) =0.994, , coefficient of determination (r^2) = 0.99.

Cu/Zn activity of red cell haemolysate- The activity of red Cu/Zn-SOD was determined from a reaction mixture containing 10 µl of the diluted red cell haemolysate, 200 µl diluted radical detector and 20 µl of diluted xanthine oxidase. After shaking and incubation at room temperature for 20 minutes, final absorption at 450 nm was read using plate reader (Fluor Star Omega, BMG Labtech). Samples and standards were assessed on the same plate under the same conditions.

The average absorbance from triplicates of each sample was calculated. The linearized rate (LR) for each sample was obtained by dividing standard A's averaged absorbance by the samples' averaged absorbance. The red cell Cu/Zn-sod activity was calculated using the equation obtained from linear regression of the standard curve (Equation 2-3) in accordance to the **equations (2-5, 2-6)**.

$$\text{Cu/ Zn-SOD (U/ml)} = \left(\frac{\text{sample LR-y-intercept}}{\text{slope}} \times \frac{0.23\ \text{ml}}{0.01\ \text{ml}} \right) \times \text{sample dilution} \quad \textbf{Equation 2-5}$$

$$\text{Cu/Zn-SOD U/g} = \frac{\text{SOD (U/ml)}}{\text{Haemoglobin (g/ml)}} \quad \textbf{Equation 2-6}$$

2.6.3 Determination of Se-Glutathione Peroxidase (Se-GPx) activity

- **Principle-** The activity of Se-GPx was measured indirectly by coupled reaction with glutathione reductase (GR) based on a modified method of Paglia and Valentine (Paglia and Valentine 1967; Pleban et al. 1982) using the kit (catalog No. 706002) obtained from Cayman (Cayman Chemical Company , 1180 East Ellsworth Road , Ann Arbor, Michigan 48108, USA). As illustrated in the **Fig 2-7**, the oxidized glutathione, glutathione disulfide (GSSG), produced upon reduction of hydroperoxide by Se-GPx, is recycled to its reduced state (GSH) by glutathione reductase (GR), in the presence of hydrogen donor coenzyme β-nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm (A340). Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A340 is directly proportional to the GPx activity in the sample.

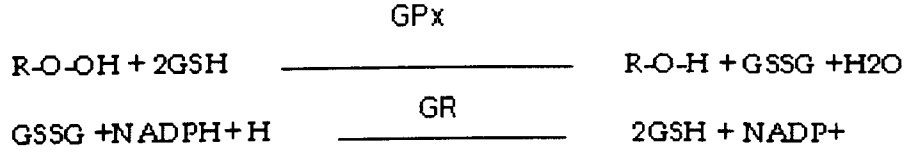


Figure 2-7 Scheme Glutathione Peroxidase of the Assay

2.6.3.1 Preparation of reagents and working standards-

Se-GPx assay buffer- The assay buffer was prepared by diluting 2ml of assay buffer concentrate (Cayman Chemical Company, US) with 18ml of HPLC-grade water to obtain the final assay buffer (50 mM Tris-HCl , pH 7.6, containing 5 mM EDTA).

Se-GPx sample buffer- The assay buffer was prepared by diluting 2ml of sample buffer concentrate (Cayman Chemical Company, US) with 18ml of HPLC-grade water to obtain the final sample buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA and I mg/ml bovine serum abumin).

Se-GPx co-substrate mixture- The supplied co-substrate mixture (Cayman Chemical Company, US) comprised of lyophilized powder NADPH, glutathione and glutathione

reductase. The co-substrate mixture was reconstituted by adding 2ml HPLC-grade water to one vial of the powder.

Se-GPx Cumene Hydroperoxidase- The reagent was ready to use as supplied by (Cayman Chemical Company, US), Cumene hydroperoxide was the source of peroxide (oxidant agent) in the reaction.

Bovine red blood cell Se-glutathione peroxidise (control) - The stock solution was prepared by diluting 10 µl of the supplied enzyme (Cayman Chemical Company, US) with 490 µl of diluted sample buffer.

Sample preparation- An aliquot (20 µl) of red blood cell haemolysate, that was prepared as explained in 2.5.1, was diluted in 60 µl of diluted sample buffer. The final dilution factor (X35) was prepared to obtain suitable target absorbance decrease between 0.02 and 0.135/min, the absorbance suitable to guarantee reproducible results according to the supplier instructions (Cayman Chemical Company,US).

Standard curve of Se-GPx- in order to assess the integrity of the provided reagents (Cayman Chemical Company, US), the activity of Bovine red blood cell Se-glutathione peroxidise (control) was measured in the reaction mixture containing 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of diluted Se-GPx (control). The absorbance was read once every minute for 8 minutes at 340 nm (Fig 2.10). The diluted enzyme caused a decrease of 0.04 absorbance unit/minute as calculated from **equation (2-7).**

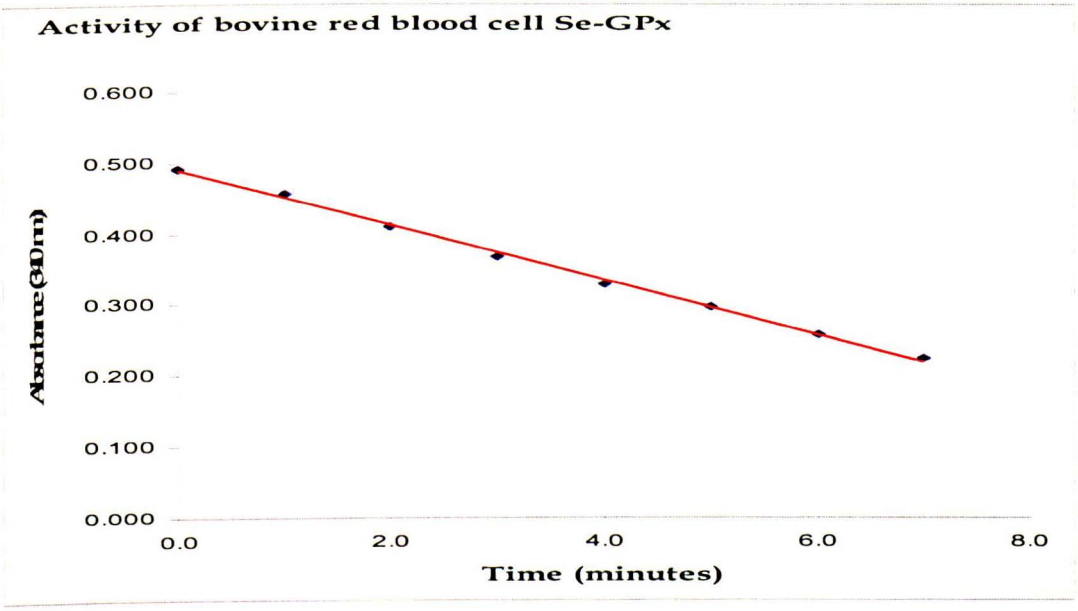


Figure 2-8 Standard curve of the activity of bovine red blood cell Se-GPx
Equation of the regression line: Absorbance (340 nm) = 0.039xTime (minutes) + 0.49) **Equation 2-7**
Correlation coefficient(r) = -0.9974, , coefficient of determination (r^2) = 0.995.

Determination of Se-GPx activity-The oxidation of NADPH to NADP and concomitant reduction of the absorbance at 340 (ΔA340) was assessed in a duplicate of a reaction mixture containing 20 µl of the diluted haemolysate, 100 µl of the diluted assay buffer, 50 µl of the co-substrate mixture and 20 µl of cumene hydroperoxide. After few seconds of careful shaking, the absorbance was immediately read at wavelength 40 nm once every minutes for 8 minutes by using plate reader (Fluor Star Omega, BMG Labtech) and soft ware (Fluor Star Omega Analysis, BMG Labtech) for data analysis.

The average absorbance of sample duplicates at each minute was calculated. The rate of reduction of the absorbance at 340 (ΔA340) was calculated using the **equation 2-8**. The rate of ΔA340/min for the non-enzymatic well (background) was calculated and subtracted from that of the sample wells. The reaction rate (GPx activity) at 340 nm was determined using the NADPH extinction coefficient of 0.0373 µM-1 (**equation 2-9**). One unite is defined as the amount of the enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP per minute at 25 °C.

$$\Delta A340/\text{min} = \frac{A340/\ (\text{Time2}) - A340/(\text{Time1})}{\text{Time2 (min)} - \text{Time 1 (min)}} \qquad \textbf{Equation 2-8}$$

$$GPx = \frac{\Delta A_{340}/\text{min}}{0.0373\ \mu\text{M-1}} \times \frac{0.19\ \text{ml}}{0.02} \qquad \textbf{Equation 2-9}$$

2.7 Determination of plasma concentrations of lactate dehydrogenase enzyme (LDH)

Principle -Plasma levels of LDH was measured by using ELISA kit (Catalog No. E91864Hu) obtained from Uscn Life Science Inc, USA. In this assay the LDH present in the samples reacts with monoclonal antibody specific to LDH which have been adsorbed to the surface of the microtiter plate. Next, a biotin-conjugated polyclonal antibody preparation specific for LDH (detection A) is bound to LDH-antibody. Afterwards, avidin conjugated to Horseradish Peroxidase (detection B) is added to the LDH-anti-body complex. Following washing step, the enzyme bound to the immunosorbnt is assayed by the addition of a chromogenic substrate, 3',3,5,5'-tetramethylbenzidine (TMB). The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of LDH in the samples is determined by comparing the absorption of the samples to the standard curve.

2.7.1 Preparation of LDH assay reagents and working standards

Anti-human LDH ELISA micro plate- Was used as supplied.

Assay diluent A and Assay Diluent B – Was prepared by diluting the supplied diluent concentrates (1:2) with deionised water.

Detection reagent A and Detection Reagent B- Was prepared by diluting the detection reagents concentrates A and B (1:100) with assay diluent A and B respectively.

Wash solution- Diluted wash solution (x1) was prepared from the supplied wash solution concentrate (x30) by adding deionized water.

TMB substrate- Was used as supplied.

Working standards- The stock standard solution (50 ng/ml) was prepared by reconstituting the dry standard with 1 ml of the supplied standard diluent. The stock standard was kept for 10 minutes at room temperature and mixed gently before use. The working standards 25 ng/ml (Std 25 ng/ml) was prepared by additing 500 µl of reconstituted standard to 500 µl standard diluent solution. The standards 12.5- 0 ng/ml (Std 12.5- Std 0 ng/ml) were prepared by serial dilution using 500 µl of the higher standard concentration, **Figure 2-9.**

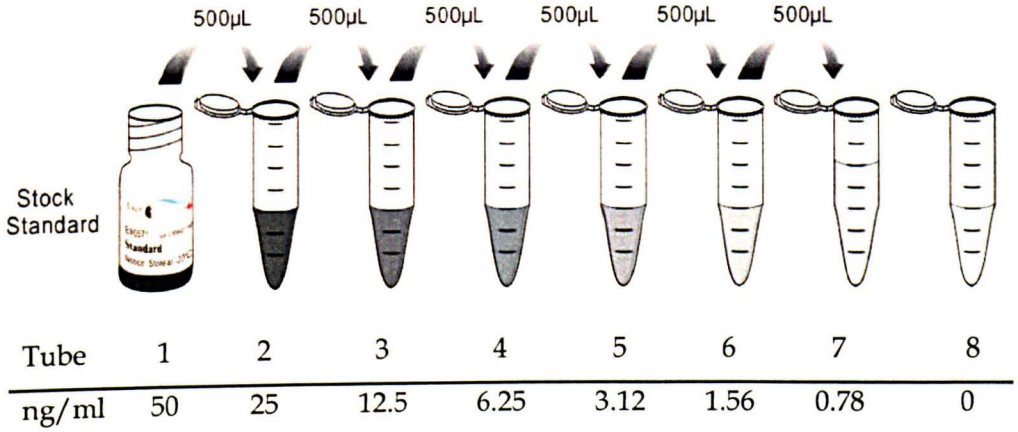


Figure 2-9 Lactate dehydrogenase working standards

Preparation of the sample- An aliquot (0.5 ml) of plasma, that was prepared as explained in (2.2.2), was gradually equilibrated to room temperature and diluted (1:10) with distilled water.

Measurement of absorbance of LDH standards and samples- 100 µl of the standards, blank and samples were added in duplicate to the appropriate wells and incubated for two hours in 37 °C. After aspiration of all contents of each well, 100µL of Detection

reagent A was added to each well and incubated for one hour in 37 °C. The plates were tightly sealed by adhesive plate cover during incubation period. Afterwards, the well's content was aspirated and washed three times with wash solution. Subsequently, 100 µl of detection reagent B was added to each well and incubated for 30 minutes at 37oC followed by five cycles of wash. Then, 90µL of substrate solution was added to each well and incubated for 20 minutes in dark at 37oC. The reaction was stopped by adding 50 µL of stop solution. The plate reader (Fluor Star Omega, BMG Labtech) was used to read the test absorbance at 450 nm.

Standard curve of LDH- The standard curve for LDH was generated by plotting the average absorbance of each standard versus the corresponding LDH concentration (**figure 2- 9& figure 2-10**). The curve was created using the GraphPad prism software (GraphPad Prism 5).

Calculation of the samples' LDH concentrations- The concentration of samples Hp was interpolated using the soft ware package GraphPad Prism (version 5), **Figure 2-10**.

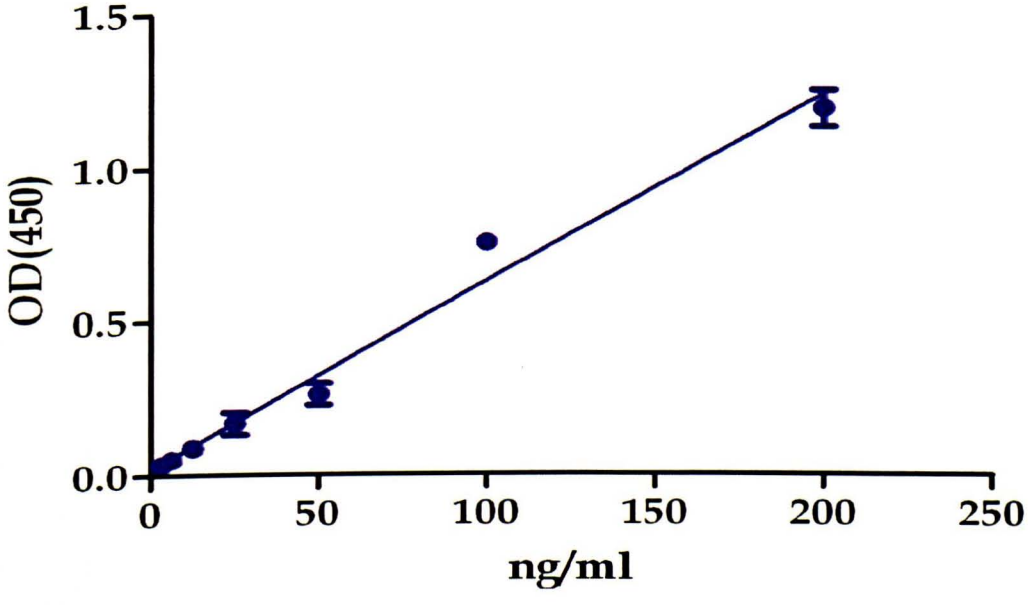


Figure 2-10 Standard curve of LDH
Regression line- y=163.7 x + 0.02

2.8 Determination of plasma concentration of haptoglobin (Hp)

Priciple- plasma levels of haptoglobin (Hp) was measured by high sensetive double anti-body sandwich ELISA using a kit (Catalog No. E-80HPT) obtained from Immunology Consultant Laboratory, Inc, USA. In this assay the haptoglobin present in the samples reacts with the anti-Hp anti-bodies which have been adsorbed to the surface of polystyrene microtitre wells. Afterwards, the Hp-anti-body complex is bound to anti-haptoglopin conjugated with horseradish peroxidase enzyme (HRP). Following washing step, the enzyme bound to the immunosorbant is assayed by the addition of a chromogenic substrate, 3',3,5,5'-tetramethylbenzidine. The quantity of bound enzyme varies directly with the concentration of Hp in the sample tested. The absorbance at 450 was used to measure the concentration of Hp in the test samples. The quantities of Hp in the samples were interpolated from standard curve.

2.8.1 Preparation of Hp assay reagents and working standards

Anti-human haptoglobin ELISA micro plate- Was used as supplied.

Diluent solution -Was prepared by diluting the diluent concentrate (1:5) with deionised water.

Wash solution- Was prepared by diluting wash solution concentrate (1:20) with deionised water.

Diluted enzyme-antibody conjugate- Was prepared by diluting the supplied enzyme-antibody conjugated with horseradish peroxidase (1:100) with diluent solution.

Chromagen-substrate solution- Was used as supplied.

Stop solution- Was used as supplied.

Working standards- The stock standard solution was prepared by adding 1 ml of deionised water to the supplied human haptoglobin calibrator and mixed gently until completely dissolved. The working standards 200 ng/ml (Std 200 ng/ml) was prepared by additing 2 µl of reconstituted human haptoglobin calibrator to 488 µl diluent solution. The standards 100- 0 ng/ml (Std 100- Std 0 ng/ml) were prepared by serial dilution using 250 µl of the higher standard concentration.

Preparation of the sample- An aliquot (0.5 ml) of plasma, that was prepared as explained in (2.2.2), was gradually equilibrated to room temperature and diluted 1:50000 with Diluent solution. The diluted samples were mixed thoroughly at each stage.

Measurement of absorbance of Hp standards and samples- The absorbance (Abs) at 450 nm of each standard concentration (Std 0- Std 200 ng/ml) and sample, was measured by incubating 100 µl in duplicate of sample and standard in the pre-coated well for fifteen minutes at room temperature. The plate was kept covered during the incubation. After aspiration of all contents of each well, they were washed manually three times with wash solution. Afterwards, 100 µl of diluted enzyme-antibody conjugate was added to each well and incubated covered in the dark at room temperature for another fifteen minutes. The plate was washed three times before 100 µl of chromagen-substrate solution was added to each well and incubated for precisely five minutes at room temperature. Subsequently, 100 µl of stop solution was added to each well. The plate reader (Fluor Star Omega, BMG Labtech) was used to read the test absorbance. The absorbence of samples and standards were calculated by subtracting the average background value from the test value.

Standard curve of Hp- The standard curve for Hp was generated by plotting the average absorbance of each standards versus the corresponding Hp concentration, using a four-parametric logistic curve fit, (**Figure 2-11**). The curve was created using the GraphPad prism software (GraphPad Prism 5).

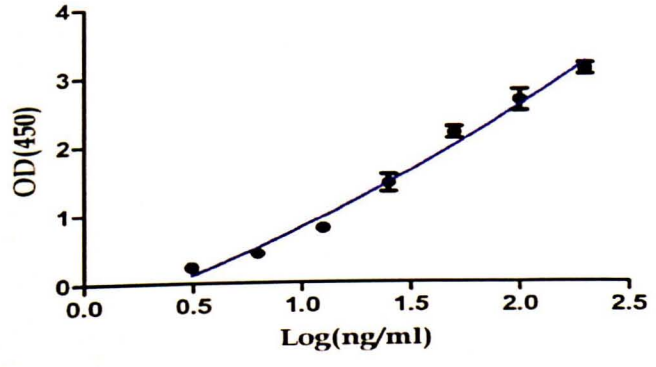


Figure 2-11 Standard curve of Hp
Equation of the regression line- $y=-0.42+0.96x+0.27x^2$

Calculation of the samples' Hp concentrations- The concentrations of samples' Hp were interpolated using the four-parametric logistic curve fit equation; **Figure (2-11)**. The soft ware package GraphPad Prism (version 5) was used for calculation of the samples' concentrations.

2.9 Determination of plasma levels of pro- and anti-inflammatory cytokines

2.9.1 Tumor necrosis factor-α (TNF-α)

Principle- The plasma levels of TNF-α was determined by enzyme-linked immunosorbent assay (ELISA) using a kit (catalog No. EH3TNFA) obtained from Pierce Biotechnology (Pierce Biotechnology, Rockford, USA). The method is based on microtiter strips coated with monoclonal antibody specific for human TNF- α. The TNF- α present in the samples (or standards) forms a complex with the anti- TNF-α antibody, and is subsequently labelled by a biotin conjugated monoclonal antibody specific to TNF-a. The unbound biotinylated anti-TNF-α is removed by multible washing. Streptavidin- horseradish peroxidise (Streptavidin-HRP) is then added to bind the remained bound biotinylated antibody. A substrate solution reactive with HRP is added, and results in the formation of a coloured product, in proportion to the amount of TNF- α present in the sample (or standards). The reaction is terminated by addition of acid and absorbance can be measured at 450 nm.

2.9.1.1 Preparation of TNF-α assay reagents and working standards

Anti-human TNF-α pre-coated 96-well strip plate- the Anti-human TNF-α pre-coated 96-well strip plate was used as supplied.

Wash buffer- wash buffer wash prepared by diluting 50 ml of the supplied concentrated (30X) solution (Pierce Biotechnology, USA) with 1450 ml of ultra pure water.

Sample diluent- The sample diluent (0.1% sodium azid) was used as supplied (Pierce Biotechnology, USA).

Biotinylated antibody- The biotinylated antibody was used as supplied (Pierce Biotechnology, USA).

Streptavidin-HRP- The Streptavidin-HRP was used as supplied (Pierce Biotechnology, USA).

HRP substrate solution (TMB) - The HRP substrate solution (3,3',5,5'-tetramethylbenzidine (TMB), hydrogene peroxide (H2O2), and proprietary catalyzing and stabilizing agents) was used as provided (Pierce Biotechnology, USA).

Stop solution- The stop solution (0.16M sulphuric acid) was used as supplied (Pierce Biotechnology, USA).

Working standard- The stock standard solution was prepared by reconstituting the supplied vial of lyophilized recombinant human TNF- α by adding 1 ml of water. The working standards (Std 1000 pg/ml- Std 0 pg/ml) were prepared by pipetting 200 µl of sample diluent into each tube. The standard (Std 1000 pg/ml) was prepared by adding 200 µl of the reconstituted standard, serial dilution was made using 200 µl to prepare the rest of the standard concentrations (Std 500 250, 125, 62.5, 31.2, 15.6, 0 pg/ml).

Preparation of the sample- An aliquot (0.5 ml) of plasma, that was prepared as explained in (2.), was gradually equilibrated to room temperature, and gently mixed before assay performance.

Measurement of absorbance of TNF- α standards and samples- The absorbance (Abs) at 450 nm and 550 nm of each standard concentration (Std 1000 pg/ml- Std 0 pg/ml) and sample, was measured by incubating 50 µl in duplicate of sample and standard in the pre-coated well for one hour at room temperature. After washing three times with the diluted wash buffer, 100 µl of biotinylated antibody was added to each wells and incubated for another one hour. The plate was washed three times as above before 100 µl of Streptavidin-HRP was added to each well and incubated for 30 minutes at room temperature. Subsequently, 100 µl of TMB substrate solution was added to each well and the reaction was allowed to develop at room temperature in the dark for 30 minutes. The reaction was stopped by adding 100 µl of stop solution to each well. A plate reader (Fluor Star Omega, BMG Labtech) was used to read the absorbance (Abs).

The 550 nm values were subtracted from at 450 values to correct for optical imperfections of the microplate. Samples and standards were assessed on the same plate under the same conditions.

Standard curve of TNF- α – The standard curve for TNF- α was generated by plotting the average absorbance (450 nm- 550 nm) obtained from each standard concentration versus the corresponding TNF- α concentration using a four-parametric logistic curve fit (**Figue. 2-12**). The curve was created using the GraphPad prism software (GraphPad Prism 5).

Calculation of the samples' TNF- α concentrations - The concentration of samples TNF- α was interpolated using the four-parametric logistic curve fit (figure 2-14). The soft ware package GraphPad Prism (version 5) was used for calculation of the samples' TNF- α concentrations.

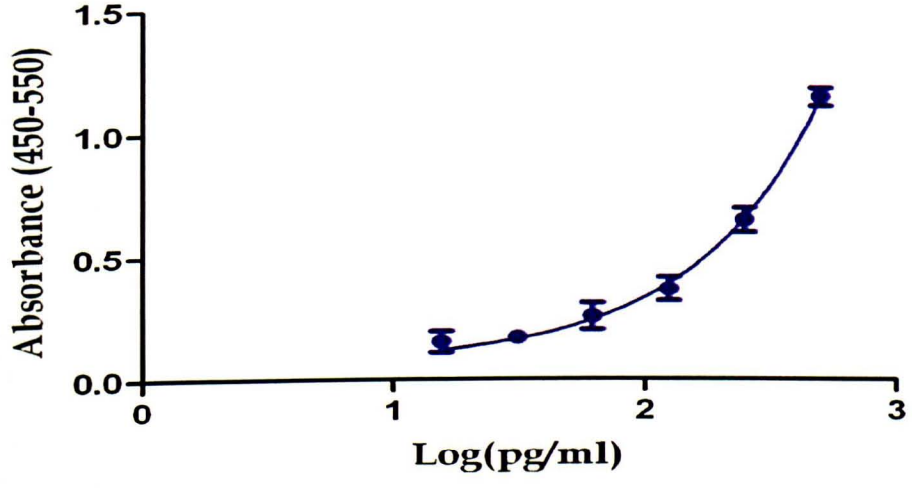


Figure 2-12 TNF- α four-parametric logistic standard curve fit
Equation of thev curve, Y=0.07+ 1433/(1+10^((6.3-x)*0.9))

2.9.2 Interlukin-10 (IL-10)

Principle-The plasma levels of IL-10 was determined by enzyme-linked immunosorbent assay (ELISA) using a kit (catalog No.EHIL02) obtained from Pierce

Biotechnology (Pierce Biotechnology, Rockford, USA). The assay principle is as described on **section 2.8.1.**

2.9.3 Preparation of IL-10 assay reagents and working standards

Anti-human IL-10 pre-coated 96-well strip plate- the Anti-human TNF-α pre-coated 96-well strip plate was used as supplied (Pierce Biotechnology, USA).

Wash buffer- wash buffer wash prepared by diluting 50 ml of the supplied concentrated (30X) wash solution (Pierce Biotechnology, USA) with 1450 ml of ultra pure water.

Standard diluent- Was used as supplied.

Biotinylated antibody- The biotinylated antibody was used as supplied.

Streptavidin-HRP dilution buffer- Was used as supplied.

Streptavidin-HRP solution- Was prepared by adding 30 µl of streptavidin-HRP concentrate to 12 ml of streptavidin-HRP dilution buffer.

HRP substrate solution (TMB)- Was used as supplied.

Stop solution- Was used as supplied.

Working standard- The stock standard solution was prepared by reconstituting the supplied vial of lyophilized recombinant human IL-10 by adding 1 ml of ultrapure water. The working standards (Std 600 pg/ml- Std 0 pg/ml) were prepared by pipetting 240 of standard diluent into pre-labelled standard tube. The standard (Std 1000 pg/ml) was prepared by adding 160 µl of the reconstituted standard, serial dilution was made using 160 µl to prepare the rest of the standard concentrations (Std 240, 96, 38.4, 15.36, 0 pg/ml).

Preparation of the sample- An aliquot (0.5 ml) of plasma, that was prepared as explained in (2.2.2), was gradually equilibrated to room temperature, and gently mixed before assay performance

Measurement of absorbance of IL-10- standards and samples- 50 µl of the standards or test samples were added in duplicate to each well. In addition, 50 µl of standard

diluents were added to two wells that do not contain samples or standard. The plate was tightly sealed by adhesive plate cover and incubated at the room temperature for two hours with 50 µl of biotinylated antibody. After aspiration of all contents, each well was washed manually three times with wash buffer. Subsequently, 100 µl of Streptavidin-HRP solution was added to each well and incubated in room temperature for thirty minutes. After washing three times with the diluted wash buffer, 100 µl of TMB substrate solution was added to each well and the reaction was allowed to develop at room temperature in the dark for 30 minutes, and stopped by adding 100 µl of stop solution to each well. The by plate reader (Fluor Star Omega, BMG Labtech) was used to read the absorbance (Abs) at 450 nm and 550 nm of standards and samples concentrations. The 550 nm values were subtracted from at 450 values to correct for optical imperfections in the microplate.

Standard curve of IL-10- The standard curve for IL-10- α was generated by plotting the average absorbance (450 nm- 550 nm) obtained from each standard concentration versus the corresponding IL-10 concentration using a four-parametric logistic curve fit (**Figure. 2-13**). The curve was created using the GraphPad prism software (GraphPad Prism 5).

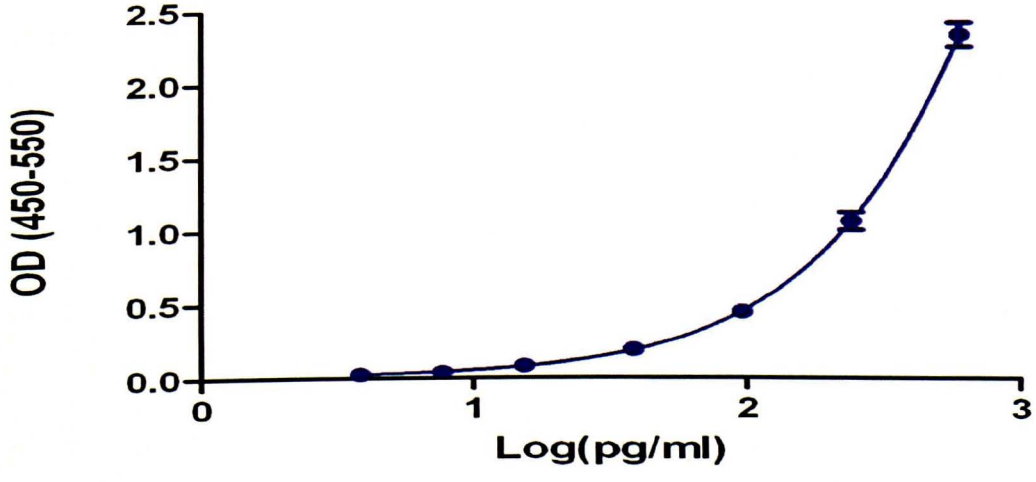


Figure 2-13 IL-10 four-parametric logistic curve fit
Equation of the curve, Y= 0.012+ 10.9/ (1+10^((3.34-x)*1.02))

Calculation of the samples' IL-10 concentrations- The concentration of samples IL-10 was interpolated using the four-parametric logistic curve fit fig (**2-13**). The soft ware package GraphPad Prism (version 5) was used for calculation of the samples' IL-10 concentrations.

