SICKLE CELL DISEASE IN YEMENI CHILDREN: CLINICAL, BIOCHEMICAL AND GENETIC ASPECTS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of

Doctor in Philosophy

BY

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DEDICATION

To my brother in law Mahmoud Al-Saqladi (35 years), early in his life departed in July 2008 before he could see the project that he supported morally and financially, successfully completed.

To my mother, my wife and my children Amjed, Mohammed, Abdullah, Sabah, Farid, Wael, and Wala, for their love, understanding, motivation and for being patient with my long absence.

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Declaration of work done

To undertake this study an arrangement was agreed for opening of a Sickle Cell Clinic in the hospital which was provided with essential staff. The researcher was responsible for the day to day running of the clinic and the clinical care of all SCD children attending and for evaluation and advice on the management of hospitalised cases.

Activity	Responsibility
Clinical history and examination	Sole
Anthropometry	Sole
Blood collection	Shared
Securing of specimen transport and storage	Sole
Blood counts and Hb electrophoresis	Laboratory staff
LFT, BUN, creatinine	Laboratory staff
Homocysteine, folate, B6, B12	Shared
sTfR, ferritin, CRP, SAA	Sole
MTHFR genotyping	Others
β^S cluster haplotyping	Others

ABSTRACT

Introduction and objectives: SCD is a global health issue and a public health problem in many countries. It is common in Yemen, but with scanty information on disease profile and outcome. This study aimed to describe the clinical characteristics and pattern of complications of SCD in Yemeni children and to investigate factors associated with disease severity.

Methods: A cross-sectional study of children (< 16 years), with SS haemoglobin confirmed by electrophoresis recruited at Al-Wahda Teaching Hospital, Aden. Data on clinical history and examination were obtained by direct interview. Severity was evaluated using a severity index (SI). Growth was assessed by anthropometry. Blood samples were collected for haematological, biochemical and genetic analysis. tHcy, folate, vitamin B6, B12, ferritin, sTfR, CRP and SAA were determined by chemiluminescent assay or ELISA. MTHFR genotype by PCR and β^S cluster haplotype by MCA.

Results: Of 102 children, 56 were male. Mean age was 7.2 years and 67% experienced disease manifestations as infants, mostly acute dactylitis. The main causes of hospitalisation were painful crisis (36%), anaemic crisis (16%) and acute chest syndrome (11%). Prevalence estimates were: hepatomegaly (72%), splenomegaly (40%), enuresis (44%), malaria (20%) and epistaxis (15%). Stroke, cholelithiasis, hepatic crisis and leg ulcers each occurred in about 5% of children. Consanguineous marriage occurred in 62.8%, with 32.5% first cousins. SI coded severe disease for 64%, who had higher frequency of pain crises, hospitalization and blood transfusion (all $p<0.001$). In severe cases Hb F % was lower ($p<0.01$), and PMN leukocytes, total and direct bilirubin were higher ($p<0.005$). Regression analysis showed association of severity with Hb F%, younger age at presentation and female sex. The homozygous TT genotype for MTHFR C677T was present in 2 %, and heterozygous CT in 10.8 %, with an allele frequency of 7.35 %. The T allele was not associated with raised plasma tHcy or increased disease severity. Mean (±SD) tHcy was 2.8 ±1.7μmol/l, increased with age and was highest in children >10 years (3.6±2.5 vs 2.5±1.2μmol/l, $p<0.05$). Whole blood folate and plasma vitamin B12 levels were normal or elevated in all children, and 4% had vitamin B6 deficiency. sTfR levels were markedly elevated (median 58.5mg/l) and negatively correlated with degree of anaemia ($p=0.004$) and positively with reticulocyte count ($p=0.002$), but not with ferritin or inflammatory markers (CRP, SAA). 25% of children had plasma ferritin < 100μg/L. Plasma CRP and SAA were elevated above reference values for the majority of children in steady state and were significantly higher during acute disease complications ($p=0.025$ and <0.001), but were not correlated with disease severity. Hypoalbuminemia occurred in 20.6%. Low weight, height and BMI for age (< -2 z score) were measured in 45%, 54% and 35% of children respectively. β^S cluster haplotyping showed the predominance of Benin haplotype in 82.6%, Bantu in 5.6% and atypical haplotypes in 11.9%. Haematological parameters and SI did not differ among the three haplotypes.

Conclusions: SCD is a serious health problem, affecting children in Yemen from an early age. Disease course and severity were similar to that in Africans and western Saudi Arabs. Severity was associated with age, gender and Hb F. Growth retardation was prevalent. Inflammatory markers were raised in steady state and more markedly during acute disease complications. sTfR was markedly elevated and correlated with degree of anaemia and erythropoiesis. The frequency of the MTHFR C677T genotype was low and the β^S Benin is the predominant haplotype in this sickle cell population. Opportunities for improving the health care of these children were identified.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACD	Anaemia of chronic disease
ACE	Acetate cellulose electrophoresis
ACS	Acute chest syndrome
ADC	Acute disease complication
ADMA	Asymmetric dimethylarginine
AGP	Alpha-1-acid glycoprotein
AI	Arab-Indian
APR	Acute phase reactant
AS	Sickle cell trait
ATP	Adenosine triphosphate
AVN	Avascular osteonecrosis
BCG	Bacillus Calmette-Guérin
BEN	Benin
BH4	Tetrahydrobiopterin
BMI	Body mass index
BMIZ	Body mass index Z-score
BUN	Blood urea nitrogen
C3c	Complement C3c fragment
CAM	Cameroon
CAR	Central African Republic
Cl^-	Chloride ion
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CSSCD	Co-operative Study of sickle cell disease
CT	Computerised tomography
CVA	cerebral vascular accident
CVS	Chorionic villous sampling
DHA	Docosahexaenoic acid
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPT	Diphtheria, Pertussis and Tetanus vaccine

DVT	Deep venous thrombosis
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
ESR	Erythrocyte sedimentation rate
FBP	Folate binding protein
FCP	F-cell production locus
FFM	Fat free mass
FSH	Follicle stimulating hormone
GDP	Gross Domestic Product
GH	Growth hormone
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAZ	Height for age Z-score
Hb	Haemoglobin
Hb A	Normal adult haemoglobin
Hb F	Fetal haemoglobin
Hb S	Sickle haemoglobin
Hib	Haemophilus influenzae type B vaccine
HIF-1α	Hypoxia-inducible factor-1α
HO-1	Haeme oxygenase-1
HPHP	Hereditary persistence of fetal haemoglobin
HPLC	High-performance liquid chromatography
HRP	Horse-radish peroxidase
HS I-V	Hypersensitive sites 1-5
hsCRP	high sensitivity C-reactive protein
HU	Hydroxyurea
HUSOFT	Hydroxyurea safety and organ toxicity
ICAM	Intercellular adhesion molecule
ID	Iron deficiency
IDA	Iron deficiency anaemia
IEF	Isoelectric focusing
IGF-I	Insulin-like growth factor-1
IgG	Immunoglobulin G

IL	Interleukin
ISC	Irreversible sickled cell
K^+	Potassium ion
KCC	Potassium-chloride co-transporter
LCR	Locus control region
LDH	Lactate dehydrogenase
LH	Luteinising hormone
Lu/BCAM	Lutheran blood group/basal cell adhesion molecule
MCA	Melting curve analysis
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MRI	Magnetic resonance imaging
5-MTHF	5-Methyltetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
MUAC	Mid-upper -arm circumference
NF-KB	Nuclear factor-Kappa B
NO	Nitric oxide
OD	Optical density
PCR	polymerase chain reaction
PHT	Pulmonary hypertension
PICP	Procollagen carboxy-terminal propeptide
PLGF	Plasma placenta growth factor
PLP	Pyridoxal-5'-phosphate
PMNs	polymorphonuclear leukocytes
PROPS	Prophylaxis with oral penicillin in children with sickle cell anaemia
PS	Phosphatidylserine
PSR	Proliferative sickle retinopathy
PUFA	Polyunsaturated fatty acids
Pyd	Pyridinoline
QTL	Quantitative trait locus
RDA	Recommended daily amount
REE	Resting energy expenditure
RFLP	Restriction fragment length polymorphism

RFLPs	Restriction site polymorphisms
SAA	Serum amyloid A
SAH	S-adenosyl-homocysteine
SC	Sickle cell C disease
SCD	Sickle cell disease
SEN	Senegal
SGOT	Serum glutamic oxaloacetic transaminase.
SGPT	Serum glutamic pyruvic transaminase
SI	Severity index
SNHL	Sensory neural hearing loss
SNPs	Single nucleotide polymorphisms
SPECT	Single photon emission computer tomography
SS	Homozygous sickle cell disease
sTfR	Soluble transferrin receptor
STOP	Stroke prevention trial in sickle cell anaemia
TCD	Transcranial Doppler ultrasonography
TfR	Transferrin receptor
Th	T helper cells
tHcy	Total plasma homocysteine
TIA	Transient ischaemic attack
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TSH	Thyroid stimulating hormone
UGT1A1	5'-diphosphate-glucuronosyltransferase 1A1
VCAM	Vascular cell adhesion molecule
WAZ	Weight for age Z-score
WHZ	Weight for height Z-score

CHAPTER ONE

INTRODUCTION AND AIMS

1.1 BACKGROUND

Sickle cell disease (SCD) is a global public health problem and one of the commonest genetic diseases worldwide. SCD is a group of hereditary haemoglobin disorders which incorporate two abnormal beta-globin gene variants of which at least one is the sickle cell gene. It includes four major genotypes: homozygous SCD (SS or sickle cell anaemia), sickle haemoglobin C (SC) disease, sickle cell β^+ thalassaemia ($S\beta^+$ thalassaemia), and sickle cell- β^0 thalassaemia ($S\beta^0$ thalassaemia) (Serjeant 1993). The most common and most severe form is the homozygous SCD condition which carried the two abnormal SS genes (Redding-Lallinger & Knoll 2006). The populations predominantly affected by SCD are those of African ancestry and also the Indian, Arab and some Mediterranean populations (Nagel & Fleming 1992). Due to population movement the disease has become a worldwide problem with about 275,000 affected children born annually (Modell & Darlison 2008). The numbers of affected individuals, in areas where the disease prevalence is considered low, as in European countries, is currently increasing (Roberts & de Montalembert 2007).

SCD is characterized by protean clinical manifestations which involve most body systems and result from a variable degree of chronic haemolytic anaemia with intermittent episodes of vascular occlusion that cause tissue ischemia and chronic organ damage (AAP. 2002). Affected peoples are prone to a wide range of acute and chronic complications and they require frequent medical care and hospitalization which causes a substantial burden on healthcare systems. Clinical features vary considerably between individuals with wide geographical diversity in disease

presentation and outcome. This variation is not fully understood and multiple genetic and environmental factors considered modulating this diverse phenotypic expression. The level of Hb F, co-inheritance of α-thalassaemia, and the β-globin gene cluster haplotype are well known modulators. New single nucleotide polymorphisms (SNPs) have been identified and have been related to particular phenotypic events (Steinberg 2009). Although, SCD was the first molecular disease described, there remains a lack of progress for many aspects of this disorder (Hagar & Vichinsky 2008)).

Current concepts in the pathophysiology of SCD shed a light on the role of red cell dehydration, cell activation and adhesion, inflammation, oxidative stress, chronic haemolysis and reduced bioavailability of nitric oxide (NO), which could provide new targets for potential therapeutic interventions (Kato *et al,* 2009).

In the Arabian Peninsula the sickle cell gene is prevalent and some areas such as Eastern Saudi Arabia, sickle cell trait frequency reaches 20% (El-Hazmi & Warsy 1999b). The initial study on the frequency of sickle cell trait in Yemeni individuals reported a frequency of 0.95% (White *et al,* 1986). The 1260 participants in that study comprised mainly pregnant females who were living in UAE, with blood specimens collected during their routine antenatal visits. A higher prevalence has been reported in a more recent pilot study which screened 1500 cord blood samples from hospitals in Sana'a city, Yemen (Al-Nood *et al,* 2004). The AS carrier prevalence was 2.2%, but varied between <1% to 10%, between different parts of the country according to parental area of origin. The estimated incidence of affected homozygous births was 20/10,000 live birth. The clinical profile and severity of SCD in Yemeni children has not been previously described, and there is a lack of information concerning biochemical and genetic aspects in this population.

1.2 AIMS

General

To describe the clinical features, severity and complications in SCD in Yemeni children and to examine their association with biochemical and genetic factors.

Specific

1. To describe the clinical characteristics, age and mode of presentation, type and frequency of clinical events and complications.
2. To assess the disease severity profile and its correlation with haematological and biochemical parameters.
3. To estimate the prevalence of MTHFR C677T mutation and to determine its association with plasma tHcy, folate, vitamin B12 and B6, and with disease severity.
4. To assess sTfR concentrations in relation to disease severity and its correlation with plasma ferritin and markers of inflammation.
5. To determine the level of acute phase proteins (CRP and SAA) in steady state or during acute disease complications, and their association with disease severity.
6. To assess growth status in relation to age, gender, disease severity, haematological and biochemical parameters.
7. To determine the type and frequency of β^S cluster haplotypes and their association with haematological and clinical profiles.

1.3 THESIS STRUCTURE

Chapter Two is a review of relevant literature and includes an historical overview of important milestones and discoveries in SCD. Current concepts in disease pathophysiology are briefly explained. The epidemiology of the disease is described with emphasis on the regional prevalence of β^s gene in countries of the Arabian Peninsula. Clinical features and systemic complications are summarised and the principles of management described. In **Chapter Three** the methods including study design, definitions of clinical events, severity scoring system, laboratory analysis and ethical clearance are described. In **Chapter Four**, the clinical profile, age and type of presenting symptoms, frequency and type of clinical events and complications are documented. Frequencies of clinical manifestations are compared with other regions. New and peculiar findings are highlighted. **Chapter Five** described the results of the severity assessment using a Severity Index (SI) with distinguish disease severity category. Factors associated with severity are analyzed and results are compared with findings from studies in other populations. In **Chapter Six**, the frequency of the MTHFR C677T mutation is determined and its association with disease severity, vascular complications, plasma level of homocysteine and related vitamins are evaluated. **Chapter Seven** examines sTfR concentration and its association with severity, degree of anaemia and inflammatory markers. The plasma levels of CRP and SAA in different clinical situation and in relation to severity are evaluated and discussed in **Chapter Eight**. Anthropometric measurements are compared to reference values and other comparative studies and are described in **Chapter Nine**. In **Chapter Ten**, the frequency and type of beta globin gene cluster haplotypes are outlined. **Chapter Eleven** discusses the main study findings, its limitations and conclusions and recommendations for future work.

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORICAL OVERVIEW

The symptoms of sickle cell crisis were known by various names in Africa, long before they were recognized in the western literature. According to Konotey-Ahulu, the disease was known in Ghana as (chwechweechwe), and in the Manaya Korbo tribe it can be traced back through three centuries (Konotey-Ahulu 1974). The first published description of sickle cell features has been attributed to Africanus (1874), who described a condition of recurrent rheumatic pain which was exacerbated during the cold rainy season and was associated with fever and symptoms of anaemia. The first case of SCD was reported by Herrick (1910) in a young anaemic student from Grenada named Walter Clement Noel. He had come to Chicago to study dentistry at the School of Dental Surgery. He was admitted to Presbyterian Hospital with severe respiratory distress under the care of the intern on duty Dr Ean E. Iron who examined the blood smear and observed many pear-shaped elongated red cells. Iron reported these findings to Herrick who was the attending physician. The blood test was repeated several times and on different occasions during follow up over the next 30 months. The appearance was considered an exaggerated form of poikilocyosis and not a characteristic of a new disease. Their clinical findings were accurate and described a typical clinical picture of sickle cell disease, including anaemia, heart enlargement, jaundice, fever, abdominal pain, arthritis and leg ulcers. They consulted other colleagues and searched the literature for similar cases, but were unable to reach a diagnosis (Savitt & Goldberg 1989). In their literature search they identified an article in a German journal written in 1905 by M. Lowitt which described a staining technique on three blood specimens from

anaemic patients identifying unusual red cells that they named "sichel formen" but without any details of the patients from whom the blood was taken (Haller *et al,* 2001).

By preparing a cell culture from a patient with this condition Emmel (1917), demonstrated that the red cells become elongated and crescentic in shape when allowed to stand in room temperature for several hours. This change was observed even in apparently normal cells before incubation, which retained a potential to transform into the sickle-shape. The same alteration occurred in the blood taken from the patient's father, which pointed to a genetic base for the disease.

Mason (1922) reviewed the three previously reported cases separately reported by Herrick (1910), Washburn (1911), and Cook and Meyer (1915), and a fourth case reported by himself. He noted strong similarity in the clinical picture and blood changes for these cases. These were specified and distinguished from other known causes of anaemia, and the possibility emerged that this was a new clinical entity and the name of sickle cell anaemia was used for the first time. Since all reported cases at that time were in black people, Mason assumed that the disease was related to the black race.

Huck (1923) noted that sickling occurred in the blood of asymptomatic parents of anaemic patients and analysis of the patients pedigree led him to conclude that the disease was genetic, resulting from dominant Mendelian inheritance, and occurring in both male and female patients. Huck also showed that allowing red cell preparations to stand for 24 hours at room temperature induced sickling changes with a variable rate, from 25% in asymptomatic, 75% in mildly symptomatic, to 100% in severe cases. Normal red cells remained unsickled when placed in sera of affected

anaemic patients, whereas red cells from affected anaemic patients became sickled in shape in normal serum, indicating that the disorder was related to the red cell.

Sydenstricke (1924) noted the difference between a "latent" form, which was more common with fewer symptoms, and an "active" form with a characteristic disease profile and blood which showed obvious sickling changes. Support for the hereditary basis for the disease was suggested by the presence of sickled cells in cord blood and in infant blood of mothers with characteristic features of sickle cell disease. In addition to the detailed clinical description of reported cases, spleens of patients were found on necropsy to be small, atrophic with multiple infarcts and abundant pigmentation. Sydenstricke also noticed the high reticulocyte count and bone marrow expansion associated with active in vitro phagocytosis of erythrocytes, suggesting an accelerated destruction of red cells by the spleen.

Hahn and Gillespie (1927) reported that sickling of these red cells could be induced by low oxygen tension, reversed by cell exposure to carbon monoxide and retarded by lowering temperature. They noted that most of the red cells in anaemic patients were sickled, whereas in those with sickle cell trait, sickling only occurred if oxygen and hydrogen ion concentration were reduced. The changes of shape and appearance of red cells were described in detail. Sickled cells started to swell, retracted and regained the normal transparency on exposure to oxygen. The authors concluded that there was a clear distinction between patients with the disease and those with trait. In vivo hypoxia was considered to cause sickling with cell distortion increasing susceptibility to haemolysis leading to anaemia. They considered there were difficulties using the term sickle cell to include the different forms of presentation and haematological findings, and proposed the term "drepanocytemia" (from Greek drepan "sickle") to replace the term sickle cell.

The occurrence of anoxia induced by sickling was demonstrated in vivo (Scriver & Waugh 1930) by tying the finger of patients proximally for 5 minutes with a rubber band in order to produce venous stasis. Blood collected from the distal end of the finger showed that the erythrocytes sickled more rapidly and in greater number than blood from controls without stasis.

The red cells from patients with sickle cell anaemia undergo a series of progressive morphological changes. Diggs and Bibb (1935) described this progressive structural transformation from discoid to irreversible sickled forms with many other bizarre shapes. This shape alteration could revert to round forms by exposure of the preparation to air, but other cells did not revert and showed fixed alteration with "irreversible sickling". In this study sickling was demonstrated in all tissues but mainly in the spleen and bone marrow. The small blood vessels appeared to be distended with sickled cells, indicating probable interference with blood circulation explaining the pathogenesis of the disease.

The role of oxygen tension in the sickling phenomena in vivo was demonstrated by the observation of a higher percentage of sickled cells in venous than arterial blood. Red cells from cases with sickle cell trait required a lower oxygen tension than those from patients with the disease which could explain why sickling occurred in patients with disease and not in those with the trait (Sherman 1940). A further important observation reported in this study, was the birefringence of deoxygenated sickle cells under polarized light, suggesting that low oxygen tension altered the structure of haemoglobin molecules although this interpretation was not considered in the original paper.

The paucity of sickled cells in the blood of young infants born to mothers with sickle cell anaemia was about 6% of red cells at birth, increasing gradually to

90% at 4-5 months. This lead to the suggestion that these differences were due to the presence of Hb F in the infant red cells and sickling was a feature of adult haemoglobin (Watson 1948).

It was several years before the hypothesis of sickle cell inheritance, which was thought to result from a single dominant gene with variable expression, was challenged by Neel (1949), who proposed heterozygous and homozygous gene inheritance. Examination of 42 parents of 29 patients with sickle cell anaemia using the sickling test found all to be positive supporting the homozygous-heterozygous hypothesis. A similar result at this time was reported by Beet (1949), who described a family from a Bantu tribe in North Rhodesia, although Mendelian dominance was still generally considered as the mode of inheritance.

Sherman suggested that the sickling process was related to the state and nature of haemoglobin, which was distributed uniformly if in a combined state, but aggregated if uncombined, in a way which caused distortion of the cell membrane leading to birefringence (Sherman 1940). Following a conversation between Linus Pauling and William Castle in a shared train compartment while returning from a meeting in Denver, Castle drew the attention of Pauling to the work of Sherman and to the birefringence of sickle red cells. He assumed that the sickling phenomena could be related to an abnormal haemoglobin in red cell (Feldman & Tauber 1997). Pauling and collaborators (1949) using the modified Tiselius electrophorotic apparatus demonstrated that the haemoglobin from patients with sickle cell anaemia and sickle cell trait was defective and termed it sickle cell anaemia haemoglobin. It was present in 100% of patient red cells, whereas in trait it was a mixture of normal with about 40% sickle cell Hb. This established SCD as the first known molecular disease in medicine. Hb S was evident when examined under the polarized

microscope and could be further demonstrated by x-ray diffraction (Perutz & Mitchison 1950, Perutz *et al,* 1951). The axis of crystals was found to be parallel to the length of the cells and the combination of reduced solubility and crystallization of Hb S was assumed to be the reason for the changes in red cell shape. The combination of electrophoresis and partition chromatography enabled the detection of the differences in peptide chains between Hb A and Hb S. Running of the same haemoglobin several times always reproduced the same pattern, by a technique which described the protein as "finger-printing" (Ingram 1956). Peptides derived from Hb A contained more glutamic acid, and those of Hb S more valine suggesting that the difference was only in a single amino acid substitution with valine replacing the glutamic acid residue in the peptide chains of Hb S (Ingram 1957). A sickle cell mutation in the beta chain at amino acid 6 was found to be consistent with a single base nucleotide transversion from GAG to GTG (Marotta *et al,* 1977).

Dried filter paper blood spots used as a method for neonatal screening were found to be applicable for Hb AS detection (Garrick *et al,* 1973), and this became the most common method of sample collection for newborn SCD screening programs. Kan and co-workers reported an approach to prenatal diagnosis of SCD by examination of amniotic-fluid cells (Kan & Dozy 1978). Subsequently the same group determined fetal globin genotypes using fetal cells isolated from the maternal circulation (Cheung *et al,* 1996).

Bone marrow transplantation in SCD was first reported in an 8 year old black girl with acute myeloblastic leukaemia who was successfully transplanted with the bone marrow of her AS brother for treatment of her leukaemia. The sickle cell condition of this girl was also improved and the Hb S level reduced to that present in the marrow donor (Johnson *et al,* 1984).

Prophylaxis with oral penicillin in children with sickle cell anaemia (the PROPS trial) was terminated 8 months early, after the occurrence of three deaths from pneumococcal septicaemia in the placebo group with no deaths in the penicillin group. Penicillin prophylaxis was associated with 84% reduction in pneumococcal septicaemia and has become a routine preventive measure in children (Gaston *et al,* 1986).

The efficacy of hydroxyurea in SCD adults was demonstrated in a multicenter study of hydroxyurea in the early 1990's (Charache *et al,* 1995), and subsequently this treatment was approved by the Food and Drug Administration (FDA) Board in the USA in 1998 and this has become a generally accepted prophylactic measure.

The usefulness of chronic transfusion for primary prevention of initial stroke episode was demonstrated in the stroke prevention trial in sickle cell anaemia (STOP) (Adams *et al,* 1998). One year into the study the trial was halted due to the occurrence of ten cerebral infarctions and one intracerebral hematoma in the standard-care group compared with a single patient with infarction in the transfusion group.

2.2 GLOBAL EPIDEMIOLOGY OF SCD

SCD is a global public health issue, and is one of the commonest genetic disorders worldwide with about 275,000 children born annually with the disease (Modell & Darlison 2008). Prevalence of SCD at birth depends on the frequency of sickle cell trait. In equatorial Africa this ranges between 10% to 40%. In Nigeria the carrier state occurs in about 24% with 150,000 affected children born annually. In spite of improving survival rates in affected children in developed countries, in Africa it is estimated that in SCD between 9% to 16% of deaths in under-fives may

be due to the disease (WHO 2006). In some West African countries 50% of children with SCD have died by the age of five years frequently from complicating infections.

Sickle cell gene is found at high frequency in the Arabia Peninsula and in some parts of India. It occurs in lower frequencies in Mediterranean populations. It is reported in Iraq, Iran, Afghanistan, Georgia, Azerbaijan, Turkmen and Tadzhikistan. In the Americas prevalence is high in blacks in the USA, Canada, the coastal areas in Mexico, central America, the Caribbean and in several south American countries including Colombia, Venezuela, Guyana, Surinam French Guinea, Brazil and Peru (Nagel & Fleming 1992).

In Europe in recent decades, with greater migration and increased ethnicity diversity enhancing the size of affected population, SCD has become more common in Northern European countries including France, the Netherlands, UK, Belgium and Germany (Modell *et al,* 2007). In the UK, SCD is estimated to affect more than 1 in 2,400 births in England, with about 240,000 healthy carriers and 12,500 with the disease (NHS 2006).

2.3 EPIDEMIOLOGY OF SICKLE GENE IN THE ARABIAN PENINSULA

The Arabian Peninsula is located in Southwest Asia at a junction with Africa and is bound in the North by Jordan and Iraq, the East by the Persian Gulf and the Gulf of Oman, the South by the Arabian Sea and the Gulf of Aden, and the West by the Red Sea. It consists of seven independent states, including Saudi Arabia, Yemen, Oman, the United Arab Emirates (UAE), Qatar, Kuwait, and Bahrain (Encyclopedia Britanica, 2009).

The initial report on the distribution of the sickle gene in the Arabian Peninsula was that of Lehman et al, (1963), who noted a high frequency of sickle cell trait (10-25%) among residents of the Eastern province of Saudi Arabia, contrasting

with 1% in settlers who had originated from other areas. An interrelation between the distribution of the sickle gene and malaria endemicity in this area, which contains a well-watered oasis in Qatif and Al-Hasa, has been assumed. The majority of affected individuals had a mild disease characterized by minimal manifestations, normal physical growth and persistent splenomegaly (Gelpi 1970). This mild expression was considered attributed to the co-presence of thalassaemia or G6PD deficiency, but subsequently a report from a different area of the Peninsula, which included six children from two unrelated Arab families from Kuwait, confirmed the occurrence of a milder variant of SCD (Ali 1970). This difference was attributed to the presence of high levels of Hb F (13-35%) in milder cases.

A detailed report which described the natural history of SCD in 270 SS patients from Eastern Saudi Arabia, (Perrine *et al,* 1978) found that many cases remained asymptomatic in early life with diagnosis delayed in some until 15 years of age. Serious complications occurred in less than 25% and mortality was low. Death was rare in early childhood with fewer deaths occurring in adolescents and young adults, who usually showed spleen enlargement into adulthood. The high levels of Hb F together with α-thalassaemia were suggested as explanations for their modified course of SCD.

Conversely, a non-benign form of SCD in the Western Province of Saudi Arabia was reported in a study of 71 patients (36 Saudis and 35 Yemeni) (Acquaye *et al,* 1985). Many of these experienced severe anaemia, recurrent infection, bone pain and other serious complications including hepatic crisis, acute chest syndrome, and stroke. A more severe picture occurred even in cases with Hb F level above 10%. Furthermore, a study of the clinical and haematological features of these two distinctive groups comprising 28 SCD from the Eastern Province of Saudi Arabia

showed that those of South-Western ancestral origin retained the severe form of the disease, compared to the milder form in those from the Eastern area (El Mouzan *et al,* 1989). Those most affected were more likely to present with anaemia, experience severe infection and require more frequent blood transfusion and hospital admission, with a similar clinical presentation to American or Jamaican patients. The authors suggested that environmental factors such as climate, exposure to infectious agents, and type of medical care, played a lesser role than genetic factors in determining severity.

In order to clarify these regional differences, comparison of haematological, clinical and some molecular genetic features of 33 SS patients from the Eastern Province and 30 patients from the South Western Province confirmed the presence of two different clinical forms of SCD (Padmos *et al,* 1991). Western patients had the more severe picture with frequent dactylitis and acute chest syndrome, whereas painful crises and avascular hip necrosis occurred equally in both groups. All patients from the Eastern region carried the Arab-Indian haplotype contrasting with the mostly African Benin haplotype in those from the Western region (21/28). Deletional α-thalassaemia was almost twice as common in cases from the Eastern region, (37.5% vs 19.6%).

Determination of the factors responsible for the high Hb F levels lead to the examination of Hb F synthesis in SS patients from Saudi Arabia (n= 22) compared to cases of an African origin (n= 22) (Wood *et al,* 1980). Gamma chain synthesis in the Saudi group ranged between 4.0% to 19.9% (mean 8.1%) and in the African group between < 0.3% to 4.6% (mean 1.7%). In both groups peripheral blood Hb F levels were 3-4 times higher than the estimated proportion synthesized indicating selective improved survival of Hb F containing red cells (F cells). Molecular analysis

of the promoter regions of the Hb F G gamma and A gamma globin genes among Saudi patients with high levels of Hb F, showed a single-base cytosine-to-thymidine substitution at position 158, 5-prime to the G-gamma-globin gene (Miller *et al,* 1987). The substitution was present in nearly 100% of patients with SCD or trait and in 22% of normal Saudis. As this mutation had no demonstrable effect on haemoglobin F production in the normal population, its role in regulating haemoglobin F production required interaction with additional factors such as haemolytic stress, or possibly with other molecular determinants linked to the sickle cell gene.

In the Arabian Peninsula the frequency of SCD is highly variable between different countries and among regions within the same country. Neonatal SCD screening in three hospitals in the Eastern Province of Saudi Arabia enabled detection of 47 affected babies with an FS phenotype in the first 17 months of the program (Al-Awamy *et al,* 1984). The prevalence of sickle cell trait was varied by locality from 4.4% in Al Khobar, 6.7% in Dammam to 17.9% in Qatif. The number of affected cases was higher than expected from the gene frequency which was attributed to high prevalence of sickle β^0 thalassaemia and effects of non-random mating from frequent consanguineous marriage.

