INTRODUCTION

β-thalassemia is an inherited hemoglobin disorder resulting from impaired production of β-globin chains of the hemoglobin tetramer ***(Olivieri, 2010)***. The resultant phenotype is chronic hemolytic anemia of varying severity, depending on the level of β-globin chain deficiency and subsequent α-globin chain accumulation. β-thalassemia major (β-TM) is characterized by severe transfusion-dependent anemia, starting from the first year of life ***(Aessopos et al., 2005; Stoyanova et al., 2012)***. The variable spectrum of sickle cell disease (SCD) is also the consequence of multiple events and genetic susceptibility that go beyond the occurrence of a single amino acid substitution in the β-globin chain of hemoglobin ***(Morris, 2008).***

A growing body of data suggests that thalassemia has many biologic and epidemiologic factors in common with SCD ***(Morris et al., 2008)***, including chronic hypoxia, long term effect of splenectomy, red cell membrane pathology ***(Atichartakarn et al., 2003; Phrommintikul et al., 2006)***, coagulation abnormalities, platelet activation ***(Eldor and Rachmilewitz, 2002; Singer et al., 2006)***, oxidative stress ***(Walter et al., 2006)***, iron overload ***(Fung et al., 2006)*** and chronic hemolysis ***(Vichinsky, 2005).*** Many mechanisms contribute to the complex pathophysiology of both thalassemia and SCD, with dysfunction of the vascular endothelium as a

unifying theme. Specifically, hemolysis-associated low arginine and nitric oxide (NO) bioavailability, amplified by NO synthase uncoupling, elevated arginase activity, superoxide production, oxidative stress, accumulation of arginine analogs, ischemia-reperfusion injury, inflammation, apolipoprotein A-1 depletion, and a hypercoagulable state lead to vasomotor instability and ultimately produce a proliferative vasculopathy ***(Morris, 2008; Rother et al., 2005)***. This vasculopathy is characterized epidemiologically by a clinical subphenotype of pulmonary hypertension, cutaneous leg ulceration, priapism, sudden death, and possibly stroke in SCD patients ***(Gladwin and Kato, 2005).***

NO is the most powerful endogenous vasodilator ever known. It can inhibit the adhesion, aggregation and recruitment of platelets; vascular smooth muscle cells migration and growth, also regulates some vessel-platelet interactions and limits the oxidation of atherogenic low density lipoproteins ***(Moncada et al., 1991).*** At the release site it mediates local vasodilatation, antagonizes platelet aggregation and inhibits vascular smooth muscle cell proliferation ***(Schmidt and Walter, 1994).*** In the kidney, NO dilates renal blood vessels and modulates renin secretion ***(Kone BC and Baylis, 1997)***. The constitutive endothelial NO synthase (eNOS), an enzyme that generates NO, is expressed in the endothelium, encoded by a 26 exon gene (NOS3) located on chromosome 7q35-36 with a total size of 21 kb and encodes an mRNA of 4052 nucleotides

(Nadaud et al., 1994). eNOS is a major determinant of endothelial function ***(Tolins et al., 1990).***

Several *eNOS* gene polymorphisms have been reported as 'susceptibility genes' in various human diseases states including cardiovascular, pulmonary and renal diseases ***(Kone, 2001).*** Among the reported polymorphisms of the *eNOS* gene, a polymorphism in intron 4 of the *eNOS* gene is a candidate gene in cardiovascular and renal diseases ***(Elshamaa et al., 2011).*** The variable number of tandem repeat (VNTR) polymorphism located in intron 4 of *eNOS* (*eNOS4b/a* polymorphism) was significantly associated with plasma NO concentration ***(Wang et al., 1997).*** There are two alleles identified in intron 4 of the *eNOS* gene. The larger allele, *4b*, consists of five tandem 27-bp repeats and the smaller one, *4a*, has four repeats ***(Wang et al., 1999)***. An association of the *4a* allele of the *eNOS* gene with coronary heart disease, renal disease and hypertension has been reported ***(Miyamoto et al., 1998; Buraczynska et al., 2004)***. Although impaired NO bioavailability is a common denominator in the pathogenesis of vasculopathy in hemolytic anemia ***(Morris, 2008)***, the specific role and clinical relevance of *eNOS* gene polymorphism remains to be elucidated.