2.10 Determination of adhesive molecules on blood cell membranes

2.10.1 Determination of monocytes and granulocytes CD11b and CD62L expression

Principle- Flow cytometry is a method allowing unique recognition of cellular patterns in fluid suspention (Hulett etal 1969, Alvarez et al 2009). One of the fundamentals of flow cytometry is the ability to order samples into a stream of single particles by employing the hydrodynamic focusing mechanism. The fluid flows past a detector point, where the stream is illuminated by a focused laser beam. The cells are usually labelled using fluorescent probes which bind to specific cell associated molecules. Subsequently, the probes fluoresce and the emitted light is detected and converted into electronic signals proportional to the amount of light collected.

An important feature of flowcytometry data analysis is gating- the ability to selectively visualize the cells of interest while eliminating results from unwanted particles. Cells can be gated according to their physical characteristics, the different physical properties of granulocytes, monocytes and lymphocytes allow them to be distinguished from each other and from cellular contaminants in the forward (FSC) and side scatter (SSC) plot (**figure 2-14**)

2.10.2 Preparation of reagents for the integrin αM (CD11b) and L-selectin (CD62L) assay

Antibodies-The mouse anti-human CD11b FITC conjugated, anti-human CD62L FITC conjugated, and the anti-IgG was used as supplied **(Appenix 2)**

Phosphate buffer saline (PBS 10X)- 100 ml of the concentrated PBS X10 (Sigma-Aldrich Co. UK, D1408) was diluted with 900 ml HPLC-grade water.

Bovine serum albumin (BSA)- The bovine serum albumin 2mg/ml was prepared by desolving 200mg (Sigma-Aldrich Co. UK., A7906) of the supplied powder in 100 ml of HPLC-grade water.

Preparation of mononuclear cells and granulocytes samples- Mononuclear cells and granulocytes, prepared as described in 2.2.3, were quickly defrosted at 37 °C. 200 µl of each defrosted sample was washed in 10 ml of phosphate buffer saline (PBS 1X) by centrifugation at 400xg for 5 minutes. Supernatant was discarded, and the pellet was suspended in 1 ml PBS 1X/BSA 2mg/ml (Sigma-Aldrich Co. UK., A7906). 50 µl of cell suspension was incubated on a shaker with 10 µl (used as supplied) of anti-human-CD11b fluorescein isothiocyanate (FITC) conjugated antibody, anti-human -CD62L-FITC, or mouse anti-IgG, at 4°C for one hour in the dark (Appenidx 2). Cells were also incubated with no antibody at the same conditions, and used as negative control. After incubation, cells were washed three times in 1.5 ml PBS 1X, by centrifugation at 150xg for 5 minutes. Cells pellet was resuspended in 200 µl PBS 1X, and used for flow cytometry analysis

Calibration of flowcytometer forward and side scatter- In order to standardise the positioning of monocytes, micro beads conjugated to anti-Human-Cd14 antibody were used for monocytes purification (Miltenyi Biotec GmbH, Germany). 107 total mononuclear cells counted by microscope visualization using a counting chamber were suspended in 80 µl of incubation buffer (0.5 % BSA, 2mM EDTA in PB1X pH 7.2), and incubated with CD14 micro beads for 30 minutes at 4 °C, on a shaker in the dark. Subsequently, cells were washed three times in incubation buffer by centrifugation at 300xg for 10 minutes. Cells were resuspended in 500 µl of incubation buffer. Cells bound to CD14 micro beads were sequestered by the used of a column placed in a magnetic field separator (Miltenyi Biotec GmbH, Germany). Cells were eluted by washing the column with 1 ml of incubation buffer. Cells were precipitated at 300xg, diluted in PBS1X /BSA 2mg/ml, and labelled with anti-human-CD11b FITC conjugated as described in section (2.7.1, iii) The fluorescent signal from the purified monocytes was compared to the same non purified sample (**Figure 2-14**).

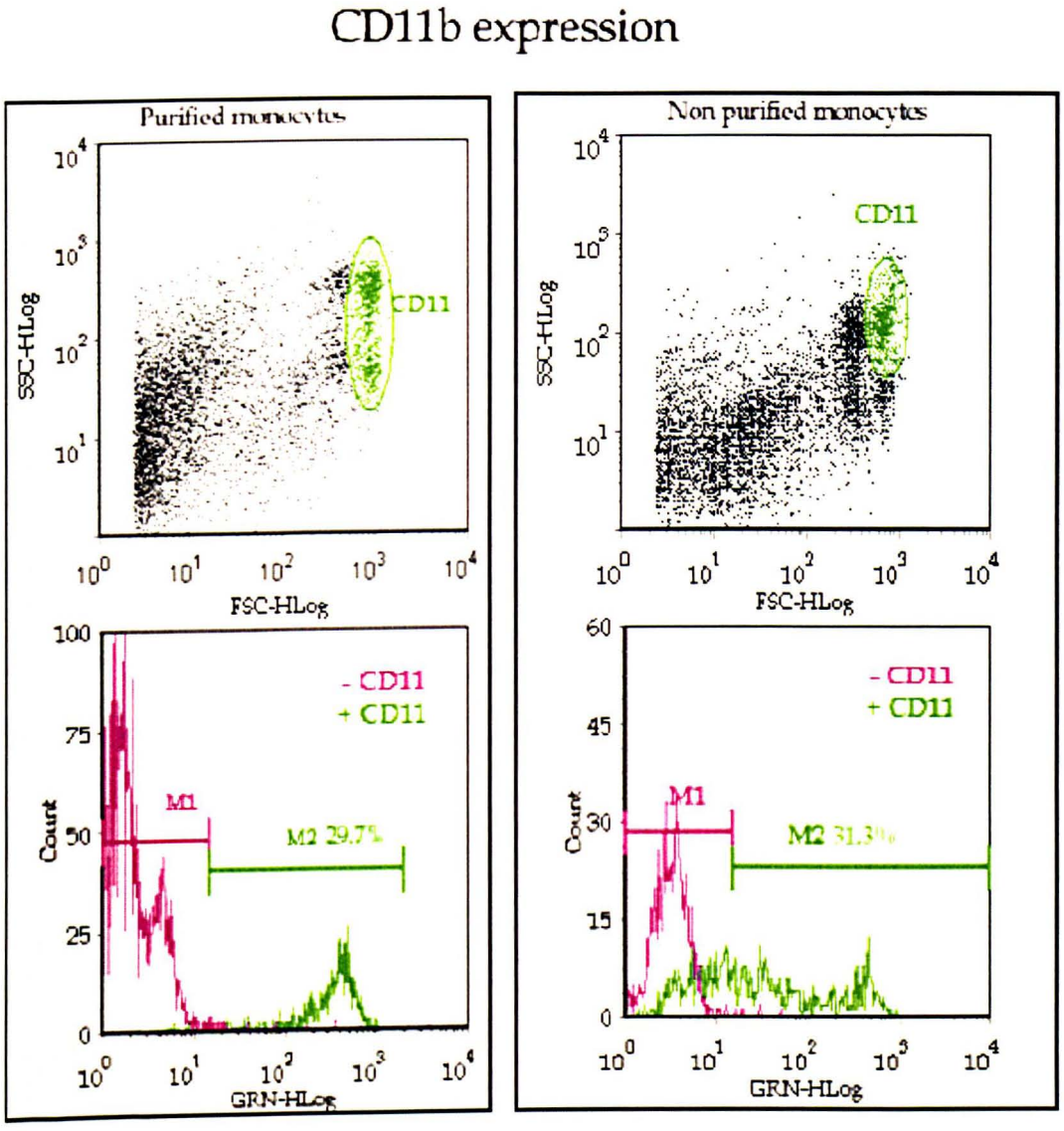


Figure 2-14 M1 Background fluorescence, M2 FITC green fluorescent, FSC-Log forward scatter, SSC-Log side scatter.

Determination of mononuclear cells and granulocytes CD11b and CD62L expression -Samples were loaded into 96-well plates in duplicates, and expression of CD11b and CD62L were measured by flow cytometry using the GUAVA PCA-96 flow cytometer, and GUAVA express plus software (Guava technologies, UK). Optical emission filter for 580-583 nm was used to detected FITC fluorescence. Over 5000 cells were submitted for analysis at each try. Unlabelled cells and IgG labelled cells were used as negative

control for non-specific flow cytometry background. Mononuclear and granulocytes cells were gated carefully based on forward and side scatter, (**Figure 2-15**)

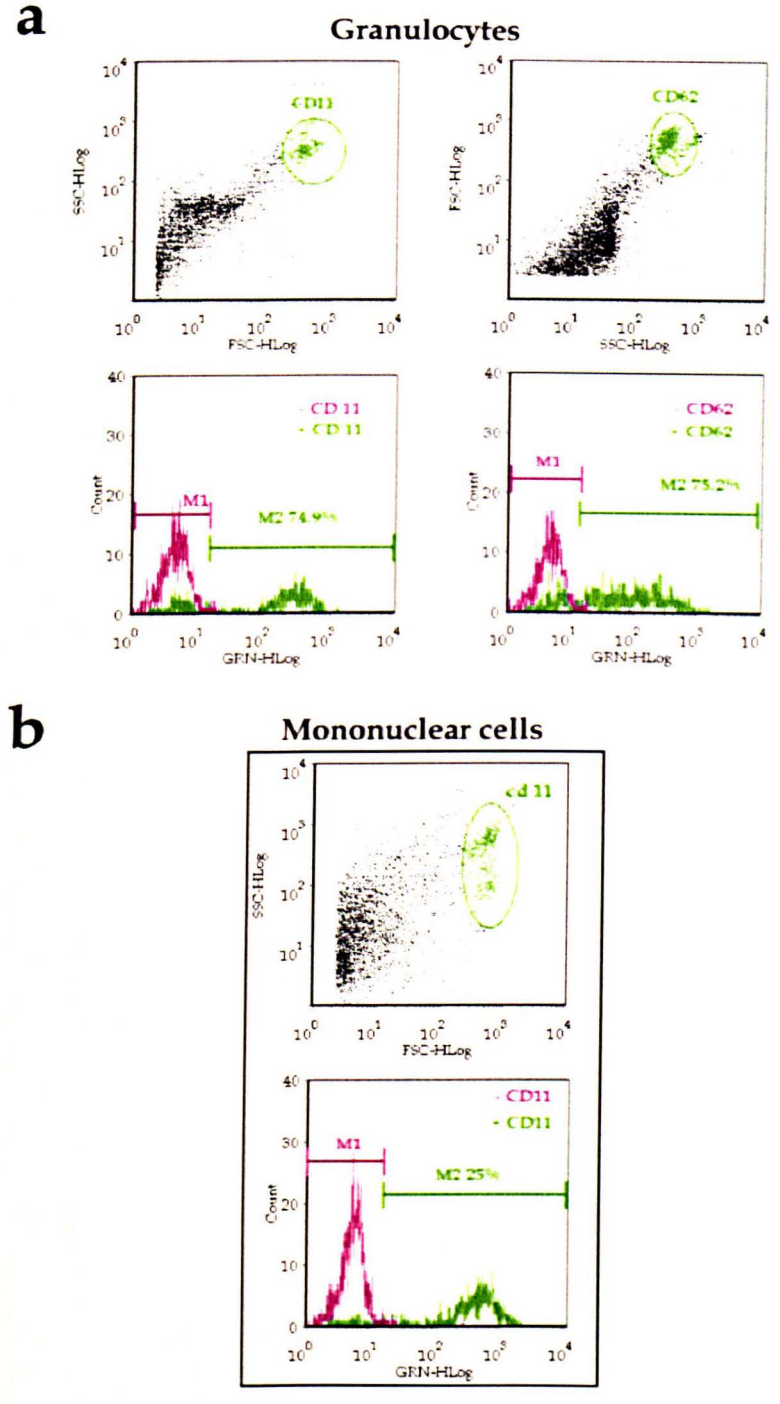


Figure 2-15 Determination of mononuclear cells (a) and granulocytes (b) CD11b and CD62L expression

2.11 Statistical analysis

The data-demographic, fatty acids, anti-oxidant vitamins, anti-oxidant enzymes, cytokines, adhesive molecules, plasma lactate dehydrogenase, plasma haptoglobine , haematological parameters were expressed as mean± Standard deviation (SD). The annual sickle cell associated crisis rates were expressed as median and percentile or inter-quartile range (IQR). Normality test was applied to all data set. Depending on the normality of the Gaussian distribution, parametric or non-parametric statistical tests will be used to investigate if there is a significant difference between test and control groups. Significance was assumed at a "p" value of less than 0.05. The statistical software, SPSS for Windows, Version 18 (SPSS Ltd., Woking, Surrey, UK) and GraphPad Prism 5 was used to analyse the data. The detailed statistical methods employed were discussed where appropriate.

Chapter Three

The effect of hydroxyhurea on red blood cell membrane fatty acid composition

3.1 Introduction

Hydroxyurea (HU) is effective therapy for adults, children and infants with severe sickle cell disease (Hoppe et al. 2000; Stuart and Nagel 2004; Segal et al. 2008; Strouse et al. 2008a). In randomised controlled clinical trails, HU decreased the frequency of painful episodes, acute chest syndrome, blood transfusion and hospital admission in severely affected adult, children and infants with SCD(Charache et al. 1995; Brawley et al. 2008; Strouse et al. 2008a; Wang et al. 2011). In addition HU might have other benefits including increasing life expectancy(Voskaridou et al. 2010), protection against cardiovascular disease(Zimmerman et al. 2007), proteinurea(Fitzhugh et al. 2005), and reduction of hypoxaemia(Singh et al. 2008). However, Despite the extensive evidence of efficacy, HU remains vastly underutilised drug due to continuous concerns about short and long term side effects(Hankins and Aygun 2009), which include myelosuppression(Lanzkron et al. 2008), malignancy(Zumberg et al. 2005), irreversible male subfertility and teratogenicity(Berthaut et al. 2008; Ballas et al. 2009). Other factor that complicat HU usage that it undergoes renal clearance, dose adjustment and close monitoring of myelotoxicity must be implemented in individual with renal impairment(Yan et al. 2005). In addition, large number of patents with SCD do not respond to treatment with HU(Stuart and Nagel 2004).

Hydroxyurea (HU) is a chemotherapeutic agent that inhibits ribonucleotide reductase and interferes with S-phase of the cell cycle(Trompeter and Roberts 2009). The myelosuppressive and cytotoxic effects of HU induce RBC regeneration and the recruitment of earlier progenitor programmed to produce higher levels of HbF(Dover et al. 1986; Fathallah and Atweh 2006). There is evidence; one of the mechanism by which HU increases HbF levels is mediated through a NO-dependant activation of soluble quanylyl cyclase in erythroid cells, **Figure 3-1**(Cokic et al. 2003; Lou et al. 2009). It was thought that it mediates its action solely through induction of foetal haemoglobin (HbF) and subsequent inhibition of polymerisation of deoxyhaemoglobin S (Steinberg et al. 1997). Nevertheless, clinical improvements do occur prior to a significant rise in levels of HbF (Charache et al. 1996). This suggests; HU may modulate the pathophysiology of the disease by other additional factors. Indeed, there is

evidence HU mediates its beneficial effects on SCD through lowering leukocytes, reticlocyte and platelets count (Ballas et al. 1999), reducing myeloperoxidase, diminishing endothelial adhesion molecule and red cell adhesion(Johnson and Telen 2008), reducing PS externalisation(Covas et al. 2004), increasing NO production(Nahavandi et al. 2002), and improving level of red cell dehydration and deformability(Ballas et al. 1989; Athanassiou et al. 2006).

Adhesion, aggregation and deformability of blood cells are strongly modulated by membrane fatty acids(Schwartz et al. 1985c; Nishiyama et al. 2000; Mukherjee et al. 2004). Steady-state sickle cell patients untreated with HU have abnormal RBC, platelets and mononuclear cell fatty acids, which is characterised by increase in arachidonic acid (AA, 20:4n-6), and decrease in linoleic acid (LA, 18:2n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)(Connor et al. 1997; Manodori et al. 2000; Ren et al. 2005a) (a). These findings have led to the postulation; "an imbalance of blood cell membrane n-3 and n-6 fatty acids may be the antecedent of loss of membrane asymmetry, blood cell adhesion, aggregation and vaso-occlusion in SCD"(Ren et al. 2005b). The aim of this study was to elucidate whether HU-treatment modulates RBC membrane fatty acids of steady state homozygous sickle cell patients (HbSS).

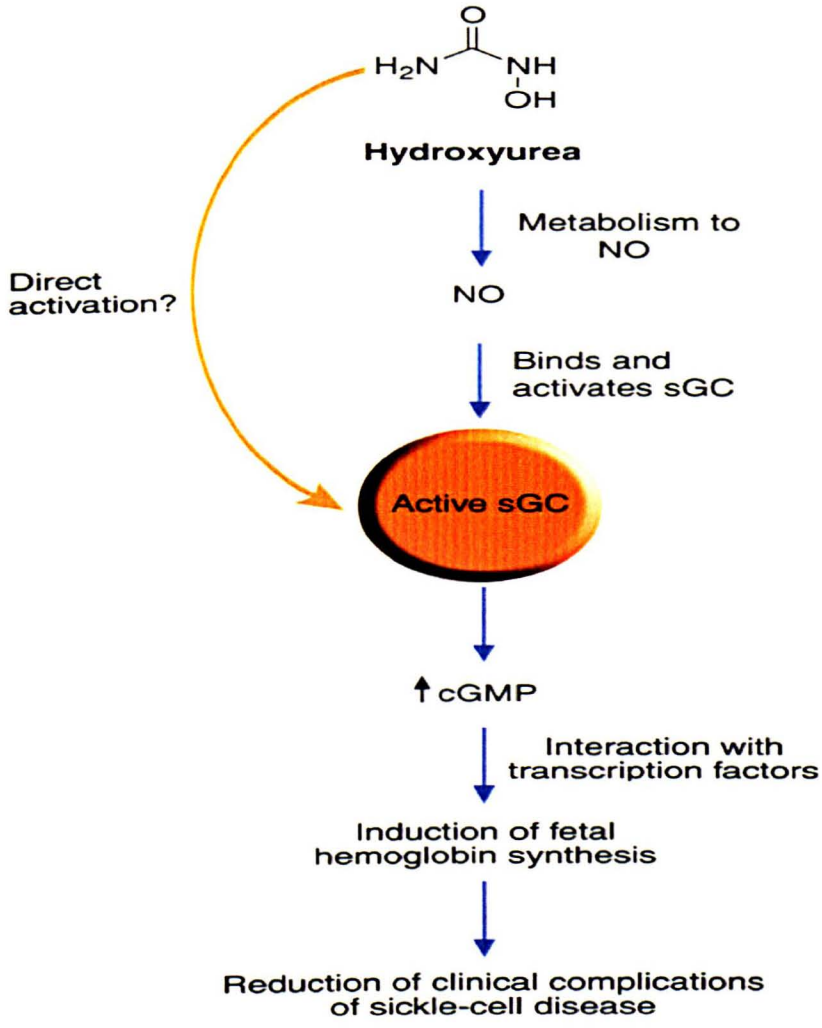


Figure 3-1 Mechanism of fetal hemoglobin induction by hydroxyurea
Adopted from(King 2003)

3.2 Subjects and methods

3.2.1 Subjects

Steady state HbSS sickle cell patients treated (age 7-26; n=19) and untreated (age 7-22; n=17) with HU, and healthy (HbAA) controls aged (age 6-21; n=20) matched for ethnicity and economic background were enrolled from Abnaof Paediatric Hospital, Khartoum, Sudan, Table 3-1. The phenotypic characteristic was confirmed by cellulose acetate electrophoresis. The treated patients were on HU for three months or longer. The exclusion criteria were: sickle cell crisis, acute illness in the pervious one-month, presence of other chronic diseases, blood transfusion in the previous four-months or

pregnancy. The Research Board of the Faculty of Medicine, University of Khartoum, Sudan, approved the study. Self- or investigator-read and explained written consent was obtained from the participants or their guardians. In addition, approval was obtained from National Research Ethics Service, Southampton & South West Hampshire Research Ethics Committee (A) UK (REC reference: 05/Q1702/48).

Table 3-1 Characteristics of the hydroxyurea treated and untreated HbSS patients and healthy (HbAA) subjects

	Hydroxyurea treated HbSS	Hydroxyurea untreated HbSS	Healthy Control (HbAA)
Number of subjects	19	17	20
Gender (Male/Female)	8/11	7/10	9/11
Age ± SD (year)	14.5 ± 4.3	14.5 ± 6.1	9.7 ± 6.7
Weight ± SD (kg)	36 ± 7.1	31.5 ± 11.8	36.1 ± 14.6
Height ± SD (cm)	146.9 ± 15.3	127.8 ± 25.5	146.3 ± 15.0

3.2.2 Blood collection and separation of blood component

After an overnight fast, 5 ml of whole blood was taken from the patients and controls in EDTA tubes. The separation of blood component is decribed in chapter 2, section 2.2.2.

3.2.3 Analysis of plasma lipids

The analysis of plasma total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol) and triglyceride concentrations is decribed in chapter 2, section 2-4.

3.2.4 Analysis of red blood cell fatty acids

The procedure of lipid extraction and fatty acid analysis of red cell and plasma is described in chapter 2, section 2-3.

3.2.5 Statistical analysis

The data are expressed as mean ± SD. Unpaired t-test was used to explore differences in concentrations of total, HDL and LDL cholesterol, and triglycerides between the HU treated patients and healthy controls. RBC fatty acids between the HU treated and untreated patients and healthy controls were compared with one- way ANOVA and the post hoc test for unequal variance, Tamhane's 2. Statistical significance was

assumed at a "p" value of less than 0.05. The statistical software, SPSS for Windows, Version 17 (SPSS Ltd., Woking, Surrey, UK) was used to analyse the data.

3.3 Results

3.3.1 Plasma lipids

Mean concentrations of total, HDL and LDL cholesterol, and triglyceride of the HU treated sickle cell patients and healthy controls are presented in **Table 3-2**. The patients, compared with their healthy counterparts, had lower total HDL ($p<0.001$) and LDL ($p<0.01$) cholesterol.

3.3.2 Red blood cell fatty acids

Percent fatty acid composition of RBC choline phosphoglycerides (CPG) and sphingomyelin (SPM), the major phospholipids of the outer membrane leaflet, and ethanolamine (EPG) and serine (SPG) phosphoglycerides, the dominant phospholipids of the inner membrane leaflet, are shown in **Table 3-3, 3-4, 3-5 and 3-6**, respectively.

3.3.2.1 Red cell CPG

The healthy controls had lower levels of palmitic acid (C16:0), oleic acid (C18:1n-9), total monoenes and adrenic acid (C22:4n-6) ($p<0.001$), and higher stearic (C18:0) and linoleic (C18:2n-6) acids and total n-6 ($p<0.001$) fatty acids compared with the hydroxyurea treated and untreated patients. In addition, they had higher docosahexaenoic acid (22:6n-3), n-3 metabolites ($p<0.01$) and total n-3 fatty acids ($p<0.05$) than the HU untreated patients. Stearic and gamma linolenic (18:3n-6, GLA) acids ($p<0.001$) and total saturated fatty acids ($p<0.01$) were reduced in the HU untreated compared with the treated patients.

3.3.2.2 Red cell SPM

Palmitic, stearic, total saturates and arachidonc were elevated ($p<0.01$), and oleic, nervonic ($p<0.01$) and total monoenes ($p<0.001$) reduced in the healthy controls than in the HU untreated patients. The HU treated compared with the untreated patients had increased palmitic, stearic and arachidonic ($p<0.05$), and decreased lignoceric (C24:0) and total monoenes ($p<.001$) and nervonic ($p<0.01$)

3.3.2.3 Red cell EPG

The healthy control group compared with the HU treated and untreated patients had reduced stearic, total saturates, adrenic and dihomo gamma linolenic (20:3n-6, DHGLA) ($p<0.001$) and increased linoleic and eicosapentaenoic (20:5n-3) acids ($p<0.001$). The HU treated patients had lower arachidonic, adrenic, osbond (22:5n-6) ($p<0.05$) and n-6 metabolites and total n-6 ($p<0.001$) than their untreated counterparts.

3.3.2.4 Red cell SPG

Both the HU treated and untreated patients had lower linoleic ($p<0.001$) and higher DHGLA, adrenic, osbond and total n-6 ($p<0.01$) compared with the healthy control subjects. The HU treated patients had lower GLA, eicosdienoic (20:2n-6), arachidonc, osbond, n-6 metabolites and total n-6 ($p<0.01$) than their untreated counterparts.

Table 3-2 Plasma lipid concentrations of steady state HU treated HbSS sickle cell patients and HbAA healthy controls.

Plasma lipids	HbAA (n=20)	HbSS (n=17)
LDL-cholesterol (mmol/L)	2.4 ± 0.5	1.7 ± 0.7**
HDL-cholesterol (mmol/L)	1.1 ± 0.3	0.8 ± 0.2***
Total-cholesterol (mmol/L)	3.71 ± 0.56	2.7 ± 0.8***
Triacylglycerol (mmol/L)	1.01 ± 0.4	1.24 ± 0.03

HbSS patients versus HbAA controls: ** P<0.01, ***P<0.001.

Table 3-3 Fatty acid composition of red blood cell choline phosphoglycerides of hydroxyurea untreated (n=17) and treated (n=19) HbSS patients and HbAA controls (n=20).

Fatty acids	HbSS (Un-treated)	HbSS (Treated)	HbAA (Healthy Control)
16:0 (Palmitic acid)	36.7 ± 1.6	36.3 ± 1.7×××	31.8 ± 2.5+++
18:0 (Stearic acid)	8.9 ± 0.7***	10.3± 1.2×××	13.2± 1.0+++
20:0 (Arachidic acid)	0.1 ± 0.03***	0.2 ± 0.1×	0.1 ± 0.1
22:0 (Behenic acid)	tr	tr	tr
24:0 (Lignoceric acid)	0.04 ± 0.01	0.05±0.01	.04±0.01
∑Saturates	46.0 ± 1.3**	47.2±0.8××	45.2 ± 1.9
16:1n-7 (Palmitoleic acid)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
18:1n-7 (Vaccenic acid)	1.6 ± 0.2	1.7± 0.3×	1.4 ± 0.2+
18:1n-9 (Oleic acid)	18.3 ± 1.1	17.7 ± 1.5×××	14.2 ± 1.5+++
24:1n-9 (Nervonic acid)	tr	tr	tr
∑Monoenes	20.2 ± 1.1	19.7 ± 1.6×××	15.9 ± 1.6+++
18:2n-6 (Linoleic acid)	16.3 ± 1.4	15.4 ± 2.6×××	20.7 ± 2.1+++
18:3n-6 (γ -linolenic acid)	0.1 ± 0.02***	0.1 ± 0.03	0.1 ± 0.05
20:2n-6 (Eicosdienoic acid)	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0.1
20:3n-6 (Dihomo-γ-linolenic)	1.9 ± 0.4	1.7 ± 0.2××	2.1 ± 0.1
20:4n-6 (Arachidonic acid)	9.6 ± 0.7	9.5 ± 1.1	9.4 ± 1.5
22:4n-6 (Adrenic acid)	0.9 ± 0.2	0.9 ± 0.2×××	0.7 ± 0.1+++
22:5n-6 (Osbond acid)	0.5 ± 0.1	0.5 ± 0.1×	0.6 ± 0.2+
∑metabolites	13.6 ± 0.7	13.1 ± 1.3	13.4 ± 1.7
∑n-6	29.9 ± 1.6	28.4± 1.9×××	34.1 ± 2.7
18:3n-3 (α-Linolenic acid)	tr	tr	tr
20:5n-3 (Timnodonic acid)	0.1 ± 0.02	0.1 ± 0.05	0.11 ± 0.03
22:5n-3 (Clupandonic acid)	0.3 ± 0.06	0.3 ± 0.07	0.3 ± 0.05
22:6n-3 (Cervonic acid)	0.7 ± 0.2	0.8 ± 0.4	1.0 ± 0.2 ++
∑metabolites	1.1 ± 0.2	1.2 ± 0.4	1.3 ± 0.3++
∑n-3	1.2 ± 0.2	1.2 ± 0.4	1.4 ± 0.3+
N6/N3	26.58 ± 5.03	24.91 ± 6.78	25.65 ± 5.09

tr- trace

HU untreated versus treated HbSS patients, *p<0.05, **p<0.01, ***p<0.001

HU untreated HbSS patients versus HbAA healthy controls, +p<0.05, ++p<0.01, +++p<0.001

HU treated HbSS Patients versus HbAA healthy controls, × p<0.05, ××p<0.01, ×××p<0.001

Table 3-4 Fatty acid composition of red blood cell sphingomyelin of hydroxyurea untreated (n=17) and treated (n=19) HbSS patients and HbAA healthy controls (n=20).