Genetic analysis of β^S globin gene using restriction site polymorphisms (RFLPs), enabled determination of beta S characteristic haplotypes in six different population groups from Africa and Asia showing that chromosomes of individuals from the Eastern oases of Saudi Arabia and from the West and the East coast of India were a different haplotype to that found in Africa. This was assumed to represent an independent Asian origin of the sickle cell mutation (Kulozik *et al,* 1986). The distribution of this Asian (Arab-Indian) β^S haplotype corresponded with the reported

geographic distribution of the mild clinical SCD phenotype associated with high levels of Hb F.

National Screening Programme for sickle cell and thalassaemia in the Saudi population commenced in 1982, and analysis of the first 30055 blood samples showed a wide variation in gene distribution amongst different geographic regions (El-Hazmi & Warsy 1999b). Frequency of sickle gene was highest in the Eastern Province (0.114), followed by the South Western (0.076), then North-Western Province (0.046), with the lowest frequency in the Central Region (0.005). Sickle cell trait (AS) showed a range 0.08% to 21.3%.

Studies from other countries of the Arabian Peninsula are fewer than those from Saudi Arabia. An early report in 1986 included 5060 subjects from three countries; Yemen (n=1260), the United Arab Emirates (n=2750), and Oman (n=1050). The frequencies of the sickle gene were 0.95%, 1.9% and 3.8% respectively (White *et al,* 1986). In the United Arab Emirates analysis of 500 blood samples of unrelated healthy adult males from various military institutions in Abu Dhabi showed that 2.4% were heterozygous AS (Kamel *et al,* 1980). A higher percentage (4.6%) was reported in a cross-sectional survey of 496 preschool children (Miller *et al,* 2003). A pilot SCD neonatal screening programme in Abu Dhabi, Al-Ain and the Western Region Medical Districts was launched at 2002 using HPLC for diagnosis (Al Hosani *et al,* 2005). Examination of 9165 newborn samples over one year period gave incidence estimates of 0.07% (SS), 1.49% (AS), 0.4% (AD) and 0.03 % (AC).

In Omani subjects analysis of 952 samples showed positive sickling tests in 58 all of which were heterozygotes AS, giving a frequency of 6.1% with an estimated incidence of affected homozygotes of 3.7 per 1000 live births (White *et al,*

1993). A review of symptomatic haemoglobinopathies from the National Register of Oman reported the birth prevalence of symptomatic beta-globin disorders as 3.1 per 1000 live births, including 2.7 per 1000 live births for homozygous SCD, with an AS carrier frequency of 10% (Rajab *et al,* 2000). A lower estimate was obtained in a community-based survey of hereditary blood disorders in Omani children (<5 years) which examined 6342 blood samples. Prevalence of sickle cell trait was 5.8% with significant regional variation, being highest in North Sharqiya (10.1%) and Al Dakhiliyah (9.3%), and lowest in Dhofar (0.2%) (Al-Riyami *et al,* 2001). First cousin consanguinity was high (33.6%) and significantly associated with all homozygous blood disorders. In a separate study clinical severity was mild in 66% of 375 Omani SCD children and associated with high Hb F levels (mean 15.84±9.5%), with few documented cases of acute chest syndrome or stroke, and about one third experiencing moderate to severe disease expression (Al-Lamki *et al,* 2000). These mixed clinical features in Omani patients reflected the co-existence of the three major haplotypes, the Benin (typical and atypical) 52.1%, the Arab-India 26.7%, and the Bantu 21.4%, (Daar *et al,* 2000). Linking this mixed polymorphism to historical records suggested that the sickle cell gene arrived in Oman through gene flow, with the Arab-India haplotype related to ancient contacts with Iraq, Iran, the Indus Valley and India. Its presence in the Arabian Peninsula strengthened the hypothesis that this Indo-European form of the beta S gene may have originated in the Harappa culture, or nearby population, and migrated during the Sassanian Persian Empire to the Eastern parts of the Arabian Peninsula. While the Benin haplotype was linked to contacts with the Sub-Saharan West Africa, and the Bantu haplotype to the more recent contacts with East Africa, Mombasa and Zanzibar, in present day Tanzania.

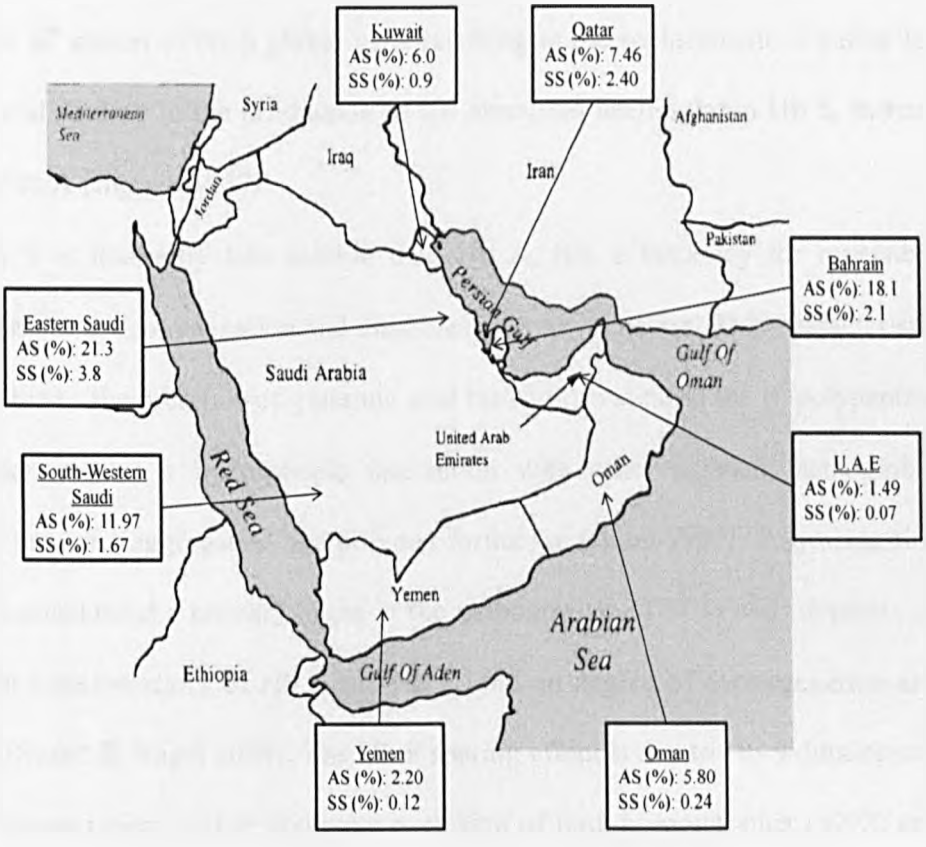
The initial report on SCD from Bahrain was by Buhazza et al., (1985). The haematological findings of 50 patients were evaluated and 21 newborns with 20 normal adults as controls. Patient mean Hb F level was 13.8%, which was lower than reported from Kuwait or Saudi Arabia. AS trait was detected in 3 of 20 adult controls (15%) and despite the small sample size this result suggested a high SCD prevalence. This was supported in a separate study using neonatal cord blood screening (n= 10,327) which gave an estimate of 2.1% homozygous SS and 11.2% heterozygous AS (Mohammed *et al,* 1992). A further neonatal analysis (n= 5503) gave a higher estimate for AS prevalence (18.1%) with the same frequency for SS (2.1%) (Al-Arrayed & Haites 1995). A screening study of 5685 students reported 1.2% SS and 13.8% AS prevalence, with regional variation and highest prevalence in the Western region (23%) and lowest prevalence in Hidd (2.7%) (Al-Arrayed *et al,* 2003). The clinical manifestations in Bahraini patients were generally mild, associated with a predominant Arab-Indian haplotype (90%), and higher levels of Hb F (2-40%).

An early study from Kuwait of 193 random blood samples from Bedouin tribes (Suluba and Ajman) and townsmen reported sickle cell trait prevalence in the general population of 2.3% with estimated Hb S gene frequency 0.01% (Al-Nassar *et al,* 1981). More recent haemoglobin electrophoresis studies (n= 2386) identified sickle cell trait in 6%, SS in 0.9%, $S\beta^0$ thalassaemia in 0.8% and $S\beta^+$ thalassaemia in 0.8% (Marouf *et al,* 2002). Except for a sub-set of patients with frequent painful crisis and osteonecrosis (detected by MRI technique) the course of SCD in Kuwaitis was usually mild, and attributed to high levels of Hb F (mean 22.8 ±5.7%), high prevalence of α-thalassaemia trait (48%), and predominance of the β^S AI haplotype (Adekile 2001). Acute painful crises were the most common cause for hospital

admissions in children and severe complications were rarely observed, such as stroke, hand foot syndrome, priapism, or leg ulcers (Akar & Adekile 2008).

In Qatar analysis of all haemoglobin electrophoreses performed in the main hospital, including 1702 Qatari nationals, reported a prevalence of AS 7.46%, SS 2.40% and Sß^{0}thal 3.94% , with 70% of SS patients showing high Hb F levels (Fawzi *et al,* 2003).

Figure 2. 1 Frequency of sickle cell gene in countries of the Arabian Peninsula



Data sources: Saudi Arabia (El-Hazmi & Warsy 1999b); UAE (Al Hosani et al, 2005); Oman (Al-Riyami et al, 2001); Bahrain (Al-Arrayed & Haites 1995); Kuwait (Marouf et al, 2002); Qatar (Fawzi et al, 2003); Yemen AS (Al-Nood et al, 2004), SS (Modell & Darlison 2008).

2.4 PATHOPHYSIOLOGY OF SCD

SCD is a group of heterogeneous disorders which incorporate two beta-globin gene variants at least one of which is the sickle cell gene. It includes four major genotypes: homozygous SCD (SS or sickle cell anaemia), sickle haemoglobin C (SC) disease, sickle cell β^+ thalassaemia ($S\beta^+$ thalassaemia), and sickle cell- β^0 thalassaemia ($S\beta^0$ thalassaemia) (Serjeant 1993). Many other compound heterozygous conditions exist, i.e. S/HPHP (hereditary persistence of fetal Hb), S/D Punjab, S/E, and S/O Arab, which are considerably less common. The basic molecular genetic defect is a single nucleotide change, with transversion of GAG-GTG in the 6^{th} codon of the β globin gene resulting in the replacement of valine for glutamic acid leading to the production of the abnormal haemoglobin Hb S, instead of normal Hb A (Ingram 1957).

Hb S is markedly less soluble than Hb A, has a tendency for reversible polymerization on deoxygenation and dissolves with oxygenation. This characteristic change is due to the presence of glutamic acid instead of valine in the β^s polypeptide chain which creates a hydrophopic interaction with other adjacent hemoglobin molecules inducing aggregation and polymer formation (Bunn 1997). Polymerazition of Hb S is considered a primary event in the pathogenesis of SCD and depends on the cellular concentrations of Hb S and Hb F, and on degree of deoxygenation and blood pH (Stuart & Nagel 2004). The Hb F sparing effect is due to Hb S dilution and reduced concentration, and to complete exclusion of both homotetramer ($\alpha 2\gamma 2$) and hybrid heterotetramers ($\alpha 2\beta S\gamma$) from polymer formation, a characteristic shared by Hb A2 (Poillon *et al,* 1993). This is in contrast to Hb A and C which have approximately 50% chances to be incorporated into the polymer. The kinetics of Hb S polymerization is suggested to start through a double nucleation model, in which

homogeneous nucleation is initiated primarily through aggregation of deoxygenated Hb S to form a nucleus, then other molecules are added to build the structured polymer. Heterogeneous nucleation is a secondary pathway which takes place on the surface of existing polymers (San Biagio *et al,* 1989). The time required for the Hb S polymer to be formed, is called the delay time, it is inversely related to the power of one third the Hb S concentration and is relatively longer than the transit time of red cells in the microcirculation (<1 second) (Mozzarelli *et al,* 1987, Cao & Ferrone 1996). Hence, about 80% of deoxygenated red cells return to the lungs and are polymer free. Any condition that leads to prolongation of the transit time can promote Hb S polymerization and sickling and would have a critical effect on the vaso-occlusive process (Bunn 1997).

Initiation of vaso-occlusion is complex, stochastic, varying over time and with anatomic site. Several clinical variables are not explained by the polymerization mechanism, particularly the marked clinical variation and the unpredictable episodes of painful vaso-occlusion (Embury 2004). Hence, Hb S polymerization alone is not sufficient to produce vaso-occlusion, which probably results from a complex interplay of several mechanisms involving RBC adhesion, endothelial cell activation, leukocyte recruitment, platelet aggregation and interaction with other plasma and tissue matrix factors (Chiang & Frenette 2005).

The sickled cells have an increased propensity to lose K^+ (mostly) and Cl^- ions following by water leading to the formation of dense dehydrated cells with markedly increased Hb S concentration. Hb S concentration is a major determinant of polymerization and if more concentrated Hb S polymerization is fast with associated cell sickling. There are at least three major suggested pathways responsible for sickle cell dehydration. These include the P_{sickle} non-specific cation

channel which opens with deoxygenation and mediates both K^+ loss and Na^+ entry and importantly allows intracellular influx of calcium ion (Ca^{++}), the main activator for the Gardos channel. Opening of the Gardos channel is activated by increased free intracellular Ca^{++} , leading to large loss of K^+ , Cl^- and water. Another pathway is the K^+- Cl^- co-transporter (KCC), which is an oxygen sensitive membrane transporter and regulated by cell swelling, reduction in blood pH, pO2 level and Mg^{++} erythrocyte content. A large portion of dense cells become irreversibly sickled cells (ISC) and maintain distorted shapes even after blood is fully oxygenated. These fixed deformed cells become mechanically fragile with short life-span of about 10-20 days, as they are rapidly removed from the circulation by the reticuloendothelial system (Gibson & Ellory 2002, Brugnara 2003).

Red blood cells at rest have an average diameter of 7.8μm and deform markedly to pass the smallest capillaries of the microcirculation (3–7μm). Rheological alteration in the sickled cell with ability to deform and flow is secondary to irreversible membrane changes. These changes occur due to factors such as binding of haemoglobin to the membrane, oxidant damage, disruption of phospholipid asymmetry and skeletal protein alteration (Stuart & Nash 1990).

In the normal red cell membrane phospholipids are asymmetrically organized with the choline containing phospholipids (sphingomyelin and phosphatidylcholine,) mainly located in the external leaflet, whereas the amine-containing phospholipids (phosphatidylethanolamine and phosphatidylserine) are confined to the inner leaflet. This asymmetry is maintained by active ATP–dependent aminophospholipid translocase or flipase. When there is deactivation of the ATP–dependent aminophospholipid translocase, this asymmetry is disrupted with externalization of phosphatidylserine (PS). PS display is sickling dependent, occurring at high levels in

dense and sickled cells and at low levels in immature cells and in cells with a high percentage of Hb F. The PS externalization is suggested to increase procoagulant activity and thrombosis, enhance cell adhesion and increase phagocytic recognition with reduction of red cell survival (Zwaal & Schroit 1997, Yasin *et al,* 2003). PS receptor on the surface of activated microendothelial cells has been determined and presumably involved in the adhesion of PS-positive erythrocytes to endothelium. It is up-regulated by relevant agonists such as cytokines, hypoxia, and haeme (Setty & Betal 2008).

The adhesion process in SCD is complex and involves multiple red cell receptors, leukocytes, plasma proteins, vascular endothelial cells and sub-endothelial matrix components. Among red cell receptors and molecules are integrin α4β1 (VLA-4) binding to fibronectin and CD36, a thrombospondin receptor expressed on stressed reticulocytes. Other molecules not restricted to reticulocytes which occur in mature red cells include Lutheran blood group/basal cell adhesion molecule (Lu/BCAM), a high affinity receptor for the extracellular matrix protein called laminin, LW (ICAM-4), receptors which bind different types of integrins, particularly αVβ3, CD44, and a hyaluronan receptor. On the endothelial side, αVβ3 integrin, vascular cell adhesion molecule-1 (VCAM-1), CD36, and P-selectin (Telen 2007).

The adhesive characteristics of sickled red cells depend on density and deformability which are heterogeneous. Cell heterogeneity varies from almost normal, to very dense discocytes, to dense sickled cells composed mainly of ISC. These diverse types of cells would contribute differently to vaso-occlusive events (Kaul 2008). In the initial phase of vaso-occlusion, which take place mainly in the post-capillary venules, there is preferential adhesion of deformable discocytes (regular and irregular), which increases transit times and induces hypoxia, followed

by selective trapping of dense discocytes and ISCs leading eventually to vessel obstruction.

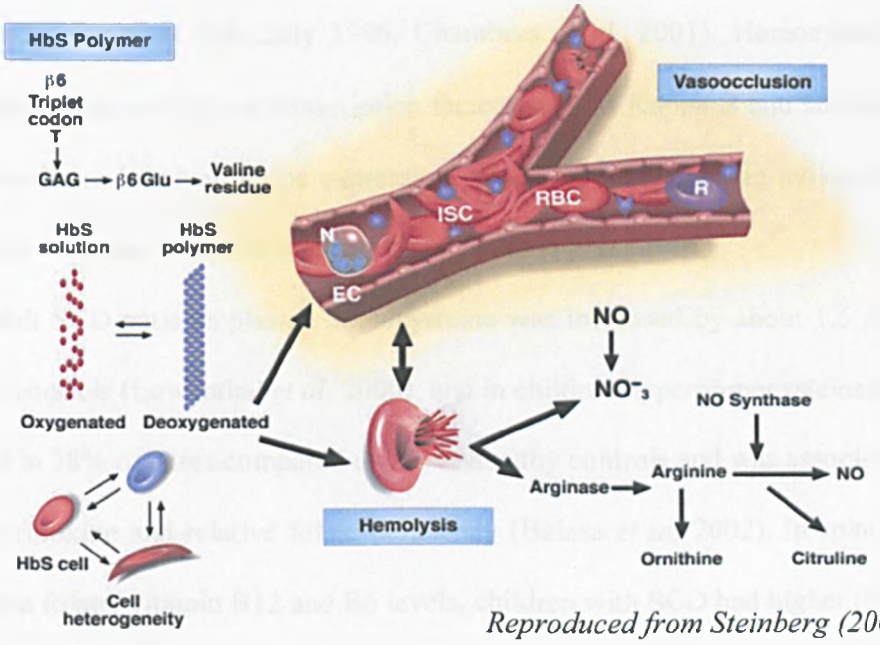
Leukocytes are larger and stiffer than erythrocytes and adhere more effectively to the wall of blood vessels. In SCD leukocyte number and activity are increased, they bind to each other, to platelets, and to erythrocytes creating a multi-cellular meshwork where ISCs become easily trapped. Leukocytes also cause activation of vascular endothelium by releasing inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1β augmenting endothelial expression for adhesion molecules on both red and white cells, (Okpala 2004). Endothelial activation becomes evident through induced nuclear factor (NF-KB) in endothelial cells which up-regulate expression of adhesion molecules such as E-selectin, VCAM-1, ICAM-1 and through increased P-selectin. These changes promote leukocyte recruitment and interaction with other blood cells.

SCD is an inflammatory state (Platt 2000), suggested to be the result of recurrent episodes of localized tissue ischemia and re-perfusion injury which induces local inflammation by stimulating cellular and humoral inflammatory mediators and this condition is maintained by secretion of high levels of cytokines including IL-1, TNFα, endothelin-1 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) through activated endothelial cells and monocytes (Kaul 2008).

Nitric oxide (NO) is a potent vaso-relaxant that binds to and inactivates soluble guanylyl cyclase in vascular smooth muscle, resulting in accumulation of cyclic guanosine monophosphate (cGMP) and muscle relaxation. NO possesses antioxidant and antithrombotic properties, reduces platelet activity, attenuates blood cell adhesion, and decreases the level of endothelin-1 the most potent vasoconstrictor (Wood *et al,* 2008). Impaired NO homeostasis can lead to vasoconstriction and

decreased blood flow potentially contributing to vessel obstruction. The bioavailability of NO in SCD is diminished as a consequence of increased scavenging by plasma haemoglobin and reactive oxygen species, or limitation of arginine substrate, which is competitively utilized by arginase enzyme liberated during intravascular hemolysis (Figure 2.2). Markers of hemolysiss including plasma Hb and serum lactate dehydrogenase (LDH) correlate closely with NO consumption and endothelial dysfunction and have been linked to hemolysis-endothelial vasculopathic complications such as pulmonary hypertension, priapism, skin ulceration and possibly non-hemorrhagic stroke (Kato *et al,* 2007a). Furthermore, NO formation is inhibited by asymmetric dimethylarginine (ADMA) the endogenous competitive inhibitor of NO synthase. This inhibitor is found to be elevated in SCD patients and correlated significantly with Hb and LDH levels (Schnog *et al,* 2005).

Figure 2. 2 The pathophysiology of sickle cell disease



Reproduced from Steinberg (2008)

Hb S: sickle haemoglobin; RBC: red blood cell; ISC: irreversible sickled cell; R: reticulocyte; EC: endothelial cell; N: neutrophil; NO: nitric oxide; NO^-_3: nitrate

Haemostatic changes manifested by decreased levels of natural anticoagulant proteins (C and S), increased markers of thrombin generation and platelet activation lead to the assumption that SCD is a hypercoagulability condition (Ataga *et al,* 2007). Moderate thromboytosis was also observed and related to functional asplenia. Among other factors possibly implicated in haemostasis and endothelial dysfunction is elevation of plasma homocysteine (tHcy) concentration, a recognized risk factor in thrombo-vascular disorders. tHcy can be elevated with deficiency of folate or B vitamin co-factors, or through reduced activity of the key enzyme in homocysteine-methionine metabolism: 5,10-methylenetetrahydrofolate reductase (MTHFR) (Castro *et al,* 2006). Hyperhomocysteinemia has been associated with vascular endothelial dysfunction. This effect may be exerted by promoting oxidative damage in endothelial cells with generation of superoxide anion radicals and hydrogen peroxide, leading to oxidation of low density lipoprotein (LDL), reduced NO bioavailability, increased levels of adhesion molecules, leukocyte adhesion, platelet activation and thrombosis (McCully 1996, Chambers *et al,* 2001). Homocysteine may also enhance the activity of transcription factors such as Kappa B and activator protein-1, which are involved in the expression of cell surface adhesion molecules, growth factors, immune-receptors and cytokines (Durand *et al,* 2001).

In adult SCD patients plasma homocysteine was increased by about 1.5 fold compared to controls (Lowenthal *et al,* 2000), and in children hyperhomocysteinemia was reported in 38% of cases compared to 7% of healthy controls and was associated with both pyridoxine and relative folate deficiency (Balasa *et al,* 2002). In spite of similar plasma folate, vitamin B12 and B6 levels, children with SCD had higher tHcy levels compared to healthy controls (12.7±4.5 vs 10.9±3.5 μmol/L p=0.04), and their plasma tHcy concentration was lowered by 53% (5.7±1.6) following oral

supplementation 2- 4mg folic acid daily (van der Dijs *et al,* 1998). Homocysteine concentration was higher in children with SCD complicated by stroke compared to those without stroke (median 13.3 μmol/L vs 9.7 μmol/L), with a 3.5 fold increased risk for stroke (Houston *et al,* 1997). Other investigators have reported no difference in plasma tHcy levels between American SCD patients and controls, who also had normal serum and red cell folate levels (Rodriguez-Cortes *et al,* 1999). This result suggested that folate status was satisfactory due to adequate consumption of folate fortified food (Rana *et al,* 2000, Schnog *et al,* 2000). MTHFR 677 TT genotype is associated with hyperhomocysteinemia particularly if folate levels are low, or if there is a state of accelerated erythropoiesis as in SCD when demand for folate is increased. If MTHFR deficiency co-exists and folate requirement is not met hyperhomocysteinemia will ensue. Few reports have studied MTHFR deficiency with SCD (Table 2.1), and some of these studies have found a positive association with vascular complications, such as avascular osteonecrosis (AVN), stroke, retinopathy and acute chest syndrome (Kutlar *et al,* 2001, Moreira Neto *et al,* 2006), whilst others have reported no association (Zimmerman & Ware 1998, Adekile *et al,* 2001).

Table 2.1 Studies of MTHFR C677T and sickle cell disease

Location & year of publication	Age* (yrs)	Case (n)	Control (n)	Genotype (n) CC:CT:TT		Allele frequency (%)		TT frequency (%)		Comments	Reference
				Cases	Controls	Cases	Controls	Cases	Controls		
Middle East											
Lebanon 2006	8-42	12	50	5:5:2	-	37.5	-	16.7	-	All cases were Sβ° thalassemia No significant difference	(Isma'eel *et al*, 2006)
Bahrain 2006	15.8±9.8	106	156	69:33:4	116:30:10	8	8.4	2.0	1.6	No significant difference, normal homocysteine in all patients	(Al-Absi *et al*, 2006)
Saudi Arabia 2004	23.1±14.15	87	105	51:28:8	79:22:4	25	14	9.0	3.8	Higher than controls but no significantly different	(Fawaz *et al*, 2004)
Kuwait 2001	2-41	41	-	25:15:1	-	20.7	-	2.4	-	No significant difference between AVN group and others	(Adekile *et al*, 2001)
Americas											
USA 2002	4-21	77	110	60:15:2	88:21:1	12.3	10.5	2.6	0.9	No correlation between genotype and homocysteine level	(Balasa *et al*, 2002)
USA 2001	15-54	107	-	83:21:3	-	11.2	-	2.8	-	Significantly higher in AVN group	(Kutlar *et al*, 2001)
USA 1999	2-17	18	35	17:1:0	25:10:0	2.8	14.3	-	-	Cases with stroke, controls without. Mutation higher in controls	(Driscoll & Prauner 1999)
USA 1999	2.1-21	40	32	31:7:2	26:5:1	14	11	5.0	3.0	Cases with stroke, controls without. No significant difference	(Balasa *et al*, 1999)
USA 1998	5-60	29	57	24:5:0	49:8:0	8.6	7	0	0	Cases with stroke or AVN, controls without. No significant difference	(Zimmerman & Ware 1998)
Brazil 2006	13-72	53	-	34:18:1	-	19	-	2.0	-	Mutation associated with vascular complications	(Moreira Neto *et al*, 2006)
Brazil 2004	1-73	69	-	52:13:4	-	15.2	-	5.8	-	Higher than local ethnic prevalence	(Couto *et al*, 2004)
Brazil 1998	14-62	73	137	- - : 0	-	-	2	0	1.6	No patient had TT genotype. No correlation with mortality	(Andrade *et al*, 1998)
Caribbean											
Guadeloupe 2002	-	314	203	264:43:7	-	9	15	2.0	-	Lower frequency than controls, no correlation with morbidity	(Romana *et al*, 2002)
Jamaica 1999	7-36	48	48	41:6:1	42:6:0	8.3	6.3	2.0	0	Cases with stroke, controls without. No significant difference	(Cumming *et al*, 1999)

* Age range or mean ±SD, - no data, AVN: avascular osteonecrosis

2.5 CLINICAL MANIFESTATIONS

SCD is characterized by protean clinical manifestations which involve most body systems and result from variable degrees of haemolysis and intermittent episodes of vascular occlusion that cause tissue ischemia and acute and chronic organ dysfunction (AAP. 2002). Studies of the natural history of SCD demonstrate considerable variation in clinical features with wide geographical diversity in disease presentation and complications. This may reflect the impact of both genetic as well as environmental factors and their interaction in modulating disease phenotype.

2.5.1 Anaemia

Sickle cell life span is shortened due to shape alteration and mechanical fragility which lead to accelerated red cell destruction. Hb concentration in affected children decreases following the neonatal period until the second year of life, when a moderate degree of anaemia persists in most patients. Anaemia is more severe in SS and S β^0 thalassaemia than SC and S β^+ thalassaemia (Serjeant 1993). Hb levels in African haplotypes are usually between 6-9 gm/dl, and higher in patients from Greece, Saudi Arabia and India. The main determinants of Hb concentration, beside variation with age and gender, are α-thalassaemia, and Hb F concentration, probably through a decreasing rate of haemolysis (Serjeant *et al,* 1996). Chronic anaemia in children may be complicated by micronutrient deficiency as a result of increased folate requirements or inadequate intake during a period of rapid growth. In adults erythropoietin deficiency, secondary to renal insufficiency is an important cause of chronic anaemia. Patients with chronic hyper-haemolysis, which compromises NO bioavailability, have an increased prevalence of leg ulcers, priapism (in males), higher systolic blood pressure and pulmonary hypertension, which increases their

risk of death. They less frequently experience vaso-occlusive pain or osteonecrosis (Taylor *et al,* 2008).

2.5.2 Anaemic crisis

Development of acute anaemic crisis with transient cessation of erythropoiesis and reticulocytopenia is caused mostly by parvovirus B19 infection. The marrow aplasia is self-limited with spontaneous recovery usually occurring within 7-10 days, although blood transfusion may be required. Parvovirus infection occurs predominantly in children and immunity appears to be life-long. Fever, pain, acute splenic sequestration, and ACS are frequent associated events (Serjeant *et al,* 2001a, Smith-Whitley *et al,* 2004). Acute anaemia may result from sudden splenic enlargement with pooling of circulating blood leading to acute splenic sequestration crises with life-threatening anaemia and hypovolemic shock. Splenic sequestration occurs in infants and young children (6 months-5 years), with approximately a 50% risk of recurrence. Parental education for early detection of pallor with palpation for an enlarging spleen and prophylactic splenectomy has reduced mortality from this condition by more than 90% (Emond *et al,* 1985, Khatib *et al,* 2009). Hyper-haemolytic crisis, a common type of anaemic crisis in tropical Africa, and malaria infection may precipitate such episodes (Juwah *et al,* 2004).

2.5.3 Acute painful crisis

Acute painful crises are common and considered the hallmark of SCD. The pain crises occur in an unpredictable manner, typically affecting the long bones, spine and abdomen and lasting from hours to many days (Serjeant *et al,* 1994). Recognized precipitating factors include exposure to cold, dehydration, physical or psychological stress and infection. Frequency and severity of painful episodes may vary widely and many of these events can be managed successfully at home (Shapiro

et al, 1995). Nevertheless, painful crisis is a major cause of recurrent hospital admission particularly in adolescents and young adults. Pain frequency tends to increase in females during the first decade and markedly in males between 15-25 years, decreasing after 30 years of age (Gill *et al,* 1995). The reasons of these age and sex differences remain unclear. Risk factors for painful crises include high baseline Hb, low Hb F and α-thalassaemia (Platt *et al,* 1991). In young children (mostly 3 months - 3 years), these crisis may commence as dactylitis (hand-foot syndrome) which is characterized by painful swelling of the dorsum of the hands and feet due to involvement of the metacarpals, metatarsals and phalanges. This is often the first presenting symptom of SCD in about 50% of young children (Stevens *et al,* 1981). Most of these episodes resolve spontaneously and only in severe cases with epiphyseal infarction will bone deformity or finger shortening occur. Dactylitis in the first year of life is considered to have prognostic significance (Miller *et al,* 2000). It is presumably caused by infarction of active red bone marrow in the peripheral small bones of younger patients. In older patients pain is mostly localized to flat bones and to juxta-articular areas of the long bones.