AIM OF THE WORK

The aim of this study was to detect endothelial nitric synthase gene intron4 Variable Number Tandem Repeat (VNTR) polymorphism in patients with β-thalassemia major and SCD and its potential involvement in disease severity and various hemolysis-associated complications.

Chapter (1)

SICKLE CELL DISEASE

Sickle cell disease (SCD) is a wide spread hemolytic anemia that is due to a point mutation leading to a valine/glutamic acid substitution in the β globin chain, causing a spectrum of clinical manifestations in addition to hemolysis and anemia. Acute painful crisis is a common sequelae that can cause significant morbidity and negatively impact the patient's quality of life ***(Mousa and Qari, 2010).***

Sickle cell disease is one of the most prevalent genetic disorders. There are more than 200 million carriers of sickle cell trait worldwide ***(Inati et al., 2008).*** Sickle cell disease is a serious and life-threatening disease that affects approximately 1 in 600 African Americans ***(Rees et al., 2010).***

Historical review

The first description of SCD, published in 1910, was followed by six decades of genetic, hematologic, pathologic, clinical and molecular observations. Since the mid-1970s, two longitudinal prospective studies of children with sickle cell disease have produced a large body of clinical data on the evolution of the disease from birth ***(DeBaun et al., 2012).***

Herrick was the first to discover sickle cell hemoglobin (Alpha2 Beta-S2) with sickle-shaped erythrocytes. In 1910, he described the case of a young black student from the West Indies with severe anemia characterized by "peculiar elongated and sickle-shaped red blood corpuscles (Figure 1)." Herrick

also noted a slightly increased volume of urine of low specific gravity and thus observed the most frequent feature of sickle cell nephropathy: inability of the kidney to concentrate urine normally ***(Herrick, 2001).***

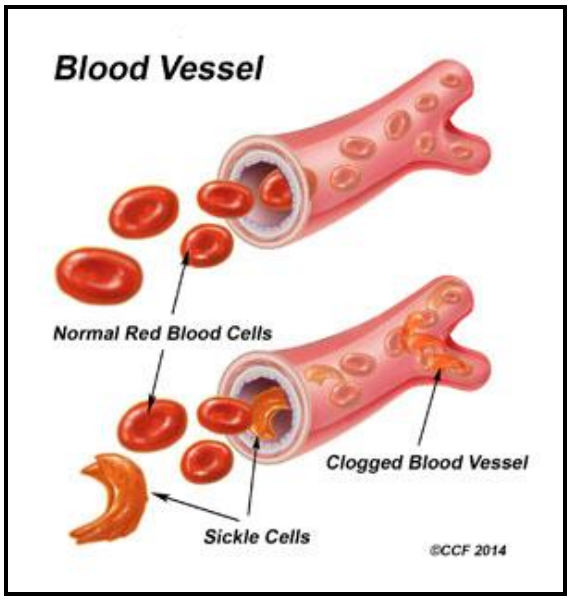

Figure (1): Shape of normal RBCs and sickled RBCs. ***(http://my.clevelandclinic.org/disorders/sickle_cell_anemia/hic_sickle_cell_anemia.aspx).***

In 1959, Ingram discovered the exact nature of the defect: substitution of valine for glutamic acid at the sixth residue of the alpha chain, establishing sickle cell anemia as a disease of molecular structure, "a molecular disease" based on one point mutation. It is most fascinating that one substitution in the gene encoding, with the resulting replacement of Alpha 6 glutamic acid by valine, leads to the protean and devastating clinical manifestations of sickle cell disease ***(Ingram, 1957).*** The chronological order of the important discoveries in SCD is shown in (Table 1).

Table (1): Important discoveries in the pathological and clinical features of sickle-cell disease in chronological order.