Fatty acids	HbSS (Un-treated)	HbSS (Treated)	HbAA (Healthy Control)
16:0	21.1 ± 4.3*	25.3 ± 3.5	26.1 ± 2.2++
18:0	8.7 ± 2.2*	11.9 ± 3.5	11.3 ± 2.0++
20:0	2.0 ± 0.2	1.00 ± 0.2×××	1.44 ± 0.2+++
22:0	8.1 ± 1.5*	6.6 ± 1.3	7.6 ± 1.1
24:0	25.3 ± 4.8***	18.5 ± 2.9××	21.6 ± 2.6+
∑Saturates	63.9 ± 3.8	63.8 ± 3.1×××	68.2 ± 1.4++
16:1n-7	tr	tr	tr
18:1n-7	0.3 ± 0.1***	0.7 ± 0.1××	0.5 ± 0.1+++
18:1n-9	3.8 ± 1.9	3.4 ± 0.6×××	2.0 ± 0.3++
24:1n-9	17.8 ± 3.6**	14.2 ± 1.7	13.9 ± 1.7++
∑Monoenes	21.9 ± 2.8***	18.3 ± 2.0×	16.5 ± 1.8+++
18:2n-6	2.1 ± 1.1	2.3 ± 0.6	2.0 ± 0.3
18:3n-6	tr	tr	tr
20:2n-6	0.4 ± 0.3**	0.1 ± 0.04××	0.09 ± 0.04++
20:3n-6	0.3 ± 0.1**	0.5 ± 0.1××	0.3 ± 0.1
20:4n-6	3.7 ± 1.3*	4.9 ± 1	5.1 ± 1.3++
22:4n-6	1.8 ± 1.0	2.0 ± 0.8×	1.3 ± 0.4
22:5n-6	1.4 ± 0.6	1.3 ± 0.6	1.1 ± 0.3
∑metabolites	7.5 ± 2.9	8.4 ± 1.9	7.9 ± 2.1
∑n-6	9.6 ± 3.5	10.8 ± 1.7	9.9± 2.2
18:3n-3	tr	tr	tr
20:5n-3	0.11 ± 0.02***	0.07 ± 0.02	0.06 ± 0.02+++
22:5n-3	0.4 ± 0.2	0.45 ± 0.2	0.4 ± 0.1
22:6n-3	1.4 ± 0.7	1.8 ± 0.8	1.7 ± 0.6
∑metabolites	1.9 ± 0.8	2.4 ± 0.9	2.1 ± 0.7
∑n-3	1.9 ± 0.8	2.4 ± 0.9	2.1 ± 0.7
N6/N3	5.3± 1.6	5.3 ± 1.7	4.9 ± 1.1

tr- trace

HU untreated versus treated HbSS patients: *p<0.05, **p<0.01, ***p<0.001

HU untreated HbSS patients versus HbAA healthy controls: +p<0.05, ++p<0.01, +++p<0.001

HU treated HbSS patients versus HbAA healthy controls: ×p<0.05, ××p<0.01, ×××p<0.001

Table 3-5 Fatty acid composition of red blood cell ethanolamine phosphoglycerides of hydoxyurea untreated (n=17) and treated (n=19) HbSS sickle cell patients and HbAA healthy controls (n=20).

Fatty acids	HbSS (Un-treated)	HbSS (Treated)	HbAA (Healthy Control)
16:0	12.8 ± 1.3	12.8 ± 1.3	12.8 ± 1.2
18:0	7.9 ± 0.9	8.7 ± 1.4×××	6.0 ± 0.7+++
20:0	0.1 ± 0.02	0.08 ± 0.03	0.06 ± 0.03
22:0	0.03 ± 0.01***	0.07 ± 0.02	0.08 ± 0.04+++
24:0	0.06 ± 0.03*	0.08 ± 0.02×××	0.06 ± 0.02
∑Saturates	21.02 ± 1.0	21.9 ± 1.5×××	19.1 ± 1.1+++
16:1n-7	0.1 ± 0.03***	0.1 ± 0.02	0.1 ± 0.04+++
18:1n-7	0.9 ± 0.2	1.2 ± 0.8	0.9 ± 0.1
18:1n-9	13.1 ± 1.2	12.1± 1.1	12.9 ± 0.7
24:1n-9	tr	tr	tr
∑Monoenes	14.0 ± 1.3	13.2 ± 1.1	13.9 ± 1.0
18:2n-6	4.9 ± 0.7	4.9 ± 0.9×××	6.7 ± 0.9+++
18:3n-6	0.2 ± 0.05***	0.04 ± 0.01	0.04 ± 0.01+++
20:2n-6	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.1
20:3n-6	1.4 ± 0.3	1.3 ± 0.2×××	1.0 ± 0.1+++
20:4n-6	24.0 ± 1.1*	22.9 ± 1.2××	24.8 ± 1.5
22:4n-6	10.8 ± 0.1*	9.89 ± 1.0×××	8.6 ± 0.7+++
22:5n-6	2.6 ± 0.4*	2.2 ± 0.5	1.9 ± 0.3+++
∑metabolites	39.4 ± 1.3***	36.8 ± 2.2	36.8 ± 1.7+++
∑n-6	44.3 ± 1.5***	41.4 ± 2.5×	43.9 ± 1.8
18:3n-3	0.08 ± 0.04	0.08 ± 0.02	0.1 ± 0.03
20:5n-3	0.11 ± 0.03*	0.15 ± 0.05×××	0.23 ± 0.06+++
22:5n-3	1.7 ± 0.3	1.6 ± 0.3	1.7 ± 0.2
22:6n-3	2.7 ± 0.4	2.9 ± 0.88	3.2 ± 0.6+
∑metabolites	4.5 ± 0.5	4.6 ± 1.1	5.1 ± 0.7+
∑n-3	4.6 ± 0.5	4.7 ± 1.1	5.2 ± 0.7+
N6/N3	9.8 ± 1.4	9.3 ± 2.34	± 1.3+

tr- trace
HU untreated versus treated HbSS patients: *p<0.05, **p<0.01, ***p<0.001
HU untreated HbSS patients versus HbAA healthy controls: +p<0.05,++p<0.01, +++p<0.001
HU treated versus HbAA healthy controls: ×p<0.05, ××p<0.01, ×××p<0.001

Table 3-6 Fatty acid composition of red blood cell serine phosphoglycerides of hydoxyurea untreated (n=17) and treated (n=19) HbSS sickle cell patients and HbAA healthy controls (n=20).

Fatty acids	HbSS (Un-treated)	HbSS (Treated)	HbAA (Healthy Control)
16:0	4.4 ± 0.9	5.5 ± 1.6	5.2 ± 1.2
18:0	41.9 ± 1.3	42.9 ± 1.9	41.6 ± 1.1
20:0	0.3 ± 0.1	0.3 ± 0.06××	0.2 ± 0.07++
22:0	0.1 ± 0.04***	0.2 ± 0.1	0.2 ± 0.07
24:0	tr	tr	tr
∑Saturates	46.9 ± 0.9***	49.2 ± 0.8×××	47.5 ± 1.0
16:1n-7	tr	tr	tr
18:1n-7	0.4 ± 0.9***	0.8 ± 0.2	0.7 ± 0.1+++
18:1n-9	4.9 ± 1.03*	6.0 ± 1.23	5.1 ± 1.0
24:1n-9	0.03 ± 0.01***	0.4 ± 0.31	0.3 ± 0.23+++
∑Monoenes	5.4 ± 1.1**	7.1 ± 1.5××	5.4 ± 1.2
18:2n-6	3.20 ± 0.6	3.3 ± 0.6×××	6.1 ± 1.6+++
18:3n-6	0.2 ± 0.06***	0.06 ± 0.03×××	0.02 ± 0.02+++
20:2n-6	0.5 ± 0.1***	0.2 ± 0.04	0.3 ± 0.09+++
20:3n-6	3.2 ± 0.8	2.9 ± 0.8×××	2.1 ± 0.3+++
20:4n-6	24.9 ± 2.3**	22.13 ± 2.2×××	25.7 ± 1.9
22:4n-6	6.7 ± 1.1	6.1 ± 1.0×××	3.9 ± 0.8+++
22:5n-6	3.4 ± 0.7**	2.7 ± 0.58××	2.1 ± 0.5+++
∑metabolites	38.8 ± 1.4***	34.1 ± 1.9	33.9 ± 2.4+++
∑n-6	42.0± 1.2***	37.0 ± 2.0×××	40.1 ± 2.7++
18:3n-3	tr	tr	tr
20:5n-3	0.07 ± 0.01	0.08 ± 0.09	0.11 ± 0.04++
22:5n-3	1.4 ± 0.3	1.2 ± 0.3	1.1 ± 0.2++
22:6n-3	3.0 ± 0.6	2.8 ± 0.8	2.7 ± 0.8
∑metabolites	4.4 ± 0.7	4.1 ± 1.0	3.9 ± 1.1
∑n-3	4.4 ± 0.7	4.1 ± 1.03	3.9 ± 0.1
N6/N3	9.7 ± 1.9	9.7 ± 2.6	11.0 ± 2.9

tr- trace
HU untreated versus treated HbSS patients: *p<0.05, **p<0.01, ***p<0.001
HU untreated HbSS patients versus HbAA healthy controls: +p<0.05, ++p<0.01, +++p<0.001
HU treated HbSS Patients versus HbAA healthy controls: ×p<0.05, ××p<0.01, ×××p<0.001

3.4 Discussion

The reduced concentrations of total, HDL and LDL cholesterol in the HU treated sickle cell patients is consistent with the low level total, HDL and LDL (Saha and Samuel 1982; Stone et al. 1990; VanderJagt et al. 2002; Marzouki and Khoja 2003; Rahimi et al. 2006) and total cholesterol (Shores et al. 2003) reported in HU untreated, steady state, sickle cell patients. The patients and healthy control subjects in the current study were broadly matched for age, gender, ethnicity and socio-economic background. Consequently, the observed reduction in cholesterol levels in the HU treated patients was unlikely to have been a reflection of genetic or environment factors. It has been postulated, hypocholesterolemia in sickle cell patients is due to haemodilution, down regulation of cholesterol synthesis, and decreased transfer of cholesterol from membrane to circulating High density lipoprotein (HDL) because of reduced activity of lecithin-cholesterol acyltransferase(Glew et al. 2003; Shores et al. 2003). In the current study, the HU patients and healthy controls had a comparable level of triacylglycerols. Hence, the hypocholesterelomia in the patients could not be explained by haemodilution. Likewise, down-regulation of biosynthesis of cholesterol may not be a factor as there is evidence that RBC of sickle cell patients have higher content of cholesterol (Muskiet and Muskiet 1984; Sasaki et al. 1986). SCD patients have lower level of apolipoprotein A-I(Sasaki et al. 1986; Morris 2008; Yuditskaya et al. 2009), and deoxygenated HbS red cells has been shown to have increased uptake of cholesterol analogue (Kavecansky et al. 1995). Hence, it is plausible; the hypocholosterelomia observed in the patients was a reflection of increased uptake by RBC membranes and reduced transfer to circulating HDL.

The current comparative study of Sudanese homozygous sickle cell patients and healthy controls revealed both HU treated and untreated patients had abnormal red cell fatty acid pattern, which was more pronounced in the latter group. The remarkable anomalies in the HU untreated patients compared with the healthy controls were: decreased linoleic, stearic, and increased palmitic and oleic acids in CPG, decreased palmitic and stearic and increased nervonic and lignoceric acids in SPM, decreased linoleic acid, EPA and DHA and increased stearic, adrenic and osbond acids in EPG and decreased linoleic and oleic and increased adrenic and osbond acids. These

findings are broadly consistent with the previous findings in serum phospholipids(Enomoto et al. 1998), red cell total phospholipids(VanderJagt et al. 2003) , red cell phospholipid classes(Connor et al. 1997; Ren et al. 2005a) and total lipids of mononuclear cells and platelets(Ren et al. 2005b). These findings in steady state homozygous sickle cell patients of different ethnic, cultural and socio-economic backgrounds demonstrate, the disease induces blood cell membrane fatty acid perturbation. This could be due to: (a) metabolic dysfunction - impaired synthesis, uptake and/or enhanced turnover; (b) peroxidation caused by iron overload. Both the HU untreated patients and healthy controls had very low n-3 fatty acids compared with the British and Nigerian subjects previously investigated by our group(Ren et al. 2005a). This difference is most likely a reflection of the n-3 fatty acid status of the populations studied since we have observed North Sudanese maternal milk(Nyuar KB 2010) and blood (unpublished) have very low content of these nutrients.

Hydroxyurea had a profound effect on fatty acid composition of membrane phospholipids, particularly sphingomyeilin, and ethanolamine and serine phosphoglycerides. The striking effect on the former phospholipid was a reduction of 18.1, 26.7 and 20.6% of behenic (C20:0), lignoceric (C22:0) and nervonic (C24:1n-9) acids, respectively. The HU-induced changes in sphingomyelin composition is significant since lipid rafts, the highly ordered membrane microdomains, which are thought to play a pivotal role in membrane trafficking, signal transduction and gene and protein expression, are rich in sphingomyelin(Brown and London 2000a; Niemela et al. 2007). Moreover, saturated and mono-unsaturated fatty acids are structural components and major functional determinants of sphingomyelin.

In addition, hydroxurea reduced arachidonc acid (AA, C20:4n-6) significantly in both ethanolamine (-4.7%) and serine (-11.1%) phosphoglycerides, phospholipids found predominately in the inner leaflet of membrane lipid bi-layer. Such reductions were not apparent in choline phosphoglycerides and sphingomyelin, which are principally found in the outer leaflet of membrane lipid bi-layer. Hydroxyurea generates nitric oxide in vivo (Glover et al. 1999; Nahavandi et al. 2002; King 2004) and in vitro(Pacelli

et al. 1996; Kim-Shapiro et al. 1998), and the functionally coupled cPLA2α and cyclooxygenase 2 (COX2)(Fitzpatrick and Soberman 2001) are activated by nitric oxide(Kim et al. 2005; Xu et al. 2008).

Prostaglandin E2 (PGE2), which is a metabolite of AA and a vasodilator, has been shown to induce the synthesis of foetal haemoglobin in peripheral blood derived from erythroid colonies from normal and sickle cell adults(Datta 1985). In rats, the HU-induced synthesis of foetal haemoglobin is obviated by aspirin, the potent inhibitor of COX(Datta et al. 1991) . Hence, it is tenable to suggest, in the HU treated patients, AA is selectively released by the activation of the arachidonic acid-selective cytosolic phosopholipase A2α (cPLA2α) and subsequently metabolised by COX2 to generate PGE2.

Consistent with our previous findings in HU-untreated HbSS patients(Ren et al. 2005b), the HU-untreated patients in this study had significantly lower levels of DHA and total n-3 fatty acids in CPG and EPG; and EPA in EPG, SPG and SPM compared with healthy controls. In contrast, in the HU-treated patients, the reduction in n-3 fatty acids was restricted only to EPA in EPG. It appears the n-3 fatty acid abnormality often observed in HU-unreated patients was partially ameliorated by hydroxyurea treatment. This modulation of membrane fatty acid composition would be expected to help enhance trans-membrane ion flux, cell hydration, rheology and deformability(Poschl et al. 1996; Ho et al. 1999; Djemli-Shipkolye et al. 2003), factors which are known to improve in hydroxyurea treated sickle cell patients (Ballas et al. 1989; Adragna et al. 1994; Athanassiou et al. 2006).

This investigation demonstrates, hydoxyurea modulates red blood cell membrane fatty acid abnormalities including the n-3/n-6 imbalance reported previously in steady state homozygous sickle cell patients. These modulations in synergy with the hydroxyurea-generated vasodilators nitric oxide and PGE2 may be play a critical role in clinical improvements which occur prior to the increased synthesis of HbF in treated patients.

The current study did not investigate the effect of hydroxyurea on metabolism of eicosanoids derived from arachidonic acid, oxidative stress and fatty acid composition of other blood cells, such as platelets and leukocytes. These limitations are potential lines of inquiry which may need to be explored in future studies.

Chapter Four

An investigation into the impact of omega-3 LC PUFA on oxidative status in Sickle cell patients

4.1 Introduction

Red blood cells (RBCs) experience continuous oxidative insults by being exposed to endogenous and exogenous reactive oxygen species (ROS)(Nagababu et al. 2003).Despite the fact that RBC lacks the mitochondrion which is the major source of ROS in other tissues(Turrens 2003), the RBC iron-containing haemoglobin (Hb) produces substantial amount of ROS through auto-oxidation, the process in which oxy-haemoglobin Hb (oxyHbS) undergoes spontaneous heterolytic dissociation into metHb and superoxide ($O^{\cdot}_2$) (Reeder and Wilson 2005), **equation 4- 1**.

$$Hb\text{-}Fe^{II}\text{-}O_2 \longrightarrow Hb\text{-}Fe^{III} + O^{\cdot}_2 \qquad \textbf{(equation.4- 1)}$$

In addition, The Hb iron, can act as a potent catalyst in reactions that generates highly reactive radicals (hydroxyl radical) in biological systems. Hydroxide radicals are generated from hydrogen peroxide (H_2O_2) through superoxide-driven, iron- catalysed Haber-Weiss reaction (equation. 4-4) , which makes use of Fenton chemistry(equation. 4-2&4-3) (Kehrer 2000).

$$Fe^{3+} + O^{\cdot}_2{}^{-} \longrightarrow Fe^{2+} + O^2 \qquad \textbf{(equation 4-2)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot} \qquad \textbf{(equation 4-3)}$$

(Fenton reaction)

The net reaction (Haber-Weiss):

$$O^{\cdot}_2{}^{-} + H_2O_2 \longrightarrow O_2 + \cdot OH + OH^- \qquad \textbf{(equation 4-4)}$$

Under normal conditions, this iron-mediated oxidative reaction is prevented by the hydrophobic globin pocket that tightly binds haem and consequently limiting (compartmentalisation) the access of the iron to the labile membrane unsaturated fatty acids(Marengo-Rowe 2006).However, it has been estimated 1-2% of normal RBCs

oxyhaemoglobin undergoes autoxidation,(Johnson et al. 2010).In order to cope with continuous production of H2O2 and superoxide, RBCs are equipped with enzymetic and no-enzymetic anti-oxidant systems(Wood and Granger 2007). The major non-enzymatic anti-oxidant include those scavenge free radicals as Vitamin E, C, caretenoids and glutathion(Chan et al. 1999; Manfredini et al. 2008). These antioxidant nutrients occupy distinct cellular compartments and among them, there are active recyclings. ROS in the aqueous phase are scavenged by the water-soluble vitamin C, while vitamin E, A and β-carotene, predominantly scavenge ROS that pervade the non-aqueous lipid membrane environment, and consequently prevent the peroxidation of PUFA by breaking the radical chain reaction (Sies and Stahl 1995; Fang et al. 2002). The most important enzymatic antioxidants are superoxide dismutase (Cu-Zn-SOD), which catalyses dismutation of the $O_2^{\cdot-}$ into H_2O_2. The H_2O_2 generated from Cu/Zn-SOD reaction is then deactivated by glutathione peroxidase (Se-GPx, **Figure 4-1**), catalase (Fe-CAT) and peroxiredoxin-2 (Prdx2)(Andersen et al. 1997; Low et al. 2007; Johnson et al. 2010). While Fe-CAT is completely specific for H_2O_2, Se-GPx is able to reduce organic peroxides and lipid hydroperoxide(Johnson et al. 2002). Therefore it has been suggested Se-GPx might have particular importance in SCD(Cho et al. 2010).

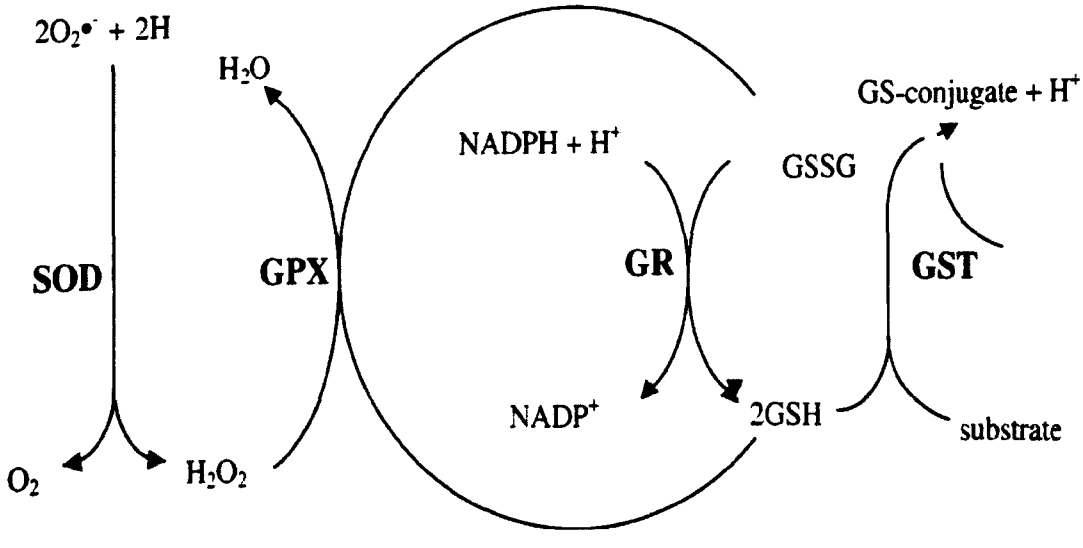


Figure 4-1 Neutralization of ROS in red blood cells by superoxide dismutase (SOD) and glutathion peroxidase (GPx),
Adopted from(Yan et al. 2008)

GR=glutathione reductase, GSH= glutathione, GSSG=glutathione disulfide.

Compared to normal Haemoglobin A (HbA) , haemoglobin S (HbS) auto-oxidises 1.7-fold faster in solution (Hebbel et al. 1988) resulting in continuous production of superoxide anion. In addition, the conversion of metHbS to oxyHbS is impaired due to insufficiency of nicotinamide adenine dinucleotide (NADH) the co-enzyme of metHb reductase in sickle red blood cells (Zerez et al. 1990). Such imbalance results in higher metHb levels and subsequent denaturation of Hb (haemachrome) and release of heme. Furthermore, the instability of HbS and its ability to interact with membrane lipids of intact sickle red blood cells(Sugihara et al. 1992b; Marva and Hebbel 1994), leads to iron decompartmentalisation and deposition in form of hemichrome, free heme(Kuross et al. 1988) and free iron associated with component of RBC membrane(Kuross and Hebbel 1988). As a result, the membrane iron provides a biologic "Fenton reagent" for the generation of hydroxyl radicals at the membrane and consequent oxidation of membrane lipids(Misra and Fridovich 1972; Repka and Hebbel 1991).

Among the factors thought to participate in systemic high OS associated with SCD is the increased intravascular haemolysis and excessive levels of cell-free haemoglobin with its catalytic action on oxidative reaction (Jeney et al. 2002; Nur et al. 2011). In addition, chronic pro-inflammatory state is a characteristic of SCD patients even in steady state(Akohoue et al. 2007), and ROS production is a main feature of and plays an important role in inflammation(Guzik et al. 2003). Hence, it has been suggested that chronic inflammatory state may contribute in OS related to SCD. A possible source of ROS stemming from SCD-related inflammation is high polymorphonuclear Cells (PMNs) count in SCD(Wun 2001; Frenette 2002). It has also been demonstrated that hypoxia-reoxygenation cycles in SCD associated with increased OS in SCD(Kaul and Hebbel 2000). Due to fact that SCD patients have high number PMNs and NADPH oxidase-mediated respiratory burst is chronically activated in (PMNs) of SCD patients (Dahlgren and Karlsson 1999; Wun 2001), more severe reperfusion injury-induced oxidative stress tend to occur in SCD(Nur et al. 2011).

On the other hand, it has been found that the anti-oxidant capacity in RBCs of SCD patients is highly impaired (Schacter et al. 1988; Wood and Granger 2007; Ren et al.

2008; Cho et al. 2010).Thus, the chronic high oxadative stress state (OS) associated with SCD(Aslan and Freeman 2007; Hebbel 2010) is an outcome of imbalance between enhanced generation of reactive oxygen species (ROS) and disrupted antioxidant system.

Different studies have shown that, red blood cell membrane of patients with SCD has abnormal cell membrane fatty acids composition characterized by low levels of n-3 long chain polyunsaturated fatty acids (n-3 FA) (Connor et al. 1997; Ren et al. 2005b).The underlying cause(s) of these abnormal levels of membrane fatty acids is not fully understood. However, one of the possible explanations of reduced levels of the n-3 fatty is their higher vulnerability to oxidation, especially docosahexanoic acid (DHA) which possesses a high un-saturation index (Richard D 2008).

It is well known that, alterations in cell membrane fatty acid composition has a tremendous effect on blood cell adhesion, aggregation, elasticity and inflammatory response (Chiu et al. 1981; Mills et al. 1993; Nishiyama et al. 2000; Mukherjee et al. 2004), the same factors underlie complications associated with SCD (Hebbel et al. 1980; Kaul et al. 1996; Rosse et al. 2000; Frenette 2002; Okpala 2006; Frenette and Atweh 2007).Hence, It is conceivable, correction of membrane fatty acids abnormality by supplementation with n-3 FA may have a beneficial clinical effect. However, the effect of supplementation with relatively highly oxidize-able polyunsaturated n-3 FA poses a theoretical concern owing to possibility of aggravation of high oxidative stress associated with SCD. Nevertheless, the in vivo effect of n-3 FA on lipid peroxidation and oxidative stress are debatable(Nenseter and Drevon 1996). Indeed, In vitro and in vivo studies have shown that supplementation with n-3 fatty in condition with high oxidative stress dose lessen markers of peroxidation(Yang et al. 1993; Supari et al. 1995; Bas et al. 2007; Richard D 2008), corroborating the possible anti-oxidant activity of n-3 in biological milieu (Bechoua et al. 1999; Yavin 2006). This study was conducted to investigate whether supplementation with LCPUFA n-3 fatty acids exacerbate the oxidatives stress in SCD patients, and the possible the effect of n-3 fatty acids supplementation on anti-oxidant enzyme activity.

4.2 Methodology

4.2.1 Design and intervention

This study was conducted in the context of single centre, two-arm, randomised, placebo-controlled, double blinded clinical trial investigating the effects of omega-3 fatty acid supplementation on Sudanese children with homozygous SCD. Forty (n=40) subjects fromed the active group aged 2-13 years (supplemented with capsules composed of 60% DHA and 18% EPA omega-3 fatty acids) and thirty-three (n=33) subjects fromed the placebo group aged 2-14 years (supplemented with high oleic acid sunflower seed oil) were investigated at base line, six-month and one-year after intervention. potential participant (HbSS patients) were excluded if they have history of sickle cell crisis, acute illness in the previous one-month, presence of other chronic diseases, blood transfusion in the previous four-months or hydroxyurea treatment. According to age ranges 2-4, 5-10 and 11-16 years participants were given daily dose of 250, 500 and 750 mg respectively of either the placebo or active supplements. To inhibit peroxidation 1.5 mg of alpha-tocopherol (vitamin E) per capsule was added to treatment and placebo capsules.

The trial was approved by the Ethics Committee of the, Faculty of Medicine, University of Khartoum, Sudan and the Research Ethics Committee of Southampton & South West Hampshire, UK (REC reference number-05/Q1702/48). All participants or their guardians provided informed written consent.

4.2.2 Blood sampling

After an overnight fast, 5 ml of whole blood was taken in EDTA tubes from all participants (n=73) at zero-time, samples of 36 and 28 participants from active and placebo groups respectively were collected at six-month and one year of intervention. Blood samples of nine patients (n=9), five from placebo (n=5) and four (n=4) from active groups were not collected at six-month and/or one-year due to drop-out, or inability to collect the blood sample at the pre-scheduled time points. The separation of blood component is decribed in chapter 2, section 2.2.2.

4.2.3 Complete blood count (CBC)

The complet blood count was measured as described in chapter 2, section 2.2.1.

4.2.4 Analysis of red blood cell fatty acids

The procedure of lipid extraction and fatty acid analysis of red blood cell is described in chapter 2, section 2-3.

4.2.5 Measurement of plasma α-tocopherol (vit E) and retinol concentration

The procedure of plasma vitamins extraction and analysis of is described in chapter 2, section 2-5.

4.2.6 Glutathione peroxidase (Se-GPx) and Cu/Zn-Super oxide dismutase (Cu/Zn-SOD) enzymes' activity assay

The measurement of Se-GPx and Cu/Zn-SOD is described in chapter 2, section 2-6.

4.2.7 Statistical analysis

The data are expressed as mean ± SD. Paired t-test was used to explore differences within the placebo or active group, fatty acids,un-paired t-test was used to test differences between the two groups. The Statistical significance was assumed at a "p" value of less than 0.05. The statistical software, SPSS for Windows, Version 17 (SPSS Ltd., Woking, Surrey, UK) was used to analyse the data.

4.3 Results

Table 4-1 gives the base-line characteristics of the 73 study participants. No significant differences were found between the active and placebo groups in demographic characteristic and haematological profile at base-line.

4.3.1 Red blood cell membrane PE and PC fatty acid composition

The effect of supplementation on fatty acids composition of PE and PC phospholipids is shown in **Table 4-2** and **4-3**. No differences in fatty acids profile of placebo and active groups were found at base line in PE and PC phospholipids.

4.3.2 PE fatty acid composition

In the active group, n-3 FA increased eicosapentanoic((C20:5n-3, EPA) by 67.7 % ($P<0.001$), Docosahexanoic ((C22:6n-3, DHA) by 71.9 %($P<0.001$), and total n-3 fatty acids by 59.6% ($P<0.001$), after six months of supplementation. These increases in n-3 fatty acids were associated with decrease in n-6 fatty acid, specifically linoleic (LA, C18:2n-6, $P<0.05$), arachidonic (AA, C20:4n-6, $P<0.01$), total n-6 ($P<0.001$) and total monoenoic ($P<0.001$). Continuation of n-3 fatty acids supplementation for one year caused no significant changes in fatty acid profile compared to six-month apart from slight but significant further reduction on AA ($P<0.05$).

The fatty acids composition of the placebo group broadly remained unchanged after supplementation, with the exception n-3 docosapentaenoic(DPA, C22:5n-3) that showed significant reduction ($P<0.01$) after six month of supplementation

4.3.3 PC fatty acid composition

Similar to PE, in the active group EPA increased by 69.6 % ($P<0.001$), DHA by 73.6 %($P<0.001$), and total n-3 by 64.4% ($P<0.001$), after six months of supplementation. Concomitantly, total saturate ($P<0.001$), total monoenoic ($P<0.05$), archidonic acid ($P<.01$) and adrenic acid ($P<0.01$) were reduced after supplementation. In contrast to PE, linoleic acid (LA, C18:2n-6) increased significantly and total n-6 showed no difference after intervention. Continuation of supplementation to one year resulted in additional significant increase in LA ($P<0.05$) and decrease in AA, adrenic acid ($P<0.001$) and n-3 DPA ($P<0.001$).