2.5.4 Splenic complications

Circulating splenic blood has a high hematocrit and viscosity with consequent sluggish flow creating a good environment for poorly deformable cells to be sickled, resulting in focal haemorrhage and infarction. In adults repeated infarction leads to fibrosis and splenic atrophy a condition known as "autosplenectomy". In children the enlarged spleen leads to blood bypassing the red pulp sinusoids blockaded by sickled cells through intrasplenic arteriovenous shunts and escape from the action of reticuloendothelial cells. This leads to "functional hyposplenism" (William & Corazza 2007, Khatib *et al,* 2009). Functional hyposplenism occurred in 90% of

African-American children between 6-36 months greatly increasing their risk of infection by encapsulated bacteria (Pearson *et al,* 1985). However, in patients from Eastern Saudi and India enlarged dysfunctional splenomegaly persisted into adult life in over two thirds of cases, carrying an increased risk of sequestration crises, hypersplenism, massive infarction and abscess formation. These complications were the main indications for splenectomy in this population (Al-Salem *et al,* 1999, Chopra *et al,* 2005). Diagnosis of hyposplenism is based on absence of uptake of Tc-99m sulphur colloid on spleen scanning, or by an increase in the number of pitted red cells (>3.5%) in peripheral blood. Recurrent transfusions or treatment with hydroxyurea should improve splenic function, but may put patients at risk for sequestration episodes (Hankins *et al,* 2008).

2.5.5 Infection

Splenic function may be impaired in children as young as 6 months, with consequent increased susceptibility to infection by capsulated organisms such as *Streptococcus pneumoniae* and *Haemophilus influenza* (Pearson *et al,* 1985). Other predisposing factors for infection in SCD are defective opsonisation and alternative complement pathway or neutrophil dysfunction (Humbert *et al,* 1990). A bias towards a Th2 sub-population of T lymphocytes resulted in the production of type 2 cytokines (IL-4, IL-5, and IL-10) which stimulate a non-cytotoxic antibody response and suppression of microphage activation (Raghupathy *et al,* 2000). Pneumococcal infection is a serious problem in early childhood causing septicaemia and meningitis with high mortality (Wong *et al,* 1992). These infections can be effectively prevented by immunization and penicillin prophylaxis, and the attack rate in the post–vaccination era has decreased greatly by a mean of 92% among children less than 5 years (Halasa *et al,* 2007). In chronically transfused patients with iron overload, with

or without deferoxamine, *Yersinia enterocolitica* is a specific risk (Blei & Puder 1993). In equatorial Africa organisms such as *Staphylococcus aureus, Klebsiella species, Haemophilus influenzae* and *Salmonella species* were the predominant causes of bacteraemia, challenging the potential effectiveness of pneumococcal vaccination in this area (Kizito *et al,* 2007). However, in view of the high burden of pneumococcal disease in children with or without SCD there is a priority for pneumococcal vaccine to be introduced into routine immunization schedules in developing countries, which would additionally benefit SCD patients as a specific vulnerable group (Levine *et al,* 2006). This view was supported by recent findings of dominant invasive bacterial infections caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* which are the same as those in developed countries and their vaccines are available (Williams *et al,* 2009). Bone marrow infarction and necrosis predispose patients to osteomyelitis and septic arthritis caused typically by salmonella species and *Staphylococcus aureus.* Urinary tract infection occurs frequently in females, caused predominantly by *Escherichia coli*, and can be a source of gram-negative septicaemia (Bruno *et al,* 2001). In general the main etiologic agents of invasive bacterial infection in SCD patients are *Streptococcus pneumoniae, Salmonella species, Haemophilus influenzae, Escherichia coli* and *Klebsiella species* (Magnus *et al,* 1999).

Malaria infection in SS children can be fatal, whereas AS heterozygote carriers have a relative resistance to falciparum malaria. Although this resistance is not absolute it present from early in childhood before natural immunity is acquired increasing survival rates for AS carriers. This advantage to AS carriers offsets the deleterious effects of SS thus maintaining a relatively steady population gene frequency, which is termed balanced polymorphism (Aidoo *et al,* 2002, Allison

2002). The high prevalence of sickle gene in regions where *P. falciparum* is or was endemic is considered to result from the protection afforded by this mutation against severe and fatal malaria infection.

Several mechanisms have been proposed to explain this, which indicate both innate (Nagel 1990) and acquired immune protection (Williams *et al,* 2005a). These include impairment of parasite growth, induced sickling with enhanced phagocytosis and early removal of parasitized red cells, and impairment of cytoadherence with reduced inflammation. Oxidative stress in parasitized red cells may also lead to enhanced phagocyosis through increased denaturation of Hb, membrane deposition of hemichromes and free iron, aggregation of band 3, and deposition of autologus IgG antibodies and complement C3c fragments. Selective complement-mediated phagocytosis of ring-parasitized red cells has the advantage of interrupting parasite growth, lowering the number of mature more adherent forms, and reducing cytokine output by phagocytic cells (Ayi *et al,* 2004, Cholera *et al,* 2008). An interesting recent observation was that malaria protection was lost in Hb AS when co-inherited with homozygous α-thalassaemia, assuming negative epistatic effects (Williams *et al,* 2005c). These are probably related to Hb S concentration in Hb AS carriers which decreases in proportion to the number of deleted α-globin genes. As malaria infection is associated with increased morbidity and mortality in homozygous SCD, anti-malaria chemoprophylaxis should be given to affected individuals in endemic areas (Oniyangi & Omari 2006).

2.5.6 Central nervous system

SCD is the most common cause of stroke in children, with a 300-fold increased risk compared to the general paediatric population. Overt stroke occurs in approximately 10% of children mostly between the ages of 2-5 years, with a 67%

risk of recurrence (Ohene-Frempong *et al,* 1998). Transient ischemic attack (TIA), seizures, coma, silent cerebral infarction, sinovenous thrombosis, leukoencephalopathy and acute demyelination are other neurological complications that may be encountered (Kirkham & DeBaun 2004). Silent infarction is detected in up to 25% of children by MRI scan. Although silent infarction is asymptomatic it has been associated with cognitive difficulties and compromised neuropsychologic function which may be progressive. Ischemic (non-haemorrhagic) infarction is the most common type in young children, whereas intracranial subarachnoid haemorrhage is more common in older cases. The underlying abnormalities in ischemic stroke are usually attributed to vasculopathy of cerebral, internal carotid or vertebral arteries leading to stenosis and vessel occlusion. Haemorrhagic stroke, which has higher mortality is often caused by rupture of the Moyamoya vessels (Japanese for puff of smoke appearance on angiogram) or aneurysms (Hoppe 2005). Determination of those at high risk for stroke and who require primary prevention can be achieved by measurement of the blood flow velocity in the distal internal carotid or proximal middle cerebral artery using transcranial Doppler ultrasonography (TCD). This is defined by a mean maximum velocity of ≥200 cm/s. Chronic blood transfusion therapy can decrease the risk of stroke by more than 90% (Adams *et al,* 1998). Doppler ultrasonography screening has provided a remarkably safe, non-invasive and relatively low-cost means of stroke prevention, which is affordable to children even in low resource settings. The appropriate choice of intervention in such settings is influenced by transfusion safety and related morbidities which may be limitations. Risk factors for stroke include low baseline Hb and Hb F concentration, high leukocyte count, a history of dactylitis, prior TIA or recent acute chest syndrome, infection, elevated tHcy, relative hypertension or

nocturnal hypoxemia secondary to upper airway obstruction (Prengler *et al,* 2002, Kirkham & DeBaun 2004). Familial predisposition is also reported (Driscoll *et al,* 2003). Analysis of genetic variation through Bayesian networks identified 31 SNPs in 12 genes associated with the occurrence and prediction of stroke in SCD patients (Sebastiani *et al,* 2005).

2.5.7 Pulmonary

Pulmonary complications are a common cause of hospital admission and result in substantial morbidity and mortality (Gladwin & Vichinsky 2008). Acute chest syndrome (ACS) defined by fever, chest pain, cough, wheezing, tachypnoea, or hypoxia, accompanied by new pulmonary infiltrates on chest radiograph, occurs in more than 50% of children with SCD. ACS is commonest between the age of 2-4 years and tends to recur with frequency decreasing with age becoming more severe in adults who show high mortality than children (Johnson 2005a). Several factors have been associated with the aetiology of ACS, which include: infection mainly in children (most frequently Chlamydia, Mycoplasma, and viral), pulmonary vaso-occlusion, pulmonary fat embolism, iatrogenic fluid overload, hypoventilation and atelectasis secondary to chest bone infarction, or to opioid administration (Vichinsky *et al,* 2000). Differential diagnosis between ACS and pneumonia can be difficult and broad spectrum antibiotics to cover a possible infectious aetiology are almost always given.

Children with SCD may also develop upper airway obstruction, nocturnal hypoxemia, airway hyper-reactivity and increased frequency of clinically diagnosed asthma (Caboot & Allen 2008). Changes in pulmonary function with an obstructive and restrictive pattern may lead to sequential reduction in lung volume during childhood (MacLean *et al,* 2008). Another serious chronic complication is

pulmonary hypertension (PHT), defined by a maximal tricuspid regurgitant jet velocity (TRV) $\geq$ 2.5 m/s. PHT occurs in 20-40% of adults and often carries a poor prognosis with 40% mortality at 40 months of follow-up (Kato *et al,* 2007b). In children PHT is under-diagnosed and has a different profile of complications to adults, being associated with history of sepsis, ACS, asthma or obstructive lung disease (Hagar *et al,* 2008). Although the true prevalence and prognosis in paediatric patients has not been determined, a recent study suggested a 30% prevalence of PHT in children, which is similar to that for adults, but with high reversibility following early diagnosis and treatment (Pashankar *et al,* 2009).

2.5.8 Cardiac

The hyperdynamic circulation is a consequence of chronic anaemia and increased stroke volume which is reflected in the strong peripheral pulse, active precordial movement and heart murmur. Cardiomegaly, chamber dilatation, interventricular septum hypertrophy and normal contractibility were reported on echocardiography (Covitz *et al,* 1995). In adults diastolic dysfunction and PHT were combined or independent risk factors for poor outcome and mortality (Sachdev *et al,* 2007). Cardiac failure, myocardial infarction and arrhythmias were uncommon complications in paediatric patients, and even autopsy cases with myocardial infarction showed normal or dilated coronary arteries with no atherosclerotic changes (Tsironi & Aessopos 2005). Myocardial hypoperfusion may be detected more frequently in selected patients by single photon emission computer tomography (SPECT) (de Montalembert *et al,* 2004), or magnetic resonance imaging (MRI), which were useful in identifying iron cardiac deposition and focal myocardial fibrosis (Raman *et al,* 2006, Westwood *et al,* 2007). Cardiac changes relate to volume overload as a result of chronic anaemia, with no evidence for a distinct sickle

cell cardiomyopathy (Lester *et al,* 1990). Other unknown factors, in addition to chronic anaemia, and unrelated to iron deposition or regional fibrosis had been implicated (Westwood *et al,* 2007). Arterial blood pressure in SCD patients is significantly lower than in the general population and higher systolic blood pressure is associated with increased risk of ischemic stroke and mortality (Pegelow *et al,* 1997). Elevated blood pressure although within the normal range for the general population, may still be considered as relative hypertension in these patients. Factors related to low pressure in SCD patients are low body weight and BMI and also attributed to increase sodium loss through hyposthenuria (reduced ability to concentrate urine). Occurrence of hypertension may indicate underlying renal insufficiency or proteinuria and if acute in onset then hyperviscosity should be suspected especially if following blood transfusion (Johnson 2005b).

2.5.9 Genitourinary

Hyposthenuria is a sign of medullary damage occurring in early life and may manifest as polyuria, nocturia and possibly enuresis with increased risk of dehydration during water deprivation or volume loss. Enuresis is a common clinical problem in children that can persist into adult life, with prevalence ranging between 20% to 69% (Akinyanju *et al,* 1989, Raedett *et al,* 1990, Barakat *et al,* 2001, Field *et al,* 2008). High urine volume may contribute to nocturnal enuresis, but a multifactorial aetiology is more likely (Readett *et al,* 1990a). Haematuria occurs in the disease state as well as in carriers. It probably results from red cells sickling and leading to vascular obstruction within the vasa recta with red cell extravasation or papillary necrosis. This process is promoted by low oxygen tension, acidosis and a high tonicity milieu in the renal medulla. The left kidney is four-fold more likely to be affected, which is probably because of the increased venous pressure in the longer

left renal vein due to kinking and compression of the aorta and superior mesenteric artery. In general haematuria is a benign problem, but may rarely be associated with a highly malignant renal medullary carcinoma. Glomerulopathy, proteinuria, nephrotic syndrome, hypertension and chronic renal failure are complications that increase with age and are usually observed in adults (Bruno *et al,* 2001, Scheinman 2009). Priapism, a painful purposeless persistent penile erection is a common problem with a reported prevalence between 28% to 38% (Adeyoju *et al,* 2002). In children the calculated actuarial probability of experiencing priapism was 12.9% by 10 years of age, 50.3% by 15 years of age, and 89.3% by 20 years of age (Mantadakis *et al,* 1999). Priapism is the result of increased arterial inflow (high flow) or, more commonly by venous outflow obstruction of the corpus cavernosum (low flow), with failure of penile detumescence and if prolonged can result in irreversible fibrosis and erectile dysfunction (Muneer *et al,* 2008). Episodes of priapism may present as short, stuttering attacks usually nocturnal or as prolonged major attacks lasting for hours or if longer with increased risk of impotence. Poor patient knowledge, with low accessibility to the health care system in low resource settings may lead to a high rate of impotence (31.6%) (Gbadoe *et al,* 2001). Priapism has been associated with chronic hyper-haemolysis and α-thalassaemia consider protective (Taylor *et al,* 2008). Genetic polymorphism of the klotho gene and other genes involved in adhesion, coagulation, inflammation, cell signalling and NO biology in association with priapism have been identified (Nolan *et al,* 2005, Elliott *et al,* 2007).

2.5.10 Hepatobiliary

Direct liver involvement may occur due to vascular occlusion with acute ischemia, sequestration, and cholestasis, or through a mixed hepatocellular-

cholestatic mechanism (Koskinas *et al,* 2007). Hepatic viral infection and iron overload from multiple blood transfusions are further causes of hepatic injury. Hepatomegaly is a common finding in children and adults and if persistent it has been associated with disease severity (Olatunji & Falusi 1994). Acute sickle hepatic crisis occurs in about 10% of patients, commonly presenting with acute right upper quadrant abdominal pain, tender hepatomegaly, jaundice and elevated liver enzymes. A more severe form with high mortality results from intrahepatic cholestasis with extremely high bilirubin levels and conjugated bilirubin exceeding 50% (Banerjee *et al,* 2001). Chronic haemolysis with high levels of bilirubin excretion may lead to the formation of pigment gallstones, biliary obstruction and cholecystitis. Gallstones which are usually asymptomatic occurring in approximately 30% of SS children by the age 15-17 years, increases to over 50% in adults (Walker *et al,* 2000). Polymorphism of the 5'-diphosphate-glucuronosyltransferase 1A1 (*UGT1A1*) gene encoding a key enzyme in bilirubin catabolism is reported to be a genetic risk factor modifying frequency and age of onset of gallstones (Chaar *et al,* 2005, Martins *et al,* 2008). Management through laparoscopic surgery in children is considered a safe option for both symptomatic and elective cholecystectomy (Suell *et al,* 2004, Curro *et al,* 2007).

2.5.11 Bone

Bone marrow infarction is common in vaso-oclusive crises. It relates to marrow hyper-cellularity with expanded marrow activity leading to impaired blood flow and regional hypoxia. Acute infarction presents with acute localized pain, swelling, and fever. Juxta-articular areas are especially vulnerable and accompanied by joint pain and effusion. The long bones, spine and pelvis are common sites of involvement in adults, whereas in young children this usually presents as dactylitis.

With vertebral marrow infarction bone collapse produces the typical 'fish mouth' appearance. Ischemic osteonecrosis may result from infarction of the articular surfaces and epiphyses of long bones. The most common site of avascular necrosis is the femoral head with overall prevalence of about 10% (Milner *et al,* 1991). MRI allows early diagnosis before symptoms of pain and limitation of movement appear permitting earlier intervention with possible preservation of joint function. The humeral head is the second most common site involved, but the knee and small joints of the hands and feet are also affected (Huo *et al,* 1990, Aguilar *et al,* 2005, Almeida & Roberts 2005). At present no effective measure can prevent progressive joint damage, and treatment of osteonecrosis is generally unsatisfactory, although bed rest and decreased weight bearing in early stages may be helpful.

2.5.12 Leg ulceration

Chronic leg ulcers are frequent in adults but rare in children before the age of 10 years. Prevalence varies widely from 2.5% in the USA, to 1.5% to 13.5% in Africa, and 30% in Jamaica. It is rare in patients from Saudi Arabia and India. Males tend to be more affected. The ulcers occur either spontaneously or following trauma and are painful with well defined margins typically affecting the skin around the medial or lateral malleoli (Serjeant *et al,* 2005). Chronic ulcers are difficult to treat, highly recurrent and impact greatly on the patient's quality of life. Risk factors for these include lower socio-economic status, venous incompetence and elevated LDH (Cumming *et al,* 2008). Leg ulcers which are associated with other complications are thought to be related to a hyperhaemolysis sub-phenotype (Halabi-Tawil *et al,* 2008).

2.5.13 Ocular

Non-proliferative ocular manifestations include hyphema, (blood in the anterior chamber) occurring secondary to minor trauma, or ocular surgery. This

enhances local red cell sickling and obstruction of anterior chamber outflow, increasing intra-ocular pressure and reducing perfusion to the optic nerve and retinal tissues aggravating the risk of visual loss (Emerson & Lutty 2005). Proliferative sickle retinopathy (PSR) is a more serious eye complication occurring in approximately 17% in SC and 1% in SS by the age of 15-17 years, and then increasing with age. The cause of the higher frequency of PSR in SC disease than in other genotypes remains unknown, although increased blood viscosity is postulated. Onset of PSR is insidious with local ischemia stimulating angiogenesis vascular endothelial growth factor and neovascularisation, producing a sea-fan appearance in the retina. PSR increases the predisposition to vitreous haemorrhage and retinal detachment which can lead to irreversible loss of vision (Downes *et al,* 2005).

2.5.14 Otologic

Patients with SCD have a much higher incidence of hearing impairment and sensory neural hearing loss (SNHL)) than the general population, with a reported prevalence between 11% to 41% (MacDonald *et al,* 1999). The degree and patterns of hearing loss are variable, although it is usually mild, and unilateral, affecting low and high frequencies, often reversible and with no clear age increment (Burch-Sims & Matlock 2005). The proposed mechanism probably relates to vascular cochlear damage, with ischemia of the stria vascularis and hypoxia of the secondary organ of Corti. Neural involvement cannot be excluded. There is a suggestion that the crucial period in the development of SNHL may be during the intra-uterine period or the first few years of life (Ajulo *et al,* 1993). Hydroxyurea (HU) may prevent cochlear damage and hearing loss (Stuart *et al,* 2006).

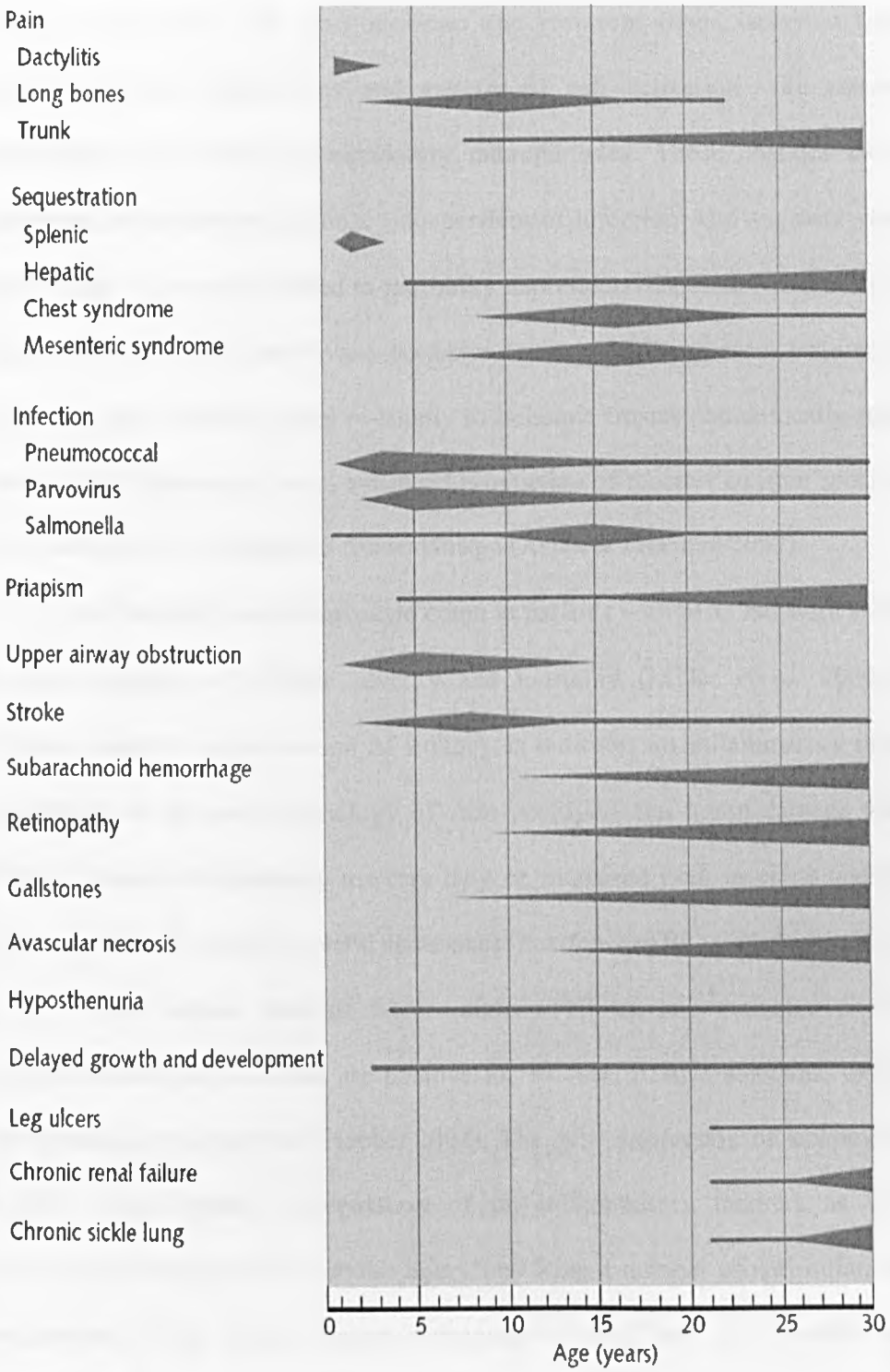
2.5.15 Growth and developmental delay

Growth and maturation delay frequently affected children with SCD and attributed to disease severity, nutritional deficiencies and endocrine dysfunction. Most growth data was from the Americas and Caribbean countries with few studies from Africa, the Middle East or Europe emphasizing the need for further studies to address this disparity, particularly from low-resource settings, where malaria is endemic and childhood malnutrition is prevalent. There is evidence that improved nutrition and better quality of care substantially improve child growth. A detailed review of this topic was completed as part of this study and published (Appendix G).

2.5.16 Psychological

Children with SCD and their families may develop psychological adaptation difficulties which affect relationships with health care providers as well as the community, with quality of life impaired for both parent and child (Thomas & Taylor 2002). Pain is an important factor which impacts on quality of life, and its recurrence and unpredictability disturb the family, school and social life often through repeated hospital admissions and even feelings of helplessness (Howard *et al,* 2008). The psychological complications relate to difficulties in coping and maladjustment of patients to their chronic illness which is non-remittent, often limiting social activity and work, leading to low self-esteem (Anie 2005). Stroke may lead to language problems, impaired attention and learning difficulties with poor academic performance. This depends on the site and extent of brain insult, and they are more severe in patients with stroke than in those with silent infarction who tend to have more subtle frontal lobe problems affecting attention and concentration ability (Berkelhammer *et al,* 2007).

Figure 2. 3 Age distribution of acute and chronic complications



Reproduced from Davies & Oni (1997)

2.6 INFLAMMATION

SCD is recognized as an inflammatory condition (Platt 2000). Repeated sickling of red cells with vaso-occlusion and recurrent tissue ischemia leads to enhanced cell-cell interactions and endothelial cell activation with release of inflammatory mediators and circulatory microparticles. These changes induce a continuous inflammatory response (independent of infection) and augment vascular injury which is probably related to morbidity expression (Chies & Nardi 2001). The progression and resolution of vaso-occlusive crisis in SCD represent features of re-perfusion injury, where oxygen re-supply to ischemic tissues paradoxically supports a more pro-inflammatory state, enhanced production of reactive oxygen species and as a consequence contributes to tissue damage (Aslan & Freeman 2007).

An elevated baseline leukocyte count in patients with SCD has been shown to be good predictor of disease severity and mortality (Miller *et al,* 2000). The increased number and activation of leukocytes indicates an inflammatory response contributing to the pathophysiology of vaso-occlusion and organ damage (Okpala 2006b). Several inflammatory markers may be increased both in crisis and during steady-state. These include several acute phase reactants (APR), interleukin IL-2, IL-4, IL-6, IL-8, tumour necrosis factor- alpha (TNF-α), and activated circulating vascular endothelial cells that are positive for VCAM, ICAM, selectins, $\alpha v\beta 3$, and have procogulant properties (Hebbel 2004). The gene expression of leukocyte cells in SCD shows global up-regulation of pro-inflammatory markers as well as compensatory responses to vascular injury involving a number of anti-inflammatory mechanisms. These include haeme oxygenase-1 (HO-1) and IL-10, which are up-regulated and which might help to protect patients from developing coronary atherosclerotic changes, a rare complication in SCD (Jison *et al,* 2004, Lanaro *et al,*

2009). Circulating micro-particles are small membrane vesicles, phospholipid rich, derived from platelets, red cells, white cells and endothelial cells and may have pro-coagulant and pro-inflammatory activity (Piccin *et al,* 2007). Microparticles were found to be significantly elevated during steady state and had higher elevation during sickle cell crises (Shet *et al,* 2003).

Inflammation in SCD is generally considered as secondary to sickling phenomena, but elevated plasma placenta growth factor (PLGF) an angiogenic growth factor primarily expressed by placental trophoblasts and a potent stimulant of monocyte and proinflammatory cytokines is released by marrow erythroid cells, and has been correlated with incidence of vaso-occlusive events, suggesting this may be a primary event (Perelman *et al,* 2003).

APR, such as CRP and SAA, increase in SCD in steady state and during crises. In steady state a sub-clinical microvascular occlusion occurs with local ischemia which is associated with endothelial and blood cell activation and production of inflammatory cytokines (IL1, IL6, IL8, TNF) (Makis *et al,* 2000a). Variation of APR concentrations have been found to correspond with periodic fluctuations in well being and tiredness during apparently crisis free periods (Akinola *et al,* 1992). Detection of changes in APR concentrations in the prodromal phase of a sickle cell crisis might be of therapeutic value as early intervention could be benificial before tissue injury is established (Stuart 1993). CRP levels have been used as a general marker of systemic inflammation in SCD patients, although a marker that reflects damage to specific cellular components is not yet available (Hagar & Vichinsky 2008). CRP and SAA showed greater elevation in SS disease more than in SC disease or normal AA controls (Singhal *et al,* 1993c). Their concentrations were reported to increase early during crises, occasionally within the prodromal

phase, and tended to persist for longer in children who longer hospitalization (Stuart *et al,* 1994). CRP has been correlated with disease severity (Hedo *et al,* 1993), showing parallel concentration with secretory phospholipase A2 in patients in vasoocclusive crisis or with ACS (Bargoma *et al,* 2005). This again indicates the potential of APR monitoring for evaluation of specific interventions.

2.7 SOLUBLE TRANSFERRIN RECEPTOR (sTfR)

Transferrin receptor (TfR) is the membrane receptor controlling iron entry into body cells, and is abundantly expressed on bone marrow erythroid precursors (Trowbridge 1989). The number of TfR sites on the cell surface is inversely related to intracellular iron concentration (Punnonen *et al,* 1997). Transferrin, the major iron carrier protein in the blood, binds to TfR forming a receptor-transferrin iron complex which enters into the cell by a process of endocytosis. The resultant endosome undergoes acidification with dissociation of the complex, leaving iron to diffuse freely into the cytosol. Residual apotransferrin is routed back to the cell membrane and released with return to the extracellular fluid physiological PH (Cook 1999).

TfR is a transmembrane glycoprotein composed of two disulfide-linked monomers with each polypeptide sub-unit containing 760 amino acids. The soluble component in blood (sTfR) is a truncated form lacking the first 100 amino acid residues of the intact receptor and exists in serum as a transferrin receptor complex (Shih *et al,* 1990).

Serum or soluble TfR represents a proportional equivalent of the total mass of tissue TfR over a wide range of erythropoietic activity and iron status. The circulatory concentration reflects total body TfR expression (R'Zik & Beguin 2001). Virtually all circulating TfR is in the form of a truncated extracellular domain with less than 1% found in normal human serum in the intact form. Although, in patients

with SCD, in whom sTfR was substantially increased, intact receptor can be detected representing an average of 3.8% of the total receptors (Shih *et al,* 1993).

sTfR levels increase with iron deficient erythropoiesis, expanded erythropoiesis including hemolytic anaemias, myelodysplastic syndromes and with the use of erythropoietin. The highest levels usually occur in chronic haemolytic anaemias including thalassaemia and sickle cell anaemia (Skikne 2008).

2.8 CLINICAL VARIABILITY AND SEVERITY

SCD is a monogenic disorder with multiple phenotypic expressions with marked individual differences in clinical manifestations or severity. It is well known that severity varies according to Hb genotype, decreasing in order from homozygous SCD (SS), $S\beta^0$ thalassaemia, Sickle C (SC), to $S\beta^+$ thalassaemia. There may be some overlap of severity categories between genotypes, although homozygous SS is the most common and most severe form, characterized by a highly variable clinical course (Redding-Lallinger & Knoll 2006). Affected individuals can be crippled by their disease with recurrent crises and serious complications, whereas others may lead a relatively near normal life with minimal disability. Variation in disease pattern and severity occurs between individuals within the same communities as well as within different socioeconomic and geographical locations (Serjeant 1989).