	Discovery	Importance
1910	Sickled erythrocytes in Grenadan dental student ***(Herrick, 1910).***	First description of disease linked to abnormal erythrocytes.
1924	Haemolysis in sickle-cell disease ***(Sydenstricker, 1924).***	Explanation for anaemia, jaundice, and cholelithiasis.
1924	Vaso-occlusion as cause of some pathological features ***(Graham, 1924).***	Explanation for ischaemic tissue damage.
1948	No symptoms in infants noted ***(Watson et al., 1948).***	Beneficial effects of high concentrations of fetal hemoglobin identified.
1949	Abnormal electrophoretic mobility of sickle haemoglobin ***(Pauling et al., 1949).***	Identified pathophysiology to have a molecular basis.
1951	Characteristics of polymerization of deoxygenated HbS ***(Perutz et al., 1951).***	Primary molecular mechanism identified.
1980s	Value of penicillin in young children with sickle-cell anaemia ***(John et al., 1984; Gaston et al., 1986).***	Reduced mortality, role of neonatal screening.
1984	Bone marrow transplant in child with sickle-cell anemia and leukaemia ***(Johnson et al., 1984).***	Identified potential cure.
1995	Efficacy of hydroxycarbamide ***(Charache et al., 1995).***	Only disease-modifying drug identified.
1998	Reduced stroke incidence in children with abnormal transcranialdopplers who were given blood transfusion ***(Adams et al., 1998).***	Primary stroke prevention with fall in stroke occurrence.

(Rees et al., 2010)

Definition and genetic basis

Sickle cell disease is an autosomal recessive abnormality of the β-globin chain of hemoglobin (Hb) that changes the sixth amino acid from glutamic acid to valine. The resulting HbS polymerizes reversibly when deoxygenated to form a gelatinous network that stiffens the erythrocyte membrane and increases viscosity, producing the characteristic sickle shape. Such sickled cells lose flexibility needed to traverse small capillaries and have "sticky" membranes that adhere to endothelium of small venules ***(Rees et al., 2010).***

Sickle cell disease was the first genetic disease to be characterized at the molecular level. The β-globin gene is located at the short arm of chromosome 11. The sole genetic problem in sickle cell anemia is a mutation of adenine to thymine in position 2 of the 6^{th} codon of β-globin gene, this change results in the substitution of glutamic acid in the 6^{th} position of β chain by valine (Figure 2) ***(Kutlar, 2007).***

The term sickle cell disease encompasses a group of symptomatic disorders associated with mutations in the hemoglobin β- globin gene (HBB gene) and defined by the presence of hemoglobin S (Hb S). Sickle cell anemia (homozygous Hb SS) accounts for 60%-70% of sickle cell disease in the US ***(Stuart and Nagel, 2004).***

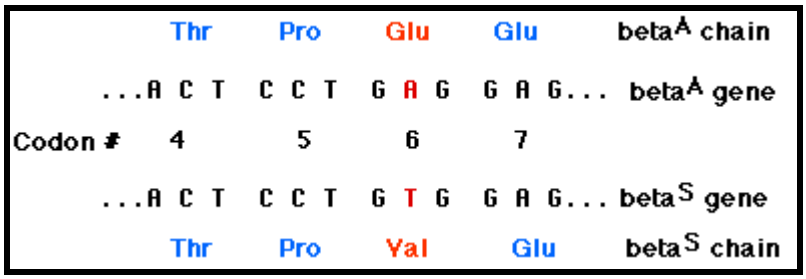

Figure (2): Amino acid position 6 substitution in beta-hemoglobin chain ***(http://www.sicklecellanemia2051.wordpress.com/structure-of-hemoglobin).***

Other forms of sickle cell disease result from coinheritance of Hb S with other abnormal β-globin chain variants, the most common forms being sickle-hemoglobin C disease (Hb SC) and two types of sickle β-thalassemia (Hb Sβ$^+$-thalassemia and Hb Sβ°-thalassemia); rarer forms result from coinheritance of other Hb variants such as D-Punjab and O-Arab ***(Tyagi et al., 2003)***.

SCD results from either the inheritance of two sickle beta globin genes or the inheritance of one sickle β gene in combination with another β globin chain defect. The most common form of SCD, hemoglobin SS (sickle cell anemia), is caused by the inheritance of hemoglobin S from both parents. Hemoglobin SC disease, hemoglobin S β$^+$ thalassemia, and hemoglobin S β° thalassemia are the other three common forms of SCD. Patients with hemoglobin S β$^+$ thalassemia and S β° thalassemia have a clinical picture that resembles SCD rather than thalassemia, because with the underproduction of normal β globin, the sickle β globin predominates. Sickle cell anemia (hemoglobin SS) and sickle β° thalassemia, conditions in which there is no normal β globin production, are generally more severe than hemoglobin SC and hemoglobin S β$^+$ thalassemia.