In contrast to active group, the total saturate, total n-3 and DHA were significantly decreased (P<0.01) in placebo group after six months of supplementation. Further reductions in n-6 and DHA occurred after one year of supplementation.

4.3.4 Comparison of the GPx and SOD activities in RBCs of the placebo and active groups

GPx and SOD activities in placebo and active groups were normally distributed at base line and six-month of supplementation. The two groups showed no differences in enzymes activities at base line. The GPx activity of placebo group remained unchanged after six-month of supplementation. After intervention, the GPx activity of the active group was reduced by 31.55% (P<0.001) and 24.5% (P<0.01) compared to base-line and placebo group respectively, **Figure 4-1**.

The SOD activates were significantly reduced after intervention both in placebo (p<0.05) and active group (P<0.01), **Figure 4-2.**

4.3.5 Relation of enzyme activity to oxidative stress

In order to assess the association of changes in enzyme activates with the redox status we analysed the effects of supplementation on levels of plasma vitamin E, A and RBC total plasmalogen contents of membrane phospholipids.

The Vitamin E levels of placebo and active group were comparable at base-line. Supplementation with omega-3 fatty acids resulted in slight insignificant increase in Vitamin E levels at six-month (P>0.05), and significant increase at one-year of supplementation compared to baseline (P<0.001) and placebo (P<0.05), **Figure. 4-3.** Vitamin A level of placebo and active group were comparable at base-line (0.64±0.2 versus 0.69±0.2), no significant difference (p>0.05) was observed in concentrations of vit A of placebo (0.6±0.1) and active group (0.63±0.2) after 6 months of supplementation. Continuation in supplement for one year showed no difference within or between the groups, vit A concentration of both placebo and active group was 0.62±0.2.

Total plasmalogens (alkenylacyl subclass) content determined as the sum of fatty aldehyde acetals (16:0DMA, 18:0DMA and 18:1DMA), which are produced after

derivatization of sn-1 fatty acids contained in plasmalogens. n-3 FA supplementation caused no significant lose in PE and PC plasmalogens Dimethyl acetals neither at six-month nor one year of supplementation, **Table 4-3& Figure 4-4**.

Table 4-1 Patients' basic characteristics

Measure	Active (n=40)	Placebo (n=33)
Demographic characteristics		
Female	18	17
Male	22	19
Age (mean in years(± SD))	6.4±3.02	6.8±3.5
Haematological profile		
Hb g/dL	6.7±1.3	7.0±1.3
Hct (%)	21.7±4.07	22.8±3.9
MCV (fL)	79.4±6.3	78.6±7.6
MCH (pg)	24.5±2.4	24.0±3.7
MCHC(g/dl)	30.8±0.95	30.8±1.8
TWBC(×103/µL)	15.8±4.2	14.8±5.4
PLTs (×103/µL)	531.3±168.6	534±118.2
RBC(×106/µL)	15.8±4.2	14.8±5.4

Hb, haemoglobin; Hct, haematocrit; MCV, mean Corpuscular Volume; MCH, mean corpuscular haemoglobin; MCHC, mean Corpuscular haemoglobin concentration; TWBC, total white blood Cell; PLTs, platelets count; RBC, red blood cell count.

Table 4-2 Fatty acids composition of red blood cell membrane phosphatidylethanolamine of the active and placebo group before and after supplementation

Major fatty acids (%)	Active group acids group			Placebo group		
	Baseline	Six-month	One-year	Baseline	Six-month	One-year
C16:0	12.0± 1.4	12.4±1.6	13.5±1.6++	12.6± 1.2	12.2± 1.2	13.4±1.2++
C18:0	8.0± 0.8*	8.3±1.19	7.7±0.9++	7.5± 1.2	7.1± 1.1	7.0±0.8
Total saturate	20.2± 1.1	20.82±1.3	21.3±1.1	20.2± 1.2	19.4± 1.6	20.5±1.3
C18:1	14.1±1.4***	13.3±1.3	13.6±1.5	14.0± 1.2	13.7± 1.7	13.3±1.3
Total monoenoic	14.4± 1.4***	13.5±1.3	13.9±1.6	14.3± 1.3	14.0± 1.7	13.5±1.3
C18:2n-6	5.0±0.8*	4.6±0.8	4.7±0.8	5.3± 0.7	5.3± 0.7	5.5±0.9
C20:4n-6	24.3± 1.1**	22.8±1.8	22.5±1.3+	24.5± 1.3	24.7± 1.4	25.0±1.3
C22:4n-6	10.9± 8.1***	6.8±1.7	7.4±2.2	10.4± 1.2	10.0± 1.6	10.2±1.3
Total n-6	44.8± 1.3***	38.0±3.6	37.6±3.9	44.6± 1.6	44.0± 3.7	44.7±2.4
C20:5n-3	0.1±0.04***	0.3±0.2	0.3±0.2	0.1±0.05	0.11± 0.06	0.1±0.05
C22:5n-3	1.7± 0.3***	1.4±0.3	1.4±0.2	1.8± 0.4**	1.5± 0.3	1.5±0.3
C22:6n-3	2.6± 0.7***	9.2±2.8	8.0±3.2	2.8± 0.6	3.1± 1.3	3.1±1.2
Total n-3	4.5± 0.8***	11.0±3.1	9.8±3.5	4.7± 0.8	4.59± 1.02	4.7±1.3

Minor fatty acids are included for calculation but not tabulated.
Baseline versus Six-month within the supplement group: *p<0.05, **p<0.01, ***p<0.001
One-year versus Six-month within the group: +p<0.05, ++p<0.01

Table 4-3 Fatty acids composition of red blood cell membrane phosphatidylcholine of the active and placebo group before and after supplementation

	Omega-3 fatty acids group			Placebo group		
Major fatty acids	**Baseline**	**Six-month**	**One-Year**	**Baseline**	**Six-month**	**One-year**
C16:0	36.49± 1.85**	34.58±1.97	36.5±2.3***	36.59± 2.11**	34.87±1.98	37.1±2.4**
C18:0	9.02± 0.90**	9.57±1.04	8.9±1.2*	9.39± 1.25	9.41±1.51	8.7±1.4**
Total saturate	46.14± 1.41***	44.88±1.32	45.5±1.5*	46.63± 1.50**	44.9±1.47	45.9±1.5*
C18:1	21.07±1.67*	20.21±1.63	20.2±2.0	19.85±1.78	19.55±1.70	18.3±2.3
Total monoenoic	21.58± 1.74*	20.66±1.70	20.5±2.1	20.33± 1.87	19.99±1.72	18.6±2.4**
C18:2n-6	15.65±2.01**	17.24±2.11	18.5±2.2*	16.75±1.93**	18.82±2.43	20.4±2.6**
C20:4n-6	9.81± 0.94**	8.81±1.31	7.8±1.4***	9.41± 0.91	9.82±1.39	8.6±1.1
C22:4n-6	1.08± 0.21**	0.69±0.19	0.6±0.2***	0.96± 0.20	0.95±0.26	0.8±0.4
Total n-6	29.44± 1.65	29.41±2.46	29.0±2.7	30.26± 2.26***	32.55±2.49	32.2±2.2**
C20:5n-3	0.085±0.039***	0.28±0.18	0.2±0.1	0.078±0.04	0.085±0.02	0.08±0.03
C22:5n-3	0.29± 0.08	0.24±0.07	0.2±0.07***	0.29± 0.07**	0.23±0.07	0.2±0.07
C22:6n-3	0.74± 0.27***	2.83±1.07	2.3±1.0	0.69±0.20**	0.88±0.32	0.7±0.3**
Total n-3	1.21± 0.33***	3.40±1.27	2.7±1.2	1.16± 0.24	1.24±0.36	1.0±0.4**
Dimethyl acetals	0.61±0.12	0.64±0.11	0.7±0.2	0.69±0.14	0.65±0.08	0.6±0.2

Dimethyl acetals correspond to the sum of 16:0+ 18:0+ 18:1 DMA. Minor fatty acids are included for calculation but not tabulated.
Baseline versus Six-month within the supplement group: *p<0.05, **p<0.01, ***p<0.001
One-year versus Six-month within the group: +p<0.05, ++p<0.01

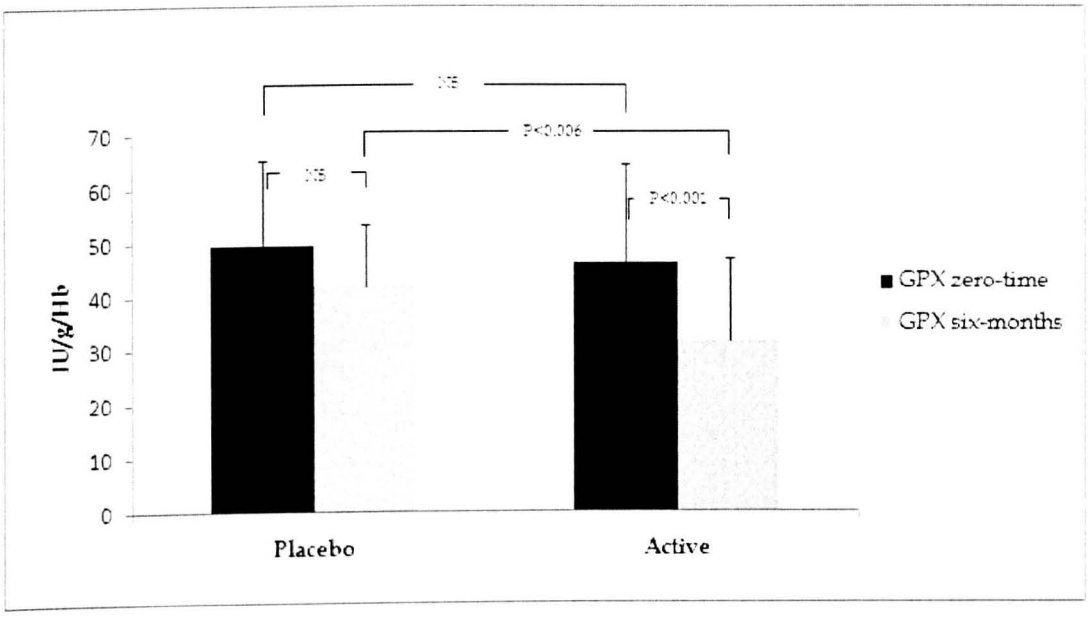


Figure 4-2 Effect of n-3 fatty acids supplementation on GPx activity in red blood cells of patients with HbSS

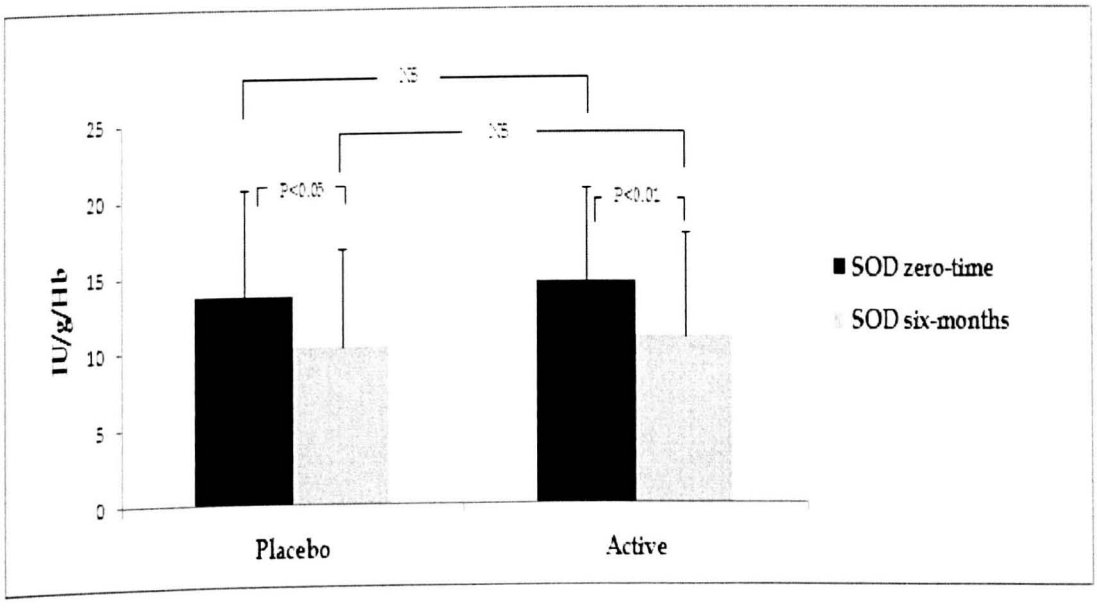


Figure 4-3 Effect of n-3 fatty acids supplementation on SOD activity in red blood cells of patients with HbSS

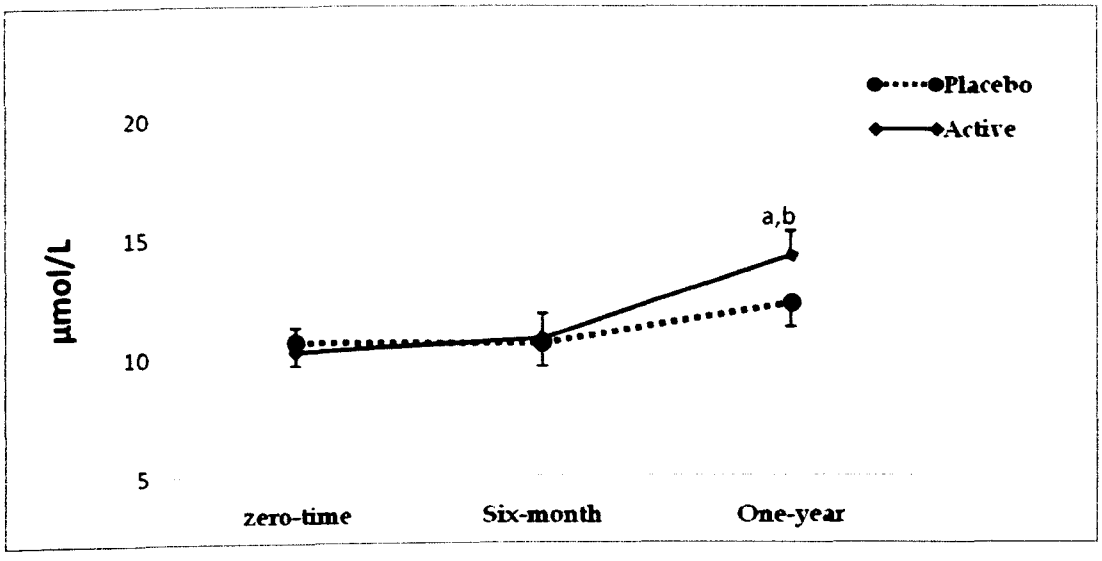


Figure 4-4 Effect of n-3 fatty acids supplementation on plasma α-tocopherol levels in patients with HbSS.
All values are in means ± SD, [a]P< 0.05 one-year vs. baseline Within the group. [b]P<0.001 one-year beteen the groups.

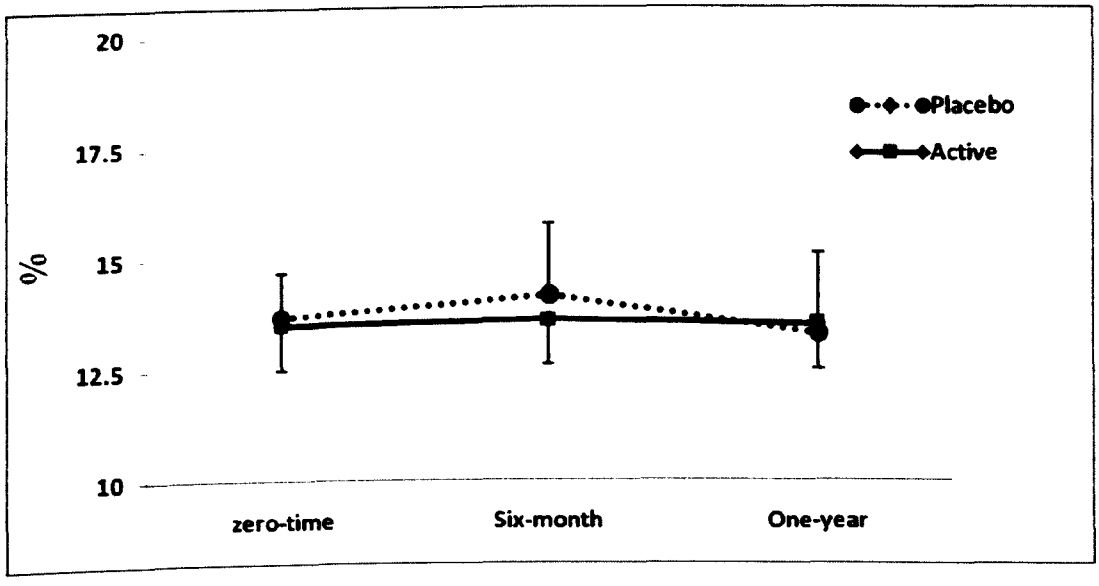


Figure 4-5 Effect of n-3 supplementation on phosphatidylethanolamine dimethyl acetals (DMAs).
All values are in means ± SD. no significan difference within or between the groups before and after Intervention.

4.4 Discussion

Although basically defined as a genetic disease, it is now becoming more evident that the pathogenesis in SCD is an out come of the concerted actions of genetic, cellular, humoral and environmental factors (Thomas et al. 1997). In this study we investigated the effects of nutritional intervention with n-3 fatty acids in RBC membrane fatty acids composition and the parameters of redox state in steady state HbSS patients. The demographic, haematological and fatty acids profile of placebo and active group were well matched at base-line, hence the observed effects of supplementation on anti-oxidant enzyme activities and markers of OS are unlikely to be due to dietary, genetic and/or sample variation.

Our study focused on the incorporation of the n-3 fatty acids DHA and EPA into RBC PE and PC, which are the main phospholipids regarding polyunsaturated fatty acids esterification(Delton-Vandenbroucke et al. 2001). The base line contents of DHA, EPA and total n-3 fatty acid are consistent with values observed on Sudanese children with or without SCD(Daak et al. 2011). After six months of supplementation the n-3 fatty acids concentration in active group increased to levels comparable to that of healthy individuals from high omega-3 intake communities(Ren et al. 2005a), the placebo group showed no significant difference compared to the base line. These results indicate reasonable compliance to supplementation, and provide evidence that the reported low levels of cell membrane n-3 fatty acids in SCD is responsive to dietary modulation(Connor et al. 1997; Ren et al. 2005b).

Plasmalogens (alkenylacyl) might be of particular importance in this study, as they are preferentially esterified with DHA(Lessig and Fuchs 2009). Moreover, Plasmalogen (endogenous anti-oxidant) are supposed to be protective against deleterious actions of ROS(Khaselev and Murphy 1999; Leray et al. 2002). It protects cells by functioning as scavengers to the free radical due to high susceptibility of vinyl ether linkage of plasmalogen to oxidative attack in comparison to unsaturated fatty acyl residues of phospholipids(Skaff et al. 2008).It have also been shown to have a role in inhibiting iron-induced peroxidation of PUFA(Sindelar et al. 1999) The pathophysilogical and clinical importances of plamalogen lie on the fact that, conditions with high oxidative stress status tend to have reduced level of plasmalogen (Brites et al. 2004; Gorgas et al.

2006). Hence, in this study we considered DMA levels as indicator of OS. In contrast to previous studies investigated effect of n-3 supplementation in DMA levels in cell lines(Delton-Vandenbroucke et al. 2001) and rats neuronal tissue(Destaillats et al. 2010), our data showed no significant effect on DMA after six-month and one-year of supplementation.This finding could indicate that n-3 FA supplementation caused no additional oxidative stress to these patients.

Vitamin E is considered as pathophysiologicaly important determinant of ant-oxidative protection(Kassab-Chekir et al. 2003; Gizi et al. 2011). SCD patients are known to have decreased levels of plasma vitamin E which is attributed hyper-consumption through excessively generated ROS(Marwah et al. 2001; Walter et al. 2006). In this study long term supplementation with n-3 caused significant increase in vitamin E levels. Although the placebo supplement contains the same amount of vitamin E no significant changes were detected, the fact which accentuates unequivocal effect of n-3 in vitamin E concentration. The observed increase in vitamin E after n-3 supplementation is consistent with previous study conducted in children and infants with or without SCD(Muskiet et al. 1991; Skouroliakou et al. 2010).

The static level of plasmalogen and significant increase in vitamin E do not support adverse effect of n-3 supplementation on overall oxidative stress in SCD. These findings are in accordance with previous studies that showed anti-oxidant effect of n-3 on healthy subjects(Guillot et al. 2009), patients with type 2 diabetes(Mori et al. 2003) and systemic lupus erythmatosus(Richard D 2008).However, a contradictory data related to n-3 FA and lipid peroxidation have been reported (Meydani et al. 1991a; Allard et al. 1997). These discrepancies could be a reflection of variation in population studied, composition of supplement used, dosage and/or the duration of the study. Indeed, there is evidence that low n-3 intake exerts an anti-oxidant effect, whereas the higher might rather promote peroxidation(Guillot et al. 2009). The positive effect of supplementation with n-3 on anti-oxidant capacity observed in this study could be attributed to relatively low dosage (250-750 mg/d) of n-3 FA with high DHA/EPA ratio.

Different mechanisms have been suggested to explain the anti-oxidant effect of n- fatty acids. The enhanced free radical scavenging capacity of DHA has been regarded as one

of the major mechanisms(Yavin 2006). Other potential mechanisms for the decrease in OS may relate to assembly on n-3 fatty acids in membrane lipids making the double bond less vulnerable to ROS attacks(Applegate and Glomset 1986). In addition, there is evidence n-3 FA modulates anti-oxidant enzymes activity(Takahashi et al. 2002; Iraz et al. 2005) and inhibits pro-oxidant enzymes NAD(P) H oxidase and phospholipase A2 (Mori et al. 2003; Richard D 2008).

Anti-oxidant enzymes provide the first line of cellular defence against toxic free radicals(Gizi et al. 2011).Studies have shown, the red blood cell anti-oxidant enzymes activities are responsive to variations in environmental factors, including diet, and degree of oxidative stress (Andersen et al. 1997; Iraz et al. 2005; Ji et al. 2006). Data about the effect of omega-3 fatty acids on superoxide dismutase (SOD) activity are contradictory (Song and Miyazawa 2001; Sarsilmaz et al. 2003; Erdogan et al. 2004). In accordance with studies conducted in rats(Iraz et al. 2005) and patients with ulcerative colitis(Barbosa et al. 2003), n-3 fatty acids supplementation in this study caused no significant difference in activity compared to placebo group. The observed significant decrease of SOD activity in both placebo and active group is intriguing. However, vitamin E which was added in a very small amount to supplement capsules may be implicated in the SOD reduction after intervention. There is evidence that vitamin E supplementation induces significant reduction in SOD activity in conditions associated with high oxidative stress status (Wang et al. 2010; Abd Hamid et al. 2011).

In contrast to other clinical trials in patients with hyperlipidemia and psoriasis(Olivieri et al. 1988; Corrocher et al. 1989; Mabile et al. 2001), Glutathione peroxidase-1 (GPx-1) activity decreased significantly after intervention. The previous studies were conducted in relatively small sample size for short period of time and used very high dose, the factors which may explain the discrepancies in the results. Similarly, Delton-Vanderbroucke etal(2001) reported decreased GPx activity in cell lines incubated in low but not high concentration of DHA. Hence, taking into account the enhanced vitamin E levels and preserved DMA concentration after n-3 supplementation, the observed reduction in GPx-1 could be interpreted as a reflection of decreased oxidative stress levels. Nevertheless, the possibility that other anti-oxidant and oxidative stress

markers not assessed in the present study were enhanced can not be excluded; further analysis and investigations may be of interest.

In conclusion, our results show that low supplementation dose n-3 FA of high DHA/EPA ratio modulated positively the RBC membrane fatty acids abnormality with no sign of additional oxidative stress. In addition, long term supplementation provided more anti-oxidant protection capacity as indicated by the significant increase in plasma vitamin E levels. This study supports the suggestions that supplementing SCD patients with n-3 fatty acids might have beneficial clinical effect.

Chaper Five

Long chain omega-3 fatty acids and clinical outcomes in patients with homozygous Sickle cell disease- Randomised, placebo-controlled, double blinded clinical trial

5.1 Introduction:

Sickle Cell Disease (SCD) is a group of autosomal recessive genetic blood disorders characterised by single point mutation in the sixth codon of the β globin gene. The resultant abnormal haemoglobin S (HbS), under low oxygen tension, polymerises and causes rigid and sickle-shaped red blood cells.(Rees et al. 2010) Homozygous sickle cell disease (HbSS), also known as sickle cell anaemia, is the major and severest form.(Serjeant 2001) In sub-Sahara Africa, the prevalence of sickle cell trait (AS) ranges between 5 and 40%(Weatherall et al. 2006) and more than 230,000 (0.74% of total birth) babies are born with sickle anaemia every year.(Weatherall et al. 2006)·(Modell and Darlison 2008) Vaso-occlusive crisis is the main clinical manifestation and cause of hospitalisation, organ damage and death.(Platt et al. 1994; Perronne et al. 2002; Ballas et al. 2010) Stroke is the most serious complication and the major cause of physical disability and cognitive impairment.(Hogan et al. 2006a; Kirkham 2007) Over 10% of patients with sickle cell anaemia develop overt stroke and about 22% show evidence of silent cerebral infarction.(Ohene-Frempong et al. 1998; Alkan et al. 2009) In Africa, life expectancy of patients with SCD is less than 20 years(Tshilolo et al. 2008) and those under 5 years of age are at a highest risk of death. (Makani et al. 2011)

It was thought that vaso-occlusive crisis is caused by a mechanical obstruction of small blood vessels by rigidly distorted (sickled) red blood cells.(Raphael 2005) There is no relationship between number of irreversible sickle cells and vaso-occlusive crisis.(Zipursky et al. 1993; Barker and Wandersee 1999) However, blood cells of sickle cell patients have a tendency to adhere to vascular endothelium and there is a correlation between blood cell-vessel wall adhesive interactions and vaso-occlusive crisis. (Hebbel et al. 1980; Zennadi et al. 2008; Kaul et al. 2009) These findings have lead to the current postulation that an enhanced tendency of red cells (sickled and non-sickled) to adhere to vascular endothelium and activation of platelets and leukocytes are the primary causative factors of vaso-occlusion.(Kaul et al. 1996; Okpala 2006; Frenette and Atweh 2007) The aforementioned factors are strongly modulated by cell membrane polyunsaturated fatty acids (PUFA).(Harbige et al. 1990; Meydani et al.

1991b; Hagve et al. 1993; Mills et al. 1993; Nishiyama et al. 2000; Mukherjee et al. 2004) Steady state sickle cell patients have abnormal red cell, platelet and mononuclear cell PUFA composition characterised by increased arachidonic (AA, 20:4n-6), adrenic (22:4ω-6) and osbond (22:5n-6), and decreased linoleic (LA, 18:2n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acid.(Connor et al. 1997; Ren et al. 2005b) The above studies(Harbige et al. 1990; Meydani et al. 1991b; Hagve et al. 1993; Mills et al. 1993; Nishiyama et al. 2000; Mukherjee et al. 2004), taken together, suggest that abnormality of blood cell membrane PUFA may contribute to the disregulation of blood cell-vessel wall interaction and vaso-occlusive crisis in patients with SCD. It has been reported that supplementation with fish oil containing n-3 fatty acids reduce the frequency of pain episodes requiring hospital presentation (2001b) and the number of sickle cell crisis.(Okpala et al. 2011) The latter did not use placebo controls, and both studies were not sufficiently powered to assess unbiased outcomes. Therefore, the aim of this double-blind placebo-controlled trial was to investigate the therapeutic potential of n-3 fatty acids supplementation in patients with homozygous sickle cell disease.

5.2 Methods

5.2.1 Design and intervention

The study was single centre, two-arm, randomised, placebo-controlled, double blinded clinical trial. The primary objective was to investigate the potential effect of omega-3 fatty acid on prevention of vaso-occlusive crisis in patients with homozygous sickle cell disease. After stratification by age and gender, the subjects were randomly assigned into treatment (n=70) or control (n=70) group. Both groups were on their respective treatment for one year.

5.2.2 Patients

Patients with homozygous sickle cell disease, aged 2 to 24 years, in regular follow up at the outpatient SCD Referral Clinic, Ibn-Aoaf Paediatrics and Khartoum Teaching Hospitals, Khartoum (Sudan) were enrolled between January 2009 and May 2010 (**Figure 5-1**), **Table 5-1**. The patients were in a "Steady State" defined as the absence of evidence of fever, infection or crisis for longer than four weeks before the start of the study. Phenotypic characteristic was confirmed with the use of cellulose acetate electrophoresis at pH 8.5. All of the patients were on regular folate supplement and those under five years on standard oral prophylactic penicillin. The exclusion criteria were: presence of other chronic diseases, blood transfusion in the previous four-months, hydroxyurea treatment, a previous history of overt stroke or pregnancy, **Appendix 6**.

The trial was approved by the Ethics Committee of the, Faculty of Medicine, University of Khartoum, Sudan and the Research Ethics Committee of Southampton & South West Hampshire, UK (REC reference number-05/Q1702/48). All participants or their guardians provided informed written consent, **Appendix 5.** The trial was conducted according to the conventional methodological standards and reported according to the standards of the CONSORT statement.

Figure 5-1 Map of Sudan
Adopted from www.lonelyplanet.com/maps

Table 5-1 Demographic and clinical Characteristics of the Patients at Base Line.

Measure	Omega-3	Placebo
Female	41	38
Male	29	32
Age (mean in years(± SD))	8.1 ± 4.6	7.8 ± 5.48
Mean weight (Kg)	21.3 ± 10.5	21.8 ± 9.65
Mean height (cm)	117.5 ± 21.7	120.0 ± 22.6
No. of crisis-induced hospitalisation/year		
No admission	9.8%	10.6%
1-2	43.7%	48.7%
3-5	24.1%	24.1%
5 >	22.4%	16.7%

5.2.3 Randomisation and blinding

Randomisation was conducted using a sequence of computer-generated random numbers at the Faculty of Life Sciences, London Metropolitan University (UK). The person who performed randomisation had no knowledge about demographic, clinical or laboratory characteristics of the patients, and staff of the SCD Referral Clinic, investigators and participants was blinded until the biochemical and clinical outcome data were analysed and the database unlocked.