Underlying reasons for this spectrum of manifestations are not clearly elucidated, as many genetic and environmental factors are interacting, modulating the disease manifestations and complications. Among genetic factors, fetal haemoglobin (Hb F) and co-inheritance of α-thalassaemia are well recognized. High levels of Hb F ameliorate disease severity through a reduction in Hb S concentration which inhibits polymerization and prevents cell sickling. Higher Hb F level is linked to the inheritance of the Senegal and Arab-Indian β^S haplotypes. These haplotypes

were found to be associated with the -158, C→T XmnI polymorphism in the γ-globin gene which influences Gγ gene expression and Hb F production, and they are associated with mild disease (Steinberg & Adewoye 2006). In addition Hb F level and production of F-cells were found to be controlled by other trans-acting regulatory elements, including the quantitative trait locus (QTL) on the X-chromosome (Xp22), 6q22.3- 23.2, and 8q (Kutlar 2007). The locus on the X chromosome may partly explain the higher level of Hb F in females. Other potential genetic modifiers include several single nucleotide polymorphisms (SNPs) which may interfere with endothelial activation inflammation, thrombosis and cell adhesion pathways (Hebbel 2004, Frenette & Atweh 2007). In addition to the XmnI polymorphism at position -158 in the Gγ –globin gene promoter (rs7482144), four other common SNPs have been detected including one which correlates strongly with Hb F expression (rs4671393). This resides in an intron of the gene BCL11A on chromosome 2. The other three (rs28384513, rs9399137, and rs4895441) are located in the inter-gene region between HBS1L and MYB on chromosome 6 (Lettre *et al,* 2008). These five SNPs explain >20% of Hb F variation and are associated with a reduced rate of pain crisis in SCD patients. Other SNPs contributing to disease variability are likely to be recognized in the future.

Co-inheritance of α-thalassaemia with SCD is a result of deletion of one or more of the four α-globin genes. The effects are most marked in SS patients with homozygous α-thalassaemia 2 (-α/-α), and intermediate in heterozygotes (-α/αα). They result in high Hb and hematocrit, low mean corpuscular haemoglobin concentration (MCHC), and higher Hb A2 with an overall decrease in cell sickling and haemolysis, although whole blood viscosity is increased (Ballas 2001). These haematological changes are associated with a reduced prevalence of leg ulcers, ACS

and stroke, but an increased prevalence of splenomegaly, splenic sequestration, AVN, retinopathy and probably painful crises.

The identification of β^S cluster haplotypes through their association with distinct combinations of polymorphic restriction endonuclease sites, lead to a recognition of five haplotypes named after the geographic regions or ethnic groups of origin. These are common, and include the Benin (BEN), Bantu or Central African Republic (CAR), Senegal (SEN), Cameroon (CAM) and Arab-Indian (AI) haplotypes and related to clinical severity. SEN and AI are the mildest, CAR the most severe, while BEN and CAM are intermediate (Powars *et al,* 1990a, Powars & Hiti 1993)

DNA haplotypes of β-globin gene have been used for determination of the origin of mutational events whether unicentric or multicentric and in tracking gene flow. Origin and distribution of β^S haplotypes is assumed to support the multicentric theory, in which the sickle gene most likely arose at least three times on separate pre-existing chromosomal haplotypes in Africa, and on one occasion outside Africa, probably in the Indus basin at the time of Harappa culture (Nagel & Fleming 1992). From these original foci of the Hb S mutation, gene flow occurred to the Mediterranean and North Africa through trade routes, to North and South America and the Caribbean during the Atlantic slave trade and recently to Europe by immigration (Serjeant 1994). In addition to anthropologic and genetic implications, the β^S polymorphisms may explain variation in the clinical manifestations and drug responsiveness.

The role of environmental and socioeconomic factors are complex and may influence disease state through many pathways, including cold exposure, extremely hot weather, exposure to malaria or other endemic infections, inadequate nutrition

leading to folate and various micronutrient deficiencies and the quality of family support and health care (Serjeant 1994, Serjeant 1995).

2.8.1 SEVERITY ASSESSMENT

Assessment of disease severity with identification of children at high risk for serious complications may allow patient selection for more vigorous therapy including hydroxyurea or bone morrow transplantation. This may assist in tailoring available therapy to the degree of clinical severity and facilitate interventions before irreversible organ damage takes place (Quinn & Buchanan 2002). In children assessment of severity is more difficult than in adults, due to age related haematological changes, progression of splenic dysfunction and a relatively late onset of clinical manifestations and complications (Bray *et al,* 1994). It is even more difficult to predict individual severity and prognosis and few predictors have been identified, such as early dactylitis (before the age of 1year), steady state leukocytosis, low basal Hb concentration (< 7g/dl) (Miller *et al,* 2000), and early ACS during the first 3 years of life (Quinn *et al,* 2007).

Clinical severity criteria classified by combinations of clinical events have been used for categorizing patients for intervention therapies. Examples of this include the occurrence of three or more painful crises within the year before entry, or at least 3 episodes of ACS requiring hospital admission within 2 years of entry, a previous history of stroke, recurrent crises without free intervals, or splenic sequestration (Ferster *et al,* 1996, Kinney *et al,* 1999, Wang *et al,* 2002). Due to the difficulties in integrating genetic and phenotypic variability in severity assessment, Sebastiani et al.,(2007) used Bayesian modelling to analyze the interaction of fourteen clinical and laboratory variables. From this analysis a computerized method was developed for calculation of individual severity scores for prediction of risk of

death. Despite these innovations clinical judgment still remains the mainstay of assessment in individual cases.

2.9 PROGNOSIS AND MORTALITY

While infants in the first 6 months of age mostly experience a period of disease free symptoms because of their high level of Hb F, the delay in diagnosis until at least the second half of their first year could result in increased subsequent mortality risk. Neonatal screening with early diagnosis, accompanied by comprehensive care and regular follow-up, has been reported to decrease early morbidity and mortality (Lee *et al,* 1995). In American studies survival probability to 20 years of age was approximately 85% (Quinn *et al,* 2004), but optimistic estimates of 98% have been reported from an East London cohort (Telfer *et al,* 2007). The main causes of death in children include infection, acute splenic sequestration, aplastic crisis, ACS and stroke (Powars *et al,* 2005). While in adults irreversible chronic organ damage of the lungs, kidney, brain and liver become more significant causes of poor survival. The median survival for SS patients in the US and Jamaican studies was estimated at 45-55 years, and near to normal for SC disease (Platt *et al,* 1994, Wierenga *et al,* 2001). For patients from Africa no data are currently available, but median survival could be less than five years (Serjeant 2005).

2.10 DIAGNOSIS

Complete blood counts and films provide useful information. Hb is low with normocytic normochromic anaemia unless there is co-existence thalassaemia or iron deficiency, when microcytic and hypochromic red cells are present. Sickled and target cells and Howell-jolly bodies are present in the blood film. Reticulocyte and white cell counts are elevated and the erythrocyte sedimentation rate (ESR) is low. A

sickling test to induce red cell changes under low oxygen, or a solubility test to detect insoluble Hb S when reduced by sodium metabisulphate, are frequently used test. Both the sickling and solubility tests do not differentiate disease from trait and are of no use in newborn babies. Hb electrophoresis at alkaline pH (8.4) with acetate cellulose (ACE) is easy, quick, and cheap and provides sharp resolution allowing quantification of the major Hb bands by densitometry. Acidic electrophoresis at pH 6.1 is capable of separating other variants not detected by ACE. Isoelectric focusing (IEF) is a more sensitive method utilizing an electric field to focus Hb fractions into sharp and distinct bands according to their isoelectric points. High-performance liquid chromatography (HPLC) separates Hb bands on the basis of charge, with excellent resolution, is highly automated and straightforward to interpret. Deoxyribonucleic acid (DNA) testing can be used to confirm diagnosis in neonatal cases and in prenatal diagnosis. For prenatal diagnosis fetal cells, obtained by amniocentesis or chorionic villous sampling (CVS) during the second trimester are tested by polymerase chain reaction (PCR) amplification of DNA. This procedure is associated with approximately a 1% risk of fetal wastage. A non-invasive method for prenatal diagnosis uses maternal blood for harvesting fetal erythroblasts with the nucleus amplified by PCR and tested for gene mutation (Cheung *et al,* 1996). Pre-implantation diagnosis by PCR analysis of a single blastomere followed by vitro fertilization, allows only unaffected embryos to be transferred to the uterus (Xu *et al,* 1999). For newborn screening, blood spot heel prick samples collected on Guthrie cards are considered the best method for sampling because of availability, ease of transport, stability and lack of maternal blood contamination (Henthorn *et al,* 2004). IEF and HPLC are the main technical methods using in neonatal screening programs, with DNA testing for confirmation of doubtful results.

2.11 MANAGEMENT

Advances in the management of SCD have been achieved following introduction of neonatal screenings with earlier comprehensive care, leading to important reduction in morbidity and mortality of affected children. Penicillin prophylaxis and full immunization are successful measures for prevention of serious and fatal infection in early childhood. Parental education and early intervention can prevent fatalities caused by acute splenic sequestration crises. Prevention of stroke by chronic transfusion and utilization of TCD has proven to be effective. Proper management of acute and chronic complications facilitates their early detection and amelioration of their impact on the patient's quality of life. Hydroxyurea has been demonstrated to reduce frequency of painful crises, number of hospitalization, frequency of transfusion and ACS episodes. It is an effective, non toxic drug, although the long-term safety has not been established. Marrow transplantation can be curative, but is limited by shortage of matched sibling donors, expensive cost and is not widely available. Gene therapy and other investigational agents hold future promise approaches for the treatment of SCD. An outline of management principles and a list of therapeutic and investigational agents for SCD are summarized in Appendices A and B.

CHAPTER THREE

METHODS

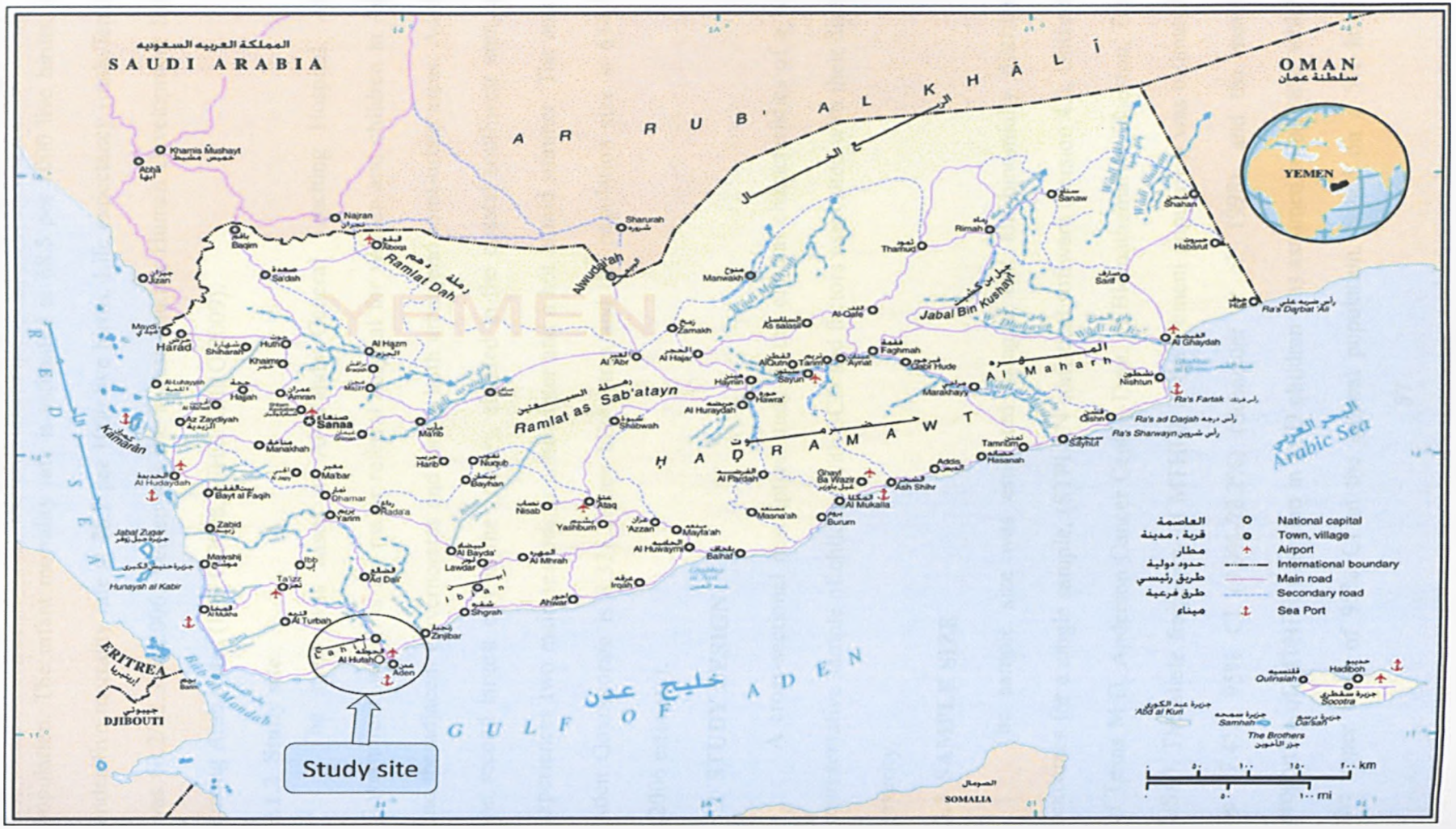
3.1 STUDY LOCATION

3.1.1 Background on Yemen

Yemen or officially the Republic of Yemen is one of the Arabian Peninsula countries and is part of the Middle East region. It is located in Southwest Asia, bordered on the north by Saudi Arabia, south by the Arabian Sea and the Gulf of Aden, east by the Sultanate of Oman and west by the Red Sea (Figure 3.1). The Republic of Yemen was established on May 22, 1990, with the union of North Yemen (the Yemen Arab Republic) and South Yemen (the People's Democratic Republic of Yemen). Sana'a is the capital, and Aden is the economic capital. Yemen has an area of 555,000 km^2, is administratively divided into 22 governorates, and is the second most populous country on the Arabian Peninsula. Its estimated population was 22.2 million in 2007 with about 61% living in rural areas. Annual population growth is 3% and the fertility rate is 6.2 (2005). About 45% of the population are less than15 years, with 3.5% $\geq$ 65 years. Arabic is the official language, and Islam is the state religion. Yemen is one of the poorest Arab countries with a Gross Domestic Product (GDP) per capita between US$ 880–US$ 904.

Health care infrastructure services in Yemen are poorly developed restricting quantity of care per capita. Annual health expenditure is currently 16 US$ per person.

Figure 3. 1 Map of Yemen showing study site



In rural areas there is only 25% health service coverage, compared with 80% in urban areas. There are three physicians and seven hospital beds per 10,000 population. The infant mortality rate is estimated at 68.5 per 1000 live births, and under-five mortality rate at 78.2 per 1000 live births. Life expectancy (male/female) was 60.2/62.3 in 2006. Yemen has the lowest ranking Human Development Index among Arab states (L.O.C 2008, WHO/EMRO 2009).

3.1.2 Study site

The study was based at Al-Wahda General Teaching Hospital, Aden Governorate, which is the major referral hospital for women and children in Aden and the adjacent Governorates and serves both urban and semi-urban areas. Aden is the second largest city in the country, situated in the coastal southwest area, and experiences two main seasons, a cool winter and a hot humid summer. The area of Aden Governorate is 8,321 square kilometres and the population size is 634,710 (2006 estimate).

3.2 STUDY DESIGN

A cross-sectional descriptive study of the clinical characteristics of a large representative sample of children with SCD and factors associated with their disease severity.

3.3 SAMPLE SIZE

The sample size was estimated using a test for binomially distributed outcomes for a single sample, (STPLAN statistical software (version 4.2, University of Texas M.D. Anderson Cancer Centre, Dept. of Biomathematics, Houston, Texas, USA). The allele frequency of MTHFR in the Yemeni population was estimated to be 17.4%, 95% CI (9.9%-28.2%) (Schneider *et al,* 1998), and the assumed proportion of MTHFR mutation in SCD children was assumed to be 28%, which is the upper band of 95% CI in the general population. Based on a 5% level of

significance with 80% power, a sample size of 90 would be required to detect this population prevalence. For the sample size of children with SS disease, the estimated prevalence of affected cases in the general population was 0.12%, and the highest expected frequency would be 5.12% (i.e ± 5% error). Accordingly, the required sample was 31 children with SS disease based on a 5% level of significance with 80% power.

For the sample size required for clinical and other descriptive purposes, the estimated prevalence of affected SS cases in the Yemeni population was 0.2% (WHO 1994), and the highest expected frequency of SS cases would be 5.2% (i.e ± 5% error). This requires a sample of 57 children based on a 5% level of significance with 80% power. The final sample was 100 children which was higher in order to allow for the sample size estimated for calculated MTHFR prevalence.

According to the estimated prevalence of affected infants with SS disease in the Yemeni population, there would be 20 new cases/ 10000 live births annually (Al-Nood *et al,* 2004). In Aden governorate 8616 infants are born annually (CSO 2005) which gives an expected frequency of 17 affected infant/annum. Over a 15 year period (the age range of enrolled cases) 255 new cases would be expected based on this annual incidence rate. Taking in consideration the estimated mortality of about 20% derived from the family history of sickle cell related deaths, the size of the SS population in this community is approximately 204 children. The sample of 102 cases included in this study therefore represents at least 50% of the estimated population.

3.4 STUDY SUBJECTS

Inclusion criteria were children aged less than 16 years with a clinical diagnosis of SCD supported by a positive sickling test and confirmed by the presence of Hb S, Hb F and Hb A2 $\leq$ 3.5% in haemoglobin electrophoresis. Symptomatic or asymptomatic children at the time of study were included. Exclusion criteria were a recent blood transfusion in the previous three months, HB A2 >3.5%, or regular intake of antifolate chemotherapy (i.e. pyrimethamine, chlorcycloguanil, or anticancer drugs). All children screened were eligible and no child satisfied the exclusion criteria.

3.5 SUBJECT ENROLMENT

The clinical study was conducted between March and August 2005. A Sickle Cell Clinic was established specifically to facilitate the study within the Out-patients Department at Al-Wahda Teaching Hospital which was staffed by two nurses. An introductory session was conducted with the study nurses in order to explain the purpose of the research, the details of study procedures, blood collection, laboratory tests and sterile procedures. The clinic was opened on a daily basis during the six month period of the study, offering free clinical and laboratory services. Information on the provision of clinical services in the clinic was provided to the general public through notices in popular local newspapers and through the Health and Education Authorities. The nature and purpose of the study was fully explained to parents or guardians, both verbally and using a written information sheet (Appendix D). If they agreed to participate then written informed consent was requested for each participant prior to enrolment (Appendix E).

Children who attended the Out-patient Department received hospital or home management based on their clinical condition when assessed and their parent's

familiarity with care pathways. Children with acute disease complications were not seen in the Out-patient Department, but enrolled following admission to the Haematology-Oncology ward, where they were directly admitted by the paediatric resident on duty. All hospitalized children also were assessed by a senior paediatric resident or consultant paediatrician. Hospital management protocols were followed on admission and children were reassessed early on the next morning following their admission and their management protocol revised as required. Once the child's condition was stabilized, the parent/guardian was approached concerning the request to include their child in the study protocol.

Hospital records for children who had previous admissions were reviewed and relevant information was extracted on past medical history, reason and duration of admission, investigations performed and treatment given.

3.6 CLINICAL DATA COLLECTION

Data was collected by parental interview using a pre-structured questionnaire (Appendix C). A detailed history and clinical examination was completed. Information on family history, past medical history and frequency of acute clinical events during the previous three years was requested. Information on previous hospital admissions, blood transfusion, severity and frequency of crises (vaso-occlusive, splenic sequestration, aplastic, hyperhemolytic, cerebrovascular) and other complications such as infection, ACS, avascular bone necrosis, skin ulcers, gall stones, priapism and urinary disorders was requested and discussed during the interviews. History of previous and current drug intake was requested.

3.6.1 Definition of steady state

The child is considered in a "steady state" if well and symptom free at the time of study enrolment and for at least the previous two weeks. The term "acute

disease complication" was used for children who were acutely ill due to sickle cell crisis or other acute clinical condition at the time of enrolment. The age and sex characteristics of studied children in these two groups are shown in table 3.1. Children with acute disease complications included those with ACS (10), acute respiratory infection (8), painful crises (5), hepatic crises (3), malaria (3), anaemic crises (2), acute dactylitis (1), and dengue fever (1).

Table 3. 1 Sample characteristics

	Steady state n=69	**Acute disease** n=33	**All** n=102
Male	35	21	56
Female	34	12	46
Age (yrs)*	7.8±1.4	5.9±4.3	7.2±4.5

*mean (SD)

3.6.2 Definitions of clinical events

Definition of clinical events were those proposed by the Co-operative Study of SCD (CSSCD) (Gill *et al,* 1995).

Acute painful crisis was defined as pain in the extremities, back, abdomen, chest, or skull for which no other explanation could be identified, and of sufficient severity that led to the request for medical care. An acute anaemic episode was defined either as acute splenic sequestration (decrease of haemoglobin or hematocrit level of at least 20% from baseline accompanied by increase in palpated spleen size of at least 2 cm from baseline), or as an acute reduction of the haemoglobin or hematocrit level of at least 30% from baseline and unrelated to non-sickle cell causes (e.g., blood loss). It was not possible to clearly separate other anaemic events into aplastic episodes or hyperhemolytic episodes and these were grouped together as anaemic events. Meningitis was defined on the basis of abnormal cerebrospinal fluid (CSF) findings

and/or a positive CSF bacterial culture. Acute chest syndrome was defined as a new chest infiltrate on chest x-ray in the presence of acute pulmonary symptoms. A skeletal event was defined as acute pain involving one or more bones or joints which lasted at least 7 days; osteomyelitis on the basis of a positive culture on aspiration or non-traumatic swelling of one or more joints associated with pain or effusion. Hand-foot syndrome was defined as pain and tenderness, with or without swelling, in the hands and/or feet of a child less than 10 years of age. Stroke was defined as onset of acute neurologic symptoms and signs lasting for 1 day. Enuresis was defined as frequent bed-wetting with a frequency of at least twice per month, in a child older than 6 years. Priapism is undesired painful erection; episode can be self limited of short duration (minor), or long duration and required intervention (major). A facility for blood culture was not available in our hospital which hampered etiological diagnosis of invasive pathogenic infection.

All children had a full clinical examination and anthropometric assessment. Information was collected from one or both parents, who were requested to bring all available medical documents (prescriptions, previous investigation records, and notes of hospital discharge). Information was cross-checked where appropriate against the admission records available through the Hospital Registration Department.

3.6.3 Anthropometric measurements

All anthropometric measurements were carried out by the researcher himself. Children were measured in light clothes without shoes or other footwear.

3.6.3.1 Length/Height

For infants and children under two years of age, recumbent (crown-heel) length was measured using a locally made wooden length board with a standardized length scale. The infant was placed on the flat board, in a supine position with their

head against the headboard and eyes looking forward. Crown-heel length was read to the nearest 0.1 cm using a sliding foot piece. In children older than two years standing height was measured using a vertical measuring rod (TTM, Japan), with a 200 cm length and accuracy of 0.1 cm. The child was asked to stand with buttocks, shoulders and the back of the head touching the upright rod with hands hanging loosely. The head was held erect with the lower border of the orbit of the eye on the same horizontal plane as the external ear canal. A metal headpiece was lowered to touch the top of the head. Measurements were in duplicate and the mean value obtained.

3.6.3.2 Weight

For infants and younger children a baby scale (MISAKI, Japan) was used with a maximum load capacity of 20 kg: 0-10 kg x 50g, and 10-20kg x100g. For older children a standing scale (TTM, Japan) with maximum capacity of 120 kg and a 100g increment was used. The scales were calibrated with a standard weight at the commencement of the study then checked weekly. Measurements were in duplicate and the mean value obtained.

3.6.3.3 Mid-upper arm circumference (MUAC)

A non-distensible fibreglass tape (LAVIGNE, France) with accuracy of 0.1 mm was used. Measurement was taken on the left arm which was hanging loosely at the mid-point between the olecranon process and the acromion.

3.7 SEVERITY ASSESSMENT

Severity was assessed based on the frequency of pain crises, hospitalization, blood transfusion and the occurrence of sickle cell-related complications. The score used was adapted from that described by El-Hazmi (1992a). The Severity Index (SI)

was calculated according to the frequency of clinical events (painful crises, hospitalization and blood transfusion) in the previous year and the occurrence of SCD related complications (Table 3.2). SI contains 15 items and maximum score of 50. The presence of each of the findings was allocated the corresponding score while its absence was scored as zero. In order to obtain complete clinical data over a full year, a child less than one year old at the time of enrolment was subsequently requested to be seen bi-monthly for medical care and at his/her first birthday. For older children, they had only assessed for once at the time of enrolment as their follow up scheduled for routine care was every six months.

Table 3. 2 Severity Index: event frequency per year or occurrence of complication

Findings	**Score***
Painful crises	0-12**
Hospitalization	0-12**
Blood transfusion	0-12**
Chest infection/ACS	0;1
Stroke	0;1
Avascular osteonecrosis (AVN)	0;1
Osteomyelitis	0;1
Leg ulcers	0;1
Gallstones	0;1
Enuresis	0;1
Priapism	0;1
Retinopathy	0;1
Deep vein thrombosis (DVT)	0;1
Haemoglobin level	
> 11g/dl	0
9-11g/dl	1
7-8.9 g/dl	2
<7g/dl	3
Bilirubin level (>35 μmol/L)	1

* Presence of each of the findings was allocated the corresponding score while its absence was scored as zero

** These were scored on the basis of the frequency recorded per year

3.8 BLOOD SAMPLE COLLECTION AND STORAGE

A blood sample management form was completed for each vein puncture sample (Appendix F). A non-fasting venous blood sample (6 ml) was collected into a heparinized Monovette tube (Sarstedt, Leicester, U.K.), at a standardized time (9-11 am), with the child in a sitting position. All samples were labelled with a unique patient identification number. Specimens collected for children admitted to hospital were placed on ice before being transported to the laboratory, which was usually within 10 minutes. Samples were divided into aliquots and plasma for each aliquot was obtained by centrifugation (2000 rpm for 10 minutes) at room temperature. A whole blood haemolysate was prepared using a freshly prepared 1% ascorbic acid solution. Samples were stored at - 4°C until transfer to a -70°C freezer within 2 hours of collection. On completion of the field study the samples were shipped frozen on dry ice to the Liverpool School of Tropical Medicine and the Academic Medical Centre, Netherlands.

3.9 LABORATORY PROCEDURES

Laboratory investigations in Yemen were completed according to standard haematological and biochemical methods.

3.9.1 Haematological

Hb concentration was measured by adding 20μL of whole blood to 5 ml of Drabkin's solution, and read by Spectrophometer with 546 nm wave length and factor of 36.8. For the leukocyte count, 20μL whole blood was added to 380μL 2% acetic acid solution and placed in a counting chamber (Neubuers). The leukocyte count was estimated by multiplying the number of cells, chamber depth and dilution for the area examined.

Differential leukocyte counts were obtained from a blood smear stained with Leishman's stain using a manual counter. Reticulocyte percentage was estimated by

counting against red cell number in whole blood using a Brilliant Cresyl blue stain. Counts were multiplied by 100 and divided by 500 RBCs. A sickling test was completed by mixing one drop of whole blood with three drops of a freshly prepared 0.1% sodium metabisulphate solution on a clean slide, using a cover slip closed by Vaseline or wax and kept at 37 °C for 30 to 60 minutes. Slides were examined under a microscope for sickled cells, which if present gave a positive test. Cellulose acetate haemoglobin electrophoresis was completed at pH 8.6, followed by scanning densitometry for determination of the haemoglobin variant fraction (Assel Electrophoresis, SELEO Engineering Densitometer, Italy).

3.9.2 Biochemical

Biochemical tests were assayed by spectrophotometery (Spectronic 20 Genesys, Spectronic Instruments, Inc, Rochester NY, USA) using commercial reagents (Randox Laboratories, Barcelona, Spain). Laboratory methods employed are summarized in table 3.3.

Table 3. 3 Biochemical tests completed in the local laboratory

Investigation	Unit	Reference range	Method	Wave length*
Total protein	g/dl	6.8-8.7	Biuret	546 nm
Albumin	g/dl	3.8-4.4	Bromocresol	610 nm
Total Bilirubin	µmol/L	Up to 18.81	DMSO	555 nm
Direct Bilirubin	µmol/L	Up to 4.27	(T&D)	555 nm
SGOT*	U/L	0-35	Reitman & Frankle	546 nm
SGPT*	U/L	0-35		
Alkaline phosphatase	U/L	73-207	Colorimetric Method p-Nitrophenyl phosphate	405 nm
Urea	mg/dl	15-40	Urease Berthelot	580 nm
Creatinine	mg/dl	0.2-1.5	Enzymatic kinetic	510 nm

* Spectronic®20 Genesys, Spectronic Instruments, USA, DMSO: Dimethyl sulfoxide, SGPT: Serum glutamic pyruvic transaminase, SGOT: Serum glutamic oxaloacetic transaminase.

3.9.3 Biochemical tests completed in Liverpool

Total plasma homocysteine, whole blood folate and plasma vitamin B12 were analysed by automated methods using the DPC commercial kits and using an Immulite 2000 analyser (DPC-UK, Glyn Rhonwy, Gwynedd, Wales, U.K.). Assays were completed following manufacturer's instructions. Procedure for machine maintenance were completed prior to and following each run as described in the Operator Manual.

3.9.3.1 Homocysteine assay

Samples, controls and adjustors require a pre-treatment step, using 300 μL of pre-treatment solution (Bovine S-adenosyl-L-homocysteine hydrolase and Dithiothreitol) added to 15μL of sample or control. Plasma homocysteine is released from its binding proteins and converted to S-adenosyl-homocysteine (SAH) by off-line 30-minute incubation at 37°C in the presence of S-adenosyl-L-homocysteine hydrolase and dithiothreitol. The treated sample and alkaline phosphatase-labeled anti-SAH antibody are simultaneously introduced into a test unit containing SAH-coated polystyrene beads. During a 30-minute incubation step, the converted SAH from the patient sample competes with the immobilized SAH for binding the alkaline phosphatase labelled anti-SAH antibody conjugate. Unbounded enzyme conjugate is removed by centrifugal wash. Substrate is added, and the procedure continues automatically by the instrument. Between-run coefficients of variation were 9.4%, 4.7%, and 4.4% at 97, 479, and 816μmol/L, respectively. The manufacturer's reference range in adult was 5.0 – 12μmol/L. In children under 15 years using the same instrument, a reference interval (2.5^{th} and 97.5^{th} percentiles) was reported to be 3-10.6μmol/L (Soldin *et al,* 2008).