SCD is a clinically heterogeneous disorder, however, and many sickle cell anemia patients may have a clinical course that is milder than that of a hemoglobin SC patient ***(Tyagi et al., 2003)***.

Inheritance

The allele responsible for sickle-cell anaemia is autosomal recessive and can be found on the short arm of chromosome 11. In people heterozygous for HbS (carriers of sickling hemoglobin), the polymerisation problems are minor, because the normal allele is able to produce over 50% of the hemoglobin. In people homozygous for Hb S, the presence of long-chain polymers of HbS distort the shape of the red blood cell from a smooth doughnut-like shape to ragged and full of spikes, making it fragile and susceptible to breaking within capillaries. Carriers have symptoms only if they are deprived of oxygen (for example, while climbing a mountain) or while severely dehydrated. Under normal circumstances, these painful crises occur about 0.8 times per year per patient ***(Ballas and Lusardi, 2005).***

In the sickle β-thalassemias, mutation of the β^{A} gene results in a total inability to produce the normal β^{A} globin chain (β^{0}) or a reduction in its production (β^{+}). The child with sickle β -thalassemia inherits an S gene from one parent and a β -thalassemia gene from the other parent ***(Wethers, 2000).***

If the child inherits an S gene from one parent and another abnormal hemoglobin, such as D, G or O, from the other parent, other rarer variants result. The normal hemoglobin present in the child with sickle β^{+} thalassemia (less than 30

percent hemoglobin A) ameliorates the clinical picture. In general, this is the mildest variant of sickle cell disease, followed in severity by sickle hemoglobin C disease. Homozygous sickle cell disease and sickle β^0 thalassemia have a comparable spectrum of severity, and specific laboratory studies are needed to distinguish between the two conditions ***(Wethers, 2000).***

Types of sickle-cell disease

The different types of SCD are shown in (Table 2).

Sickle Cell Trait

There is a long-standing controversy in the literature as to whether sickle-cell trait (SCT) should be viewed as a benign carrier state or as an intermediate disease phenotype. Because SCT is routinely detected by neonatal screening for sickle-cell disease, it becomes imperative that consensus on this issue be achieved in order to provide the best medical advice to affected individuals ***(Key and Derebail, 2010).***

Sickle-cell trait (SCT) is the term used to describe the presence in an estimated 300 million individuals worldwide of a heterozygous glutamic acid-to-valine substitution in the β-globin gene on chromosome 11 (HbAS). In the United States, 6% to 9% of the African-American population and 0.01% to 0.05% of the remaining population (primarily those of Arab, Indian, Hispanic, and Mediterranean descent) are carriers of the β^s mutation ***(Key and Derebail, 2010).***

Overall, the evidence suggests that SCT may be neither a completely benign carrier state nor a true disease entity, but rather a risk factor for certain adverse outcomes that result from the interplay between genetic and environmental influences. Venous thrombosis and renal disease are among the manifestations under reevaluation ***(Key and Derebail, 2010).*** Until such time as these observations have been confirmed, expanding screening efforts must be considered to be of little benefit. Nonetheless, with ongoing newborn screening identifying individuals with SCT, furthering research to better characterize the consequences of SCT is of paramount importance to providing better counseling on any associated health risks ***(Mitchell, 2007; Cavanaugh and Lanzkron, 2010).***