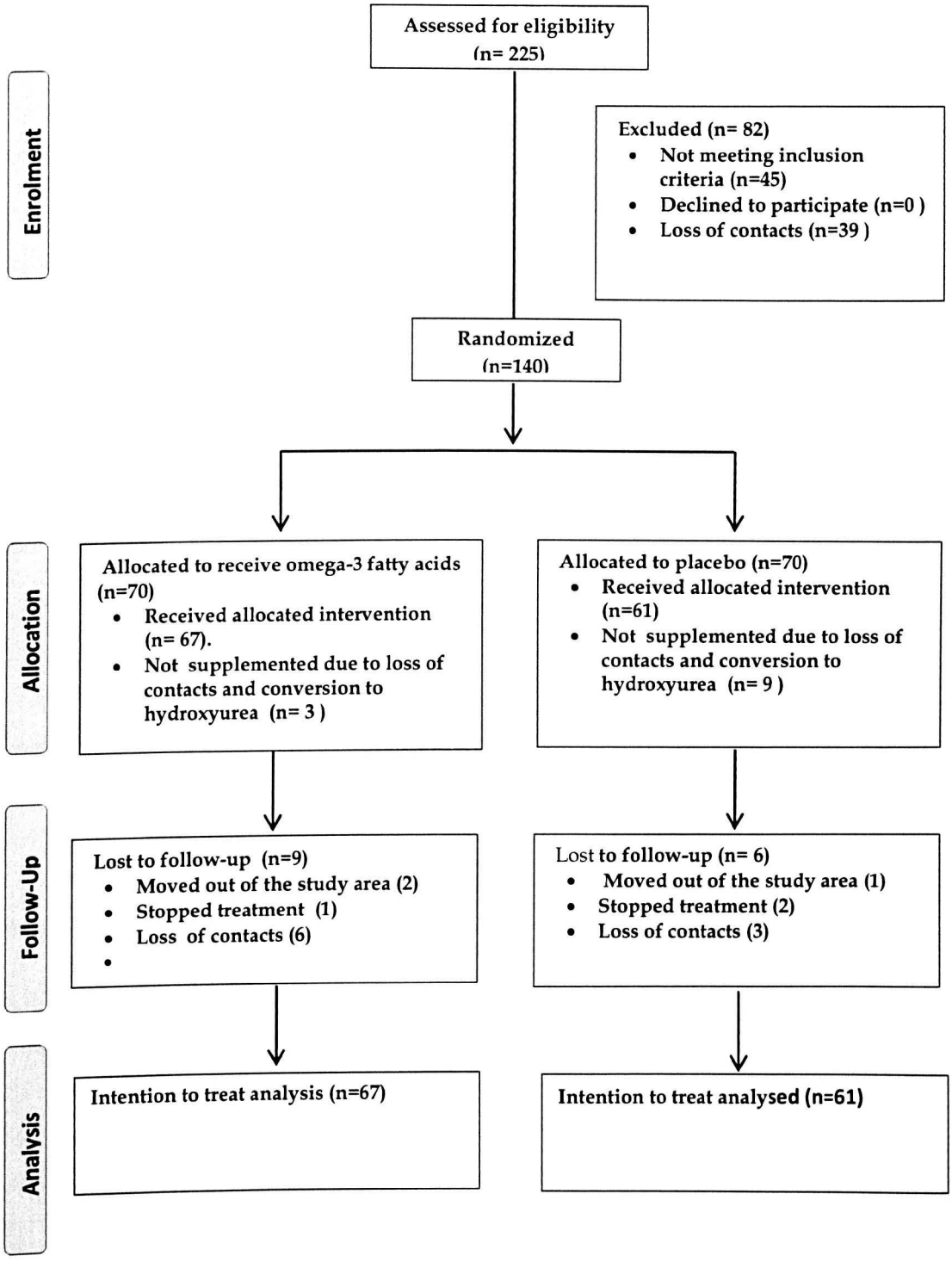


Figure 5-2 Flow chart of the patients considered for the trial and randomisation

5.2.4 Procedure

Subsequent to randomisation, the patients were given, daily, for one year, one (2-4 year old), two (5-10), three (11-16) or four (≥ 17) omega 3 or placebo capsules. The omega 3 capsule contains 277.8 mg DHA and 39.0 mg EPA and the placebo high oleic acid (41%) oil blend. The antioxidant vitamin E, 1·5mg/capsule, was added to both types of capsules to prevent peroxidation

The base line data were collected by completing a pre-structured questionnaire, **Appendix 6.** The data collected were the name, enrolment identity number, sex, residence, ethnic group, weight, height, number of hospital admission due to sickle cell disease during last year, history of blood transfusion and sickle cell complications. Whole blood (about 10 ml) was collected from both groups at recruitment and one year for measurement of Complete Blood Count (CBC) and fatty acids profile. The extraction of red blood cell lipid and analysis of red blood cell phospholipids fatty acid composition is described in chapter 2, section 2-3.

The patients were provided with monthly self-assessment health records to be filled in daily basis, **Appendix-7.** The record assessed pain frequency, compliance to supplementation and hospitalisation. In a monthly follow-up appointment, patients' health records were reviewed by a medical doctor, and the patients underwent comprehensive clinical assessment; findings were documented in structured form. After one year of follow up, 1203 health records were collected. A telephone number to contact the medical doctor in charge was given to all patients to provide advice and help in case of experiencing sickle cell crisis or severe complications.

5.2.5 Outcome assessment

Vaso-occlusive crisis was defined as a painful event characterised by musculoskeletal and/or visceral pain not otherwise explained, usually associated with mild pyrexia and the passage of dark or red urine (Serjeant 1985). The primary end point was annualised rates of clinical vaso-occlusive crisis, which is defined as painful that leads to hospitalization. Secondary end points were incidence of severe anaemia (haemoglobin concentration < 5.0 mg/dl), rate of blood transfusion, school attendance, Hb levels and mean cell volume (MCV).

The design did not include active surveillance of harm; the participants' observations about the supplementation effects were systemically recorded.

5.2.6 Blood sampling and complete blood count (CBC)

Blood sample collection and complete blood count procedure are described in chapter2, section 2.2.1.

5.2.7 Statistical analysis

The data are presented as mean± standard deviation (sd), median and percentile or median and inter-quartile range (IQR). The treatment effects were compared on the basis of annualised vaso-occlusive crisis rate computed by dividing the total number of crisis experienced by the number of follow up months and multiplication by 12 (A patient who experienced 3 crisis and followed up for 11 months will have a crisis rate of 3·3/year). Statistical differences of continuous variables of the two groups were examined with Mann-Whitney-Wilcoxon or t-test depending on the homogeneity of variance (data distribution). Categorical data differences were explored with Fisher's exact test for expected frequency of less than five or chi-squared test for five or more. All patients who started the treatment regardless of the duration of supplementation or follow-up period were analysed. Statistical analysis was performed with the use of the software, SPSS for Windows, version 17 (SPSS Ltd., Woking, Surrey, UK).

5.3 Results

Two hundred twenty-five homozygous sickle cell patients were screened for eligibility. Of these, one hundred forty were randomised on the basis of fulfilling the inclusion criteria, and one hundred twenty-eight received omega 3 or placebo, followed up and analysed (**Figure 5- 2**). Seventeen patients, eleven omega 3 and six placebo groups, had a follow up period of less than six months and the median follow period was 10 months.

There were no significant differences with regard to gender, age, ethnic background, disease severity, haemoglobin phenotype (HbSS), haematological profile (**Table 5-1 & Table5-2**) and red blood fatty acid composition before the start of supplementation (**Figures 5-4, 5-5, 5-6 and 5- 7**).

After one year of supplementation, n-3 fatty acids EPA and DHA were significantly higher in the red blood cells of the active group compared to the placebo group both in ethanolamine phosphoglyceride (**Figure 5-4**) and choline phosphoglyceride(**Fig 5-6**). On the other hand, the omega-6 linoleic acid (LA) and arachidonic acid (AA) were significantly lower in the red blood cells of the active group in comparison to placebo group in EPG and CPG (**Figure 5-5 and 5- 7**).

In intention to treat analysis, the clinical vaso-occlusive crisis (primary outcome) was significantly lower in omega-3 supplemented group, with median rates 0.00 per year on omega-3 fatty acids group compared to 1 in the placebo group (P< 0.0001) (**Table 5-3**) and (**Figure 5-3**). In the omega 3 group, the odds ratio of having clinical vaso-occlusive crisis at least once during the study period was reduced to 0·21 (95%CI: 0·09-0.47, p<0·001). The median annual rates of vaso-occlusive crisis regardless of hospitalisation were also significantly lower in omega-3 supplemented groups (2.7 Vs 4.6; P < 0.01) (**Table 5-3**). the number of inpatient days due to sickle cell disease crisis and its associated complications were significantly lower in omega-fatty acid supplemented group (median=0.00, IQR=1) compared to placebo group (median=0.00, IQR=6, P< 0.05).

There were no significant differences in risk of stroke, sequestration crisis and vascular necrosis between two groups. However, the incidence of severe anaemia and the

percentage of patients who required blood transfusions were significantly lower in n-3 fatty acids supplemented group compared to placebo group, **(Table 5-4).**

In analysis of effect of supplementation on the school attendance only n=95 Participants were included (n=46 on placebo and n=49 on active supplements) as the rest n=45 were not of school age at the time of the study. The median of absence from school due sickle cell complications was significantly lower on omega-3 fatty acids supplemented group (P<0.02), **(table 5-4).** The odds ratio of inability to attend school at least once during the study period due to the disease was significantly reduced in omega-3 fatty acids group to 0.40 (95%CI: 0.18-0.92; P< 0.03).

Haemoglobin level (Hb), mean corpuscular volumes (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were significantly higher in both groups after supplementation. Whereas the total white blood cell counts (TWC) were significantly lower in omega 3 fatty acids supplemented group but not on placebo group. Platelet (PLTs) counts remained unchanged on both groups, **(Table 5-2).**

5.3.1 Harms

No allergies, or adverse effects attributed to supplementation were recorded. There were no symptoms of easy bruising or episodes of spontaneous haemorrhage. Very few patients on placebo or active treatment complained of dyspepsia. Some patients from the active group reported increased appetite especially during the first two month of usage.

Table 5-2 Complete blood count at base line and one year after intervention

	Omega-3 fatty acids group		Placebo group	
	Baseline	**One year**	**Baseline**	**One year**
Hb g/l	67.10± 8.52	76.30± 8.50***	67.45± 8.32	77.00± 10.15***
Hct (%)	21.12± 3.29	21.04±2.32	22.26± 3.02	21.67± 3.61
MCV (fl)	80.10± 5.55	82.70± 6.14***	79.34± 6.55	80.62± 7.23*
MCH (pg/L)	24.85±2.11	30.36±2.55***	24.73±2.38	29.37±3.08***
MCHC	30.85± 0.84	36.62± 1.43***	31.22± 1.11	36.69± 1.30***
TWBC(×103/µL)	15.63± 3.97	14.40± 3.34*	14.53± 5.28	14.00± 5.13
PLTs (×103/µL)	516.78± 148.78	487.43± 114.72	496.8± 146.55	450.65± 165.05

Table 5-3 Annual rates of vaso-occlusive crisis and clinical vaso-occlusive crisis

Measure	Omega-3 (n=67)	Placebo (n=61)
Vaso-occlusive crisis *		
Minimal Value	0.0	0.0
25th percentile	0.9	3
Median Value	2.7	4.6
75th percentile	4.8	6.4
Maximal value	12	12
Clinical Vaso-occlusive crisis**		
Minimal value	0.0	0.0
25th percentile	0.0	0.0
Median Value	0.0	1.0
75th percentile	0.9	2.4
Maximum Value	4.0	6.00

*p<0.01, **P<0.0001

Vaso-occlusive crisis -painful event regardless of hospitalisation as reported by the patients.

Clinical vaso-occlusive crisis- Vaso-occlusive crisis which leads to hospitalization.

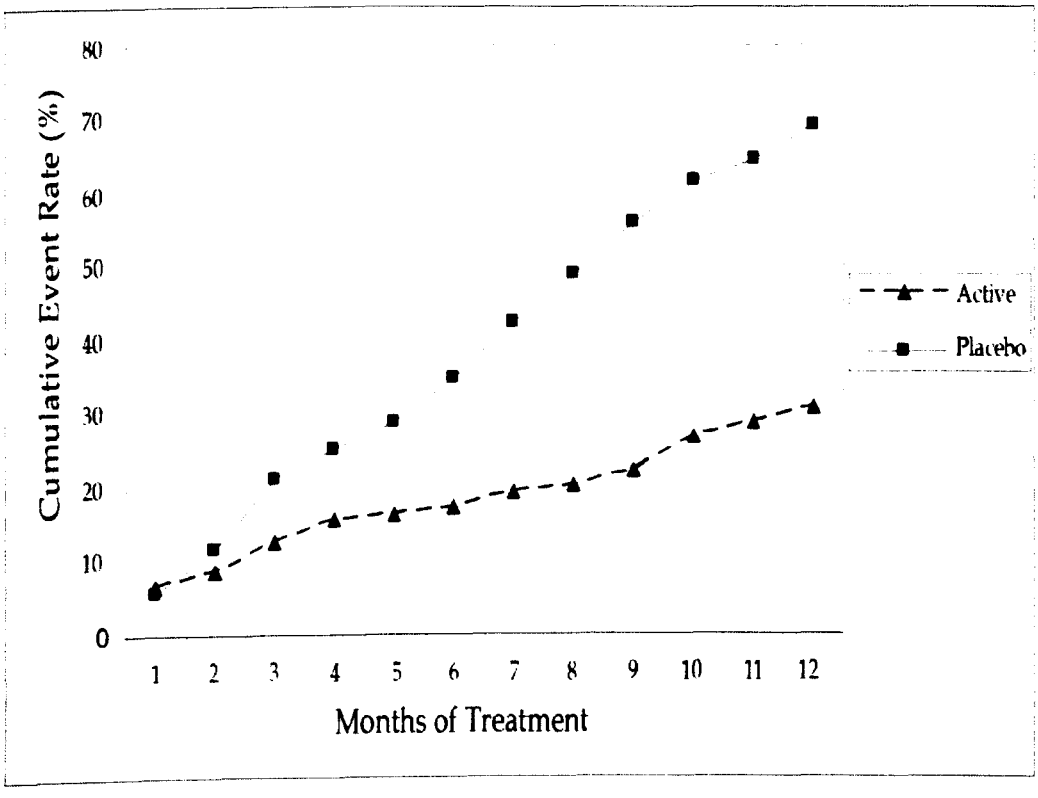


Figure 5-3 Comparison of the cumulative clinical vaso-occlusive crisis of active and placebo groups

Table 5-4 Sickle cell-associated Complications in active and placebo group after intervention

Measure	Omega-3	Placebo	P value
Severe anaemia (%) (%)	(2/67)3·2	10/61)16	0.03
Sequestration crisis (%)	(1/67)1·5	(2/61)3·3	0·5
A-vascular necrosis (%)	(1/67)1·5	(2/61)3·3	0·5
Stroke (%)	(0/67)0·0	2/61)3·3	0.5
Blood transfusion (%)	(3/67)4·5	(10/61)1	0.03
Absence from School per year-days (Median (IQR))	0·0 (7·6)	4·3 (21·1)	0.02

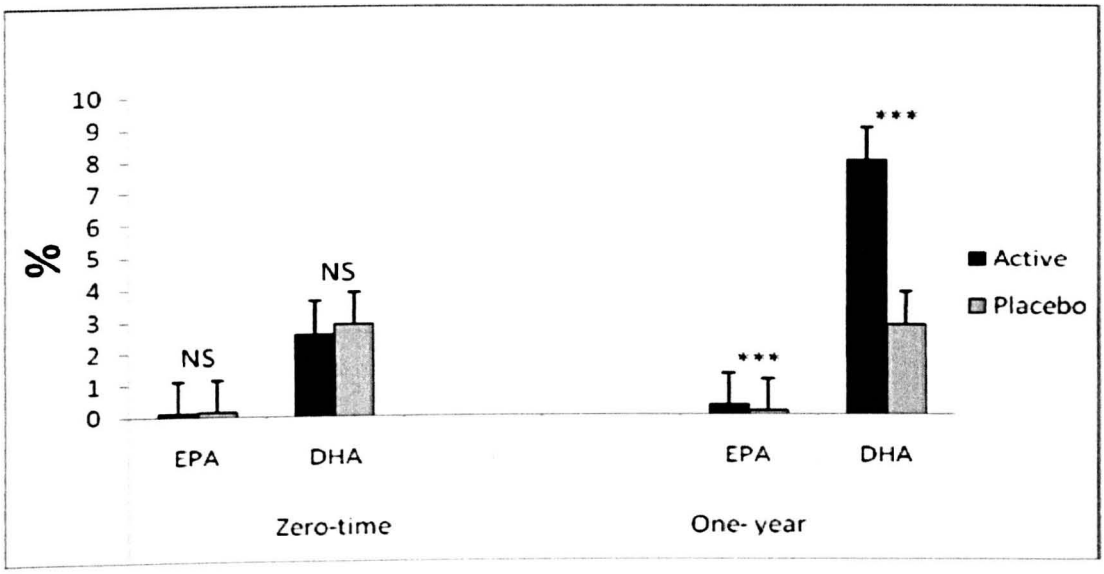


Figure 5-4 Red blood cell EPA and DHA percentages of ethanolamine phosphoglyceride before and after the intervention.

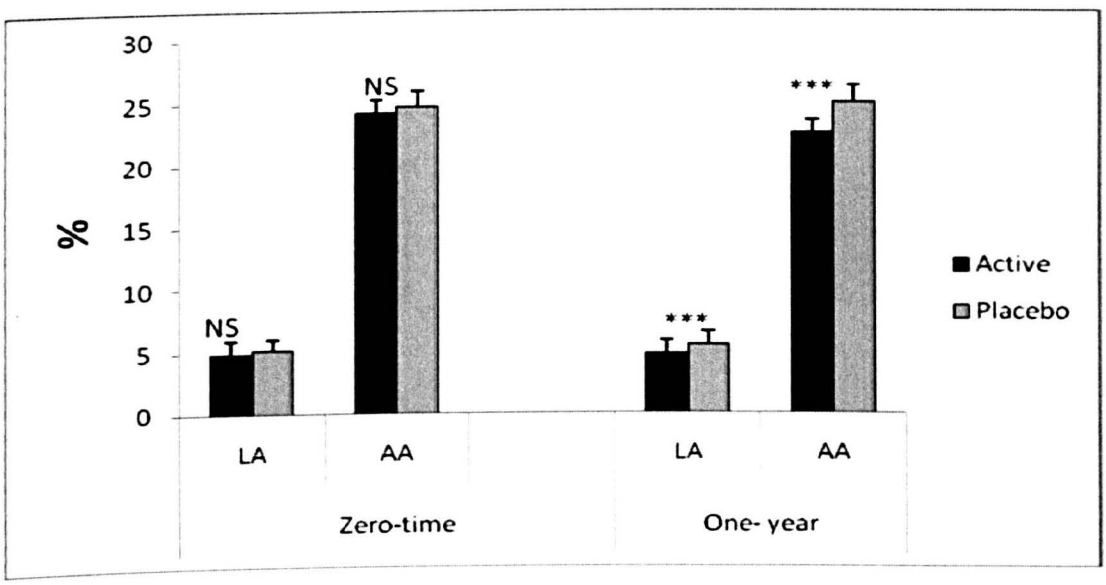


Figure 5-5 Red blood cell LA and AA percentages of ethanolamine phosphoglyceride before and after the intervention.

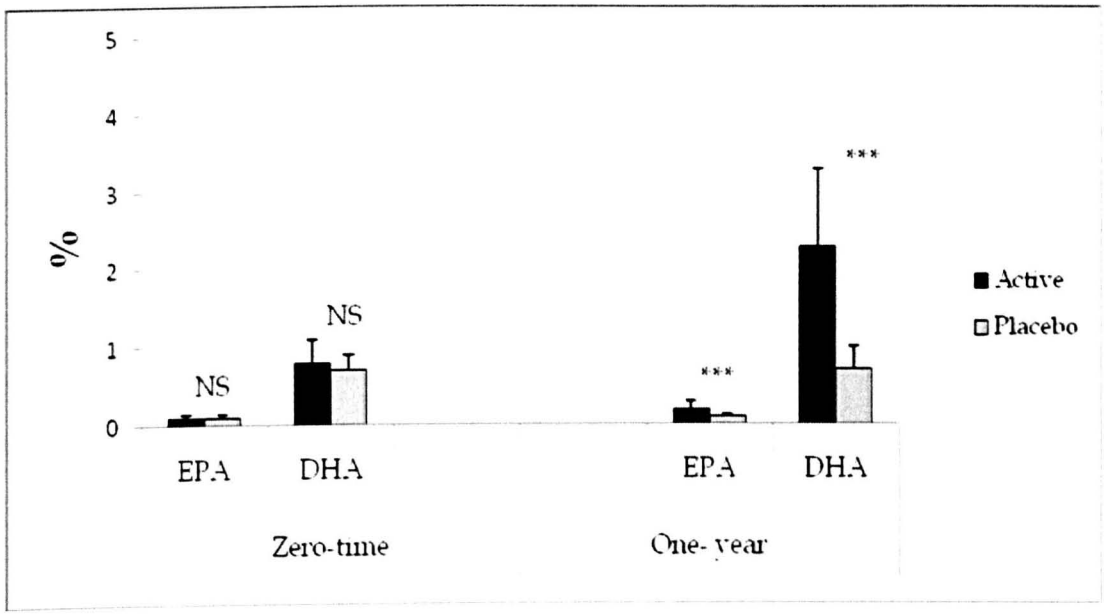


Figure 5-6 Red blood cells EPA and DHA percentages of outer cell membrane choline phosphoglyceraide before and after the intervention.

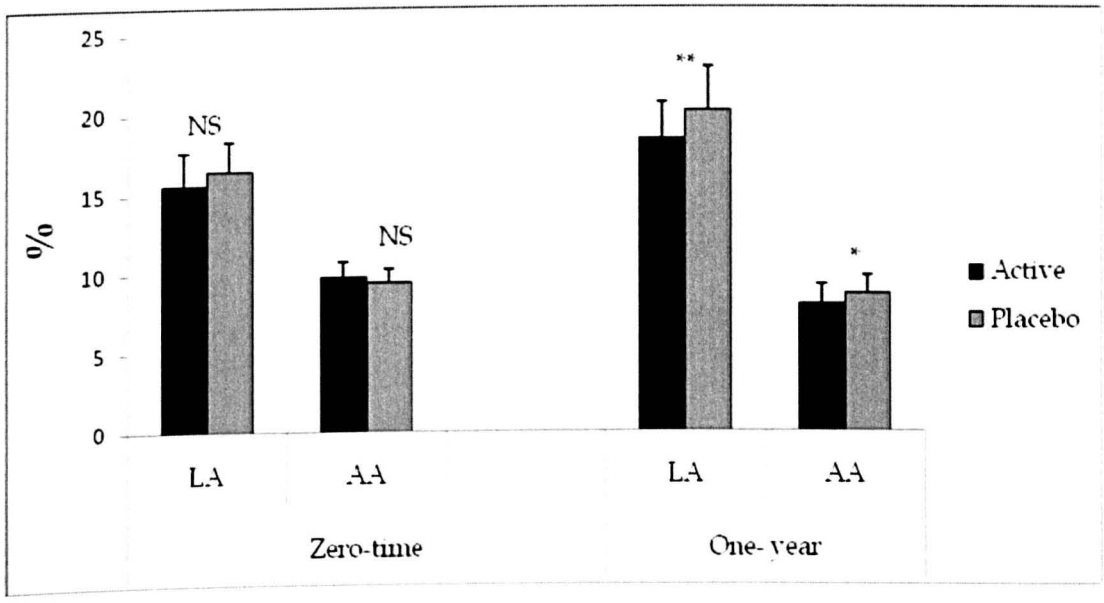


Figure 5-7 Red blood cells LA and AA percentages of outer cell membrane choline phosphoglyceride before and after the intervention

5.4 Discussion:

This randomised clinical trial signifies that omega-3 fatty acid supplementation on homozygous sickle cell disease reduces the median annual rate of vaso-occlusive crisis by 41.3 % and a 100% reduction in median annual rate of clinical vaso-oocclusive crisis, 81.7% reduction on haemolytic crisis and 72.7% reduction on annul rate of blood transfusion. These results show that DHA and EPA improve the clinical course of sickle cell patients and decrease the severity of the disease. These beneficial effects of DHA and EPA reflected on the quality of life of the patients as measured by the significant reduction of days out of school due to the disease and the risk of inability to attend school at least once due to disease.

The outcomes of supplementation on pain frequency are in accordance with the results reported by Tomer et al (2001b) in adult patients. The previous study based on a very small sample size (n=10, 5 patients in each arm) and only patients of severe course were included. These two limitation factors were well controlled in this study. Moreover, the participants were both children and young adults, overcoming one of the major pitfalls of clinical trial in sickle cell disease (Debaun and Field 2007).

The effect of supplementation on stroke, sequestration crisis and vascular necrosis showed no significant differences. This is intelligible, taking in account that the trial was not powered to detect the effect on these complications. However, the results revealed general positive trends that deserve more investigation. Especially in regard to stroke as some other emerging evidence indicates fish consumption (omega-3 rich diet) may have a good preventive effect (He et al. 2004; Larsson et al. 2011).

The observed decrease rate of haemolytic rate and blood transfusion in active group are in accordance with previous studies in rabbits(van den Berg et al. 1991) and healthy people (Mabile et al. 2001). These studies demonstrated reduced levels of haemolysis after omega-3 supplementation. Moreover, in SCD patients (in print) we observed a significant reduction in markers of haemolysis after omega-3 fatty acids supplementation. These findings do emphasis the predicted anti-haemolytic role of omega-3 fatty acids.

Intriguingly, the Hb concentration and MCH levels were increased to equivalent levels in placebo and active group. These results indicate that factors other than omega-3 fatty acids might have influenced the increase in Hb levels. A singular factor that may have played a role is vitamin E, which was added in a very small concentration to protect supplemented fatty acids from peroxidation. There is evidence, that supplementing SCD patients with vitamin E increases haemoglobin concentration, percent foetal haemoglobin, forearm blood flow and resistance of the cell to lysis (Gbenebitse et al. 2005; Jaja et al. 2005). Hence, we postulate that a combination of omega-3 with higher doses of vitamin E may provide extra beneficial effect to patients with SCD.

The omega-3 fatty acids supplement used in this study were composed of high DHA (54.9%) and low EPA (7.7%). This was particularly designed to ameliorate the observed reduction of DHA and EPA concentration on blood cell membrane in SCD. Among the omega-3 fatty acids, DHA reduction was more pronounced (Connor et al. 1997; Ren et al. 2005b). The second reason for using relatively lower EPA percentage is to anticipate the possibility of haemorrhage due to EPA anti-aggregatory effect (Park Y 2002; Nomura et al. 2003). Further more, the unsaturation index of DHA is higher than EPA, providing greater influence on cell membrane deformability and fluidity (Hashimoto et al. 1999).

The base line red blood cell omega-3 fatty acids concentration of sickler Sudanese patients were lower than we had previously observed in patients from Nigerian and British populations (Ren et al. 2005a; Ren et al. 2006). This difference is most likely a reflection of the n-3 fatty acid status of the population studied since we have found North Sudanese maternal milk (Nyuar KB 2010) and blood of healthy Sudanese children(Daak et al. 2011) have very low content of these nutrients. These low levels of base line omega-3 fatty allowed massive change in fatty acids profile due to omega-3 supplementation. The EPA & DHA concentrations of red blood cell from active group increased by three fold accompanied with significant reduction on arachidonic acid. Whereas the EPA and DHA percentages of placebo group remained unchanged with slight increase in omega-6 linoleic and archidonic acids, which may be due to placebo supplement. These notable modulations in fatty acid profile may have played a part in

ensuring the explicitness of effects observed owing to omega-3 fatty acid supplementation. On the other hand, a very valid question should be raised, whether the same evident response is reproducible in high omega-3 intake communities? And whether high habitual intake of omega-3 fatty acids has any role in modifying the clinical course of the disease? Indeed, these two questions warrant proper investigation.

Taking in account the myriad biochemical effects of omega-3 fatty acids, many mechanisms may be involved in amelioration of vaso-occlusive crisis and lessening the rate of haemolytic crisis. However, according to pathophysiology of SCD some mechanisms may be of grater relevance than others. Firstly, DHA and EPA proved to be effective in treatment of some chronic inflammatory disorders and cardiovascular diseases (Cleland and James 2000; Calder 2002; Ruxton et al. 2005). These effects attributed to decrease in n-6/n-3 ratio which in favour of production of more anti-coagulant and anti-inflammatory n-3 -series prostaglandins (Fischer et al. 1988; Knapp 1990; Mantzioris et al. 2000; Bagga et al. 2003). Moreover, it has been shown that omega-3 fatty acids supplementation do reduce the expression of adhesive molecules on blood and endothelial cells (Mayer et al. 2002; Tian H 2009). Second, the significant increase in MCV in active group can not be explained merely by the increase of Hb and MCH, as the same amount of increase in Hb occurred in the placebo group without causing equal increase in MCV. It is possible that the increase in MCV is a reflection of improved cell membrane prosperities and hydration state. Indeed, there is evidence, DHA reduces oxidative damage in SCD and improves red cell deformability and hydration state (Relton et al. 1993; Maccoll et al. 1996; Bechoua et al. 1999; Valentine and Valentine 2004; Yavin 2006; Brand et al. 2008; Ren et al. 2008).