3.9.3.2 Folate assay

A haemolysate was prepared immediately after blood sample collection by adding 1ml of freshly prepared 1% ascorbic acid to 50μL of whole blood. A working solution (Borate-KCN Buffer Solution, Ligand-Labeled Folate, Dithiothreitol Solution) was prepared on a daily basis. 200μL of this solution was added to same quantity of the adjusters, controls and samples. All tubes were placed in a covered boiling water bath (100°C) for 15 – 20 minutes, and after cooling for 5 minutes, at least 350μL of the treated sample was added to an IMMULITE Sample Cup. The solid phase, a polystyrene bead enclosed within an IMMULITE Test Unit, was coated with a murine monoclonal antibody specific for folic acid binding protein. The ligand-labeled folate and folate binding protein (FBP) were added to the reaction tube containing a bead coated with a monoclonal antibody against FBP. After introduction the test tube was incubated for approximately 30 minutes at 37°C with intermittent agitation. Folate from the sample competed with the ligand-labeled folate for the FBP, and ligand-labeled folate-FPB complex bound to the anti-FBP antibody on the bead. Alkaline phosphatase-labeled anti-ligand was added and bound to the ligand-labeled folate in the complex immobilized on the bead. Substrate addition initiated the chemiluminescent reaction, producing a result that relates to the folate concentrations in the samples. Due to high folate levels which resulted from the patient's regular folic acid supplement intake, samples were diluted 4-8 times and results adjusted for the dilution factor. Between-run coefficients of variation were 9.0%, 4.2% and 5.2% at 2.1ng/ml, 5.2ng/ml and 13ng/ml respectively. The whole blood folate normal reference range provided by the manufacturer was 43–295ng/ml.

3.9.3.3 Vitamin B12

A working solution (Borate-KCN Buffer Solution, Ligand-Labeled Folate, Dithiothreitol Solution) was prepared on a daily basis. 200 µL of this solution was added to the same quantity of the adjusters, controls and samples. All tubes were placed in a covered, boiling water bath (100°C) for 15–20 minutes, and after cooling for 5 minutes, at least 350 µL of the treated sample was added to an IMMULITE Sample Cup. Vitamin B12 in the patient sample is released from carrier proteins by a heat denaturation step, consisting of incubation at 100°C in the presence of dithiothreitol and potassium cyanide to inactivate vitamin B12-binding proteins, as well as antibodies to intrinsic factor. After introduction into the IMMULITE test unit, which contain a polystyrene bead coated with a B12 analog, and hog intrinsic factor, vitamin B12 in the treated sample competes with the B12 analog on the solid phase for a limited number of vitamin B12 binding sites on the purified intrinsic factor. Alkaline phosphatase-labeled anti-hog intrinsic factor is introduced, and the unbound enzyme conjugate is removed by a centrifugal wash. Between-run coefficients of variation were 11.3%, 10.3% and 6.7% at 159pg/ml, 204pg/ml and 401pg/ml respectively. The manufacturers recommended reference range for vitamin B12 was 174-878pg/ml.

3.9.3.4 Vitamin B6

The term vitamin B6 encompasses three naturally occurring pyridine derivatives: pyridoxine, pyridoxamine and pyridoxal and their 5'-phosphate esters. These esters are transformed to pyridoxal-5'-phosphate (PLP), the active co-enzyme form and the major component of the above derivatives.

A vitamin B6 HPLC kit was used to measure PLP (Immunodiagnostik, AG, Benshelm, Germany). The specific determination of the active coenzyme form PLP

requires an isocratic HPLC system using fluorescence detection. The PLP was derivatized to a fluorescent product and extracted from plasma following a single precipitation step. This step was accomplished by adding of 16 µl of derivatisition reagent (semicarbazide/glycine) to 200 µL of plasma sample or calibration standard or control, into brown light-protected microcentrifuge tubes. Precipitation reagent (70% Perchloric acid) was added; then the supernatant solution was withdrawn into an auto-sampler vial containing 15 µL neutralization reagents (0.5M NaOH). A mobile phase consisting of 97% 25mM K_2HPO_4 pH 3.0: 3% MeCN, was used at an adjusted flow rate of 1.75mL/min (LDC ConstaMetric III pump). Detection was accomplished using a fluorescence detector (ThermoSeparation Products FL2000) with an excitation wavelength set at 320 nm and an emission wavelength of 415 nm. Prepared unknown samples are compared to a set of plasma calibration standards to quantify the level of PLP. The manufacturer's reference level for vitamin B6 in our laboratory was (5-30ng/ml).

3.9.3.5 Ferritin

Ferritin was assayed by enzyme-linked immunosorbent assay (ELISA), using commercial kits (Fortress Diagnostics Ltd, Antrim, UK). A diluted sample, control, and calibrator (25 µl each) were added to streptavidin coated wells, then mixed with 100µL biotinylated anti-ferritin monoclonal antibody. Immune complex is formed and is immobilized on the coated well. After removal of the unbound serum proteins by a washing procedure, the antigen-antibody complex in each well is detected with specific anti-ferritin horse-radish peroxidase (HRP)-enzyme labelled antibody. After removal of the unbound conjugate, the strips are incubated with a chromogen solution containing tetramethylbenzidine (TMB) and hydrogen peroxide. The enzymatic reaction is stopped by the addition of a 1N sulphuric acid solution. Colour

change, developed in proportion to the amount of immune-complex bound to the wells of the strips, represents the concentration of ferritin in the sample, and optical density (OD) absorbance values at 450nm ± 2nm were determined. The OD for low and high calibrators was less than 0.1 and more than 1.3 respectively and results for controls fall within the established range. Unknown values were calculated by comparison to the diluted standards using a calibration curve. The final concentration of ferritin read from the standard curve was multiplied by the dilution factor. The reference range for children (6 months to 16 years) is 10-160ng/ml or µg/L.

3.9.3.6 Soluble transferrin receptor (sTfR)

For sTfR determination, a non- competitive "sandwich type" immune-enzymatic assay was used (IDEA sTfR IEMA, Orion Diagnostics Oy, Espoo, Finland). 20 µL of calibrator, control and patient's sample were placed in appropriate wells coated with monoclonal sTfR antibody, followed by added of 200µL of sTfR assay buffer. Plates were incubated for one hour on a fast plate shaker. In order to wash away the unbounded antigens, four times aspiration and washing of the strips with washing solution were completed on a plate washer. To complete the sandwich formation 200µL of enzyme conjugated monoclonal antibody was added to each well and incubated on a fast plate shaker for one hour. The unbound enzyme conjugate was removed by washing for four times, which left the bound conjugate inside the wells, the amount being directly proportional to the concentration of sTfR in the sample. 200µL of diluted substrate solution was added to all wells, incubated for half an hour, followed by the addition of 50µl of the stopping solution to stop the enzymatic reaction. The plate was read on plate reader at a wavelength of 405nm. For each test run, controls were included and results were within the recommended manufacturers range. Due to high levels of sTfR in patient samples, dilutions were

used to allow readings within the detection range. The reference ranges: 0.5–4 years, 1.6–4.0mg/L; 4–10 years, 1.5–3.7mg/L; 10–16 years, 1.4–3.4mg/L, calculated from the population described by Suominen *et al*, (2001)

3.9.3.7 C-reactive protein (CRP)

CRP measurement was conducted using ELISA kit, (DPC-UK, Glyn Rhonwy, Gwynedd, Wales, UK.). Diluted standard sera and patient samples were incubated with microtitre strips coated with anti-CRP antibody. During this incubation step CRP in the samples is bound specifically to the wells. After removal of unbound serum proteins by a washing procedure, the antigen-antibody complex in each well is detected with specific peroxidase-conjugated antibody. Unbound conjugate was removed by a washing procedure and then the strips were incubated with a chromogen solution containing tetramethylbenzidine (TMB) and hydrogen peroxide. A blue colour develops in proportion to the amount of immune-complex bound to the wells. The enzymatic reaction is stopped by the addition of sulphuric acid solution 1N H_2SO4 and the absorbance values at 450 nm ± 2 nm were determined. The unknown values were calculated by comparison to diluted standards by using a calibration curve. The final concentration of CRP read from the standard curve was multiplied by the dilution factor.

3.9.3.8 Serum Amyloid A (SAA)

SAA was assayed by an ELISA method (Biosupply Ltd, UK.). A diluted 100 µL of standard provided in the kit and samples were added to each well in the microtitre strips pre-coated with monoclonal antibody specific for SAA and incubated for one hour. During this incubation step SAA in the samples binds specifically to the wells and become immobilized. After removal of the unbound serum proteins by a washing procedure, a standardized preparation of horseradish

peroxidase (HRP)-conjugated polyclonal antibody specific for SAA is added to each well to "sandwich" the immobilized SAA. All wells were washed thoroughly after an hour second incubation. A substrate solution containing tetramethylbenzidine (TMB) and hydrogen peroxide was added. A blue colour develops in proportion to the amount of immune-complex bound to the wells. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution 1N H_2SO4. The colour developed was measured on a plate reader at a wavelength of 450 nm ± 2 nm. The unknown values were calculated by comparison to the diluted standards by using a calibration curve. The final concentration of SAA read from the standard curve was multiplied by the dilution factor. The SAA reference range was 1-5mg/L (mcg/ml).

3.9.4 MTHFR mutation detection

Testing for MTHFR was performed in the Department of Blood Coagulation, Sanquin Laboratory, Amsterdam, the Netherlands, by Dr. Jan A. van Mourik. In brief, DNA analysis was carried out by restriction fragment length polymorphism after conventional polymerase chain reaction (PCR). The DNA encoding for the MTHFR gene was amplified using the following primers: sense primer 5'-TGAAGGAGAAGGTGTCTGCGGA-3'; anti-sense primer 5'-AGGACGGTGCGGTGAGAGTG-3'. The PCR product was incubated with restriction enzyme HinfI and the presence of the MTHFR 677T allele was assessed by gel-electrophoresis.

3.9.5 β^S haplotype determination

Sequencing of β^S haplotype was performed in the Haemoglobinopathies and Red Cell Diagnostics, Department of Human and Clinical Genetics, Leiden University Medical Centre, Leiden, the Netherlands, by Dr. Cornelis L. Harteveld.

A high resolution melting curve analysis (MCA) was used. Asymmetric PCR to obtain templates for MCA for each fragment F1 to F7 was done using 8μL of a PCR-mix containing 1μL 10x PCR buffer with $MgCl_2$, 0.2mM dNTPs, 1μM forward primer, 10μM reverse primer, 5μM of the relevant MCA-probe, 1μL of LC Green plus (Bioké, The Netherlands), 1 unit Fast Start Taq Polymerase and 4μL Aqua B. Brown. The PCR was performed in a Framestar 96 wells PCR plate from 4titude, per reaction. 15μL mineral oil (to prevent evaporation of the PCR product during melting in the Lightscanner), 8μL PCR mix and 2μL genomic DNA (10ng/μL) were added and amplified 10 min at 95^0C, followed by 40 cycles of denaturation (20 sec 95^0C), annealing (30 sec 58-68^0C) and extension (40 sec 72^0C) and subsequent one step of 5 min at 72^0C and 1 min at 95^0C. DNA samples of which the genotypes are known were included as positive controls and a water sample as a blank. The products were melted in the LightScanner in 8-10 minutes (LightScanner®, Idaho Technologies Inc., USA). The starting temperature was set at 55°C, the hold temperature at 50°C and the end temperature at 98°C. Analysis of the melting curves was performed with LightScanner software (Idaho Technologies Inc., USA) following manufacturer's instruction.

3.10 QUALITY CONTROL

Clinical history and physical examination were undertaken and recorded for all children by the researcher. Anthropometry was measured in duplicate and the mean values obtained. The scales used were calibrated with standard weights at the initiation of the study, and re-checked weekly. Information was collected from the closest carer of the child, mostly the mother, or occasionally both parents, who were requested to bring to the clinic all available medical documents (doctor prescriptions,

previous investigation papers, notes of hospital discharge). For children previously admitted, relevant information was cross-checked against the hospital admission records. Collected data was transferred into the electronic database on a daily basis after being checked and verified. All haematological and biochemical tests were completed according to standard laboratory methods with strict adherence to manufacturer's recommendations. Pre-analytic measures including accurate labelling, specimen handling, time of collection, patient condition and position, storage and transportation were maintained according to a prepared blood sample management form which was completed for each specimen. To minimise inter-observer variability all blood counts were carried by the same senior qualified haematology technician and the biochemical tests by one senior biochemist technician. Slides examined for malaria parasites were re-examined in the centre of the Roll Back Malaria project by experienced laboratory technician expert in malaria slide diagnosis, to confirm the results. Plasma samples were aliquoted on collection to avoid repeated thawing. Calibrators and controls with low and high concentration values were used in each run of laboratory tests and the results were checked for correspondence within the acceptable detection range. Values outside the range were reanalysed to check their values in duplication run.

3.11 DATA ANALYSIS

Clinical and laboratory data were entered and electronically stored, after cleaning data and double checking by the researcher. The SSPS software package was used and statistical analyses were carried out with version 16 for Windows (SPSS Inc., Chicago, IL, USA). Results are expressed as means and standard deviations for normally distributed data, or median with interquartile range for non-

normal data. The independent sample *t* test was used for comparison of means in data with normal distribution, and Mann-Whitney-U test for non-parametric data. Chi-square tests were used for comparison of categorical variables. The Fisher's exact test was used for comparison with cell frequency of five or less. Pearson's or Spearman's correlation coefficients were calculated. Univariate analysis was used to describe variable distribution. Backward stepwise multiple logistic regression was completed to assess the associations between clinical, biochemical variables and severity. Variables with $p<0.2$ identified by univariate analysis were included and Odds ratios and adjusted odd ratios (AOR) with 95 % CI were calculated. Hardy–Weinberg equilibrium between expected and observed genotype distributions was assessed using the Chi-square test. All statistical tests were 2-tailed with a *p value* < 0.05 considered significance.

3.12 STUDY CONSENT

Before recruitment the nature and purpose of the study was explained to the child's parents or legal guardian(s). This was usually the child's mother or father. A patient information sheet was provided written in Arabic in plain language (Appendix D). If parent/guardian agreed to participate then the researcher read out the informed consent and written consent was requested (Appendix E). In cases of illiteracy the consent form was read by the investigator in the presence of a member of the nursing staff and verbal agreement was requested. The nurse then requested permission to witness the consent form on their behalf in the presence of parent/guardian.

Participant data was held in secure files in a locked cabinet accessible only to the researcher. Confidentiality was maintained and data analysis was conducted using an anonymous data base file.

3.13 ETHICAL APPROVAL

The study protocol was approved by the Ethical Committees of the Liverpool School of Tropical Medicine, and the National Ethical Committee in Yemen, Ministry of Public Health and Population.

CHAPTER FOUR

CLINICAL CHARACTERISTICS

4.1 INTRODUCTION

Clinical manifestations of SCD are markedly heterogeneous and there is wide variation in disease pattern and severity between individuals from different socioeconomic and geographical locations (Serjeant 1989). Although factors modulating phenotypic expression of SCD such as Hb F level, co-inheritance of α-thalassaemia, and β^S globin cluster haplotypes are well known, other potential genetic modifiers and several single nucleotide polymorphisms (SNPs) that may affect cell adhesion, thrombosis and inflammation are also proposed (Frenette & Atweh 2007).

The specific symptoms attributed to SCD usually do not occur in young infants before the age of 3 months and it is only by 6 months of age that about 6% of children will have started to develop disease related manifestations. This coincides with the rapid reduction in Hb F during early infancy. Painful crisis is the most common manifestation of SCD and in young children it often presents as acute dactylitis (hand-foot syndrome), and is the initial presenting symptom in about 50% of affected children (Stevens *et al,* 1981, Mulik *et al,* 1991).

Many acute and chronic complications occur in patients with SCD and these are reviewed in Chapter Two. Acute complications such as fulminant bacterial infection and acute splenic sequestration crisis often arise as life-threatening events, and chronic haemolysis leads to the formation of pigmented gall stones and contributes to chronic organ damage (cerebro-vasculopathy, pulmonary and renal damage, leg ulcers and avascular osteonecrosis) (Serjeant 1993).

In the Arabian Peninsula, SCD is common (White 1983). The frequency of the β^S gene in Yemen was estimated to be up to 0.04, with an expected birth incidence of 20/10,000 per year (Al-Nood *et al,* 2004). There is lack of information about SCD morbidity in Yemeni children and this study is the first to describe its clinical pattern and characteristics in this population. The aim of the present analysis was to describe the clinical characteristics and profile of the children with SCD in the Yemeni sample.

4.2 SPECIFIC OBJECTIVE

- To describe the clinical characteristics of SCD in Yemeni children, including age and clinical features at presentation, frequency of clinical events and complications.

4.3 METHODS

The general study methods are outlined in Chapter Three. The sample included all study children below 16 years of age with SCD who were either symptomatic or asymptomatic at the time of enrolment. All children had been diagnosed by a positive sickling test confirmed by SS haemoglobin electrophoresis. Subjects were recruited from the clinic or following admission as outlined in Chapter Three. A detailed history, clinical examination and review of case records were completed. Data on family history, past medical history and frequency of acute clinical events during the previous 3 years was collected using a standard prorforma. Information on previous hospital admissions, blood transfusions, severity and frequency of crises (vaso-occlusive, splenic sequestration, aplastic, hyperhemolytic, cerebrovascular) and other complications such as infections, acute chest syndrome, avascular bone necrosis, skin ulceration, gall stones, priapism or urinary disorders were recorded. Definitions of clinical events were those proposed by the Cooperative

Study Group of SCD (Gill *et al,* 1995). Information was collected from one or both parents, who were requested to bring to the clinic all available medical documents (doctor prescriptions, previous investigation papers, notes of hospital discharge). Information reported on previous admissions was cross-checked against the hospital admission record.

Standard laboratory methods were employed for evaluation of haematological and biochemical tests (Dacie & Lewis 1991). Radiography, ultrasound, and CT scan examinations were performed when requested by the responsible medical team.

4.4 RESULTS

4.4.1 Age at diagnosis and presenting features

A total of 102 children were included in the evaluation, (46 females and 56 males). The mean age at enrolment was 7.2 years, (range 6 months-15 years) (Figure 4.1). Forty five children (44%) were ≤ 5 years of age (Table 4.1).

Figure 4. 1 Distribution sex specific age groups

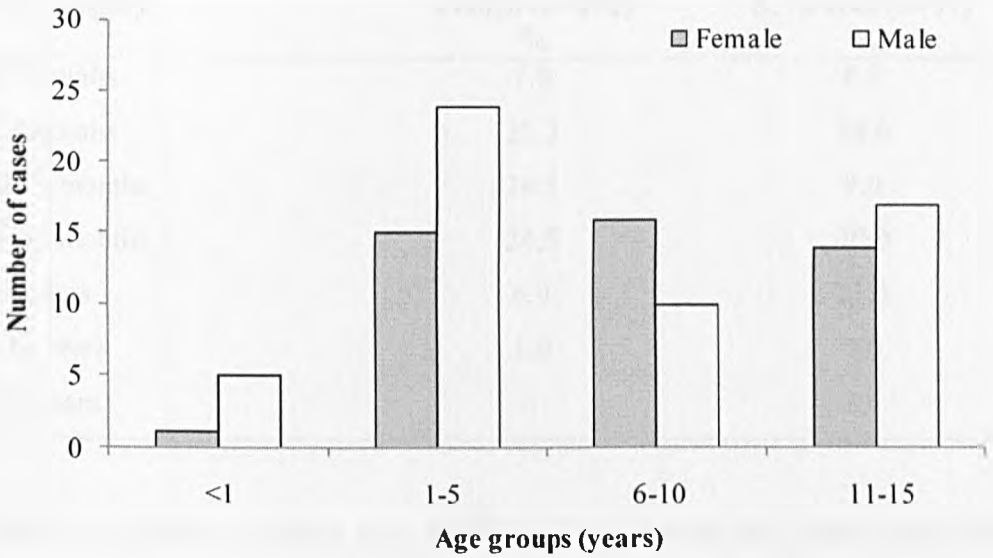


Table 4. 1 Age and sex distribution

Age (years)	Female n=46 (%)	Male n=56 (%)	Total n=102 (%)
<1	1(2.2)	5 (8.9)	6 (5.9)
1-5	15 (32.6)	24 (42.9)	39 (38.2)
6-10	16 (34.8)	10 (17.9)	26 (25.5)
11-15	14 (30.4)	17 (30.4)	31 (30.4)

All patients were Yemeni and most (96%) were from Aden and Lahj, the adjacent Governorate. Mean age at initial diagnosis was 17 months (range 2 months-7 years). Initial clinical manifestations had occurred in 20% by the age of 6 months, and in 67%, 88%, 92%, by the age of 1, 2, and 3 years respectively (Table 4.2). Approximately two thirds of these children (67%) presented during the first year of

age compared with one third (33%) of children from Saudi Arabia. One child had presented by 2 months of age, and another had reported no symptoms until 7 years. Children who presented by 6 months of age all had at least one-affected sibling and family members were already aware of the common presenting features.

Table 4. 2 Age at first diagnosis in comparison with data from Saudi Arabia (Mulik *et al,* 1991)

Age category	Yemen (n=102) %	S. Arabia (n=99) %
3-5 months	7.8	6.0
6-9 months	35.3	18.0
10-12 months	24.5	9.0
13-36 months	24.5	40.0
4-6 years	6.9	21.0
7-10 years	1.0	4.0
>10 years	-	1.0

Dactylitis occurred as early as 2 months of age, peaked at 2 years and became infrequent after 4 years, and it was the most frequent initial symptom reported in 54% of children (Table 4.3). Symptoms related to acute respiratory infection (15%) or other acute febrile illnesses (11%) were the second and third most common presenting features, followed by painful crisis (10.8%), and anaemia (8.8%).

Table 4. 3 Frequency of presenting features

Feature	Number	%
Hand-foot syndrome	55	53.9
Acute respiratory infection	15	14.7
Acute febrile illness	11	10.8
Painful crisis	11	10.8
Anaemia	9	8.8
Malaria	1	1.0
Total	102	100.0

Compared to Jamaican cohort, dactylitis was the presenting symptom during first three years of age in 54% of Jamaican children, which was in close similarity to the 52% of Yemeni children (Table 4.4).

Table 4. 4 Dactylitis as initial symptom by age in comparison with data from Jamaica (Stevens *et al,* 1981)

	Yemen		Jamaica	
Age (months)	n	%	n	%
0-5	5 (7)	71.4	8 (16)	50.0
6-11	24 (39)	61.5	40 (79)	50.6
12-23	15 (31)	48.4	39 (84)	46.4
24-35	9 (15)	60.0	12 (43)	28.0
36-47	0 (2)	0.0	2 (17)	11.7
≥48	2 (8)	25.0	1 (17)	5.8
Total	55 (102)	53.9	102 (256)	39.8

Brackets: age-specific sample size

4.4.2 Morbidity and co-morbidity

Painful crisis is a major cause of acute morbidity affecting 90.2 % of children and only 9.8% denied a previous history of a pain episode. In the three year period there were 647 painful episodes. The rate of painful crisis (episodes/year) was zero in 11.8%, 1-2 crises in 63.7% and ≥3-10 crises in 24.5%. The group with ≥3 crises, represented only one quarter of children although they accounted for more than two thirds (67.7%) of all episodes. The rate of painful crisis was significantly correlated with Hb concentration ($r=+0.22$, $p=0.028$), and PMN count ($r=+0.3$, $p=0.002$). A negative non-significant correlation was observed with Hb F%.

Eighty five patients (83.3%) were previously admitted to hospital and seventeen gave no prior history of hospital admission. There was a history of two hospital admissions for 32 patients, three and more for 18 patients, and a total of 159

previous admissions giving overall admission rate of 0.6 episodes per child/year. Table 4.5 shows that painful crisis was the predominant reason for hospital admission (36%), followed by anaemic crises (16%). Of those with acute respiratory symptoms, 11% fulfilled the definition of acute chest syndrome defined as a new pulmonary infiltrate on chest x-ray associated with acute respiratory symptoms.

The hospital admission rate was the same for both sexes (male 86/46, female 73/39). More male patients were admitted with ACS, cerebral vascular accident (CVA), malaria and acute hepatic crises, although these differences were not statistically significant.

Table 4. 5 Diagnosis at time of hospital admission

Diagnosis	Number = 159 (%)	Male n=46	Female n=39	M:F ratio
Pain crisis	57 (35.9)	29	28	1
Anaemic crisis	25 (15.8)	12	13	1
Acute chest syndrome	17 (10.7)	11	6	1.8
Malaria	13 (8.2)	9	4	2.25
Lower respiratory tract infection	12 (7.6)	7	5	1.4
Hand-foot syndrome	6 (3.8)	2	4	0.5
CVA	5 (3.2)	4	1	4
Hepatic crisis	5 (3.2)	4	1	4
Diarrhoea	5 (3.2)	2	3	0.67
Typhoid fever	2 (1.3)	1	1	1
Cardiac failure	2 (1.3)	1	1	1
Osteomyelitis	1 (0.6)	1	0	1
Meningitis	1 (0.6)	1	0	1
Other*	8 (5.0)	2	6	0.3

* Including acute febrile illnesses, epistaxis, dengue fever
Brackets: percentage

In 27 children there was no previous history of blood transfusion, while 33 children received no transfusion in the last 3 years and no child was on regular

transfusion. Blood transfusion was required on 160 occasions. Thirty nine children receiving 2-4 transfusions and 7 children received five or more transfusions (Table 4.6).

Table 4. 6 Frequency of blood transfusions by age category

	Age (years)				Total
Blood transfusion	**<1**	**1-5**	**6-10**	**11-15**	
0	2	11	4	16	33
1	3	14	11	5	33
2	0	5	6	5	16
3	0	1	3	2	6
4	0	5	0	2	7
5 and more	1	3	2	1	7
Total	6	39	26	31	102

On physical examination, pallor was present in 92% and jaundice in 20%. Mean haemoglobin level (± SD) was 7.7 ± 1.5g/dl, and only seven children had Hb concentration above 10g/dl. Most children (71.6%) had Hb values between 6-9g/dl. Severe anaemia (Hb < 5g/dl) was present in four patients.

Haemolytic facial bone change including frontal bossing and/or prominent upper jaw were observed in 28% of children. A cardiac murmur was present in 34 cases (33.3%), which was mostly systolic in timing. For one child the presence of cardiac murmur as well as painful arthropathy lead to initial diagnosis of rheumatic heart disease prior to establishing the correct diagnosis of SCD. Cardiac failure developed in two children. Hepatomegaly on palpation was present in 74 cases (72.5%), which was more than 2 cm below the right costal margin in 42 (41.2%). The spleen was enlarged in 41 patients (40.2%), (Table 4.7). Hepatosplenomegaly was present in 37 patients. Both liver and spleen size decreased with age, with a significant negative correlation with increasing patient age (Liver $r=-0.29$, $p<0.01$; Spleen $r = -0.47$,

p<0.001). Spleen size was correlated negatively with Hb concentration *(r=-0.4, p<0.01),* and positively with transfusion frequency *(r=+0.24, p<0.05)* and reticulocyte count *(r=+0 .31, p<0.01).*

Table 4. 7 Liver and spleen enlargement

Size (cm/BCM)*	Hepatic border		Splenic border	
	n	%	n	%
<2 cm	32	31.4	10	9.8
2-4 cm	34	33.3	19	18.6
>4 cm	8	7.8	12	11.8
Total	74	72.5	41	40.2

* Below costal margin

Plasmodium falciparum malaria confirmed by microscopy, was diagnosed in 21 patients (20%), in the year prior to enrolment in this study. Malaria infection was reported as the reason for hospitalization on 13 occasions. Splenomegaly was present in 8 children and there was no significant difference in the rate of splenomegaly between those with and without a history of malaria infection.

4.4.3 Complications

Complications related to SCD are summarised in Table 4.8. Seventeen children had acute chest syndrome (11 males, 64.7% and 6 females 35.3%), with a mean age (± SD) of 4.6 ± 3.3 years (range 6 months-12 years). Three children 17.6% had ACS episodes before 2 years of age, 9 (53%) between 2-5 years, 3 (17.6%) between 6-10 years, with only two children (11.7%) were older than 10 years. Broad spectrum parenteral antibiotics were given empirically to all patients, usually ampicillin, or one of the second or third generation cephalosporins, in addition to an oral macrolides (erythromycin or azithromycin). Eight children received blood transfusions, although none received exchange transfusion. The mean duration of

hospital admission was 8.6 days. The patient with the shortest stay (4 days) was discharged against medical advice.

Table 4. 8 Sickle cell disease complications

Complication	All n, (%)	Age		Male n	Female n	M:F ratio
		≤5 yrs n=45, (%)	≥ 6 yrs n=57, (%)			
Acute chest syndrome	17 (16.7)	12 (26.7)	5 (8.8)	11	6	1.8
CVA	5 (4.9)	2 (4.4)	3 (5.3)	4	1	4
Gall bladder stone	5 (4.9)	0 (0)	5 (8.8)	2	3	0.67
Hepatic crisis	5 (4.9)	2 (4.4)	3 (5.3)	4	1	4
Leg ulcer	5 (4.9)	0 (0)	5 (8.8)	2	3	0.67
Priapism (56 males)	7 (12.5)	1/29 (3.4)	6/27 (22.2)	-	-	-
Epistaxis	15 (14.7)	4 (8.9)	11 (19.1)	10	5	2
Haematuria	9 (8.8)	3 (6.7)	6 (10.5)	3	6	0.5
Avascular necrosis	1 (1.0)	0 (0)	1 (1.7)	1	0	1
Enuresis	25 (24.5)	-	25 (43.9)	13	12	1

CVA occurred in 5 children, (4 males, 1 female), all aged less than 8 years, with a mean age for the first episode of 3.5 years (range 8 months-7years). Hemiparesis occurred in all these children (3 left, 2 right-sided), convulsions in 3, and speech impairment, either aphasia or dysartheria, in 4 cases. One case recovered completely without apparent residua, while the other 4 children had some residual weakness. Recurrent stroke occurred in 3 children, one after 2 months, and in 2 a year after the initial episode. All patients with CVA received blood transfusion between 2-5 times, and none received regular transfusion regime for secondary stroke prevention. Cerebral infarction was diagnosed in 4 cases by CT scan. Other neuroimaging modalities were not available.