Table (2): Different types of sickle-cell disease

Type	Characteristics
Severe sickle-cell disease:	
1- HbS/S (β6Glu>Val/β6Glu>Val); sickle-cell anaemia	• The most common form of sickle-cell disease
2- HbS/β^0thalassaemia	• Most prevalent in the eastern Mediterranean region and India.
3- Severe HbS/β^+thalassaemia	• Most prevalent in the eastern Mediterranean region and India; 1-5% HbA present.
4- HbS/OArab (β6Glu>Val/β121Glu>Lys)	• Reported in north Africa, the Middle East, and the Balkans; relatively rare.
5- HbS/D Punjab (β6Glu>Val/β121Glu>Gln)	• Predominant in northern India but occurs worldwide.
6- HbS/C Harlem (β6Glu>Val/β6Glu> Val/β, β73Asp>Asn)	• Electrophoretically resembles HbSC, but clinically severe; double mutation in β-globin gene; very rare.
7- HbC/S Antilles (β6Glu>Lys/β6Glu>Val, β23Val–Ile)	• Double mutation in β-globin gene results in severe sickle-cell disease when co-inherited with HbC; very rare.
8- HbS/Quebec-CHORI (β6Glu>Val/β87Thr>Ile)	• Two cases described; resembles sickle-cell trait with standard analytical techniques.
Moderate sickle-cell disease	• 25–30% cases of sickle-cell disease in populations of African origin.
1- HbS/C (β6Glu>Val/β6Glu>Lys)	
2- Moderate HbS/β^+thalassaemia	• Most cases in the eastern Mediterranean region; 6–15% HbA present.
3- HbA/S Oman (βA/β6Glu>Val, β121Glu>Lys)	• Dominant form of sickle-cell disease caused by double mutation in β-globin gene; very rare.
Mild sickle-cell disease	• Mostly in populations of African origin; 16–30% HbA present.
1- Mild HbS/β^{++}thalassaemia	
2- HbS/E (β6Glu>Val/β26Glu>Lys)	• HbE predominates in southeast Asia and so HbSE uncommon, although frequency is increasing with population migration.
3- HbA/Jamaica Plain (βA/β6Glu>Val, β68Leu/Phe)	• Dominant form of sickle-cell disease; double mutation results in Hb with low oxygen affinity; one case described.
Very mild sickle-cell disease	• Group of disorders caused by large deletions of the β-globin gene complex; typically 30% fetal haemoglobin.
1- HbS/HPFH	
2- HbS/other Hb variants	• HbS is co-inherited with many other Hb variants, and symptoms develop only in extreme hypoxia.

(Rees et al., 2010)

Incidence and geographic distribution:

The inherited disorders of hemoglobin are the most common gene disorders, and it is estimated that 7% of the world's population are carriers. Approximately 300.000 children worldwide are born with documented sickle cell disease every year. Sickling disorders are found frequently in the Afro-Caribbean population and sporadically throughout the Mediterranean regions, India, and the Middle East ***(Jeremiah, 2006).***

Four region-specific African haplotypes (the Senegal, Benign, Bantu, and Cameron haplotypes) and one Asian haplotype (the Arab-India haplotype) have been defined, providing support for the hypothesis that the mutation causing HbS has occurred, and been locally amplified, on at least two, and possibly several separate occasions ***(Rees et al., 2010).***

In addition to the close geographic correlation between the frequency of the HbS gene in populations and the historic incidence of malaria, evidence for the partial resistance of carriers to all forms of plasmodium falciparum malaria has been reported in many population ***(Williams et al., 2005).***Although the mechanism of this protection is yet to be fully understood, it probably includes both innate and immune mediated mechanisms ***(Wellems et al., 2009).***

The prevalence of SCD is highest in sub-Saharan Africa which is about 80% of the global total (more than 230000 affected children are born every year in sub-saharan region). In Egypt, along the Nile Valley, the HbS gene is almost non

existent, but in the western desert near the Libyan border variable rates of 0.38 per cent in the coastal areas to 9.0 per cent in the New Valley oases have been reported ***(Mohsen et al., 2011)***. HbS carrier rates vary from 9 to 22 per cent in some regions ***(El-Beshlawy and Youssry, 2009).*** By comparison, the yearly estimate of affected births in North America is 2600 and 1300 in Europe ***(Modell and Darlison, 2008).***

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

The highest frequency of sickle cell disease is found in tropical regions, particularly sub-Saharan Africa, India and the Middle-East ***(Weatherall and Clegg, 2001).*** Migration of substantial populations from these high prevalence areas to low prevalence countries in Europe has dramatically increased in recent decades and in some European countries SCD has now overtaken more familiar genetic conditions such as hemophilia and cystic fibrosis ***(Roberts and de Montalembert, 2007)***.