The current data obtained from this RCT did not provide an answer about the long term effects of supplementation, specially the potential role of DHA and EPA in prevention of neurological and cognitive complications in children with SCD. Moreover, in this study we excluded children less than two years, and knowing the fact that organ damage starts when SCD children as young as 9 months, investigating of effect of supplementations in these group of patients is of great importance.

The advantages of this study include the randomised double blind design and the duration of supplement. Among the disadvantages, only homozygous SCD patients were enrolled excluding other phenotypes and no objective measures were used to assess the severity of vaso-occlusive besides the hospitalization.

In conclusion, our data suggest that DHA and EPA could be safe and effective therapy for prevention of vaso-occlusive crisis and decrease the rate of haemolysis in homozygous sickle cell disease.

Chapter Six

Long chain omega-3 fatty acids and markers of haemolysis in patients with homozygous Sickle cell disease

6.1 Introduction

Chronic haemolytic anaemia is one of the major clinical manifestations of homozygous sickle cell disease (SCD), both during steady state and acute exacerbation events of the disease (Ballas and Marcolina 2006). Several studies suggest haemolysis is a primary determinant of cardiopulmonary complications(Gladwin and Kato 2005), glomerulopathy(Maier-Redelsperger et al. 2010), priapism(Nolan et al. 2005), leg ulcer and death(Kato et al. 2006).

Haemolysis in sickle cell disease is often precipitated by inflammation and increased endothelial-leukocyte-erythrocyte adhesive interaction (Rees et al. 2010), high oxidative stress(Wood and Granger 2007) and changes in red blood cell deformability(Ballas and Mohandas 2004). Inflammation, cell adhesion, red cell deformability and oxidative stress are known to be strongly modulated by cell membrane fatty acids (FA) (Mills et al. 1993; Nishiyama et al. 2000; Saito and Kubo 2003; Mukherjee et al. 2004). Hence, it is conceivable that fatty acids composition of red cell membrane might influence the degree of haemolysis in SCD. In accordance to this assumption, previous studies have shown that red blood cell membrane of patients with SCD have abnormal fatty acid composition. The abnormality is typically characterized by high n-6 and low n-3 long chain polyunsaturated fatty acids (Connor et al. 1997; Ren et al. 2005b; Daak et al. 2011). In addition, Ren et al (2005a) reported that Hb concentration in SCD patients correlates positively with omega-3 fatty acid in red blood cell membrane. Interestingly, there is evidence supplementation with omega-3 fatty acid reduces haemolysis in rabbits and healthy human(van den Berg et al. 1991; Mabile et al. 2001). Hence, it is plausible that correction of the imbalanced cell membrane fatty acid abnormality by supplementation with n-3 eicosapentanoic (EPA) and docosapentanoic (DHA) fatty acids could ameliorate anaemia and reduce haemolysis in patients with SCD.

In this study we have investigated the potential effect of n-3 fatty acid supplementation on haemolysis in children with homozygous sickle cell disease.

6.2 Patients, Material and Methods

6.2.1 Subjects

Steady state HbSS sickle cell patients age 3-12 (n=21), twelve male (n=12) and nine female (n=9) were enrolled from Abnaof Paediatric Hospital, Khartoum, Sudan. Steady state was defined by absence of evidence of fever, infection or crisis for at least four weeks before the study. The phenotypic characteristic was confirmed by cellulose acetate electrophoresis. All of the patients were on regular folate supplement, those younger than five years were receiving daily prophylactic dose of phenoxypenicillin before and during the study. The exclusion criteria were: sickle cell crisis, acute illness in the pervious one-month, presence of other chronic diseases, blood transfusion in the previous four-months or hydroxyurea treatment. According to age group 2-4 years, 5-10 years and >11 years , the enrolled subjects were supplemented daily with 250 mg, 500 mg, 750 mg of high docosahexanoic n-3 fatty acid respectively. The subjects were on continuous supplementation for one year. To prevent lipids peroxidation 1.5 mg/capsule of α-tocopherol (vit E) was added to each capsule. The Research Board of the Faculty of Medicine, University of Khartoum, Sudan, and The Research Ethics Committee of Southampton & South West Hampshire, UK (REC reference number-05/Q1702/48) approved the study. The guardians of all participants provided informed written consent.

6.2.2 Blood sample collection and processing

After an overnight fast, 5 ml of whole blood were taken in EDTA tubes from patients at zero-time and one-year of supplementation. The separation of blood component is decribed in chapter 2, section 2.2.2.

6.2.3 Complete blood count (CBC)

The complet blood count was measured as described in chapter 2, section 2.2.1.

6.2.4 Analysis of red blood cell fatty acids

The procedure of lipid extraction and fatty acid analysis of red blood cell is described in chapter 2, section 2-3.

6.2.5 Measurement of plasma lactate dehydrogenase (LDH) and haptoglobin (Hp)

The procedures of plasma LDH and Hp are described in chapter 2, section 2-7 and 2-8 respectively.

6.2.6 Measurement of plasma α-tocopherol concentration

The procedure of plasma α-tocopherol extraction and analysis of is described in chapter 2, section 2-5.

6.2.7 Statistical analysis

The data are expressed as mean ± SD. Paired t-test was used to explore differences in concentrations of LDH, fatty acids, vitamin E and blood parameter before and after supplementation. Pearson correlation was used to investigate the association between fatty acids. The Statistical significance was assumed at a "p" value of less than 0.05. The statistical software, SPSS for Windows, Version 17 (SPSS Ltd., Woking, Surrey, UK) was used to analyse the data.

6.3 Results

6.3.1 Serum lactate dehydrogenase and haptoglobin

Mean plasma LDH (fig.6-1a) was significantly lower after one year of supplementation with n-3 fatty acids (P= 0.005). The difference between female and male was not significant either before (907.35± 321.31 IU/l vs. 751.27± 219.43; P=0.2) or after (588.73± 128.9 IU/l vs. 651.20± 257.70; P= 0.005) supplementation.

Plasma haptoglobin levels showed no difference before and after supplementation (P=0.3) (Fig.6-1b).

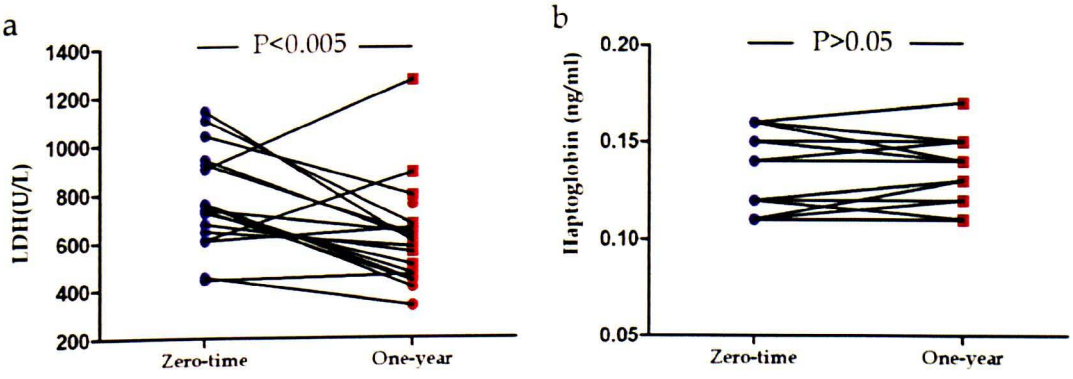


Figure 6-1 a)Lactate dehydrogenase (LDH) b)Haptoglobin levels before and after one year of supplementation with n-3 fatty acids.

6.3.2 EPA DHA and AA levels of Red blood cell membrane

N-3 fatty acids EPA and DHA were significantly higher after one year of supplementation with n-3 fatty acids (P< 0.0001), Table. 1. In contrast, n-6 fatty acids arachidonic acid (AA) was significantly lower after supplementation (P< 0.0001), **Table 6- 1.**

After supplementation, n-6/n-3 ratio was significantly decreased (10.4±2.3 vs 4.0±2.1, P= 0.001), AA and total n-6 fatty acids were inversely correlated with DHA and total n-3 fatty acids (R=0.53; P= 0.02 & R=0.85; P=0.0001 respectively)

6.3.3 Haematological parameters

Haemoglobin (Hb, P< 0.01), mean corpuscular volume (MCV, P<0.004), mean corpuscular haemoglobin (MCH, P<0.0001,) and mean corpuscular haemoglobin concentrations (MCHC, P<0.0001) were significantly higher after one year of

supplementation. The total white blood cell and platelets were significantly lower after one year of supplementation (P=0.05, P=0.04). Red blood cell count (RBC) and haematocrit (Hct) showed no difference before and after supplementation, Table 6-1.

Table 6-1 Paired haematological and biochemical data measures of steady-state HbSS patients studied before and after supplementation with n-3 fatty acids.

Measure	HbSS Zero-time	HbSS One-Year	HbAA Reference values (range)	P value
Blood count				
Hb g/L	66.73 ± 13.2	76.00 ± 9.40	102.0- 152.0	0.01
Hct (%)	20.81 ± 4.13	20.84 ± 2.47	36.0- 46.0	0.40
MCV (fL)	79.92 ± 5.61	82.85 ± 5.5	78.0- 94.0	0.004
MCH (pg/L)	24.73 ± 2.17	30.25 ± 2.57	23.0- 31.0	0.0001
MCHC	30.9 ± 0.74	36.5 ± 1.76	32.0 -36.0	0.0001
RBC count (×106/μL)	2.54 ± 0.50	2.51 ± 0.31	4.0 -5.2	0.3
PLTs (×103/μL)	574.2 ± 239.	469.8 ± 146.9	150.0 -450.0	0.04
TWB (×103/μL)	17.62 ± 9.38	13.99 ± 2.69	5.0- 17.0	0.05
PE fatty acids composition				
EPA (%)	0.11 ± 0.04	0.34 ± 0.19	0.5- 1.6	0.0001
DHA (%)	2.57 ± 0.73	8.89 ± 2.85	6.0- 11.0	0.0001
AA (%)	24.47 ± 1.03	22.09 ± 1.66	19.0- 26.0	0.0001
Vitamin E μmol/l	**10.20 ± 3.62**	**14.54 ± 2.62**	**8.4- 26.1**	**0.001**

* PE- Ethanolamine phosphoglyceride

6.4 Discussion

Chronic haemolysis is a principal feature of HbSS (Ballas and Marcolina 2006). In this study we found that supplementation with omega-3 fatty acids caused a remarkable effect on haematological parameters and significant reduction in haemolysis rate, as indicated by the decrease of plasma lactate dehydrogenase levels. The observed responses due supplementation are unlikely to be a reflection of variation in the clinical state, as all participants were on steady state at recruitment, and none of them showed sign of infection or malaria. Moreover, the after-supplementation blood samples were collected exactly at the same time of zero-time sampling. Hence, excluding the potential effect of seasonal temperature (Smith et al. 2009), and hydration state(Fowler et al. 2010).

In contrast to previous studies(Muskiet et al. 1991; Tomer et al. 2001b), the supplementation with omega-3 resulted in increased haemoglobin by 13.9%, MCH by 22.5%, and MCHC by 18.1%. Conversely, the total white blood cell (TWBC) and platelets (PLTs) count were reduced by 20.5% and 18.2% respectively. These differences in haematological outcomes from previous studies may be due to composition of the supplement used in this study (high DHA), or the relatively long duration of supplementation period.

The improvements in MCV can be merely a reflection of increased red cell haemoglobin content (MCH) and/or enhanced hydration as a result of omega-3 induced membrane modifications (Ballas 2000; Djemli-Shipkolye et al. 2003). Considering the concomitant increase in MCHC as determinate of red cell density, the detected increase in MCV is more likely an outcome of increased MCH. The increase in MCHC with concomitant decrease in overall haemolysis is intriguing. However it may support the line of argument proposing that, no relation exist between the percentages of dense cell and disease severity(Billett et al. 1986).

The observed decrease in PLTs and TWBC owing to omega-3 fatty acids supplementation is constituent with findings obtained from conditions other than SCD (Hamazaki et al. 1984; Mukaro et al. 2008; Park and Harris 2009). It is tenable; these

reductions in blood cell count is a manifestation of an improved inflammatory state (Ataga and Orringer 2003; Semple and Freedman 2010) or diminished haemopoietic activity of the bone marrow(Tancabelic et al. 1999). Interestingly, red blood cell count remained unchained after supplementation despite decrease in haemolysis. This finding together with reduced PLTs and TWBC suggests reduced bone marrow activity. There is evidence, omega-3 fatty acids supplementation reduces proliferation of myeloid progenitors and bone marrow expansion and promote differentiation (Varney et al. 2009).

In accordance to previous studies, the haptoglobin level in zero-time was very low signifying predominant haemolysis effect in steady state SCD patients (Singhal et al. 1993; Bourantas et al. 1998). Startlingly, haptoglobin levels remained unchanged after supplementation despite remarkable decrease in haemolysis. These paradoxical result can be explained by considering the dual function of Haptoglobin, an acute phase protein (Kato 2009) as well as primary Hb scavenger in the circulation level (Ascenzi et al. 2005). Therefore, the plasma concentration is an outcome of the balance between degree of consumption determined by haemolysis rate and production that increases in inflammatory state. The significant decrease in TWBC implies a decreased inflammatory state due omega-3 supplementation. Moreover, Wigmore et al(1997) observed in cultured human hepatocytes treated with omega-3 fatty acids that the production of haptoglobin is decreased. Hence, it is possible the expected increase in haptoglobin was disguised by coincident decrease in production due to overall decrease in inflammatory state or the direct omega-3 fatty acid suppressive effect.

High steady state Lactate dehydrogenase(Kato et al. 2006; Gurkan et al. 2010), platelets(Sarris et al. 2008), increased white cell count and low Hb(Miller et al. 2000) are widely considered adverse risk factor for survival and sever clinical course. In this study, omega-3 fatty acids affected positively the four parameters; suggesting a beneficial effect on long term clinical course. Moreover, Tomer et al(2001b) in a pilot study has reported fish oil (rich in omega-3) reduces markers of thrombosis. It would be of interest to investigate the short and long-term clinical effect of omega-3 fatty acids supplementation in SCD.

The measurement of omega-3 fatty acids after one year of supplementation showed threefold increase in EPA and DHA and parallel reduction in AA and nomega-6/omega-3 ratio. These findings are broadly similar to previous studies(Muskiet et al. 1991; Tomer et al. 2001b).The question is; How these profound alteration in red cell membrane fatty acid composition could contribute in reduction of haemolysis rate? Firstly, enrichment cell membrane with long chain omega-3 fatty acids increases the total unsaturation index(Hashimoto et al. 1999), enhance red cell deformability(Terano et al. 1983; Mills et al. 1993) and resistibility of red blood cell to haemolysis(Mabile et al. 2001). Second, oxidative damage of the cell membrane is one of the major mechanism of red cell fragmentation and haemolysis(Hebbel 2011), and it is known DHA not EPA to have an anti-oxidant activity (Relton et al. 1993; Bechoua et al. 1999; Yavin 2006; Brand et al. 2008). Moreover, Ren et al. (2008) reported, DHA levels in red blood cell phospholipids of SCD patients correlates positively with anti-oxidant enzyme activity. Thus, it is plausible the detected reduction in haemolysis is a reflection of reduced oxidative damage due to high DHA contents after supplementation. In agreement with this assumption, Vitamin E concentration increased significantly after supplementation, the thing which may indicate improvement in oxidative stress status(Gbenebitse et al. 2005) and it may explain partially the increased levels of Hb and decreased rate oh haemolysis(Jaja et al. 2005; Chaves et al. 2008). Finally, increased cell adhesion is provocative factor to voso-occlusion and haemolysis in SCD, and omega-3 fatty acid supplementation proved to reduce adhesive molecules expression in non-SCD subjects (Mayer et al. 2002; Thies et al. 2003) and. Therefore, it is conceivable the observed reduction in haemolyse rate could be influenced by decrease in blood cell adhesiveness.

In conclusion our study demonstrates, supplementing HbSS patients with omega-3 fatty acids do reduce markers of haemolysis and improve haematological parameters. In order to investigate the clinical implications of these laboratory findings, well designed clinical trials are needed.

Chapter Seven

Markers of adhesion and inflammation, and membrane fatty acid composition in patients with homozygous Sickle cell disease

7.1 Introduction

The classical paradigm of Sickle cell (SCD) pathophysiology has always considered haemoglobin S (HbS) polymerisation and red cell sickling as the primary causative factors of the acute and chronic complications associated with disease(Platt 2000). However, emerging evidence indicates initial events in vaso-occlusion may involve complex array of factors, both-polymerization dependent and polymerization-independent(Hebbel 1991; Embury 2004; Kaul et al. 2009). The presence of haemolysis(Kato et al. 2007), intense oxidative stress(Wood and Granger 2007) and chronic inflammatory state are among the abnormalities contribute to pathophysiology of the SCD(Hebbel et al. 2004).In vivo studies have shown that, inflammation and increased leucocyte-erythrocyte-endothelial interaction are the major potential initiating mechanisms in vaso-occlusion(Frenette 2002; Kaul and Fabry 2004; Rees et al. 2010).

Consistent with the concept that SCD is a state of chronic inflammation, sickle cell subjects even in "steady stat" exhibit chronically elevated levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β)(Pathare et al. 2004; Lanaro et al. 2009), acute phase proteins (Krishnan et al. 2010), prostaglandin E2(Graido-Gonzalez et al. 1998). In addition, studies showed SCD patients to have activated coagulation system, endothelium(Solovey et al. 2004; Ataga et al. 2008a) and leukocytosis with activated adhesive circulating blood cells(Platt 2000; Kato et al. 2009), including monocytes(Belcher et al. 2000; Chaar et al. 2010), neutrophil(Canalli et al. 2008), reticulocytes(Brittain and Parise 2008) and platelets(Villagra et al. 2007). A growing body of evidence suggests that adhesive molecules involved in granulocytes and monocytes increased adhesiveness are the cytokines-induced L-and P-selectin (CD-62L), β2-integrin CD11b/C18 (CD-11b) and LFA-1 (CD11a/CD18)(Okpala 2004; Assis et al. 2005; Canalli et al. 2008). Moreover, Turhan et al. demonstrated that recruitment of leukocytes was reduced in sickle mice lacking P- and L-selectin, resulting in protection from vaso-occlusion and increased survival(Turhan et al. 2002). Interestingly, experimental and pilot studies tested the potential anti-inflammatory (sulfasalazine, statins) (Solovey et al. 2001; Laufs et al. 2002; Kaul et al. 2004; Solovey et

al. 2004) and anti-adhessive molecules immunoglobulin therapies (Turhan et al. 2004; Chang et al. 2008) in SCD have shown reduction in vaso-occlusion by decreasing blood and endothelial cells adhesion.

The mechanisms that implicate persistence of inflammation and increased blood adhesion are not well understood. However, some unique aspects of the disease seems to be likely proximate stimulus to inflammation(Hebbel et al. 2004). A number of studies suggest that reperfusion injury (ischemia/reperfusion pathology) is one of the fundamental factors inciting inflammation in SCD(Solovey et al. 1997; Kaul and Hebbel 2000; Hebbel et al. 2009). Metabolic changes accompanying ischemia allow an excessive burst of oxidative stress during reoxygenation and activation of potent inflammatory factors such NFκB(Carden and Granger 2000). Subsequently, this results in production of inflammatory cytokines, increased activation of leukocytes and vascular endothelium(Osarogiagbon et al. 2000). In addition, monocytes in SCD seem to have a significant role in inflammatory state associated with SCD(Wun et al. 2002). This role is mainly attributed to increased monocytes expression of IL-1β/TNF-α(Belcher et al. 2000) and monocyte-mediated vasculopathic lesions in SCD(Hebbel et al. 2004). Other pathophysiological aspects of SCD that are thought to contribute to increased adhesiveness of blood cells and hypercoagulable state are the myriad abnormalities of blood cells membrane(Schwartz et al. 1985c; Hebbel 1991; Setty et al. 2002). The major cell membrane abnormalities reported in SCD are disruption of normal membrane asymmetry and exposure of the reactive serine phosphoglyceride (SPG)(Tait and Gibson 1994; Wood et al. 1996), abnormalities of phospholipids molecular species(Connor et al. 1997), and fatty acids composition of erythrocytes(Ren et al. 2005a), platelets and mononuclear cells(Ren et al. 2005b). The fatty acids composition abnormality characterised by presence of high n-6 and low n-3 long-chain polyunsaturated fatty acids (LCPUFA)(Ren et al. 2005b).

The n-3 and n-6 LCPUFA are vital structural and functional components of cell and sub-cellular component. They are precursors of eicosanoids which act as key regulators of inflammation.(Tilley et al. 2001).In contrast to n-6 arachidonic acid- derived eicosanoids, n-3 eicosapentanoic acid-derived ecosanoids are anti-inflammatory and anti-aggregatory. It is well documented that increased concentration of n-3 fatty acid in

cell membranes shift the balance to production of more anti-inflammatory mediators (Calder 2002). Moreover, new studies identified a novel series of mediators (resolvines, protectins, maresins) formed from EPA and DHA with potent anti-inflammatory actions in neutrophils and macrophages(Calder 2009; Serhan et al. 2011). In monocyctes, there is evidence that n-3 fatty acid inhibits production of TNF-α and IL-1β and reduces adhesion with endothelium (Caughey et al. 1996; Mayer et al. 2003).Hence, it is plausible the observed perturbation in fatty acids composition of blood cells membrane of SCD patents might have a significant role in chronic inflammatory and increased blood cells adhesiveness associated with the disease.

In this study we have investigated whether a) cell membrane fatty acid composition influence the inflammatory state and blood cell adhesion b) supplementation with DHA and EPA n-3 FAs ameliorate the inflammatory state and reduces the blood cell adhesion

7.2 Methods

7.2.1 Subjects

Steady state HbSS sickle cell patients (not experienced any painful crisis or other acute medical condition for at least 1 month) age 2-11 (n=27), sixteen male (n=16) and eleven female (n=11) were enrolled from Abnaof Paediatric Hospital, Khartoum, Sudan. The patients characteristics is described in chapter 5, section 5.22.

7.2.2 Samples collection and preparation plasma, rd blood cell, monocytes, granulocytes

After an overnight fast, 10 ml of whole blood was taken into EDTA tubes and 3 ml in serum preparation tube from the patients at zero-time and one-year of supplementation.

Plasma, red blood cells, monocytes, granulocytes and platelets were separated as described in chapter 2, section 2.2.2 and 2.2.3.

7.2.3 Analysis of red blood cell fatty acids

The procedure of lipid extraction and fatty acid analysis of red blood cell is described in chapter 2, section 2-3.

7.2.4 Complete blood count (CBC)

The complet blood count was measured as described in chapter 2, section 2.2.1.

7.2.5 Measurements of plasma tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10)

The procedure of plasma TNF-α and IL-10 determination is described in chapter 2, section 2.9.1 and 2.92 respectively.

7.2.6 Assessing β2-integrin CD11b/C18 (CD-11b) and L-selectin (CD62L) expression in granulocytes and mononuclear cells

The procedure of measurement of CD11b/C18 (CD-11b) and L-selectin (CD62L) expression in granulocytes and mononuclear cells is illustrated in chapter 2, section 2-10.

7.2.7 Statistics

The data are expressed as mean ± SD. Non parametric test Related-Samples Wilcoxon Signed Ranks used was used to explore differences in concentrations of TNF-α, IL-10 and adhesive molecules before and after supplementation. Independent sample Mann-Whitney U test was used to test the effect of supplementation on adhesive molecules. Paired t-test was used to test differences in fatty acids. Pearson correlation was used to investigate the association between fatty acids and other parameters of interest. The Statistical significance was assumed at a "p" value of less than 0.05. The statistical software, SPSS for Windows, Version 17 (SPSS Ltd., Woking, Surrey, UK) was used to analyse the data.

7.3 Results

7.3.1 Fatty acid composition of red blood cell ethanolamine phosphoglyceride (EPG) and choline phosphoglyceride (CPG).

The fatty acids composition of red blood cells PE and PC at baseline and after one year of supplementations were included in **Table 7-1**.

7.3.2 Total white blood cellcount (TWBC) and platelets (PLTs) count

The supplementation with n-3 fatty acids caused significant reduction on TWBC ($P<0.05$), whereas PLTs showed no significant difference, **Table 7-1**.

7.3.3 Tumour necrosis factor-α (TNF-α) and Interleukin-10 (IL-10)

The patients gender and age have no significant effect on TNF-α and IL-10 concentrations either before or after supplementation ($P>0.5$). No significant differences were detected on plasma TNF-α (18.6±12.8 vs 17.8±9.1) ($P=0.47$, **Figure.7-1, a**) and IL-10 (11.0±4.3 vs 11.3±5.8) (P=, 0.7, **Figur.7-1, b**) after one year of supplementation with n-3 fatty acids.

7.3.4 Relationships between plasma TNF-α levels and percent of RBC EPG& CPG fatty acids at baseline

TNF-α level positively correlated with red blood cell membrane PE- AA ($R=0.6$, $P=0.005$) (**Figure 7-2, a**). No significant correlations were found between PE-LA, PC-LA and PC-AA and plasma TNF-α.

No significant correlations ($P>0.5$) were found between TNF-α and EPG and CPG n-3 fatty acids EPA (R=0.2& 0.3) and DHA (R= 0.01&0.3) respectively.

7.3.5 Relationships between plasma IL-10 levels and percent of RBC PE& PC fatty acids at baseline

IL-10 level positively correlated with red blood cell membrane PE-LA ($R=0.7$, $P=0.004$) (**Figure 7-2, b**). No significant correlations were found between PE-AA ($R=-0.3$, $P=0.3$), PC-AA ($R=-0.1$, $P= 0.7$) and PC- LA ($R=0.1$, $P=0.6$) and plasma IL-10.

No significant correlation were fount between PE -EPA ($R=0.4$, $P=0.07$)(**Figure 7-2, c**) , PE- DHA ($R=0.2$, $P=.4$), PC-EPA ($R=-0.1$, $P=0.7$), PC-DHA ($R=0.2$, $P=0.5$) and IL-10.

7.3.6 Expression of β2-integrin CD11b/C18 (CD-11b) in monocytes

Adhesive molecules β2-integrin CD11b/C18 (CD-11b) and L-selectin (CD62L) analysis were made on radomely selected ten samples (n=10) from placebo and samples from active group at base-line. After one year of intervention six samples were collected from from active group (n=6) and eight samples from placebo (n=8). The drop out at one year (n=6) is due to inability of the patients to present at the pre-scheduled time for sample collection and processing. The level of adhesive molecules of placebo and active group were comparable at baseline ($p>0.05$), Figure 7-3 and 7-4.

After one year of Supplementation with n-3 fatty, CD11b expression in monocyte was significantly lower compared to placebo and baseline ($P<0.05$), **Figure 7-3 and 7-5 respectively.**

7.3.7 Expression of β2-integrin CD11b/C18 (CD-11b) and L-selectin (CD62L) in granulocytes

Supplementation with n-3 fatty acid or placebo caused no significant effect on CD11b and CD62L expression ($P>0.5$), **Figure 7-4 and 7-5.**

7.3.8 Adhesive molecules and fatty acids correlations at baseline

At base -line, negative correlations were established between monocytes CD11b and PC-EPA ($R=-0.6$, $P=0.04$), PC-LA($R=-0.6$, $P=0.02$), **Figure 7-6, a** and **figure 7-6, b** respectively. The PE n6-metabolites/n3-metabolites ratio (n-6-met/n-3-met) negatively correlated with granulocytes CD11b ($R=-0.7$, $P=0.04$), **Figure 7-7, a**. Negative but not significant correlation was found between PC n-6-met/n-3-met and granulocytes CD11b ($R=-0.6$, $P=0.06$), Fig 7-7, b. A strong negative correlation was found between granulocytes CD62L and PE-LA($R=0.8$, $P=0.02$), **Figure 7-8.**

Table 7-1 Effect of supplementation with n-3 FA on Fatty acids composition of red blood cell membrane phosphatidylethanolamine (PE), phosphatidylecholine (PC), total white blood cells (TWBC) and platelets (PLTs).

	n=20	
Measure	**Baseline**	**One-year**
Fatty acids- PE		
C18:2n-6	4.9±0.7	4.5±0.6
C20:4n-6	24.3±1.1	22.2±1.6***
C22:4n-6	10.7±1.2	7.2±2.3***
Total n-6	44.5±1.2	36.9±3.7***
C20:5n-3	0.1±0.04	0.3±0.2***
C22:5n-3	1.7±0.3	1.4±0.2***
C22:6n-3	2.5±0.7	8.8±2.8***
Total n-3	4.4±0.9	10.6±3.2***
Dimethyl acetals	**13.3±1.1**	**13.5±1.4**
Fatty acids- PC		
C18:2n-6	15.7±2.3	18.1±2.18***
C20:4n-6	9.9± 1.0	7.9±1.2***
C22:4n-6	1.1± 0.2	0.6±0.2***
Total n-6	29.5± 1.9	28.8±2.7***
C20:5n-3	0.1±0.04	0.24±0.1***
C22:5n-3	0.3± 0.06	0.2±0.1***
C22:6n-3	0.7± 0.2	2.6±0.9****
Total n-3	1.2± 0.3	3.1±1.1***
Dimethyl acetals	0.6±0.2	0.7±0.2
TWBC	15.3±3.5	14.1±2.8*
PLTs	524.3±141.4	487.6±141.4

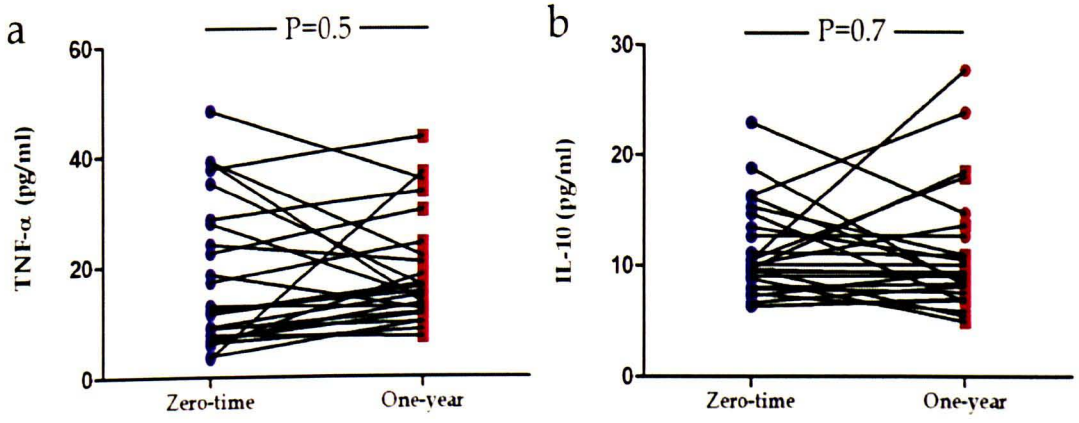


Figure 7-1 Plasma TNF-α concentrations (a) and IL-10 concentrations (b) of patients with n-3 fatty acids before and after one year of supplementation with n-3 fatty acid.