Gallbladder stones, diagnosed by abdominal ultrasound, were identified in five patients, mean age 10.2 years (range 6-14 yrs), none of whom required cholecystectomy. Five patients developed hepatic crisis, 3 of whom were admitted to hospital throughout the study period. During hepatic crisis, these children had abdominal pain, deep jaundice and hepatomegaly. Their mean±SD bilirubin was 543± 331μmol/L, with over 50% (371 ± 198 μmol/L) as conjugated bilirubin. Their mean haemoglobin concentration was 8.8 g/dl (range 7.7-10.5g/dl); thus excluding severe haemolysis as the cause of their hyperbilirubinemia. Although, all these children improved with supportive therapy and transfusion, their improvement was slow with deep persistent jaundice and they required hospitalization for between 19-34 days.

Nocturnal enuresis was present in 43.9% (25/57) of children ≥ 6 years of age (13 boys, 12 girls). A total of 55 % who were between 6 and 9 years had enuresis, with the frequency falling to 42% for those between 10-12 years, and 32% for those 13-15 years of age. Priapism was reported in 7 males, and all episodes were of minor type, repeated infrequently and resolving spontaneously without medical intervention.

History of epistaxis was reported in 15 children (14.7%) (10 males; 5 females). Four were 2-5 years (11.8%), 5 between 6-10 years (19.2%) and 6 between 11-15 years (19.4%). Epistaxis was severe in one case requiring hospitalization. Gum bleeding occurred in 3 patients.

4.4.4 Consanguinity

The study sample comprised 86 families. Five patients had one affected sibling, four patients had two affected siblings, and one patient had three affected siblings, all of whom were included in the study. Hb genotype was unknown for the

majority of parents, (66 (76.7%) fathers, 63 (73.3%) mothers). For the 83 families with more than one child, 42 siblings had AS trait and Hb genotype was unknown in the other 115 siblings. Consanguineous marriage occurred in 62.8% of parents, with the majority of children (32.5%) the progeny of consanguineous parents (first cousin), 16.3% second cousin, or a distant relative in 14%.

4.4.5 Mortality

One death occurred during the study period in a 12 year old girl, which was due to severe dengue fever, occurring during a dengue epidemic in Yemen in 2005. Information on SCD mortality from the family history showed that 18% of families had a history of a SCD related death. More than quarter of the reported deaths (27.6%) occurred during the child's first 2 years, and more than 75% during the first 15 years of life.

4.4.6 Patient medical care

The medical management of cases followed standard hospital practice at Al-Wahda Teaching Hospital. There were hospital norms for the medical management of SCD cases. Management outside the hospital was unregulated and dependent on the knowledge of a particular practitioner and their experience. All children with haemoglobinopathies including those SCD cases enrolled in the study continued to receive their regular medical follow up in the paediatric out-patient clinics mostly under the care of paediatric specialists working in the paediatric haematology/oncology division. Aden is covered by relatively good public health facilities comprising four hospitals and ten polyclinics along with many private health services, all of which support better care for these patients. Nevertheless, there is no registry for patients with SCD and special care facilities are not available.

Neonatal screening or comprehensive care packages do not exist either at a local or national level, with no prenatal testing, and little if any genetic counselling. Infection prophylaxis comprises penicillin administration (penicillin VK 125 mg given orally twice a day, increasing to 250 mg twice daily at three years of age), or use of intramuscular long acting benzathine penicillin if compliance is not ensured. Oral folic acid 5mg/daily is given routinely. Penicillin and folic acid supplementations are provided irregularly by the public health facilities and in most instances patients purchase their drugs by themselves from private pharmacies or drug stores without requiring a prescription. The National Expanded Programme of Immunization includes BCG (Tuberculosis), OPV (Poliomyelitis), DPT (Diphtheria, Pertussis, Tetanus), Hib (Haemophilus influenzae type b), hepatitis B and measles, but does not include pneumococcal vaccines. Children with SCD are not screened routinely for retinopathy or gallbladder stones. Screening for risk of stroke by TCD is also not available; therefore there is no chronic transfusion programme for stroke prevention. The main indications for blood transfusion were severe anaemic episodes, acute management of ACS and stroke. Blood collection is based mainly on replacement donors and occasionally volunteer blood donation, with minimal group phenotyping limited mainly to ABO and Rh compatibility. Routine screening for HIV, hepatitis B and malaria are done before donated blood is collected. Due to blood safety issues and difficulties with patient monitoring, doctors at Al-Wahda hospital were reluctant to use chronic blood transfusion or hydroxyurea therapy.

4.5 DISCUSSION

4.5.1 Study sample and presenting features

Most reports of SCD in the Arabian Peninsula, came from Saudi Arabia (Lehmann *et al,* 1963, Gelpi 1970, Perrine *et al,* 1978, Perrine *et al,* 1981, Acquaye *et al,* 1985, El-Hazmi & Warsy 1986, El-Hazmi *et al,* 1987, Miller *et al,* 1989, El-Hazmi 1990) and other Gulf countries including Oman (White *et al,* 1993, Daar *et al,* 2000, Rajab *et al,* 2000, Jaiyesimi *et al,* 2002), Kuwait (Ali 1970, Adekile *et al,* 1994), United Arab Emirates (El-Kalla & Baysal 1998, Al Hosani *et al,* 2005), and Bahrain (Buhazza *et al,* 1985), but information is lacking from the Yemen. The present study aimed to define the clinical pattern of SCD among Yemeni children, who were mainly from the South-Western part of the country. Although this was a hospital-based study, we tried to include all children with SCD within our catchment area by notifying all known affected individuals and opening the clinic daily with free treatment. We were able to include 69 patients who were symptom free and in a "steady state" condition and 17 without a previous hospital admission.

Although there is no neonatal screening program for diagnosing SCD in our hospital or elsewhere in Yemen, the majority of affected individuals (92.1%) who presented have been diagnosed before the age of three years. This early presentation reflects the severity of SCD in Yemeni children, which is comparable to that found in Western and South-Western Saudi Arabia, where the disease is generally considered to be severe and 73% of children are diagnosed before the age of 3 years (Mulik *et al,* 1991). Initial symptoms of SCD have been reported for 50% of American children by one year of age, and in nearly all patients by the age of 5-6 years (Powars 1975). In a Jamaican cohort about one third of children developed symptoms by the age of one year and more than 90 % by six years (Bainbridge *et al,* 1985). In this study, 67.6% of children were symptomatic by one year, and 99 % by 6 years of age.

4.5.2 Clinical characteristics and complications

Painful crisis was the most frequent and troublesome symptom, which occurred in almost 90% of children and this was the commonest reason for hospitalization both in this study, as well as elsewhere (Ballas 2005). Overall, 25% of patients reported a frequency of 3 or more painful episodes per year, with only 10% reporting no history of painful crisis. The rate of ≥3 episodes is closer to the 34.5% reported for Guadeloupian children (Tarer *et al,* 2006) but higher than the 5.2% reported for American children (Platt *et al,* 1991). These differences may be related to study design differences or to variability in the disease severity.

Dactylitis (hand-foot syndrome) is an early manifestation of vascular obstruction in the active bone marrow of the small distal bones in young children and leads to recognition of SCD in 54% of children in this study. This pattern was similar to findings in Saudi Arabia (55-66%) (Mulik *et al,* 1991), India (52%) (Kar *et al,* 1986), and Jamaica (45%) (Stevens *et al,* 1981), but more frequent than in American blacks (11%) (Watson *et al,* 1963), This variation could be attributed to patient selection and heterogeneity of disease severity, or might relate to haplotype characteristics. For example dactylitis was reported to be very rare in Eastern Saudi Arabia where this disease is generally a mild variant and AI β^S haplotype is predominant (Padmos *et al,* 1991). Infants who have developed dactylitis by the first year of life were more likely to have an adverse outcome, with a relative risk for severe disease estimated to be 2.6 (Miller *et al,* 2000). Dactylitis is also considered to be an initial manifestation of vasculopathy and carries a significant risk for development of CVA in older children (Powars *et al,* 2005) .

Splenomegaly was detected in 40% of patients, which is within the 32-77% prevalence reported from Saudi Arabia (El-Hazmi 1992a). The spleen is a site of red cell destruction and if this function is exaggerated (hypersplenism), which happens in

some sickle cell patients, then splenectomy could be helpful and should lead to haematological and clinical improvement (Emond *et al,* 1984). However, spleen size is not a reliable indicator of its function, and reticuloendothelial dysfunction can occur in SCD in the absence of a greatly enlarged spleen due to "functional asplenia" (Pearson *et al,* 1969). A palpable spleen at or before a year of age has been reported to be a risk factor for severe and recurrent bacterial infection, especially pneumococcal infection which is a consequence of functional asplenia (Rogers *et al,* 1978). In 72 Omani children aged between 5-12 years, splenomegaly was present in 55%, and only 30 % of SS individuals had normal spleen function, whereas 40% had severe asplenia with complete absence of splenic visualization on scintigraphy (Wali *et al,* 2002). It would be of helpful to assess splenic function in these Yemeni children in view of their high rate of splenomegaly and variable disease severity.

Despite the high prevalence of splenomegaly in our patients, sequestration crises were not encountered, a pattern similar to that reported from Nigeria (Juwah *et al,* 2004). However a family history of a sickle cell related death with sudden pallor and collapse during normal activity in an apparently asymptomatic child raised the possibility of an acute sequestration crisis as a cause of death at home. Parents may be not aware of the manifestations of sequestration crisis and be unable to quickly transfer their child to hospital. Death due to such fatal events can occur even before the diagnosis of the underlying SCD is described (Jenkins *et al,* 1960). Instructing parents about the method of spleen palpation, the manifestations of sequestration crisis and the need to immediately report any sudden change in spleen size, especially if associated with pallor, is important for early diagnosis and management and could save children's lives. In the Jamaican cohort parental awareness was found to increase the detection rate of acute sequestration crisis and as a consequence the

incidence rate, increased from a mean of 4.6 per 100 patient-years, to 11.3 per 100 patient-years. The fatality rate for the same period decreased from 29.4 per 100 events to 3.1 per 100 events (Emond *et al,* 1985).

The occurrence of ACS in 16.7% of this sample is higher than the 6.6% in Kuwait (Akar & Adekile 2008), 5-7.7% in Eastern Saudi (Al-Dabbous 2002), 10% in Western Saudi (Al-Hawsawi 2004), but comparable to the 22% reported from Oman (Jaiyesimi *et al,* 2002). ACS may occur in up to 50% of patients with SCD (Leong & Stark 1998). This variation in frequency could be attributed to differences in study methodology, criteria for case definition, and differences in suspicion index for diagnosis. It was noted that up to 50% of patients diagnosed with ACS were initially admitted to the hospital for other reasons, most often painful crisis, and subsequently they had developed the radiographic and clinical symptoms of ACS within 3 days of hospitalization (Vichinsky *et al,* 2000). The peak incidence of ACS is in children between age 2 and 5 years (Castro *et al,* 1994a, Vichinsky *et al,* 1997). In our study the most affected age group was 2-5 years (53%), although the youngest child was only 6 months of age. The lower ACS rate in infants under 2 years of age could be attributed to the protective effect of their higher Hb F concentration. The high rate in young children could be influenced by the high incidence of upper respiratory viral infection in the early years of life (Castro *et al,* 1994a). Males were more affected than females (1.8:1), which is consistent with other reports from Saudi Arabia (Al-Dabbous 2002, Al-Hawsawi 2004, Alabdulaali 2007). The American national ACS study showed a significant gender difference with males affected more than females, mainly in children less than 10 years of age (63% vs 38%, p=0.02) (Vichinsky *et al,* 2000). The reason for the male predominance remains unknown.

Clinically overt stroke occurred in 5 patients (4.9 %) in their first decade. The estimated prevalence and affected age group are consistent with other reports (3.3-11%) (Hoppe 2005). The reason for this age-specific variation is unclear. Cerebral blood flow is mostly determined by age, being higher in young children. It is worsened by anaemia and hyperaemia with increased flow and decreased vascular reactivity leading to impairment of vasodilatory capacity and with a limited vasomotor reserve this predisposes the brain tissue to hypoxia and ischaemic insult (Prohovnik *et al,* 2009). Because local neuro-imaging facilities were limited, it was not possible to diagnose silent infarction, which is linked to cognitive impairment (Armstrong *et al,* 1996), and increased risk of stroke (Miller *et al,* 2001). With improvement of brain imaging technology, the prevalence of silent stroke detected by MRI has been reported in up to 35% of 185 American children (Steen *et al,* 2003). For the prevention of stroke, a prophylaxis programme using TCD and chronic transfusion would be required. In the Yemen at present there are insufficient resources to facilitate the development of such a programme.

Hepatomegaly occurred in 72.5%, which is very closed to the 69% reported from Western Saudi (Acquaye *et al,* 1985). Liver abnormalities in SCD range from mild alterations of liver function in asymptomatic patient to dramatic clinical crises with extreme hyperbilirubinemia and liver failure (Banerjee *et al,* 2001). Hepatic crisis is considered when a patient develops conjugated hyperbilirubinemia, with hepatomegaly but with no other signs of serious illness, liver tenderness or anorexia which are the prominent symptoms of viral hepatitis (Kaine & Udeozo 1988). Hepatic crises can be mild and self limited with a total bilirubin in the range of 10-30mg/dl, or severe and often fatal with a bilirubin above 30-80mg/dl and associated with hepatic dysfunction, coagulopathy, encephalopathy with or without renal failure

(Ahn *et al,* 2005). In the three cases admitted during the study period, all presented with deep jaundice and hepatomegaly and conjugated bilirubin between 8.5-30mg/dl. Liver enzymes were normal or mildly elevated, serum glutamate pyruvate transaminase (SGPT) (18, 35, 70 U/L) and serum glutamic-oxaloacetic transaminase (SGOT) (29, 45, 60 U/L). Serology for acute viral hepatitis (A and B virus) was negative and obstructive gall stones were excluded by abdominal ultrsonography. The incidence of viral hepatitis in SCD children is no higher than the incidence in the general population (Soliman *et al,* 1995). However, vascular liver damage in SCD is assumed to be reversible and chronic damage could be caused by associated viral infection. A histopathological study of 16 SS Brazilian children (15 months to 18 years) revealed a preserved lobular structure in all the non-viral cases, with sinusoidal dilatation with numerous sickled cells, hypertrophied Kupffer cells, focal necrosis, and inflammatory infiltrates without fibrosis or bile duct damage. Whereas five children with hepatitis C virus showed a histopathological picture of chronic hepatitis, and two with hepatitis B virus a picture of cirrhosis (Teixeira *et al,* 2002).

Cholelithiasis is rare in the paediatric population but is a commoner complication in patients with SCD, with the frequency varying with age and diagnostic approach. The 5% prevalence of gallbladder stones in the present study is similar to that reported from Nigeria (Adekile 1985), but lower than that for Saudi Arabia (19.7%) (Al-Salem *et al,* 1996) and Kuwait (15.6%) (Haider *et al,* 1998). In the present study children were referred for abdominal ultrasound examination only if they complained of abdominal pain or had exaggerated jaundice. Abdominal ultrasound was not done routinely, and for this reason cholelithiasis may have been underestimated as asymptomatic cases would not be screened.

A frequent complication was nocturnal enuresis (43.9%), which was reported to be higher in prevalence in SCD than the normal population (Barakat *et al,* 2001). Males and females were equally affected in the present study. This is comparable with data from Nigeria, where 49.5% of sicklers had enuresis at 6-15 years of age but with no sex difference (Akinyanju *et al,* 1989) , but contrasts with a Jamaican study which reported enuresis to be more common in males than females (52% vs 38%) (Readett *et al,* 1990b). The cause of the increased frequency of enuresis in children with SCD is not fully explained. Hyposthenuria resulting from repeated renal medullary infarction which leads to high urine volume and polyuria is thought to be an underling causative factor. However, hyposthenuria affects all SS children and the analysis of urine osmolality after a water deprivation test of those with and without enuresis showed no significant differences (Readett *et al,* 1990a). Multiple factors have been suggested which include low maximum functional bladder capacity, deep sleep with decreased arousal, as well as social and environmental factors. This assumption could explain the partial response reported in ten SCD patients with enuresis to intranasal desmopressin, an agent that exerts an antidiuretic action and reduces the overnight production of urine (Figueroa *et al,* 1995). The effects of cerebral infarction and other neurological complications as potential causative factors have not been investigated. Among the five cases with overt stroke only two children (40%) reported enuresis. Enuresis also declined with age throughout childhood from 55% to 32% but continued to be a persistent problem in adolescence. It is likely that many of these adolescents will carry this problem into adulthood as previously reported (Field *et al,* 2008). Psychological and emotional effects, as a function of disease and enuresis, might have significant impact on treatment efficiency and

adherence and on the overall quality of life of affected individuals (Jordan *et al,* 2005).

Epistaxis may occur up to one third of cases in SCD, and occasionally is very severe (Konotey-Ahulu 1965), leading to profound anaemia, which might be fatal (Seeler 1972). Repeated epistaxis would lead to high iron loss and iron deficiency in some cases (Davies *et al,* 1983). Epistaxis was reported to be rare among patients from Western Saudi and Yemen (Acquaye *et al,* 1985), in contrast to our findings (14.7%), which were similar to those reported from India at 15.6% (Kar & Devi 1997), or Africa at 20% (Mouele *et al,* 1999). Vascular or haemostasis changes are probably implicated in the causation but the underling mechanism of epistaxis in SCD is not elucidated. Gum bleeding is not often reported and one study from Nigeria (Kaine 1983), described its occurrence in four SS children, two of whom had both nasal and gum bleeding, unlike in the Yemeni children where gum bleeding was isolated. The high prevalence of clinical malnutrition in the present study might be associated with vitamin and micronutrient deficiencies. Gum bleeding is associated with vitamin C deficiency and despite apparently adequate intake, vitamin C deficiency has been reported in 50% of 18 American SS patients, assessed by measurement of leukocyte vitamin C levels, a sensitive index of stores which if depleted, lead rapidly to scurvy (Chiu *et al,* 1990). Oxidative stress in SCD would increase utilization and demands for vitamin C as a potent antioxidant. Furthermore, increased renal excretion of ascorbate has been described (Westerman *et al,* 2000). These factors could all contribute to the low levels of vitamin C in SCD patients.

Sickle cell trait has been reported to be approximately 50% protective against mild clinical malaria, 75% protective against hospitalization for malaria, and almost 90% protective against severe or complicated malaria (Williams *et al,* 2005b). This

protection is not complete and they still acquire malaria infection although they are less likely to die from severe disease or cerebral malaria. Despite the relative protection offered by the heterozygous AS state, SS cases remain highly vulnerable and malaria infection can precipitate severe hyperhemolysis with marked fall in haemoglobin concentration, and induction of painful crisis (Fleming 1989, Juwah *et al,* 2004). It is not clear why patients with homozygous SS, with high levels of Hb S are not protected from malaria to a greater degree than in the heterozygous state, or why a low level of parasitaemia may present with severe malaria manifestations in homozygous cases (Makani *et al,* 2007). Malaria is endemic in Yemen with 60% of the total population exposed to infection and a 1% estimated mortality (NMCP 2006). A documented malaria episode in the previous 12 months was reported for 20 % of the children. Anaemic sickle cell crisis was triggered by malaria in some of these cases. Malaria remains a major cause of morbidity and mortality in sickle cell patients living in malaria endemic areas (Ambe *et al,* 2001). Residents from developed countries with AS or SS conditions can still be at high risk of malaria when travelling to endemic areas if they receive inappropriate and inadequate chemoprophylaxis (Glikman *et al,* 2007).

Major serious clinical infections in our sample were either unusual or underreported. A low rate of major infection has been observed in Saudi children from the Eastern (El Mouzan *et al,* 1990), and Western provinces (Pejaver *et al,* 1995). Factors likely to be associated with a low level of major infections are the common practice of rapid institution of parenteral antibiotics for febrile children with SCD, and regular penicillin prophylaxis. Diagnosis is limited by available laboratory diagnostic facilities for the isolation of causative pathogens. Invasive bacterial infections such as septicaemia and meningitis occurring in young infants and

children may be fatal before the underlying haemoglobinopathies have been detected (Williams *et al,* 2009). Only post-mortem electrophoresis and bacteriological studies would establish the diagnosis (Powars 1975). Meningitis occurred in one case in the Yemeni sample. Although, osteomyelitis is considered to be more frequent in children with SCD, its clinical signs are not specific and there are difficulties in distinguishing between bone infection and infarction. In this sample only one case with osteomyelitis was reported. In Eastern Saudi Arabia a 10 years retrospective review showed 14 cases of osteomyelitis among a community of 4940 children with SCD diagnosed through newborn screening programme, with an estimated prevalence rate of $283/10^5$ (Narchi 2000). A similar study in USA detected 10 cases among 2000 consecutive hospitalized SCD patients over 24 years (Chambers *et al,* 2000), and in Nigeria 17 cases have been reported over a period of 11 years (Thanni 2006). The lack of routine blood culture in the present study limited the diagnosis of systemic infections. A recent study from Kenya has reported a high rate of bacteraemia in children with SCD, many of whom would not have been taking regular antibiotic prophylaxis (Williams *et al,* 2009).

4.5.3 Consanguinity

Consanguineous marriage is a frequent practice in the Middle Eastern countries as well as in Yemen. A study of 1050 Yemeni couples in Sana'a city showed a related marriage in 56.8% with 32% first cousins, 12.6% second cousins and 12.2% remote relatives (Gunaid *et al,* 2004). The results of the present study are comparable with that for the general population with corresponding consanguinity rates of 62.8%, 32.5%, 16.3% and 13.9% respectively. First cousin marriage was the predominant type of consanguinity accounting for almost one-third of all marriages and more than half of all consanguineous marriages. Consanguineous marriage

increases the chance that a couple both have the recessive trait, giving a 25% chance of a homozygous offspring (Modell & Darr 2002). The first cousin is the closest form of consanguinity and the closer the relative the higher the risk of genetic similarities. In some communities with a high prevalence of consanguinity, the incidence of SCD is higher than expected. For example in Oman with a high consanguinity rate of 56%, (24% first cousin), the estimated attributable fraction is 17% of SCD cases attributed to consanguinity (Rajab & Patton 1997). However, the magnitude of effect of consanguinity in influencing genetic profiles of the sickle cell gene (trait and disease rate) has not been systematically studied.

4.5.4 Mortality

A death of one case during study period occurred due to severe dengue fever. Although serological confirmation was not available, the epidemiological history of the outbreak and the clinical findings of profound haemorrhage and shock were supportive of this diagnosis. SCD has been reported to be a risk factor for the occurrence of the severe clinical form of dengue fever, and sickle cell patients run a shorter course than other fatal cases, with 50% dying less than 24 hours after presentation (Bravo *et al,* 1987). In dengue fever, induced programmed cell death (apoptosis) of endothelial cells has been suggested as an important mechanism for vascular injury and is probably associated with increased vascular leakage. Endothelial cells are activated as part of the pathophysiology of SCD and any further dysfunction or injury could lead to alterations in the apoptosis pathway and the immune response resulting in aggravation of vascular damage (Limonta *et al,* 2009).

The low death rate in this sample may not reflect the overall mortality of SCD in this population. Infants and young children can die from SCD before diagnosis and many deaths may occur outside the hospital and be unreported, thus

mortality can only be reliably assessed through a cohort study of children from birth. However, the information obtained by family history showed that 18% had reported SCD related death. In the Jamaican cohort study there was a mortality of 17.6% by age 10 years and 25.9% by the age 19 years in SS children (Serjeant & Serjeant 1993). The mortality risk in SCD was much improved in developed countries and can be improved even in low resource settings by the implementation of a comprehensive clinical care programme with well organized team work in dedicated sickle cell clinics (Rahimy *et al,* 2003, Akinyanju *et al,* 2005).

A limitation of this study was that it was hospital-based and ascertainment bias could lead to the inclusion of a greater number of symptomatic than asymptomatic patients. Considerable effort was made to obtain adequate information on cases from informants or parents/guardians, supported by the documentary evidence from the case records. This does not exclude recall bias for clinical events. Lack of effective laboratory facilities, restricted the accurate diagnosis of bacterial or viral acute febrile illnesses, limiting the etiological classification of infection episodes.

4.6 CONCLUSIONS

This study has shown that SCD is a serious health problem affecting children in Yemen from early life. The overall disease profile and complications were similar to those reported in African and American blacks and in children from the Western part of Saudi Arabia.

Further research is required to determine the extent of SCD geographically in Yemen, and to identify the factors leading to related morbidity and mortality. Evaluation of the contribution of different environmental or genetic factors which may explain disease variability in this population is required. Early diagnosis and

management through the implementation of a neonatal screening program combined with comprehensive medical care should be considered, in order to ensure appropriate and effective early management for these children. An improved immunization schedule is required as well as better facilities for diagnosis of bacterial infections. The high prevalence of consanguinity in this society requires parental education and pre-marital testing with appropriate genetic counselling.

CHAPTER FIVE

SEVERITY ASSESSMENT

5.1 INTRODUCTION

SCD is a monogenic disorder with multiple phenotypic expressions. There are four common haemoglobin genotypes, ordered in decreased clinical severity; homozygous SCD (SS), $S\beta^0$ thalassaemia, Sickle C (SC), and $S\beta^+$ thalassaemia. Although there may be some overlapping of severity between different genotypes, homozygous SS is the most common and most severe form, and is characterised by a highly variable clinical course (Redding-Lallinger & Knoll 2006). Some affected individuals can be crippled by their disease with recurrent crisis and serious complications, while others lead relatively a near normal life with minimal disabilities. This variation in disease pattern and severity may occurs between individuals living in the same community as well as between different socioeconomic or geographical locations (Serjeant 1989). The underlying causes of the wide spectrum manifestations are not fully understood. Many genetic and environmental factors interact which can lead to modulation of disease expressions and complications.

Among the genetic factors, Hb F production and co-inheritance of α-thalassaemia are well recognized. A high level of Hb F ameliorates disease severity through reduction of Hb S concentration, preventing polymerization and cellular sickling. The β^S haplotype polymorphism is also related to this clinical and haematological variation. An increased level of Hb F is linked to the inheritance of Senegal and Arab-Indian β^S haplotypes, which are also associated with the -158, C→T XmnI polymorphism in the γ-globin gene and relates to expression of the Gγ gene and production of Hb F (Steinberg & Adewoye 2006). Other potential genetic

modifiers include several single nucleotide polymorphisms (SNPs) that may interfere in the pathways of inflammation, thrombosis and cell adhesion (Frenette & Atweh 2007). Factors such as endothelial activation, inflammation and vasculopathy are important components of the pathophysiology of SCD and assumed to be associated with disease severity (Hebbel 2004). The role of environmental and socioeconomic factors are complex, and may exert their influence through precipitation of painful crises during exposure to cold weather, increased exposure to malaria and other endemic infections, inadequate folate or other micronutrient deficiency, trauma induced leg ulcers, as well as quality of family support and health care (Serjeant 1995).

Assessment of severity in children with SCD and better identification of those at risk of serious complications may offer a useful tool for sub-group selection for more vigorous therapy such as hydroxyurea or bone morrow transplantation. It could also assist in guiding management appropriate for the degree of clinical severity (Quinn & Buchanan 2002). In children assessment of severity is more difficult than in adults, due to age related haematological changes, progression of spleen dysfunction and the varied onset of some clinical manifestations and complications (Bray *et al,* 1994). It is difficult to predict individual severity and prognosis, as only a few predictors have been identified, for example early dactylitis (before the age of 1 year), steady state leukocytosis, a low basal Hb concentration of < 7g/dl (Miller *et al,* 2000), and early onset ACS during the first 3 years of life (Quinn *et al,* 2007).

Clinical severity criteria using combinations of clinical events and organ damage have been used as indicators for selection of patients need aggressive intervention therapy in several studies. These have included: the occurrence of three or more pain crises in the year prior to entry; at least 3 episodes of ACS requiring

hospital admission within 2 years of entry; a previous history of stroke, recurrent crises without free interval; or splenic sequestration as classifications of severity (Ferster *et al,* 1996, Kinney *et al,* 1999, Wang *et al,* 2002). Due to the complex nature of SCD pathophysiology and difficulties in integrating genetic and phenotypic variability in severity assessment, Sebastiani et al.,(2007) used a Bayesian networks complex model to analyse interactions of 14 clinical and laboratory variables. They developed a computerized method to be used for calculating individual severity scores and prediction of the risk of death within 5 years.

The determination of severity in SCD is complex, particularly in absence of a completely acceptable definition (Serjeant 2004). Prediction of the disease course is complex and few predictors are available and there is lack of definitive severity biomarkers. Different criteria and scoring systems used by different investigators which limit comparability between studies and there is a need for reproducible quantitative approach to standardization of severity assessment. A scoring system which is straightforward and does not require sophisticated laboratory investigations will be appropriate for low resource settings where the majority of SCD patients live.

5.2 OBJECTIVES

In Yemen the severity of SCD has not been characterized, and the objectives of this analysis were to assess clinical disease severity in children, symptom correlation with haematological and biochemical parameters, and to compare their severity index assessment with comparative studies from other populations.

5.3 METHODS

The study methods are described in detail in Chapter Three. The sample included all symptomatic or asymptomatic children attending the Sickle Cell Clinic aged below 16 years with SCD, who had been diagnosed by a positive sickling test,

confirmed by SS haemoglobin electrophoresis. Subjects were recruited from the clinic or following hospital admission. Data was collected through a direct interview following a pre-structured questionnaire. A detailed medical history and clinical examination were attained by the researcher. Data on family history, past medical history and frequency of acute clinical events during the previous year was collected. Information was sort on previous hospital admissions, blood transfusions, severity and frequency of crises (vaso-occlusive, splenic sequestration, aplastic, hyperhemolytic, cerebrovascular) and other complications such as infections, acute chest syndrome, avascular bone necrosis, skin ulcers, gall stones, priapism or urinary disorders. Definitions of clinical events were those proposed by CSSCD (Gill *et al,* 1995). Information was collected from either or both parents, who were requested to bring to the clinic all available medical documents (medical prescriptions, previous investigation papers, and notes from previous hospital discharges). Information related to previous admissions was cross-checked against the patient's hospital admission records.

Disease severity was assessed according to the frequency of pain crises, hospitalization, blood transfusion and sickle cell-related complications using the schema of El-Hazmi et al., (1992a, 1993) as described in Chapter 3. A Severity Index (SI) was calculated for each child, by estimating the total score during the previous 12 months. If this total was ≤ 6 then the disease was considered mild, and severe if >6. The cut-off value of 6 had been used previously because the score frequency distribution was shown to be bimodal and this distinguished milder cases from those with severe manifestations with score values of above 6 (El-Hazmi *et al,* 1993). The distribution of the Severity Index in the Yemeni sample was unimodal, and the lower quartile boundary was located exactly at a value of 6 which represented the lowest

25% of the scored data. This finding was considered appropriate for categorising cases below that value as mild and those above (in the upper three quartiles) as severe. Although we used such categorisation to facilitate analysis and comparability with previous reports, a detailed description of score distribution and its relationship to clinical, haematological and biochemical parameters was considered. The severity score is a continuous variable and was used for correlation analyses.

5.3.1 Blood investigations

Standard laboratory methods were employed for evaluation of haematological and biochemical tests (Dacie & Lewis 1991). All patients had a positive sickling test and the diagnosis of homozygous SCD was confirmed by haemoglobin electrophoresis on cellulose acetate at pH 8.6, followed by scanning densitometry for haemoglobin variants. The patient was diagnosed as SS disease only if Hb S, Hb F and Hb A2 $\leq$ 3.5% were present. Diagnosis of $S\beta^0$ thalassaemia was excluded on the basis of Hb A2 $\leq$3.5%, but family studies or globin chain syntheses were not performed.

5.3.2 Other investigations

Imaging examination including X-ray, Ultrasonography, and CT scan were performed when clinically indicated.

5.4 RESULTS

One hundred and two children were included in this analysis, 78 were enrolled from the Sickle Cell Clinic and 24 following hospital admission. There were 46 female and 56 male patients, with a mean age of 7.2 years. Females were older than males (7.7± 4.6 vs 6.8± 4.5, *p=0.3*). About one third (34.8 %) of female children were ≤ 5 years compared to approximately half (51.8 %) male children.

Early clinical presentation occurred in 20% during the first 6 months of life and in 67% during the first year. The commonest presenting symptom was dactylitis (hand–foot syndrome) which occurred in 54%, followed by acute infection (25.5%) and painful crises (10.8%). None of the children had received chronic transfusion or hydroxyurea treatment which could modify their clinical course or disease severity.

The sex distribution of the severity score grouped into 5 class intervals is shown in figure 5.1, and for the rate of clinical events and haematological indices in figures 5.2 and 5.3.

Figure 5. 1 Distribution of severity score group by sex

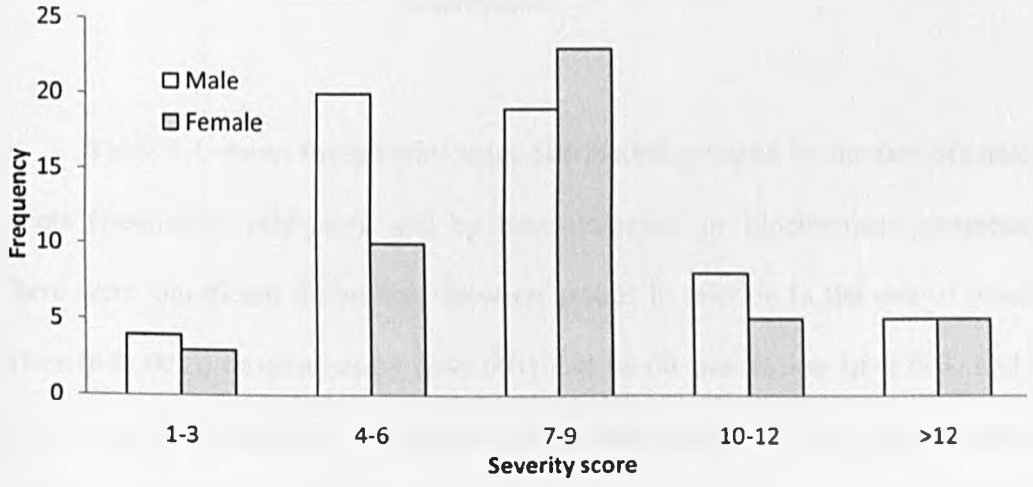


Figure 5. 2 Rate of clinical events by severity score class

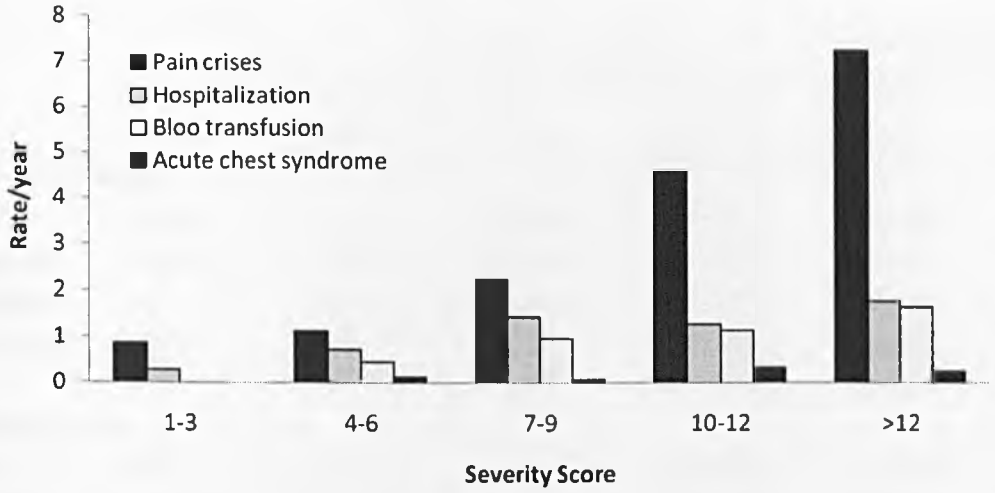


Figure 5. 3 Haematological indices by severity score class

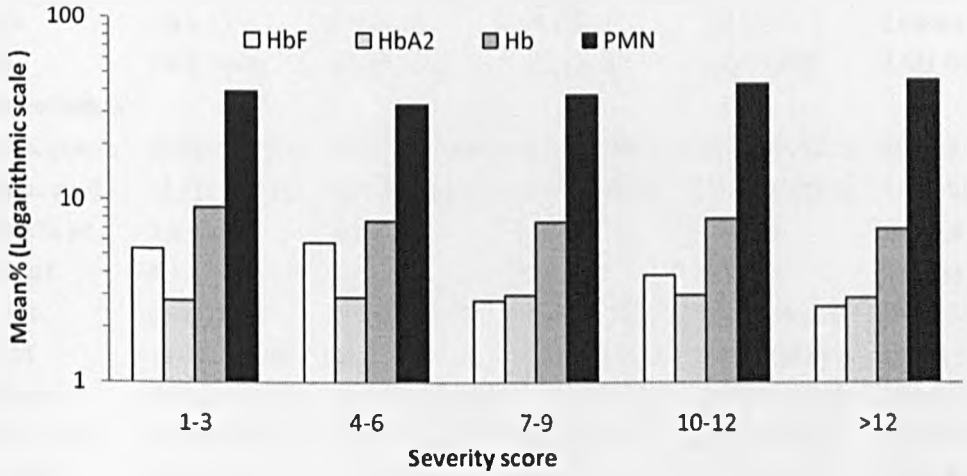


Table 5.1 shows the severity score distribution grouped by the rate of clinical events (frequency/child/year), and by haematological or biochemical parameters. There were significant differences between groups in relation to the rate of painful crises (p<0.001), hospitalisation (p<0.001) and blood transfusion (p=0.004) and to the level of Hb F (p=0.012) and polymorphonuclear cells (p=0.035). In biochemical indices differences were associated with concentration of total and direct bilirubin (p=0.003, and 0.044), and plasma ferritin (p=0.010).

Table 5. 1 Severity score group by rate of clinical events, haematological and biochemical parameters

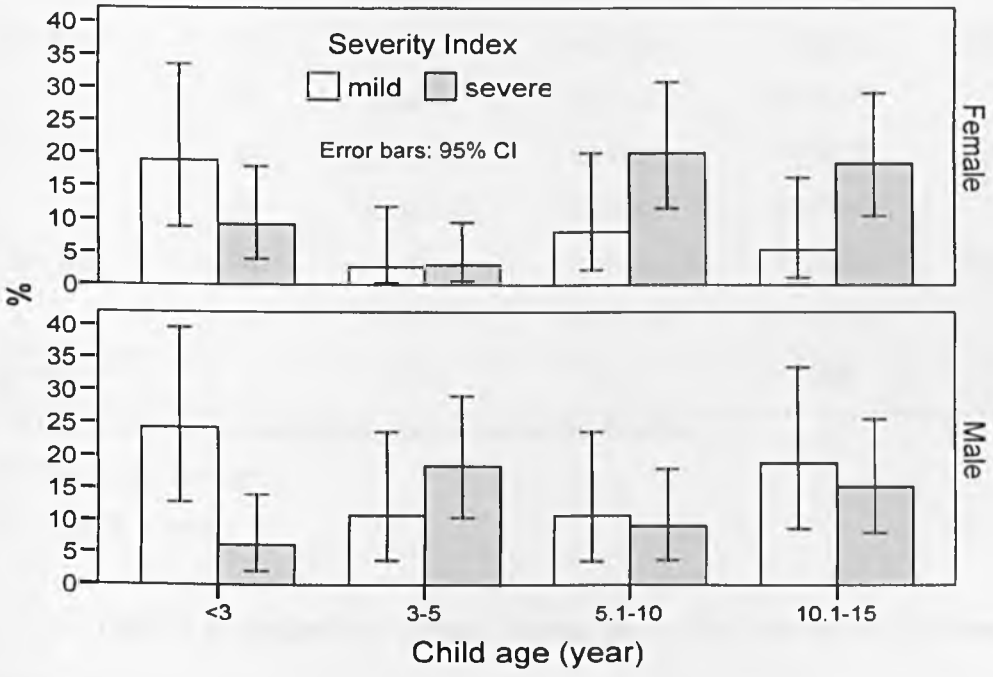
	Severity score					
	1-3 (n=7)	4-6 (n=30)	7-9 (n=42)	10-12 (n=15)	>12 (n=8)	*P value*
Clinical event, n (rate/year)						
Painful crisis	6 (0.86)	34 (1.13)	94 (2.24)	69 (4.6)	58 (7.25)	**<0.001**
Hospitalization	2 (0.29)	22 (0.73)	60 (1.43)	19 (1.27)	14 (1.75)	**<0.001**
Blood transfusion	0	14 (0.47)	40 (0.95)	17 (1.13)	13 (1.63)	**0.004**
Infection episode	6 (0.86)	71 (2.37)	108 (2.57)	35 (2.33)	20 (2.50)	0.125
ACS	0	4 (0.13)	3 (0.07)	5 (0.33)	2 (0.25)	0.088
Haematological indices						
Hb, g/dl	9.2 ± 1.0	7.7 ± 1.5	7.6 ± 1.6	7.9 ± 1.3	7.1 ± 1.0	0.102
WBC, $x10^9$/L	8.0 ± 3.7	10.9 ± 5.8	10.8 ± 5.7	8.8 ± 3.0	9.6 ± 4.5	0.505
PMN, %	39.1 ± 8.4	33.3±13.2	37.5 ± 11.4	43.3 ± 9.7	45.9 ±16.4	**0.035**
Reticulocytes, %	4.0 [2.0-7.0]	3.0 [1.5-5.5]	4.0 [2-7]	3.5 [1.3-8.0]	2.0 [1.0-7.6]	0.615
Hb A_2, %	2.8 ± 0.2	2.9 ± 0.4	3.0 ± 0.3	3.0 ± 0.3	2.9 ± 0.4	0.371
Hb F, %	5.8 [5.3-6.4]	4.6 [2.1-7.0]	2.8 [1.4-5]	3.6 [2.7-4.9]	2.5 [1.7-3.5]	**0.012**
Biochemical indices						
T.Bilirubin, µmol/L	25.7 [13.9-29.0]	24.8 [15.6-38.0]	48.7 [28.7-67.0]	38.1 [21.4-54.7]	36.9 [20.7-66.3]	**0.003**
D.Bilirubin, mol/L	13.7 [5.1-27.0]	13.9 [8.4-24.4]	22.0[13.7-32.2]	17.1 [13.7-25.7]	13.9 [10.0-29.5]	**0.044**
Total Protein, g/L	7.5 ± 1.0	6.9 ± 1.0	7.4 ± 1.3	7.3 ± 0.9	7.6 ± 1.0	0.382
Albumin, g/L	4.3 ± 0.7	3.8 ± 0.5	3.9 ± 0.7	4.0 ± 0.7	3.6 ± 0.5	0.397
SGOT, U/L	20.0 [15-30]	15. [12.0-22.3]	16.5 [9.5-30.3]	12.0 [10.0-28.0]	19 [10.5-34.0]	0.798
SGPT, U/L	15.0 [8.0-30.0]	10.0 [8.0-15.3]	12.0 [10.0-24.3]	8.0 [8.0-25.0]	14.5 [8.5-31.5]	0.522
Urea, mg/dl	25.0 [20.0-28.0]	25.0 [18.0-30.0]	25.0 [20.0-30.0]	28.0 [22.0-30.0]	27.5 [22.0-30.0]	0.855
Creatinine, mg/dl	0.4 [0.25-0.75]	0.35 [0.2-0.5]	0.45 [0.2-0.8]	0.5 [0.25-0.8]	0.46 [0.31-0.76]	0.234
tHcy, µmol/L	1.9 [1.9-4.3]	1.9 [1.9-4.3]	1.9 [1.9-4.2]	1.9 [1.9-4.3]	1.9 [1.9-1.9]	0.379
Folate WB, µg/ml	370 [269-571]	453 [340-663]	571 [290-756]	972 [420-1604]	697 [262-958]	0.348
Vitamin B6, µg/ml	41 [30-53]	40 [34-49]	35 [24-47]	33 [23-47]	43 [37-49]	0.217
Vitamin B12, pg/ml	375 [240-460]	404 [313-938]	477 [371-899]	450 [338-531]	414 [343-712]	0.606
Ferritin, µg/L	93.1 ± 65.7	262.5 ± 177	269.5 ± 229	270.4 ± 148	242 ± 160.8	**0.010**
sTfR, mg/L	61.0 [47.0-67.0]	61.5 [38-97.8]	62.0 [36.8-86.3]	85.0 [31.0-69.0]	44.0 [25.3-54.8]	0.431
CRP, mg/L	6.3 [3.8-21.8]	9.4 [4.9-25.8]	10.6 [3.4-40.7]	4.5 [1.9-48.7]	18.3 [4.7-81.9]	0.982
SAA, mg/L	15.0 [11.0-76.0]	16.0 [6.8-39.0]	14.0 [7.0-32.3]	14.0 [5.0-38]	40.5 [8.0-77.0]	0.627

Values: mean ±SD, median [interquartile range]; *P* value: ANOVA or Kruskal-Wallis tests; ACS: acute chest syndrome; T.Bilirubin: total Bilirubin; D.Bilirubin: direct Bilirubin; tHcy: total plasma homocysteine (for 88 children); Folate WB: whole blood folate; sTfR: soluble transferrin receptor; CRP: C-reactive protein; SAA: serum amyloid A

Using the Severity Index score to calssified patients into two main groups showed 65 patients (63.7 %) were severe and 37(36.3%) as mild. Thirty three (71.7%) female and 32 (57.1%) male cases were in the severe category. The distribution of SI by age and gender categories is shown in figure 5.4.

Children with severe disease tend to be older than those with mild severity score, the mean age of severe compared to mild was 8.0±4.3 vs 5.8±4.6 years, p=0.01. The severe group presented at an earlier age than the mild group, with age at first presentation (median [IQR], 10.00 [6.5-21.00] vs 12.00 [7.5-18.5] months). These differences were not significant ($p>0.05$). There was no correlation between SI and age at initial episode of hand-foot syndrome.

Figure 5. 4 Distribution of child age by sex and severity index



Severe symptoms were more frequent in female children (71.7 % vs 57.1%, p=0.09), who had a higher rate of painful crises (crises/child/year) (3.16 vs 2.0, $p<0.05$), and their severe symptoms increased more rapidly from 5 years of age ($p<0.01$), (Table 5.2). Females had slightly higher levels of Hb (7.9±1.5 vs 7.6±1.5),

and Hb F median (IQR) 3.6 (2.8-5.4) vs 2.8 (1.8-4.8), but lower Hb A2 (2.8±0.36 vs 3.0±0.27) than males, although the differences were not significant except for Hb A2 (p<0.001).

There was no difference in SI scores between those in steady state and those with acute disease complication at the time of study enrolment (SI 7.65±3.36, vs 7.97±2.86, p=0.64), and between non-hospitalized and hospitalized children (7.6±3.1 vs 8.2±3.6, p=0.39).

Table 5. 2 Mild and severe disease categories by child age and gender

Age (years)	Disease severity	Female n=46 (%)	Male n=56 (%)	All n=102 (%)	*p value**
< 3.0	M	7(53.8)	9 (69.2)	16 (61.5)	0.68
	S	6 (46.2)	4 (30.8)	10 (38.5)	
3.0-5.0	M	1 (33.3)	4 (25.0)	5 (26.3)	0.98
	S	2 (66.7)	12 (75.0)	14 (73.7)	
5.1-10.0	M	3 (18.8)	4 (40.0)	7 (26.9)	0.36
	S	13 (81.3)	6 (60.0)	19 (73.1)	
10.1-15	M	2 (14.3)	7 (41.2)	9 (29.0)	0.13
	S	12 (85.7)	10 (58.8)	22 (71.0)	
*p value***		0.01	0.12	0.09	-

* Fisher exact test for severity comparing males and females

** Chi-square for trend

M: mild, S: severe

Table 5.3 summarises clinical events and complications by SI category. Painful crisis was the most common acute clinical event, occurring 221 times in severe cases (3.4 crises/patient/year) and 40 times in mild cases (1.1 crises/patient/year). Among those with a history of 3 or more crises, 47.7% were severe and 2.7 % mild. Of the 117 hospital admissions, severe cases were admitted

93 times and mild cases 24 times, ($p<0.001$). Eighteen (48.6%) mild compared to seven (10.8%) severe cases had not been admitted to hospital during the previous year.

Table 5. 3 Clinical events and complications by Severity Index category

Event frequency†	Mild (n= 37)	Severe (n= 65)	All (n=102)
*Painful crisis****			
0	7 (18.9)	5 (7.7)	12 (11.8)
1-2	29 (78.4)	29 (44.6)	58 (56.9)
≥ 3	1 (2.7)	31 (47.7)	32 (31.4)
*Hospitalization****			
0	18 (48.6)	7 (10.8)	25 (24.5)
1-2	19 (51.4)	53 (81.5)	72 (70.6)
≥ 3	0 (0)	5 (7.7)	5 (4.9)
*Blood transfusion****			
0	24 (64.9)	22 (33.9)	46 (45)
1- 2	13 (35.1)	37 (56.9)	50 (49)
≥ 3	0 (0)	6 (9.2)	6 (5.9)
Infection episodes			
0	5 (13.5)	4 (6.2)	9 (8.8)
1-2	18 (48.7)	37 (56.9)	55 (53.9)
≥ 3	14 (37.8)	24 (36.9)	38 (37.3)
Acute chest syndrome	4 (10.8)	10 (15.4)	14 (13.7)
Stroke	0 (0)	5 (8.8)	5 (4.9)
Gallstone	0 (0)	5 (8.8)	5 (4.9)
Enuresis	9 (24.3)	23 (35.4)	32 (31.4)
Leg ulcer	0 (0)	5 (7.0)	5 (4.9)
Gross haematuria	1 (2.7)	8 (12.3)	9 (8.8)

Brackets: percentage, † Number of episodes reported in the previous year or occurrence of complication.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, for comparing mild and severe.

The primary reasons for hospital admission by severity index category were painful or anaemic crises, followed by ACS and malaria (Table 5.4).

Table 5. 4 Reason for hospital admission in relation to Severity Index category

Diagnosis	Mild n (%)	Severe n (%)	All n (%)
Pain crisis*	6 (5.2)	34 (29.0)	40 (34.2)
Anaemic crisis	3 (2.5)	16 (13.7)	19 (16.2)
Acute chest syndrome	4 (3.4)	10 (8.5)	14 (13.7)
Malaria	2 (1.7)	10 (8.5)	12 (10.2)
Lower respiratory tract infection	2 (1.7)	6 (5.9)	8 (7.8)
Hand-foot syndrome	2 (1.7)	3 (2.5)	5 (4.3)
CVA	0 (0)	4 (3.4)	4 (3.4)
Hepatic crisis	2 (1.7)	2 (1.7)	4 (3.4)
Diarrhoea	2 (1.7)	2 (1.7)	4 (3.4)
Acute febrile illness	0 (0)	4 (3.4)	4 (3.4)
Meningitis	1 (0.8)	0.0	1 (0.8)
Typhoid fever	0 (0)	1 (0.8)	1 (0.8)
Dengue fever	0 (0)	1 (0.8)	1 (0.8)
Total	24 (20.5)	93 (79.5)	117 (100)

*$p<0.05$

Blood transfusion requirements were higher in children with severe disease ($p<0.01$), with 56.9 % receiving one or two transfusions, and 10.5% three or more transfusions, compared to 35.5% and 0% respectively for the mild group.

Stroke and gallbladder stones occurred only in cases with severe disease. Other related complications including ACS, leg ulcers, nocturnal enuresis and gross haematuria which were more frequent in the severe group, although these did not differ significantly by SI category.

Infection episodes were recorded 240 times with an estimated rate 2.3 episodes/child/year. Three or more infection episodes occurred in 38 (37.2 %) children. Most infections were respiratory and occurred with almost equal frequency in mild and severe categories (severe: 2.5 episodes/child/year, mild: 2.0 episodes/child/year). Meningitis occurred in one child. A facility for blood culture was not available in our hospital which hampered etiological diagnosis of invasive pathogenic infection. Penicillin prophylaxis was used regularly by 64.4 % (29/45) of children less than five years. No child had been vaccinated against pneumococcal infection as this vaccine was unavailable in the public health facilities, the only source of all vaccines. *P. falciparum* parasitaemia confirmed by microscopy was diagnosed in 21 children during the previous year, 16 (24.6%) in the severe and 5 (13.5%) in the mild group ($p= 0.21$).

Results of haematological and biochemical tests are summarized in Table 5.5. Percentage Hb F was lower in severe cases ($p<0.01$). There was no difference in Hb A2 level between severity categories. Mean (SD) haemoglobin was 7.7 (±1.5) g/dl, and only seven children had a Hb level >10 g/dl, (4 mild and 3 severe). There was no correlation between Hb and disease severity ($r = -.17$, $p>0.05$). Leukocyte count ranged between 3.300 to 34.400/mm^c, with the differential count showing a higher percentage of polymorphonuclear leukocytes (PMNs) in the severe group ($p<0.05$). Mean values for total and direct bilirubin were significantly higher in the severe group ($p<0.05$). There were no differences in mean values for serum total protein, albumin, serum liver transaminases, and BUN between mild and severe cases. Creatinine differences were marginally significant ($p=0.053$).

Table 5. 5 Haematological and biochemical results by Severity Index

Parameter	Mild	Severe	*P value*
Mean Severity Index ± SD	4.7 ± 1.3	9.5 ± 2.6	<0.001
Haematological indices			
Hb (g/dl)	7.9 ± 1.5	7.6 ± 1.4	0.28
WBC (x10^9/L)	10.4 ± 5.6	10.2 ± 5.1	0.87
Polymorphs (%)	34.4 ± 12.5	39.9 ± 12.0	0.03
Lymphocytes (%)	59.1 ± 12.7	54.8 ±12.3	0.09
Monocytes (%)	2.6 ± 1.7	2.4 ± 1.5	0.50
Eosinophils (%)	2.5 ± 2.8	2.9 ± 2.1	0.39
Reticulocytes (%)†	3.0 [1.5-6.0]	4.0 [1.5-7.0]	0.69
Hb A_2 (%)	2.9 ± 0.3	3.0 ± 0.3	0.88
Hb F (%)	5.8 ± 5.6	3.0 ± 1.9	0.001
Biochemical indices			
T. Bilirubin (µmol/L)†	25.6 [14.9-35.9]	42.7 [27.4-64.9]	<0.001
D. Bilirubin (µmol/L)†	13.7 [6.7-22.2]	20.5 [13.7-30.8]	0.005
T. Protein(g/L)	7.0 ± 1.0	7.4 ± 1.2	0.13
Albumin (g/L)	3.9 ± 0.6	4.0 ± 0.6	0.81
SGOT (U/L)	18.0 ± 8.8	19.8 ± 12.1	0.44
SGPT (U/L)	14.2 ± 8.8	16.6 ± 12.0	0.29
Urea (mg/dl)	24.7 ± 6.3	25.3 ± 5.6	0.62
Creatinine (mg/dl)	0.4 ± 0.2	0.5 ± 0.3	0.053

SD: Standard deviation, † Median [interquartile range, IQR]

Table 5. 6 Logistic regression for factors associated with severity**

Variable	OR	95% CI	*P value*	AOR	95% CI	*P value*
Hb F%	0.744	0.62-0.9	0.002	0.734	0.59-0.92	0.007
Female	1.904	0.83-4.4	0.129	4.969	1.6-15.9	0.007
Age at presentation	0.994	0.97-1.2	0.585	0.962	0.9-0.99	0.013
U. Bilirubin	1.681	1.04-2.7	0.035	1.654	1.04-2.64	0.035
Hb A2%	2.759	0.85-8.9	0.092	5.266	1.08-25.6	0.04
Ferritin	1.002	0.99-1.0	0.179	1.002	1.0-1.005	0.086
PMNs%	1.040	1.0-1.1	0.036	1.032	0.99-1.08	0.16
Age at first HFS	1.009	0.98-1.04	0.570	1.03	0.99-1.6	0.19
sTfR	0.993	0.98-1.0	0.149	0.99	0.98-1.0	0.34
Age (months)	1.009	1.0-1.2	0.021	1.025	0.89-1.18	0.74
WBC	1.000	1.0-1.0	0.872	1.0	1.0-1.0	0.92

** Variables included in the model: gender, age, age at first presentation, age at first hand-foot syndrome (HFS), white blood cell (WBC), PMNs%, unconjugated bilirubin, Hb F%, HbA2%, ferritin, sTfR.

Backward stepwise multiple logistic regression was used to analyse the association of clinical and laboratory parameters with disease severity (Table 5.6). Variables included in the model were those with $p<0.2$ identified by univariate analysis, potential confounders or of presumptive predictive value from past researches. These were: gender, age, age at first presentation, age at first hand-foot syndrome (HFS), white blood cell count (WBC), PMNs%, unconjugated bilirubin, Hb F%, HbA2%, ferritin and sTfR. Significant correlates with severity were Hb F% *(p=0.007)*, female gender *(p=0.007)*, age at first presentation (*p=0.013*), unconjugated bilirubin *(p=0.035)*, and Hb A2% *(p=0.04)*.

5.5 DISCUSSION

Scoring systems as a means to assess severity have been widely utilized. Although, there is no general consensus about the definition of severity and how it can be accurately measured, severity assessment continues to be a dilemma and until now no widely applied scoring index is available for SCD (Serjeant 2004). Validation of a scoring method to be used in SCD is supported by comparison with expert opinion and evidence for good correspondence (Cameron *et al,* 1983, Day 2004, Pearson *et al,* 2005). Satisfactory content validity was demonstrated through the revision of a point rating scale by 15 clinicians and scientists experienced in the field of SCD (Pearson *et al,* 2005), and by use of a content validity index by another group of five experts (Day 2004). Subjective ranking based on the evaluator's perception of disease severity was compared with objective numerical score and showed a significant correlation ($r=.82$, $p< 0.001$) with adequate reproducibility (Cameron *et al,* 1983).

In the present study we used the SI proposed by El-Hazmi (1992a). This index was selected because it covers almost all relevant items related to SCD severity and incorporated the most important clinical events and complications. The items included have been repeatedly used in severity assessment of patients with SCD and validated in several studies (Steinberg *et al,* 1973, Cameron *et al,* 1983, Day 2004, Pearson *et al,* 2005). This type of SI has been used more frequently in adult patients with limited studies evaluating its utility in children, and no score have been developed specifically for use in the paediatric age group. The current SI was previously applied in children in Saudi Arabia (El-Hazmi 1992a, El-Hazmi 1992b, El-Hazmi *et al,* 1992, El-Hazmi *et al,* 1993), and in Africa (Diop *et al,* 1999). It was found to be simple, applicable and allows some degree of standardisation. Other

additional merit of this SI relates to its ability to discriminate degrees of severity correctly into two distinct groups, delineated by genetic and phenotypic characteristics. This characterisation was possible in Saudi Arabia where the two forms of the disease (mild and severe) typically co-exist. The index has been used widely in Saudi Arabia the neighbouring country of Yemen, and these populations are closely related geographically, historically and in anthropological origin. Studies of Yemeni expatriates in Saudi Arabia showed that several haematological, molecular and genetic determinants of sickle cell severity in these patients had many similarities with those from the south-western Saudi population (El-Hazmi & Warsy 1999c, el-Hazmi & Warsy 1999d, El-Hazmi & Warsy 1999a, El-Hazmi & Warsy 2000). In the present study, the beta globin cluster haplotype analysis confirmed the predominance of the Benin haplotype in Yemeni children, which was similar to that in South and North Western areas of Saudi Arabia. In spite of these genetic and environmental similarities, it is worth mentioning that the health care systems in the two countries are different and this may result in confounding effects on severity score assessment, particularly towards lower frequency of hospitalization or blood transfusion. This can be considered as a basis for grouping cases into two main categories, which would also help to facilitate analysis and comparability. Using the same cut off point of 6 in the current study was capable of distinguishing those in the lowest quartile from those in the three upper quartiles and this is consistent with the approach used previously and justified its use in the classification of cases. In addition, differences between the mean SI of the mild group (lower quartile) and those in the upper two and three quartiles were highly significant ($p<0.001$). Hb F concentration was not included as part of the SI, but its level significantly differed between the two groups ($p=0.001$). In terms of medical care and follow-up those with

more severe manifestations require more attention and health care due to the severity of their disease.

The present findings can be considered further evidence of the reproducibility of this SI, although further validation using different score weightings would be valuable. This may help in improving score accuracy and performance. Score weighting and quantifying impact on disease severity and prognosis requires a level of consensus as a prerequisite for objective classification. The utility and appropriateness of this SI in Yemeni patients should be further evaluated and its reliability and reproducibility assessed. Validation can be attained by involvement of an independent panel of paediatric haematologists and clinicians who have good experience in the care of children with SCD and are familiar with the surrounding circumstances.

There were no significant differences in SI values between asymptomatic compared with symptomatic children and those admitted to hospital, which indicates that the index is not affected by the child's current clinical condition. This suggests that SI may be used as an assessment tool for all children with SCD independent of their clinical status.