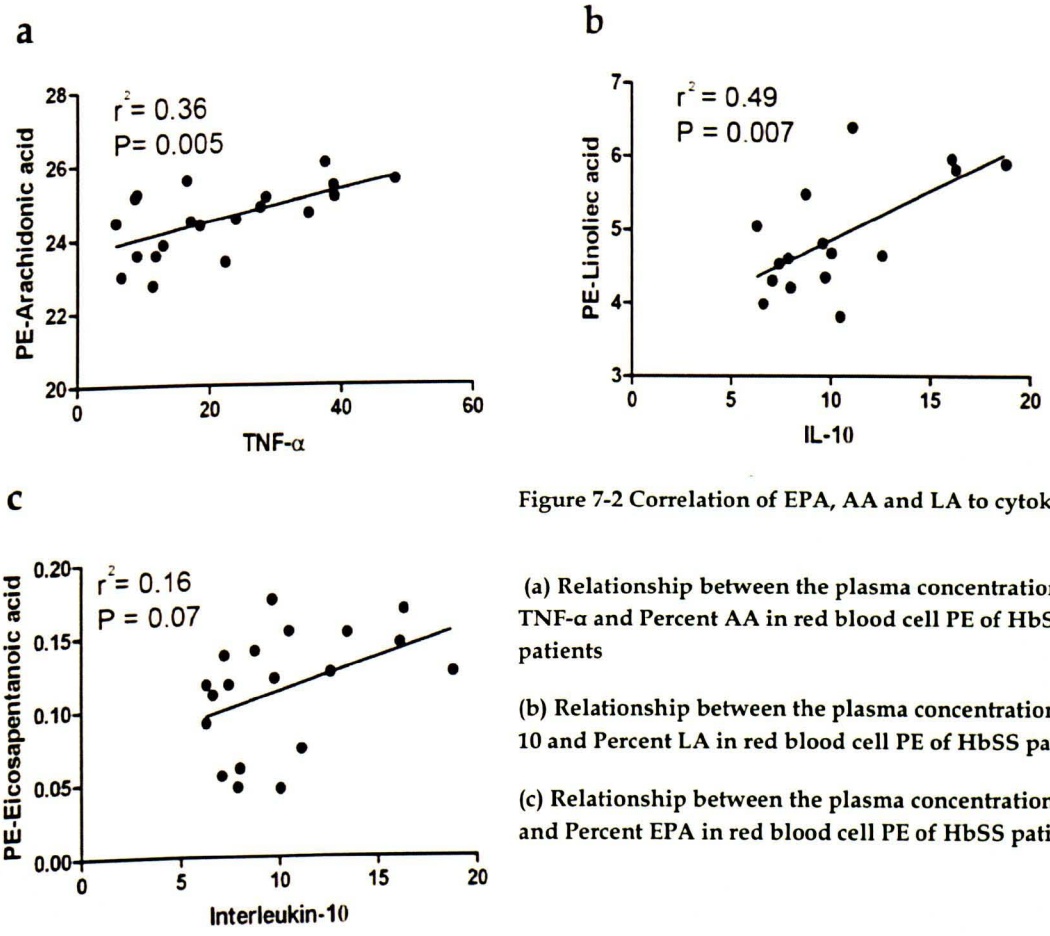
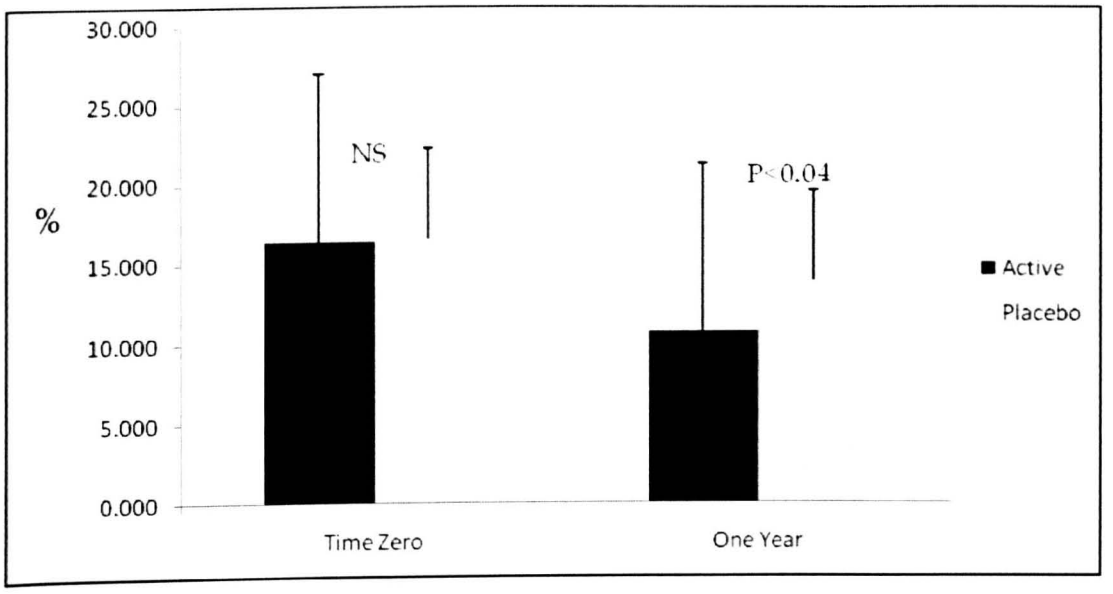


Figure 7-2 Correlation of EPA, AA and LA to cytokines

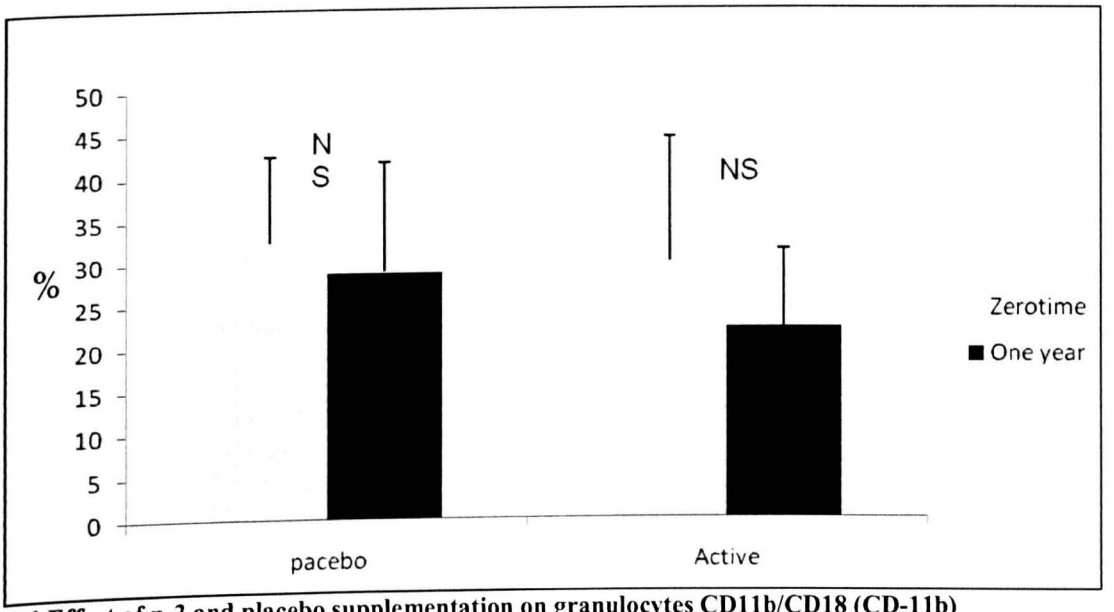
(a) Relationship between the plasma concentration TNF-α and Percent AA in red blood cell PE of HbSS patients

(b) Relationship between the plasma concentration IL-10 and Percent LA in red blood cell PE of HbSS patients.

(c) Relationship between the plasma concentration IL-10 and Percent EPA in red blood cell PE of HbSS patients



7-3 Effect of n-3 and placebo supplementation on monocytes integrin CD11b/C18 (CD-11b)



7-4 Effect of n-3 and placebo supplementation on granulocytes CD11b/CD18 (CD-11b)

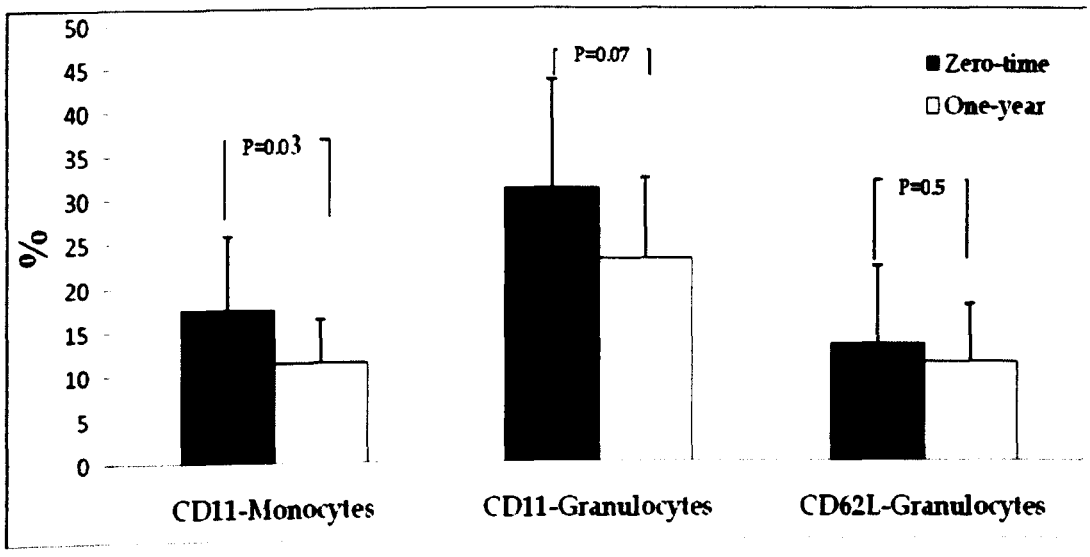


Figure 7-5 Effect of n-3 fatty acid supplementation on monocytes and granulocyctes CD11 β2-integrin CD11b/C18 (CD-11b) and granulocytes Selectin (CD62L)

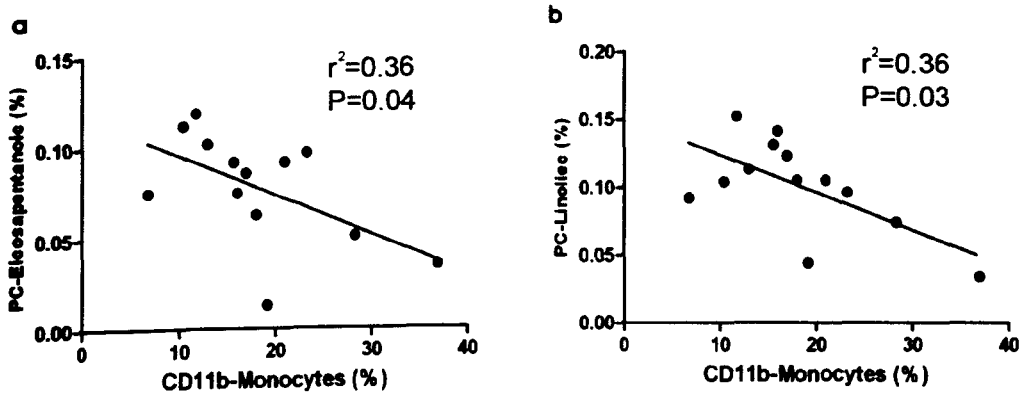


Figure 7-6 Relationship between CD11-Monocytes level and eicosapentaenoic acid (a) linoleic acid (b) percent in red blood cell PC of patients with HbSS.

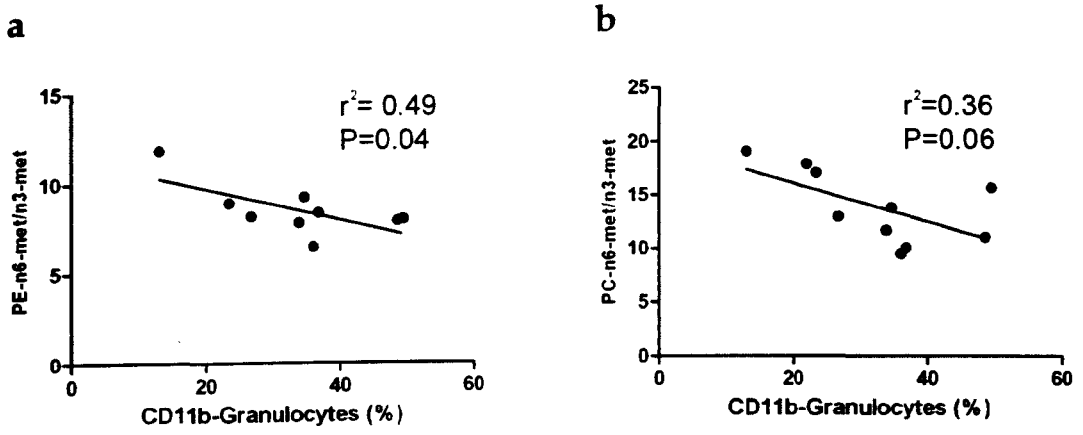


Figure 7-7 Relationship between CD11-Granulocytes level and n-6-meta/n-3-met in red blood cell PE (a) and PC (b) of patients with HbSS.

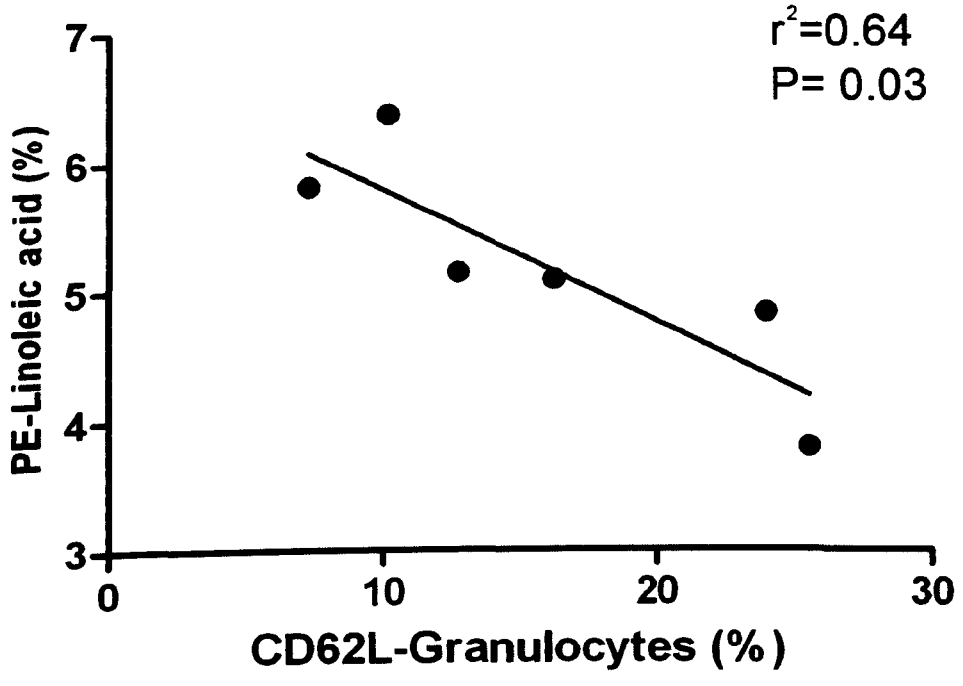


Figure 7-8 Relationship between CD62L-Granulocytes level and Linoleic acid in red blood cell PC of patients with HbSS.

7.4 Discussion

The relationship between fatty acid composition of blood cells and inflammatory status is well documented(Calder 2008b). Although the fatty acids anomalies observed in red blood cells reflect similar abnormalities in white blood cells and plasma of SCD patients (Ren et al. 2005b; Ren et al. 2006), few studies if not any have examined the effect of fatty acids status on biomarkers of inflammation and blood cells adhesive molecules in sickle cell disease. The findings in this study suggest strong association between fatty acids composition of sickle red blood cells and inflammatory state, and support partially the hypothesis that n-3 fatty acids have anti-inflammatory prosperities.(Farzaneh-Far et al. 2009; Cottin et al. 2011).

The positive association between base-line PE-AA and TNF-α is of particular importance. TNF-α is generally considered pro-inflammatory cytokines, and AA is precursor of strong pro-inflammatory eicosanoid , particularly PG E2 the most abundant eicosanoid in human body(Tilley et al. 2001; Serhan and Levy 2003). Hence, it is conceivable (Tanaka et al. 1996)that high concentration of AA in blood cell membrane may aggravate the inflammatory response (Karck et al. 1988). Indeed, there is evidence, reduction of AA in blood cells is associated with concomitant reduction of PG E2 and TNF-α both in healthy subject and patients with rheumaroid arthritis(James et al. 2000; Sundrarjun et al. 2004; Ferrucci et al. 2006; Rees et al. 2006; Farzaneh-Far et al. 2009). From these clinical studies it has been suggested that the modulatory effect of blood cell fatty acids composition on inflammatory response is a role of PGE2(James et al. 2000). However, in vitro studies investigated the effect of PG E2 upon production of TNF-α have shown inhibitory rather than the predicted stimulatory effect (Dooper et al. 2002; Miles et al. 2003; Serhan and Levy 2003). This inhibitory effect in experimental studies have been attributed to differences between the in vivo environment and the ex vivo conditions, as the overall impact of eicosanoid in inflammatory response depend on several factors including the level of immune cell activation, presence of other mediators and the physiological state(Tilley et al. 2001; Pischon et al. 2003).

In contrast to AA, the n-6 parent fatty acids Linoleic acid (LA) is positively correlated with anti-inflammatory cytokines IL-10(de Waal Malefyt et al. 1991; Sabat et al. 2010), and negatively correlated with adhesive molecules CD11b-Monocytes and CD62L-

granulocytes. These correlations signify that LA acid might have an anti-inflammatory role. Recently, Poudel-Tandukar et al(2009) reported similar inverse association between LA and inflammatory marker C-reactive protein. Moreover, clinical trials and comprehensive reviews of epidemiologic investigations on n-6 and cardiovascular diseases (CVD) concluded that higher intake of LA is not associated with elevated levels of inflammatory markers and tend to decrease cardiovascular disease (CVD) risk(Djousse et al. 2001; Willett 2007; Harris et al. 2009). In addition, PE and PC n-6/n-3 ration in this study were negatively correlated with CD11b-granulocytes.These observations do challenge the predominant conception that all n-6 fatty acids are fundamentally pro-inflammatory by necessity (Hennig et al. 2006; Calder 2009).

In SCD disease, the role of LA in pathophysiology of the disease is intriguing. Previous studies investigated the fatty acids composition of sickle red blood cells have demonstrated constantly lower LA, whereas the n-6 fatty acids metabolites are significantly higher compared to controls(Ren et al. 2005a; Ren et al. 2006). This may indicate FA metabolic dysfunction- impaired synthesis, uptake or enhanced turnover of LA to long chain n-6 metabolites. In this study LA strongly negatively correlated with AA and supplementation with n-3 fatty acids decreased AA and increased LA. Hence, it is tenable to postulate that increased LA conversion to AA may contribute to pro-inflammatory state associated with SCD by generating higher concentration of AA, and in turn higher levels of pro-inflammatory eicosanoids.

In contrast to Ferrucci et al(2006) that reported positive correlation between EPA and IL-10 and negative correlation between n-3 and TNF-α on healthy individual, our results showed no association between DHA, total n-3 and IL-10 or TNF-α. Moreover, supplementation with n-3 fatty acids caused no effect on TNF- α and IL-10. The effect of supplementation on plasma TNF-α is in accordance with that reported from interventional studies conducted on patients with diabetes mellitus (Molvig et al. 1991; Mori et al. 2003), rheumatoid artheritis(Adam et al. 2003; Sundrarjun et al. 2004) and crohn disease(Trebble et al. 2004). Similar to TNF-α, no previous studies reported significant effects of n-3 supplementation on systemic IL-10(Sijben and Calder 2007).On the other hand, some studies that measured the effect of supplementation on cytokines production by monocytes (Caughey et al. 1996; Mayer et al. 2003) or local

rather than systemic levels of cytokines showed significant effects(Matsuyama et al. 2005). This discrepancy between the systemic and cell or tissue-specific levels of cytokines may indicate that systemic plasma cytokines is not sensitive enough to reflect the possible variability in sensitivity of different tissues to the immunomodulatory effects of n-3 (Sijben and Calder 2007). Therefore, further investigation considering the effect of supplementation on cytokines production from blood cells of patients with SCD might be of interest.

Interaction of leukocytes with endothelial cells involves expression of adhesive molecules(Tan et al. 1999). L-selectin (CD62L) facilitate rolling and temporary arrest of circulating leukocytes on endothelium surface(Strausbaugh et al. 1999), whereas β2-integrin (CD11b) is involved in subsequent stable adhesion(Nathan 2002). Therefore, therapeutic approaches to reduce expression of CD62L and CD11B have been important strategies to prevent vaso-occlusion in sickle cell disease(Canalli et al. 2008). In this study, EPA negatively correlated with CD11b-monocytes at baseline, and supplementation with n-3 reduced significantly total number of leukocytes and expersion of CD11b- monocytes, indicating improvements in the chronic inflammatory states. These results are consistent with previous in vitro (De Caterina and Libby 1996; Mayer et al. 2002) and in vivo(Mayer et al. 2003) studies that showed supplementation with n-3 fatty acids reduce inflammatory markers and expression of adhesive molecules. The mechanism (s) by which n-3 fatty acids regulates the expression of adhesive molecules remains elusive. However, studies showed that modifying cell membrane lipids composition by n-3 supplementations alters lipid microdomains, such as lipid rafts and caveolae, involved in the compartmentalisation, modulation and integration of cell signalling components(Stillwell et al. 2005; Massaro et al. 2008). Moreover, there is evidence the EPA-derived resolving E1 induces L-selectin shedding together with reduction in CD18 surface expression on human polymorphonulear cells and monocytes(Dona et al. 2008). In SCD multiple mechanisms are thought to be involved in pathophysiology of blood cell adhesion and chronic inflammatory stat. Therefore the n-3 FA multi-level actions with broad adhesion preventing ability render it a promising therapy for SCD that may be able to meet the criteria of the "vascular lubricants" proposed by Hebbel et al(2004) as a preferable therapeutic approach over specific anti-adhesive agents.

In conclusion, the current study demonstrates that fatty acids status of patients with sickle cell disease has a significant effect on the inflammatory state, and supplementation with omega-3 fatty acids does modulate adhesive molecules on blood cells. Therefore, further studies investigating the effect of omega-3 fatty acids supplementation on clinical outcomes, PG2, acute phase proteins and tissue-specific cytokines might be of interest

Chapter Eight

Overview conclusion and future research

8.1 Overview

Sickle cell disease (SCD) is a debilitating monogenic blood disorder, which is characterised by recurrent episodes of vaso-occlusive crisis and chronic haemolytic anaemia. It causes short and long term complications that affect all organs and systems of the body. The pattern of the complications and long-term survival vary considerably among patients but the reason for the variations is yet to be elucidated. However, it is now thought that the pathophysiology of the disease is an out come of the concerted actions of genetic, cellular, humoral and environmental factors. Indeed, there is evidence that leucocyte-erythrocyte-endothelial interaction and inflammation are the primary predisposing factors of vaso-occlusion.

Ahesion, aggregation, elasticity of blood cells and inflammatory response are strongly modulated by cell membrane omega-3 and omega-6 fatty acids. Indeed, there is evidence steady state patients with SCD have abnormal erythrocytes, platelets and mononuclear cell fatty acid composition. The abnormality is characterised by high omega-6 arachidonic (AA), adrenic and osbond acids, low omega-3 docosahexanoic (DHA) and eicosapentnoic (EPA) acids and n-6/n-3 imbalance. Besides their vital role as integral part of the cell membranes AA and EPA are precursors of eicosanoids, a group 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 and formation of thrombus. In contrast, EPA-derived eicosanoids are relatively anti-inflammatory and anti-aggregatory. In addition EPA and DHA are precursors of a novel class of lipid mediators, resolvins (Rv), and maresin and neuroprotectin D1 from DHA, with strong anti-inflammatory effects.

This research programme investigated whether **(a)** HU, the commonly used treatment for the disease, ameliorate red cell membrane fatty acid abnormalities observed in previous studies; **(b)** Supplementation with n-3 FA reduce the frequency of painful crisis through correction of the membrane fatty acid perturbation and reduction of inflammation and blood cell adhesion.

Study 1(Chapter 3) investigated the effect of hydroxyuea on red blood cell membrane fatty acids composition of gender and aged matched HU-treated and untreated homozygous sickle cell patients and healthy controls. The HU treated compared with the untreated patients had lower arachidonic (AA) acid level in EPG and SPG. The treated patients and healthy controls had comparable levels of docosahexaenoic (DHA) and total n-3 fatty acids in EPG and CPG. In contrast, the untreated group had significantly lower DHA and total n-3 compared with the controls in EPG. HU is known to activate cytosolic phospholipase A2 and cyclooxygenase 2, and from this study it appears to induce mobilisation of AA from the inner cell membrane PE and PS. Hence, it is possible the clinical benefits of HU can be partially mediated through correction of membrane fatty acids composition.

Study 2(Chapter 4) - Sickle patients are under continuous oxidative stress (OS) and LCPUFA n-3 fatty acids are susceptible to peroxidation because of their high unsaturation index. This study assessed whether supplementation with n-3 FAs exacerbates oxidative stress by comparing n-3 FA supplemented and unsupplmented homozygous sickle cell patients. The base line contents of DHA, EPA and total n-3 fatty acid were consistent with values observed on Sudanese children with SCD. After six months of supplementation the n-3 fatty acids concentration in active group increased to levels comparable to that of healthy individuals from high omega-3 intake communities, with concomitant decrease in n-6 fatty acids particularly AA acid. Continuation of n-3 fatty acids supplementation for one year caused no significant changes in fatty acid profile compared to six-month apart from further reduction on AA. These results provide evidence that the reported low levels of cell n-3 and high n-6 membrane fatty acids in SCD is responsive to dietary modulation. After one year of supplementation, vit E level was significantly increased in active group whereas the total plasmalogens content remained unchanged. In accordance with previous studies, n-3 fatty acids supplementation in this study caused no significant difference in activity of Cu/Zn-Superoxide dismutase (SOD) compared to placebo group. The observed significant decrease of SOD activity in both placebo and active group compared to base-line activities is intriguing. However, vit E which was added in a very small amount to supplement capsules may be implicated in the SOD reduction after intervention. In contrast to the reported results of clinical trials in patients with

other chronic inflammatory state, Glutathione peroxidase-1 (GPx-1) activity decreased significantly after intervention in our study. Hence, taking into account the enhanced vitamin E levels and preserved plamalogen (DMA) concentration after n-3 supplementation, the observed reduction in GPx-1 could be interpreted as a reflection of decreased oxidative stress levels. Therefore, we concluded that our findings do not support the anticipated adverse effect of n-3 supplementation on overall oxidative stress in SCD, and long term supplementation provides more anti-oxidant protection capacity.

Study 3 (Chapter five)- In order to examine the potential therapeutic effects of omega-3 fatty acids in sickle cell disease, we have conducted single centre, two-arm, randomised, placebo-controlled, double blinded clinical trial. One hundred forty (n=70 in each arm) Sudanese SCD patients participated in the study. The primary end point was annualised rates of clinical vaso-occlusive crisis, which is defined as painful crisis that leads to hospitalization. Secondary end points were haemolytic crisis, rate of blood transfusion, school attendance, Hb levels and mean cell volume (MCV). Intention to treat was the analysis strategy and encompassed all randomised patients regardless of the duration of follow-up. The monthly self-assessment health records and the 0, 6 and 12 months red blood cell membrane fatty acids composition showed high compliance and tolerability to supplementation. The primary end point was successfully met and the number of inpatient days due to sickle cell disease crisis and its associated complications were significantly lower in omega-fatty acid supplemented group. Moreover, the beneficial effects of DHA and EPA were reflected on the quality of life of the patients as measured by the significant reduction of days out of school due to the disease. In contrast to the placebo group, no patient in active group developed stroke, the finding which suggest a potential preventive effect of n-3 FA. The observed decrease rate of haemolytic crisis and blood transfusion is consistent with the reported anti-haemolytic role of omega-3 fatty acids. Enigmatically, the Hb concentration and MCH levels were increased to comparable levels in placebo and active group. These results indicate that factors other than n-3 fatty acids might have influenced the increase in Hb levels. A singular factor that may have played a role is vitamin E, which was added in a very small concentration to protect supplemented fatty acids from peroxidation. Interestingly, the high increase in MCV of the active can not be

explained merely by the increase of Hb and MCH, as the same amount of increase in Hb occurred in the placebo group without causing equal increase in MCV. It is possible that the increase in MCV is a reflection of improved cell membrane prosperties and hydration state. In conclusion, this study provides strong evidence that DHA and EPA could be safe and effective therapy for prevention of vaso-occlusive crisis and decrease the rate of haemolysis in homozygous sickle cell disease.

Study 4 (Chapter six) - Study 3 showed that n-3 fatty supplementation reduced the haemolytic crises and blood transfusion rate. In this study we investigated the effect of n-3 FA supplementation on biochemical markers of haemolysis lactate dehydrogenase (LDH) and haemoglobin concentration in sub-group of patients at steady state. Intervention with n-3 fatty acids caused significant reduction in LDH, which signifies improvement in haemolysis rate. In contrast to previous studies reported by other groups, the supplementation with omega-3 resulted in significant increase in haemoglobin, MCV, MCH and MCHC. Conversely, the total white blood cell (TWBC) and platelets (PLTs) count were reduced by 20.5% and 18.2% respectively, indicating lessened inflammatory state and/or diminished haemopoietic activity of the bone marrow. These differences in haematological outcomes from previous studies may be due to composition of the supplement used in this study (high DHA), or the relatively long duration of supplementation period. High steady state Lactate dehydrogenase, platelets, increased white cell count and low Hb are widely considered adverse risk factor for survival and sever clinical course. In this study, omega-3 fatty acids affected positively the four parameters; the findings which agree with the observed beneficial clinical effects off n-3 fatty acid supplementation in SCD.

Study 5 (Chapter seven) - In this study, we investigated the effect of DHA and EPA supplementation on fatty inflammatory state and blood cells adhesion. The patients contributed in this study were sub-group of the placebo and the active group of the clinical trial (study 3) discussed above. Supplementation with n-3 FAs showed no effect on IL-10 or TNF-α. However, other studies investigated the effect of n-3 supplementation showed significant reduction in tissue or local TNF-α rather than in systemic levels. Hence, we have postulated that discrepancy between the systemic and local or tissue-specific levels of cytokines may indicate that systemic plasma cytokines

are not sensitive enough to reflect the possible variability in sensitivity of different tissues to the immunomodulatory effects of n-3 FA. As an indication of improvement in inflammatory state, DHA and EPA supplementation caused a significant reduction in total number of leukocytes and expression of CD11b- monocytes. These results are consistent with previous studies that showed supplementation with n-3 fatty acids do ameliorate inflammatory markers and expression of adhesive molecules. In conclusion, our data provide a limited evidence that supplementation with DHA and EPA does modulate adhesive molecules on blood cells and mitigate the inflammatory state.

8.2 Conclusion

This study which was based on a significant number of homozygous sickle cell patients clearly demonstrated that supplementation with DHA and EPA reduces significantly the frequency of vaso-occlusive crisis, and it suggested that the effect may be mediated by reductions of blood cell adhesion, haemolysis and inflammation. In addition, it revealed that DHA and EPA, which are highly labile and prone to peroxidation, do not exacerbate oxidative stress.

8.3 Future studies

As it is evident from the above conclusions, DHA and EPA have the potential to be effective and affordable treatment option for patients with SCD. However, before such recommendation could be made, further studies would need to be undertaken to establish universality and possible mechanisms.

- **Multi-centre clinical trial**

This study provided strong evidence that DHA and EPA supplementation reduces vaso-occlusive crisis in patients with SCD. However, because of the influence of environmental and genetic variation on the clinical course of the disease, a multi-centre study involving patients from different genetic and environmental backgrounds must be conducted to test the generalisability of the current findings.

- **N-3 fatty acids and neurological and cognitive complications.**

The current study has suggested that supplementation with DHA and EPA reduces the risk of SCD-induced stroke. A well-powered, long-term, study is needed to delineate the efficacy of EPA and DHA supplementation in reduction of silent and overt stroke, brain vascular pathology and cognitive impairment. To obtain objective data, MRI, TCD and standardised cognitive assessment test should be employed.

- **N-3 fatty acids and prevention of organ damage in infants.**

Sickle-cell anaemia is associated with substantial morbidity from acute complications and organ dysfunction beginning in the first year of life. The current investigation did not included patients less than two years of age. Hence, it is important to assess whether supplementation with DHA and EPA prevents or delays the onset of organ damage.

- **Effect of n-3 fatty acids on organ-specific complications**

The pattern of organ-specific complication varies among sickle cell patients. These variations suggest interplay between environment and genetic factors. As consumption of fish, which is the main source of n-3 fatty acids, differs between regions (coastal versus inland), the effect of n-3 fatty acids on variations on organ-specific complications (avascular necrosis, kidney function, retinopathy and cardiopulmonary complication) is warranted.

- **Synergistic effect of hydroxyurea and n-3 fatty acids**

Hydroxurea (HU) is the commonly used drug for SCD. About 30% of patients are not responsive. Moreover, as HU is a cytotoxic drug, its long term adverse effects are of concern. It would be instructive to unravel whether a combination therapy of n-3 fatty acids and HU enhances responsiveness; and reduces the therapuetatic dosage.

- **Mechanisms of efficacy of n-3 fatty acid supplementation**

Our findings in this study provide limited evidence that DHA and EPA may have anti-inflammatory and anti-adhesive effects in SCD. Thus, studying the effect on additional markers of inflammation, endothelium function and co-agulation state will shed light on mechanisms through which n-3 FAs mediate its effects in SCD.

- **Hydroxyurea treatment and arachidonic acid metabolism**

Data from the current study show that arachidonic acid (AA) is selectively mobilised from the inner cell membrane amino-phosphoglycerides, ethanolamine and serine, in patients treated with HU. There is evidence which suggests that PGE2, metabolite of AA, plays a role in HbF production. A study that which explores the potential role of eicosanoids generated from the AA would help to understand the mechanism of action of HU treatment in SCD and the role of lipid mediators in the pathophysiology of the disease.

- **Metabolism of n-3 and n-6 fatty acids**

The current Sudanese study consistent with our earlier investigations in British and Nigerian patients demonstrate that sickle cell disease is associated with abnormal levels of red cell n-3 and n-6 polyunsaturated fatty acids (PUFA). Levels of n-3 and n-6 PUFA are determined by Single nucleotide polymorphism of the lipid desaturase (FADS1 and FADS2) genes, which along with the beta globin gene are located in chromosome 11. A study should be undertaken to elucidate the genetic variations in FADS1 and FADS2, and the activity of the expressed enzymes in steady state patients.

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Appendices

Appendix 1- List of instruments

Gas-Liquid chromatography:-

Product: 1800 Series gas chromatography model 8533 (HRGC MEGA 2 Series) with dual flame ionisation detector, split/splitless, amplifiers with A 200S autosampler.

Supplier: Fisons Instruments (UK organic sales, Crewe Road, Wythenshaw, Manchester, M23 9BE)

Column:	BPX-70 column
Supplier:	SGE Europe Ltd (UK)
Length:	60 metre
Physi properties:	Highly Polar
Material	70% Cyanopropyl Polysilphenylene-siloxane
Type:	BPX70
Film Thickness:	0.25 µm
Operating temperature	150°C to 250°C
Conditioning temperature	250 0C for 5 minutes

Hydrogen generator:-

Product:	H_2 Flow
Supplier:	In house Gas

This hydrogen generator produces up to 400 cc/min of pure dry hydrogen gas using deionised water and electricity.

UV/VIS spectrometer:-

Product: Lambda 35

Supplier: Perkin Elmer Inc.

96-well microplate reader:-

Product: Fluor Star Omega

Supplier: BMG Labtech

Flow Cytometry system:-

Product: Guava PCA-96 System

Supplier: Guava technologies

High Performance Liquid Chromatograph:-

Model Agilent 1100 series high performance liguid chromatograph system with a quaternary pump connected to an autosampler, fraction collector and diode-array detector.

Diode Array Detector:-

Model Agillent 1100 Series diode array detector

Light source Deuterium and tungsten lamps

Wavelength range 190-950 nm

Diode Width < 1nm

Autosampler:-

Model	Agilent 1100 series autosampler
Dimentions	200x345x435 mm
Ambient Operating Temperature	4-40 oC
Humidity	95%, at 25-40 oC

Fraction collector

Model	Agilent 1100 series fraction collector
Dimentions	200x345x440 mm
Ambient Operating Temperature	4-55 oC
Humidity	95%, at 25-40 oC

Supplier Agilent Technologies(Agilent Technologies Deutchland GmbH, Waldbronn analytical division, Waldbronn, Germany)

Hypersil GOLD HPLC Columns:-

(Cat.No. 25005-25630)

Length	250 mm
Diameter	4.6 mm
Particle size	5µm
Pore size	175Å
pH range	1-11
Supplier	Thermo Scientific, UK

Appendix 2 List of reagents and kits

Name	Company	Code
Acetic Acid	Fisher Scientific UK	A/0400/PB08
Acetone	Fluka	00568
Acetyl chloride	Acros organic	151270010
ACOX1	Applied Biosystems	Hs01074240_m1
Boric acid	Sigma-AldrichCo. UK	B6768
Bovine serum albumin	Sigma-AldrichCo. UK	A7906
Butylated hydroxytoluene	Sigma-AldrichCo. UK	B-1378
Chloroform, HPLC grade	Fisher Scientific UK Ltd	C/4966/17
CPG standard	Sigma-AldrichCo. UK	P5394
2, 7-Dichlorofluorescein	Sigma-AldrichCo. UK	410217
Diethylpyrocarbonate	BDH	441703D
Dried methanol	Fisher Scientific UK Ltd	M/4050/15
EPG standard	Sigma-AldrichCo. UK	P7693
Ethanol	Hayman Ltd.	64-17-5
FAME Mix	Sigma-AldrichCo. UK	18919-IAMP
GUAVA via count flex reagent	Guava technologies	4700-0060
Heptane	Fisher Scientific UK Ltd	H/0106/17
Hexane	Sigma-AldrichCo. UK	H/0406/17

Histopaque-1077	Sigma-AldrichCo. UK	1077-1
Histopaque-	Sigma-AldrichCo. UK	1119
Hydrochloric Acid 37%	Sigma-AldrichCo. UK	7647-01-0
IPG standard	Sigma-AldrichCo. UK	P6636
Isopropanol	Fisher Scientific UK Ltd	P/7490/08
Methylamine, extra pure, 40 wt%	Acros organic	126230010
Petroleum ether	Fisher Chemical	P/1800/17
PBS	Sigma-AldrichCo. UK	
Potassium bicarbonte	Fisher Scientific	P/5120/53
Sodium chloride	Sigma-AldrichCo. UK	S 7653
Sodium sulphate anhydrous, granular	Fisher Scientific	S/6600/53
Vacutainer Serum Separator	Becton, Dickinson and Company (BD)	367954
Vacutainer K2E	Becton, Dickinson and Company (BD)	367525

Analysis kit:

Kit	Company	Catalog No.
Se-GPX	Cayman	703102
Cu/Zn-SOD	Cayman	706002
LDH	Life science Inc	E91864Hu
Hp	Immunology Consultant Laboratory	E-80HPT
TNF- α	Pierce Biotechnology	EH3TNFA
IL-10	Pierce Biotechnology	EHIL02

Anti-bodies:

Name	Company	Code
Mouse Anti-human CD11b FITC conjugate	Beckman Coulter	IM0530
Mouse Anti-human CD62L FITC conjugate	Beckman Coulter	IM1231U

Thin-Layer Chromatography Plates:

Product	TLC silica gel
Cat No	1.5721.0001
Size	20x20 cm
Layer thickness	210-270μm
Pore volume	0.74-0.84 m/g
Supplier	Merck KGaA, Germany

Appendix 3

Fatty acids standard mix: 100 mg ampule contains the following with weight percentages indicated

Trivial Name	Abbreviation	Weight (%)
Butyric acid Methyl Ester	4:0	4
Caproic acid Methyl Ester	6:0	4
Caprylic acid Methyl Ester	8:0	4
Undecanoic acid Methyl Ester	10:0	2
Lauric acid Methyl Ester	12:0	4
Tridecanoic acid Methyl Ester	13:0	2
Myristic Acid Methyl Ester	14:0	4
Myristoleic acid Methyl Ester	14:1	2
Pentadecanoic acid Methyl Ester	15:0	2
Cis-10-Pentadecenoic acid Methyl	15:1	2
Palmitic acid Methyl Ester	16:0	6
Palmitoleic acid Methyl Ester	16:1	2
Heptadecanoic acid Methyl Ester	17:0	2
Cis-10-Heptadecenoic acid Methyl	17:1	4
Stearic acid Methyl Ester	18:0	4
Oleic acid Methyl Ester	18:1	4
Elaidic acid Methyl Ester	18:1	2
Linoleic acid Methyl Ester	18:2	2
Linolelaidic acid	18:2	2
α- linolenic acid Methyl Ester	18:3	2
γ- linolenic acid Methyl Ester	18:3	2
Arachidic acid Methyl Ester	20:0	4
Cis-11-eicosenoic acid Methyl	20:1	2
Cis-11,14-eicosadienoic acid	20:2	2

Cis-11,14,17-eicosatrienoic acid	20:3	2
Arachidonic acid Methyl Ester	20:4	2
Cis-5,8,11,14,17-eicosapentaenoic	20:5	2
Heneicosanoic acid Methyl Ester	21:0	2
Behenic acid Methyl Ester	22:0	4
Erucic acid Methyl Ester	22:1	2
Cis-13,16-docosadienoic acid	22:2	2
Cis-4,7,10,13,16,19-	22:6	2
Tricosanoic acid Methyl Ester	23:0	2
Lignoceric acid Methyl Ester	24:0	4
Nervonic acid Methyl Ester	24:1	2

Appendix 4

Vitamin Standards:

Retinol, purity >99.0% (HPLC	Fluka	95144
α-Tocopherol, purity >97.0% (HPLC)	Fluka	95240

Appendix 5

Information Letter and Consent

Information

- *You are invited to participate in a study on Sickle Cell Disease (SCD) to be conducted by staff from the Faculty of Medicine, University of Khartoum in collaboration with researchers from London Metropolitan University, London, United Kingdom*

- *Read carefully the information provided below before deciding whether or not to participate in the study. If the information provided is not clear or you have any other question related to the study, please feel free to ask.*

-

Title of study - Omega-3 Fatty Acid Therapy for Prevention of Vaso-Occlusive Crisis in Patients with Homozygous Sickle Cell Disease (SCD).

Investigators - Dr Ahmed Daak and Professor Mustafa El Bashir, Department of Medical Biochemistry, Faculty of Medicine, University of Khartoum, Sudan.

What is the purpose of the study? Previous studies conducted in other countries (America and Venezuela) indicate omega 3 fatty acids have beneficial effects for patients with sickle cell disease. These findings are very important since omega 3 fatty acids, which are obtained from fish and fish products, are cheap and easily accessible to poor patients. Unlike the drugs which are currently used for sickle cell patients, omega 3 fatty acids do not have serious side effects. The studies conducted in the past were based on a small number of patients and the supplementation period was short. Hence, we are unsure whether omega 3 fatty acids (a) will be beneficial for Sudanese patients who are ethnically different from those previously studied; (b) Have adverse effects when given for a longer period of time. The purpose of this study is to find out if omega-3 fatty acid supplementation reduces the frequency of painful crises in Sudanese sickle cell patients.

What do have to do if I volunteer to participate? You are invited to participate in the study described above. if you volunteers to take part in the study, you will be asked to:

(a) take omega 3 fatty acid capsules for one year (capsules shown); (b) Keep written record about your medial condition; (c) Provide a teaspoon of blood (5 ml) before you start to take the capsules and subsequently after 6 and 12 months; (d) Attend the monthly clinic appointment for clinical and biochemical assessments of your condition.

Do omega 3 fatty acids have side effects? Omega-3 fatty acids are nutrients extracted from fish and do not have side effects. However, there very few people who develop allergic reaction if they eat fish. If you are one of those individuals, you must inform the investigators before deciding to volunteer.

What will happen if I decide to withdraw from the study? You are free to withdraw from the study at anytime without giving any reason. Withdrawal from the study will not affect the treatment you receive from the doctors in the clinic.

Consent

Title of study - Omega-3 Fatty Acid Therapy for Prevention of Vaso-Occlusive Crisis in Patients with Homozygous Sickle Cell Disease (SCD).

Investigators - Dr Ahmed Daak and Professor Mustafa El Bashir, Department of Medical Biochemistry, Faculty of Medicine, University of Khartoum, Sudan.

Declaration of consent - I confirm I have read and understood the information provided regarding the study and I had the opportunity to ask questions. Also, I know that I can withdraw from the study without giving a reason for my decision.

I agree to take part in the study.

Name (Patient/Guardian) **Signature** **Date**

Investigators Dr Ahmed Daak Professor Mustaf El bashir

Signature

Date

Telephone Number 091231515331

If you have questions or require advice please phone Dr Ahmed Daak

Appendix 6

Enrollment Form:

Khartoum University

Faculty of Medicine

Homozygous Sickle cell Diseases Assessment form

From No. .....

Date of enrollment: .....

Demographic Data:

- **Name:** ..... **Age:**
- **Sex:** Male ☐ Female ☐
- **Place of birth:** .....
- **Original home:** ..... **Tribe:** .....
- **Father name:** ..... **Mother name:**
- **Consanguinity:** Yes ☐ No ☐
- **Permanent address:**

Geographic area .....

Prominent sign.....

Tel No. ..... - E. mail

Person in Kin: .....

Address .....

Homozygous SCD past history:

- **Age when the disease firstly diagnosed:-** .....
- **Diagnostic tests:**

- Sickling test Paraffin wax: Yes ☐ No ☐

- Sickling test sodium metabisulphate: Yes☐ No☐

- Haemoglobin electrophoresis: Yes ☐ No ☐

- **Symptoms of first presentation:** .....

- **Past history of hospital admission (last year):** Yes ☐ No ☐

- **If "Yes", Number of hospital admission (per year):**

1- 2☐ 3 - 5 ☐ > 5 ☐ (Specify): ☐

- **Causes of hospital admission:**

- Pallor	☐	- Hand food swelling and pain	☐
- Jaundice	☐	- Long bones pain	☐
- Fever	☐	- Abdominal pain	☐
- Chest pain	☐	- Back pain	☐
- Limb weakness	☐	- Visual impairment	☐
- Leg ulcer	☐	- Limbing	☐
- Priapsim	☐	-	☐

Others:.....

- **Past history of blood transfusion:** Yes ☐ No ☐

- **If "Yes" number of blood transfusion:** ☐

- **Duration from last blood transfusion (in months):**

<3 months ☐ 3- 6 ☐ 7 - 12 ☐ > 12 ☐

- **Drug history:**

Phenoxy penicillin: Yes ☐ No ☐

- Folic acid: Yes ☐ No ☐

- NAID: Yes ☐ No ☐

- If "Yes" How many per month: .....

- Others (specify): .....

• **Vaccinations:**

• No ☐ - Started ☐ - Completed ☐

Family and Social history:

	Father	Mother
Age		
Tribe		
Occupation		
Residence		
Education		
Income/month		
History of disease		

Siblings:

No.	Age	Sex	State of health	No.	Age	Sex	State of health
1				7			
2				8			
3				9			
4				10			
5				11			
6				12			

Is the family living in its own house: Yes ☐ No ☐

Residential area class:

First ☐ - Second ☐ - Third ☐ - Slumps ☐

Schooling:

- School attendance: Good ☐ Moderate ☐ Poor ☐

- School Performance: Good ☐ Moderate ☐ Poor ☐

Is poor performance due to Sickle cell disease: Yes ☐ No ☐

Clinical examination:

Wt.- .....**kg** **Ht.-** .....**cm**

General condition: Good ☐ Satisfactory ☐ Poor ☐

C.V.S:- Pulse...../min BP...../mmHg

Heart:

.....

.....

Chest:

.....

Abdomen: - Liver:

.....

Spleen: .....

Others: .....

CNS: - Limb weakness: Yes ☐ No ☐

If "Yes" specify:.....

Sensory loss: Yes ☐ No ☐

If "Yes" specify:.....

Hand & foot syndrome: Yes☐ No ☐

Sickle cell disease complications:

Priapism: Yes ☐ No ☐

Osteomylitis: Yes ☐ No ☐

Leg ulcer: Yes☐ No ☐

Enuresis: Yes☐ No ☐

Other signs: .....

Investigations:

1-Haematological analysis:

Total Hb.....mg/dl PCV .....% MCV .....fm

MCH..... TWBC .....

Platelets.....

% of sickle cells.....

diff.: Nitrophils..... Lymphocytes.....

D. dimmer concentration ..... Prothrombin fragment.....

Bilirubin .....

Urine:- pH ..... R.B.Cs ..... Pus cells .....

Casts.....

Urine specific

gravity.....

Radiology:-

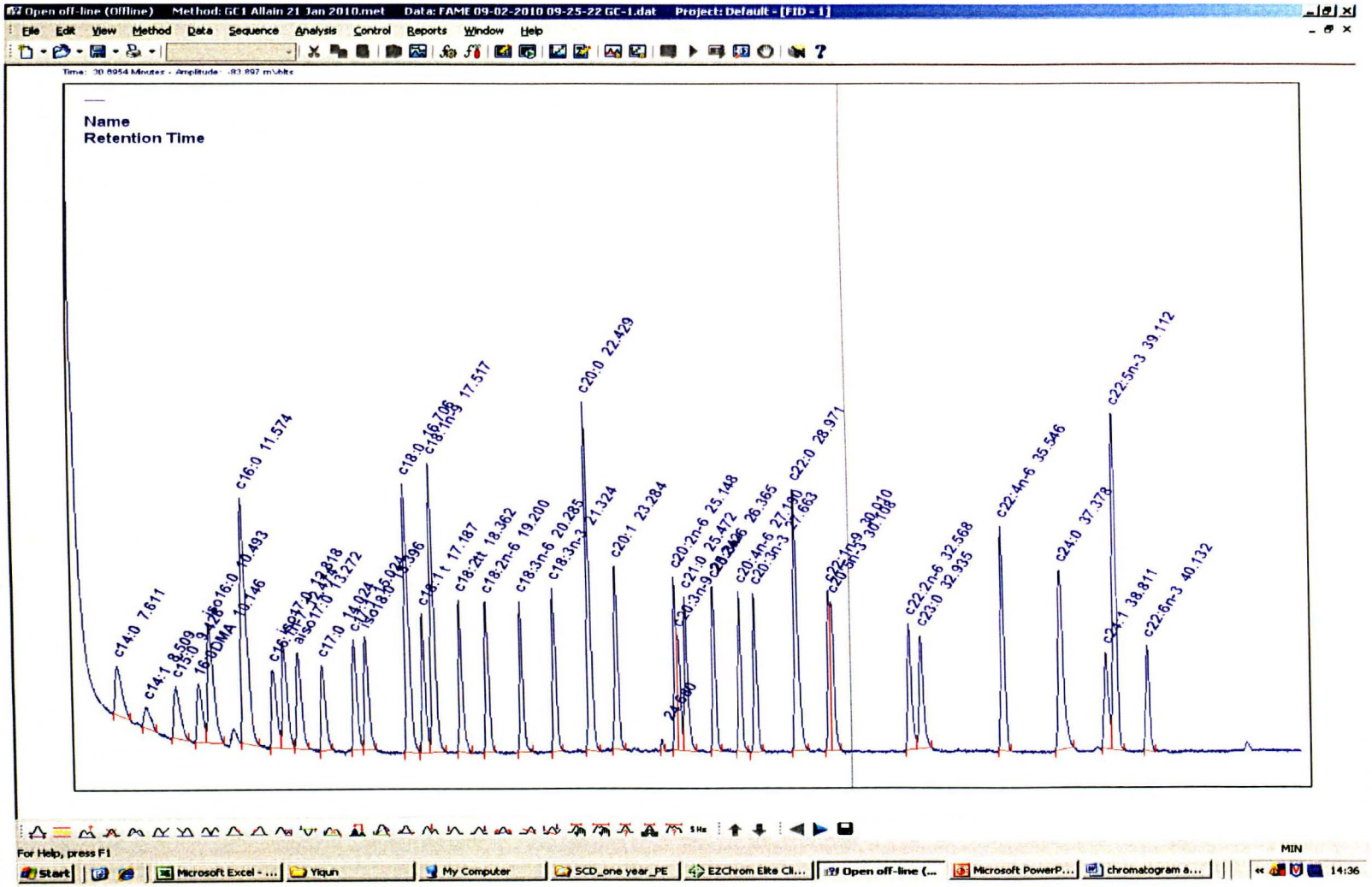
Chest:.....

Others: .....

Abdominal ultrasound:-

.....

Appendix 8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Retention Time
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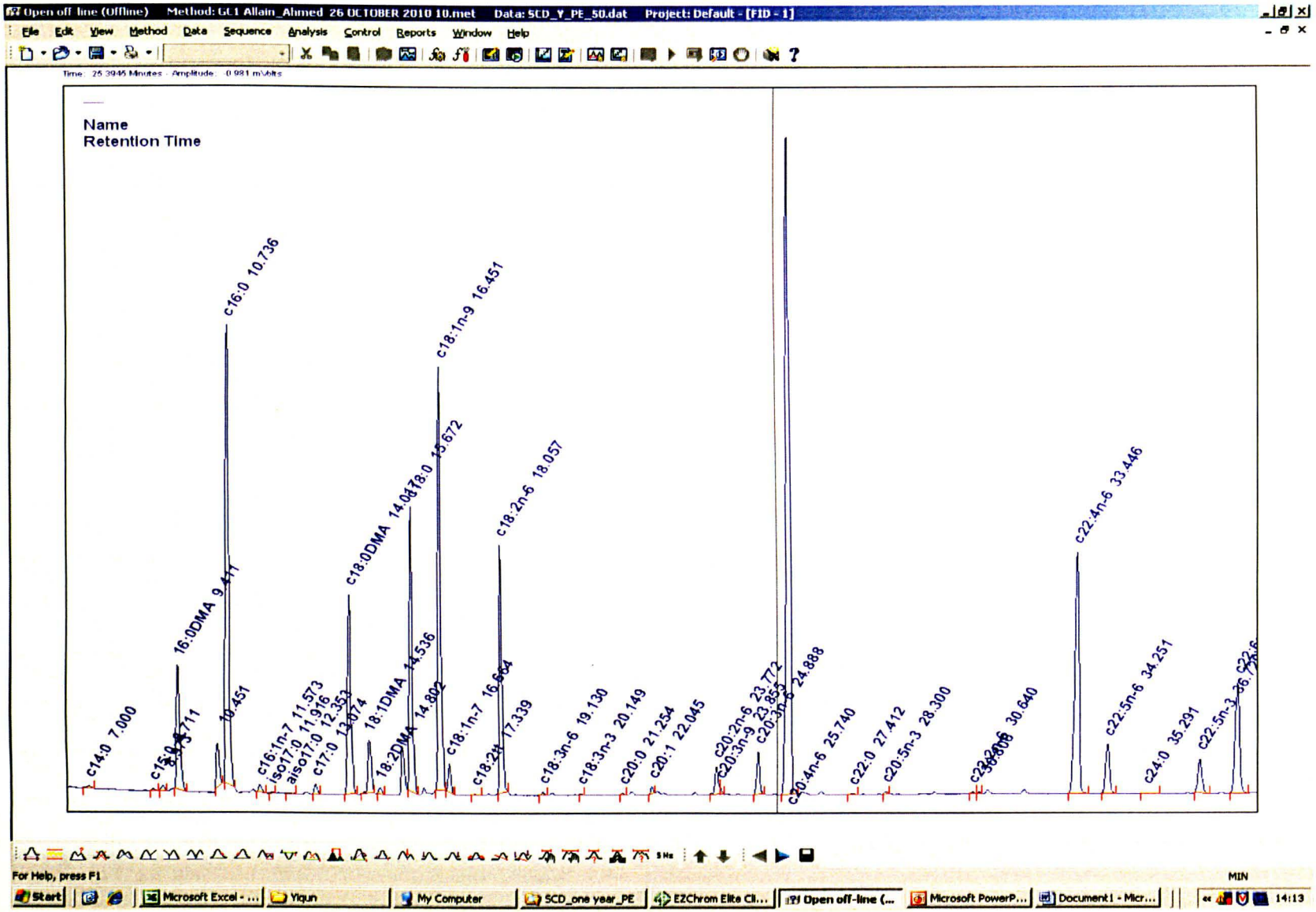
c14:1 8.509
c15:0 9.428
16:0DMA 10.146
c16:0 11.574
c16:1n-9 13.272
c17:0 14.924
c18:0 16.706
c18:1n-9 17.517
c18:1t 17.187
c18:2t 18.362
c18:2n-6 19.200
c18:3n-6 20.285
c20:0 22.429
c20:1 23.284
c20:2n-6 25.148
c21:0 25.472
c20:4n-6 26.365
c22:0 28.971
c22:2n-6 32.568
c23:0 32.935
c22:4n-6 35.546
c24:0 37.378
c22:5n-3 39.112
c24:1 38.811
c22:6n-3 40.132
For Help, press F1
MIN
Start
Microsoft Excel - ...
Yiqun
My Computer
SCD_one year_PE
EZChrom Elite Cli...
Open off-line (...
Microsoft PowerP...
chromatogram a...
14:36

Appendix 9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File Edit View Method Data Sequence Analysis Control Reports Window Help
Time: 25.3945 Minutes Amplitude: 0.981 mVolts
Name
Retention Time
c14:0 7.000
c15:0 8.711
16:0DMA 9.411
c16:0 10.736
10.451
c16:1n-7 11.573
iso17:0 11.916
aiso17:0 12.353
c17:0 13.074
c18:0DMA 14.018
18:1DMA 14.536
18:2DMA 14.802
c18:0 15.672
c18:1n-9 16.451
c18:1n-7 16.664
c18:2n-6 18.057
c18:2n-6 17.339
c18:3n-6 19.130
c18:3n-3 20.149
c20:0 21.254
c20:1 22.045
c20:2n-6 23.772
c20:3n-9 23.853
c20:3n-6 24.888
c20:4n-6 25.740
c22:0 27.412
c20:5n-3 28.300
30.640
c22:4n-6 33.446
c22:5n-6 34.251
c24:0 35.291
c22:5n-3
For Help, press F1
MIN
Start
Microsoft Excel - ...
Yiqun
My Computer
SCD_one year_PE
EZChrom Elite Cli...
Open off-line (...
Microsoft PowerP...
Document1 - Micr...
14:13