The SI showed significant differences in relation to age and gender, and females had higher rate of painful crisis than males. Previous studies have reported a significantly higher incidence rate of painful crisis in female than male patients during the first decade of life and this difference increased with age (Gill *et al,* 1995). After the age of fifteen painful crisis sharply increased in males (Platt *et al,* 1991). Girls tend to report higher pain intensity and more body areas with pain (Franck *et al,* 2002), and older children reported higher levels of pain than younger children (Sporrer *et al,* 1994). It may be difficult for younger children to report pain,

and some episodes are likely to be managed at home. An elevated Hb level, which is a risk factor for painful crisis, has been reported to be higher in females from 1-10 years then in males after 15 years (Hayes *et al,* 1985). Age and gender differences have been attributed to several haematological and hormonal factors and recently to nitric oxide bioavailability with different levels of haemolysis releasing plasma haemoglobin, a potent NO scavenger (Reiter & Gladwin 2003). The exact reasons for these age and gender differences remain unclear. Our findings indicated that gender and age-adjusted analyses are preferable than pooling of data for males and females of all ages.

Hb S gene frequency in the Arabian Peninsula is one of the highest in the world (Weatherall & Clegg 2001). Mild and severe clinical forms of homozygous SCD have been reported from Saudi Arabia, (El Mouzan *et al,* 1989, Padmos *et al,* 1991) (mild in eastern and severe in western areas of the country), and from the United Arab Emirates (El-Kalla & Baysal 1998) and Oman (Jaiyesimi *et al,* 2002). The mild variant has also been reported from Kuwait (Ali 1970, Adekile 2001) and Bahrain (Al-Arrayed *et al,* 2003). Searching for the genetic determinants for the clinical heterogeneity revealed two major βs globin polymorphisms, the Benin and the Arab-Indian haplotypes, which have different clinical profiles and the later is associated with high Hb F levels and generally milder disease (El-Hazmi 1993). In Oman, as well as the Arab-Indian haplotype two further African haplotypes (Bantu and Benin) are present indicating the multicenteric origin of the beta S mutation in that region (Daar *et al,* 2000). Yemen is located between Oman and Saudi Arabia, and geographically is considered a crossing or bridging between Africa and Asia. Although β^S haplotypes are not described from the Yemen, its geographical and historical relation with the surrounding countries and with Africa, and the

heterogeneous SCD clinical profile, suggests the presence of mixed haplotype polymorphisms. An interesting finding in this study was the existence of the full clinical spectrum of severity with mild and severe forms occurring in individuals living on the same south-western area of the country.

Among factors modulating SCD, co-inheritance of α-thalassaemia has beneficial effects in some respects and deleterious in others (Ballas 2001). On one hand there is reduction of mean corpuscular haemoglobin concentration, number of dense cells, reticulocytes and the haemolysis rate, while on the other there is increased haemoglobin and hematocrit concentration leading to elevation of whole blood viscosity. Clinically there may be reduced risk of stroke, leg ulcer, acute chest syndrome and improved survival, but with a greater degree of splenomegaly, retinopathy and avascular necrosis, and a possible increased risk of acute painful episodes. We did not examine the prevalence of α-thalassaemia in the present study, although a high frequency of α-thalassaemia has been reported in a study of 26 Yemeni SCD children living in Saudi Arabia (El-Hazmi & Warsy 1999a). Double α-gene deletions were detected in 6 children, and a single α-gene deletion in 9 children, with an overall frequency of about 0.577. Haematological findings, compared to those without an α-gene deletion, showed their red cell count was higher and Mean Corpuscular Volume (MCV) and MCHC levels were lower with no differences in Hb F level. The clinical relevance of these findings has not been fully evaluated in Yemeni children.

The mean Hb F level in our patients was low with wide variation (4.0 ± 3.9), which is similar to findings reported from Riyadh (3.6 ± 2.9) and Qunfuda (5.0 ± 3.9) in Saudi Arabia (El-Hazmi & Warsy 2001). Despite these low Hb F concentrations, significant differences were still detected distinguishing the mild and

severe disease profiles. This finding is consistent with the conclusion of Platt et al, (1991) that even with low Hb F levels small increments may have beneficial effects in ameliorating painful crisis which is a core component of the severity scoring system. Higher Hb F concentration was found in sickle cell individuals who had the XmnI polymorphism, and values tended to be lower in those without this mutation. While about 50% of Hb F variation is not known, XmnI SNP is a major quantitative trait locus underlying Hb F heritability (Thein & Menzel 2009). The XmnI polymorphism was reported to be of low prevalence in Yemeni children with the β^S mutation (El-Hazmi & Warsy 2000, Al-Nood *et al,* 2004). In the study of El-Hazmi and Warsy (2000), among 30 Yemeni sickle cell patients, only 3.3% had the XmnI polymorphic site, and wide variation of Hb F levels was reported. This low frequency was similar to that in South-Western Saudi Arabia and in other Arab countries, where severe SCD is described. The wide variation of Hb F concentration did not consistently correspond with disease severity, as not all cases with high Hb F had mild disease, or those with low levels had severe disease (Donaldson *et al,* 2001, Inati *et al,* 2003).

Polymorphonuclear leukocytosis was more frequent in children with severe disease. Elevated PMNs have been related to increased early mortality, acute chest syndrome, and stroke (Platt *et al,* 1994, Miller *et al,* 2000). In patients with crises, PMNs are activated and exhibit greater vascular endothelium adhesion which could contribute to initiation and propagation of vaso-occlusive crises (Fadlon *et al,* 1998). Decline in the rate of PMN production and reduction in endothelial adhesion was associated with reduced severity and frequency of vaso-occlusive events in children treated with hydroxyurea (Benkerrou *et al,* 2002).

Malaria in Yemen is of unstable endemicity and anti-malaria prophylaxis is not given routinely to children with SS disease. It was of interest to note the higher frequency of documented malaria in the severe compared to the mild category (24.6 % vs 13.5%). Children with severe SCD who are infected with malaria may be more symptomatic, have different haematological and inflammatory responses, or splenic and immune responses, altering their ability to clear malaria parasites. This finding needs further confirmation in a larger sample and in areas with different levels of malaria exposure.

A lack of correlation between clinical severity and several haematological and biochemical parameters has been previously reported (Keidan *et al,* 1989). Dissociation between haematological indicators and clinical severity has been observed and this association is not straightforward (Serjeant 2004). For example a low level of Hb may be a risk factor for stroke and early death, whereas a high Hb level may increase the risk for painful crises, ACS, avascular necrosis and possibly retinopathy.

The severity profile of SCD in the present study was similar to that reported from South Western Saudi Arabia and Other Arab countries (El-Hazmi *et al,* 1999). Using the same scoring system the average SI in our patients was 7.8, with 63.7% scored as severe, compared with 73.8 % in Saudi Arabia (El-Hazmi *et al,* 1993). A lower score was observed in Senegalese patients, who had a SI average of 5.8, with 51.7% having severe disease (Diop *et al,* 1999).

The adjusted logistic analysis of factors associated with SCD severity demonstrated that early presentation, female gender, levels of un-conjugated bilirubin, Hb F and Hb A2 were significant determinants. Age at presentation and Hb F% had an inverse association with severity. In the absence of a neonatal screening

programme, early presentation seems to be an ominous indicator of disease severity. Levels of Hb A2 and un-conjugated bilirubin may be related to haemolysis stress and an increased in haemolytic rate. Accelerated vasculopathy in SCD has been attributed to hyper-haemolysis and reduced NO bioavailability with predisposition to complications such as pulmonary hypertension, stroke, leg ulceration and priapism (Kato *et al,* 2009).

The problem of severity assessment in the paediatric age group is that it takes time for the occurrence of chronic organ damage and complications to develop. Other factors which may not necessarily be related to the disease itself and may affect items of severity assessment score include: patient pain coping skills and tolerance, parental knowledge and experience, family socioeconomic status and ability to cover cost of medical care, time of acute event (night or week end), accessibility to health facilities, availability of health care, doctor experience and preference, and quality of health care afforded.

The clinical course in SCD does not follow specific pattern overtime, and changes may occur abruptly. For this reason it is uncertain to which extent disease severity assessment is capable of predicting the long-term clinical course in individual patients.

A limitation of this study was that data was collected retrospectively which could lead to variable degree of recall bias. This might be randomly distributed between mild and severe cases, although recall may be better for more severe symptoms. Only a single reading of laboratory investigations was obtained for each child, whether symptomatic or asymptomatic "steady state", and duplicate measurements would be preferable. Although, alkaline electrophoresis was used as the primary investigation for the diagnosis of SCD, available DNA sequencing for 72

children confirmed homozygous SS. The SI classified them into mild (34.7%) or severe (65.3%), which were comparable proportions to those of the whole sample.

5.6 CONCLUSIONS

This analysis reports for the first time the presence of mild and severe forms of SCD in Yemeni children. In the majority of children (63.7 %) the disease was classified as severe and severity was associated with age, gender and Hb F level. The frequency of malaria infection in these children is an important finding and may reflect chronic parasitaemia and altered susceptibility. The usefulness of the severity index score allowed objective clinical comparison with studies from other geographical locations.

This work highlights the need for research on the genetic and environmental factors affecting disease characteristics and phenotypic expression. Many aspects of SCD in Yemen are poorly studied and the extent of this problem is underestimated. The condition is really a neglected tropical disease.

CHAPTER SIX

MTHFR C677T POLYMORPHISM

6.1 INTRODUCTION

Vaso-occlusion plays a central role in the pathophysiology of SCD. Elevated plasma total homocysteine (tHcy) concentration is a recognized risk factor for vascular and thrombotic disease in the general population (McCully 2007). Therefore, tHcy may influence the clinical phenotype/severity of SCD. Raised homocysteine levels have a detrimental effect on endothelial tissues and could contribute to the organ damage observed in individuals with SCD as early as the first year of life (Schnog *et al,* 2000). Elevated levels of plasma tHcy may result from deficiency of folate or B vitamin co-factors, or by reduced activity of the key enzyme in homocysteine-methionine metabolism: 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) (Castro *et al,* 2006). A thermolabile variant with 50-60% reduced activity of this enzyme is caused by a missense point mutation at nucleotide 677 with thymidine replacing cytidine (C677T), resulted in alanine substitution for valine at position 222 of amino acid sequence (Ala222Val, rs1801133) (Kang *et al,* 1988, Goyette *et al,* 1994). Normal MTHFR activity is crucial for maintenance of the pool of circulating folate and to prevent accumulation of homocysteine (Frosst *et al,* 1995). The MTHFR C677T allele is associated with decreased blood folate and raised plasma tHcy (Jacques *et al,* 1996). This mutation has also been reported to increase the risk for neural tube defects and pregnancy complications and decrease the risk for colon cancer and adult acute lymphocytic leukaemia (Ueland *et al,* 2001).

Worldwide prevalence of the MTHFR C677T polymorphism shows extensive geographic variation, with frequency of the homozygous (TT) genotype among Caucasians between 10-30%, and the highest prevalence in Mexicans and the lowest

in blacks (Wilcken *et al,* 2003). Individuals with this mutation may have higher folate requirements for regulating homocysteine metabolism (Jacques *et al,* 1996), and with sub-optimal folate intake tHcy concentration is raised by about 50% (Malinow *et al,* 1997).

Patients with SCD suffer from chronic haemolysis, leading to accelerated erythropoiesis and increased folate requirements, therefore patients with a mutated MTHFR have increased folate requirements with risk of hyperhomocysteinemia if these requirements are not met. Administration of folic acid can lower plasma tHcy levels in a dose dependent manner (Clarke 2005), supposedly limiting endothelial damage and thrombosis in SCD (Ballas & Saidi 1997). Increased plasma homocysteine is reported in children with SCD (van der Dijs *et al,* 1998, Balasa *et al,* 2002), and in those who develop stroke (Houston *et al,* 1997). MTHFR deficiency has been associated with sickle cell vascular complications, including avascular osteonecrosis (AVN), stroke, retinopathy and ACS by some (Kutlar *et al,* 2001, Moreira Neto *et al,* 2006), but not all investigators (Andrade *et al,* 1998, Zimmerman & Ware 1998, Balasa *et al,* 1999, Cumming *et al,* 1999, Driscoll & Prauner 1999, Adekile *et al,* 2001, Romana *et al,* 2002).

6.2 OBJECTIVES

- To estimate the prevalence of MTHFR C677T mutation in Yemeni children with SCD.
- To evaluate the association of MTHFR polymorphism, plasma tHcy and circulating folate, vitamin B12 and B6 with vascular complications and disease severity.

6.3 METHODS

All symptomatic or asymptomatic children (<16 years) with SCD were recruited from the clinic or following hospital admission as described in detail in Chapter Three. Diagnosis was based on clinical manifestations, a positive sickling test, and confirmation by Hb electrophoresis. Data were collected through a direct interview using a pre-structured questionnaire. A detailed health history was obtained and clinical examination was conducted by the researcher. Information on family history, past medical history and frequency of acute clinical events covering a one year retrospective period was collected, including previous hospital admissions, blood transfusion, severity and frequency of crises, infection episodes, acute chest syndrome, avascular bone necrosis, skin ulcer, gall stone, priapism and urinary disorders. Definitions of clinical events were those proposed by the CSSCD (Gill *et al,* 1995). One or both parents were interviewed and requested to provide all available medical documents (medical prescriptions, previous investigations, and notes of hospital discharge). Previous admissions were cross-checked against hospital records. Intake of folic acid or multivitamin supplements was recorded.

Disease severity was assessed on the basis of frequency of painful crises, hospitalization, blood transfusion and sickle cell-related complications. A severity index (SI) was calculated for each child using the total score for the previous one year (El-Hazmi 1992a). A score of ≤ 6 disease was considered mild, and > 6 severe. This cut-off facilitated comparison with previous studies categorising mild or severe cases (El-Hazmi *et al,* 1993).

6.3.1 Blood investigations

A non-fasting venous blood sample of 6 ml was collected into a heparinized tube, at a standardized time (9-11 am), with the child in a sitting position. A fasting blood sample is preferred for homocysteine determination, although collection after three hours of food intake is satisfactory (Rasmussen & Moller 2000). Blood specimens were centrifuged at room temperature within 5-10 minutes of collection and plasma aliquots stored at -70° C. A whole blood haemolysate was produced using freshly prepared 1% ascorbic acid solution. Biochemical analyses were completed at the Liverpool School of Tropical Medicine, UK, and genetic analysis at the Department of Blood Coagulation, Sanquin Laboratory, Amsterdam, the Netherlands.

Total plasma homocysteine, whole blood folate and plasma vitamin B12 were analysed by automated methods using the DPC Immulite 2000 analyser (DPC-UK, Glyn Rhonwy, Gwynedd, Wales, UK). Analysis of tHcy was performed through a solid-phase, two-site chemiluminescent, immunometric assay (normal reference range in adult 5.0 – 12 μmol/L). The reference range in children under 15 years was reported to be lower (3-10.6 μmol/L) (Soldin *et al,* 2008). Whole blood folate and plasma vitamin B 12 were measured by competitive, liquid-phase chemiluminescent assay. Manufacturer's reference values for whole blood folate and vitamin B12 were 43-295 ng/ml, and 174-878 pg/ml respectively. Plasma pyridoxal-5'-phosphate (PLP) was determined by isocratic High Performance Liquid Chromatography (HPLC) using fluorescence detection. The PLP is fluorescinated and extracted following a single precipitation step. A mobile phase consisting of 97% 25mM K_2HPO_4 pH 3.0: 3% MeCN is used at a flow rate of 1.75 mL/min (LDC ConstaMetric III pump). Detection is accomplished using a fluorescence detector (ThermoSeparation Products FL2000)

with an excitation wavelength set at 320 nm and an emission wavelength of 415 nm. Prepared samples are compared to a set of plasma calibration standards to quantify the level of PLP. The reference level for vitamin B6 in our laboratory was (5-30 ng/ml).

6.3.2 Detection of MTHFR mutation

DNA analysis was performed by restriction fragment length polymorphism after conventional polymerase chain reaction (PCR). The DNA encoding for the MTHFR gene was amplified using sense and anti-sense primer. The PCR product was incubated with restriction enzyme HinfI and the presence of the MTHFR 677T allele was assessed by gel-electrophoresis.

6.4 RESULTS

Of the 102 SCD children blood samples were obtained in 69 children while in steady state, and in 33 during acute disease complications, 24 of whom required hospital admission.

The prevalence of homozygous TT for the *MTHFR* 677gene was 2 % (2/102), and heterozygous CT 10.8 % (11/102), giving an allele frequency of 7.35 %. The observed homozygote to heterozygote ratio was consistent with the Hardy–Weinberg equilibrium ($p^2 = 0.76$, $2pq = 0.216$, and $q^2 = 0.04$) with similar allelic frequency between males and females (7.1% vs 7.6 %, *p=0.92*), (Table 6.1). This ensures that there is panmixia and no selection against or in favour of certain genotypes.

Table 6. 1 Frequency of MTHFR C677T

Genotype	Female n=46	Male n=56	Total n=102	*P value*
CC	40	49	89 (87.2)	Ref
CT	5	6	11 (10.8)	0.97
TT	1	1	2 (2.0)	0.88
TAF	7/ 92	8/112	15/204 (7.3)	0.90

CC: Normal
CT: Heterozygous
TT: Homozygous
TAF: Total allele frequency
Brackets: Percentage

Using the severity index, 65 children (63.7 %) scored as severe and 37 as mild (36.3%). Table 6.2 summarises the association of SCD severity with MTHFR genotype and allele frequency. There was no homozygous variant type in mild cases. No significant differences were observed between mild and severe cases in relation to either genotype distribution or allele frequency.

Table 6. 2 MTHFR genotype distribution and allele frequency by severity groups

Degree of SCD	Genotype frequency, % (n)			Allele frequency, % (n)		
	CC [a]	CT [b]	TT [c]	C	T	*p value* [d]
Mild	30.4 (31)	5.9 (6)	(0)	91.9 (68)	8.1 (6)	0.75
Severe	56.9 (58)	4.9 (5)	2 (2)	93.0 (121)	7.0 (9)	-
p value [e]	0.42	0.21	0.43	-	-	-

[a] Homozygous wild type
[b] Heterozygous variant type
[c] Homozygous variant type
[d] Difference between C and T alleles
[e] Difference between mild and severe

There was no association between vascular complications (painful crisis, stroke, acute chest syndrome) with MTHFR genotype. A child with AVN and five children with stroke all had the wild MTHFR genotype (Table 6.3).

Table 6. 3 Association between MTHFR 677T allele and vascular complications

Complication	CC (n=89)		CT /TT (n=13)			
	Yes	No	Yes	No	OR (95% CI)	*p value*[a]
Painful crisis	80	9	12	1	0.90 (0.83-0.96)	0.23
Stroke	5	84	1	12	0.94 (0.89-0.99)	0.38
ACS	10	79	2	11	0.70 (0.12-5.28)	0.66

[a] Fisher exact test

The tHcy plasma concentration was available for 88 subjects; 59 in steady state, and 29 with acute disease complications or when admitted to hospital. The mean (±SD) tHcy was 2.8 ± 1.7 μmol/L (range 1.9 to 9.8 μmol/L) and median 1.9 μmol/L. There was no difference in mean concentration between males and females (2.9±1.8 vs 2.7±1.6 μmol/L, $p>0.05$). The plasma tHcy increased with age and was

higher in children older than ten years compared to younger children (2.5±1.2 vs 3.6±2.5 μmol/L, *p<0.05*) (Table 6.4). Mean tHcy plasma concentration during acute disease complications or hospitalization was 2.75 ± 1.44 μmol/L which did not differ from that in the steady state of 2.86 ± 1.89 μmol/L, (*p= 0.89*). Twenty one children had tHcy over the upper IQR, although no child had hyperhomocysteinemia including three cases with stroke. The mean severity score was significantly increased with increasing tHcy concentrations from the lower quartile (3.8) to upper quartile (5.3), with a mean of 4.6 μmol/L ± 3.7, *p<0.001*. Two children with homozygous TT had normal tHcy levels.

Table 6. 4 Mean homocysteine concentration (μmol/L) in relation to demographic and clinical characteristics

	n	Mean ± SD	*P value*
Sex			
male	41	2.7±1.6	0.73
female	47	2.9±1.8	
Age (years)			
< 10	62	2.5±1.2	0.04
>10	26	3.6±2.5	
Clinical status			
Steady state	59	2.9±1.9	0.89
ADC/hospitalization†	29	2.8±1.4	
Hb concentration (g/dl)			
<9.0	71	2.8±1.8	0.85
>9.0	17	2.9±1.7	

*Homocysteine analysis for 88 cases, †acute disease complication

There was no association between tHcy concentration with MTHFR genotype, disease severity, haemoglobin level, or whole blood folate concentration (Table 6.5).

The median plasma pyridoxine level was 38 ng/ml (range 3.9-96) and four children had values below the reference range, three of these were scored as severe disease and one who had the highest tHcy level had a CC genotype. Only one child had a vitamin B12 assay value below the normal range.

The median level of whole blood folate was 530 ng/ml (range, 111-6720), with 22% of cases within the normal range, and 78% above this range. Ninety one children reported regular intake of oral folic acid in a dose of 5-10mg daily and a few children intermittently used non-iron multivitamin supplements There was no significant difference in mean tHcy values between those who had taken folic acid on a regular basis (n=81) and those who had not (n=9) tHcy (2.8 μmol/L ± 1.8 versus 1.9 μmol/L ± 1.0, p=0.14). In univariate analysis there was no correlation of child age, whole blood folate, plasma vitamin B6, B12, or tHcy with MTHFR genotype. Multiple regression analysis showed no association between these variables with MTHFR genotype. With disease severity as a dependent variable, child age had a significant negative association, (adjusted OR= 0.21, 95 % CI, 0.06-0.74, *p=0.01*).

Table 6. 5 Plasma concentrations of homocysteine, folate, vitamin B6, B12 by MTHFR genotype and disease severity

	MTHFR genotype		*p value* [a]	Clinical severity		*P value* [a]
	CC (n=89)	CT/TT (n=13)		Mild (n=37)	Severe (n=65)	
tHcy, μmol/L	1.9 [1.9-2.7]	1.9 [1.9-5.1]	0.54	1.9 [1.9-4.3]	1.9 [1.9-2.7]	0.96
Folate, ng/ml*	521 [311-823]	605 [344-739]	0.77	437 [311-622]	571 [319-924]	0.15
Vitamin B6, ng/ml	38 [31-48]	40 [28-52]	0.75	40 [33-49]	36 [27-47]	0.49
Vitamin B12, pg/ml	439 [355-723]	395 [295-970]	0.61	402 [312-804]	460 [363-735]	0.52

[a] Difference between CT/TT and CC genotypes, and between mild and severe (Mann-Whitney U test)

*Whole blood folate

Values given are median [interquartile range]

6.5 DISCUSSION

The worldwide frequency of MTHFR homozygous TT genotype varies extensively according to ethnicity and geographical location, with the highest estimate in Mexico (32%) and lowest in Africa (0-1%) (Botto & Yang 2000, Wilcken *et al,* 2003). Among Arab populations the frequency is intermediate between Caucasian and African populations (Abu-Amero *et al,* 2003, Bu *et al,* 2004), with geographic gradients of 2-4% in the South (Yemen and Saudi Arabia) to 11-18% in the North (Lebanon and Syria) (Schneider *et al,* 1998, Herrmann *et al,* 2003, Ameen *et al,* 2005).

Among Yemeni children with SCD MTHFR C677T genotype prevalence was 12.7% with an allele frequency of 7.4%. This is similar to prevalence estimates among SCD patients from Bahrain 8% (Al-Absi *et al,* 2006), Jamaica 8.3% (Cumming *et al,* 1999), USA 8.6% (Zimmerman & Ware 1998) and Guadeloupe 9% (Romana *et al,* 2002). A previous study of 46 subjects from Yemen, showed the CT heterozygous frequency to be 30.4% and homozygous TT to be 2.2 %, with an allele frequency of 17.4% (Schneider *et al,* 1998). In the current study the MTHFR TT frequency was not significantly higher than in the Yemeni general population (2% vs 2.2%), which is consistent with reports from other countries in the Arabian Peninsula (Adekile *et al,* 2001, Fawaz *et al,* 2004, Al-Absi *et al,* 2006), and elsewhere (Balasa *et al,* 1999, Romana *et al,* 2002) . The finding of overall allele frequency lower than that for the general population (7.4% vs 17.4%), was similar to results reported by Romana et al., (2002) who observed an allele frequency of 9% in SCD patients compared to 15% in controls. There was a lack of an association with specific clinical events, suggesting little clinical impact of this gene variant on the course of SCD. The young age of many of these children with SCD would have resulted in a

disease profile with few severe disease complications which are common in older children.

In this analysis there was no association between the MTHFR C677T genotype and overall SCD severity, or with individual clinical complications. This is in agreement with other studies which reported no difference in genotype distribution in relation to vascular complications (Andrade *et al,* 1998, Zimmerman & Ware 1998, Balasa *et al,* 1999, Cumming *et al,* 1999, Driscoll & Prauner 1999, Adekile *et al,* 2001, Balasa *et al,* 2002, Romana *et al,* 2002). However, the number of cases with the TT genotype in all of these studies was small (range 0-8 cases), limiting their statistical power and make it difficult to draw definitive conclusions (Table 2.1). A single study reported a significant MTHFR association with occurrence of the combined vascular complications (stroke, AVN, retinopathy and ACS), although this was not significant when each complication was considered separately (Moreira Neto *et al,* 2006). Kutlar et al., (2001) compared 45 SCD patients (> 15 years) with AVN and 62 patients without this complication, and observed a significant positive association between the MTHFR mutation and AVN (35.6% vs 12.9%, $p=0.006$). In the present study, only one child (15 years old) with the wild MTHFR genotype developed AVN. A larger number of older children would be required to further assess this association.

Mean tHcy was reported to be increased in 46 SCD Caribbean patients from Curacao compared to controls, even though both cases and controls had comparable levels of plasma folate, vitamin B12 and B6. Administration of folic acid (2-4 mg/day) decreased tHcy concentration by 53% (van der Dijs *et al,* 1998). This observation indicates the ability of folic acid supplementation to lower plasma Hcy even if folate deficiency is not present. Elevated tHcy in SCD was also related to

pyridoxine deficiency (Balasa *et al,* 2002). Other studies have reported no difference in plasma tHcy, folate or B12 concentrations between children with SCD and controls (Balasa *et al,* 1999, Rodriguez-Cortes *et al,* 1999, Segal *et al,* 2004). In this study all participants had received folate supplementation which may blunt the effect of MTHFR on tHcy.

Serum or plasma folate represents the circulatory folate. It fluctuates rapidly and reflects recent dietary intakes, while red cell folate indicates long term intracellular folate. The whole blood folate reflects both plasma as well as RBC folate, and its value is intermediate between the two levels (Sauberlich 1995). Whole blood folate values in the majority of children were higher than the reference range, which probably indicates good patient compliance with long-term folate supplementation. The presence of a younger red cell population resulting from accelerated erythropoiesis, would increase folate levels as younger red cells have higher folate concentration (Muskiet *et al,* 2000, Schnog *et al,* 2001).

With sub-optimal folate status plasma tHcy and folate are highly correlated in children with SCD (van der Dijs *et al,* 1998), although this correlation is not maintained when folate status is satisfactory (Rodriguez-Cortes *et al,* 1999, Segal *et al,* 2004), as observed in the present study. In a study of 49 adult SCD patients, receiving oral folic acid supplementation (1mg/day), plasma Hcy concentration was elevated despite their significantly increased plasma folate, which was 1.5 fold higher compared to controls and with similar levels of vitamin B12. The authors suggested that higher folate concentrations may be required to normalize plasma Hcy and patients with SCD might have a higher nutritional requirement for folic acid than the general population (Lowenthal *et al,* 2000).

In individuals with MTHFR C677T, the inverse relation between plasma tHcy and plasma folate was intensified with the number of T alleles (Guttormsen *et al,* 1996, Hustad *et al,* 2007) and homozygous TT has been associated with lower folate level and higher tHcy concentration (Jacques *et al,* 1996, Moriyama *et al,* 2002). Folic acid supplementation reduced and often normalized elevated tHcy in individuals with this enzyme defect (Malinow *et al,* 1997, Ashfield-Watt *et al,* 2002, Ulvik *et al,* 2007). The two children with homozygous TT in this study had normal tHcy levels. Folate deficiency and hyperhomocysteinemia were absent in these children, probably reflecting their adequate level of compliance with folic acid supplementation.

We observed that parents tended to escalate the dose of folic acid according to recurrence of clinical events or painful crisis. For this reason, children with severe disease had higher levels of blood folate reflecting their increased intake. Furthermore there was no significant difference in circulatory folate between those taking or not taking folic acid regularly, which may indicate a satisfactory folate level even with intermittent intake.

Folic acid administration, in a higher dose (>5000 μg/day) may have a direct beneficial effect on vascular endothelial function independent of Hcy lowering mechanism (de Bree *et al,* 2007). The active circulating form of folate, 5-methyltetrahydrofolate (5-MTHF) has a structure similar to that of tetrahydrobiopterin (BH4), an essential co-factor of the enzyme endothelial nitric oxide synthase (eNOS). Diminished availability of BH4 leads to eNOS uncoupling and subsequently decrease NO formation. Folate can help to maintain the bioavailability of NO by stabilization and restoration of BH4 through its regeneration from the inactive form BH2 and by directly interacting with eNOS (Moens *et al,*

2008). Endothelial dysfunction was demonstrated in patients with SCD and suggested to be mediated by impaired NO activity with failure of vessel diameter adjustment in response to variations in wall shear stress, a major stimulus of eNOS activity and NO production (Belhassen *et al,* 2001). Impairment of NO dependent vasodilatation has been demonstrated in SCD during both acute crises and during steady state (Blum *et al,* 2005). Although the endothelial dysfunction was mostly reported in adult patients, it has been confirmed to occur in children (de Montalembert *et al,* 2007). A recent study on the effect of MTHFR polymorphism showed that vascular 5-MTHF was the most important determinant of endothelial dysfunction and vascular oxidative stress rather than the plasma or vascular Hcy concentration. The effect of 5-MTHF on vascular BH4, NO bioavailability and eNOS coupling seems to be independent of vascular Hcy, suggesting that plasma Hcy is an indirect marker of 5-MTHF rather than a primary regulator of endothelial function (Antoniades *et al,* 2009).

A lack of information on nutrient content of locally consumed foods and on vitamin dietary intakes in the Yemeni population limited estimation of dietary intake of these vitamins. However, an evaluation of folate dietary intakes in American SCD children, where food is folate fortified and dietary intake is better, showed that 53-57 % received lower daily folate intakes than recommended, and 15 % had low RBC folate levels despite receiving 1 mg folic acid supplements daily (Kennedy *et al,* 2001, Segal *et al,* 2004). In the Yemen, as in many other developing countries, malnutrition is highly prevalent and nutritional assessment of these children showed that the majority were undernourished, signifying a marked calorie deficiency with likelihood of concomitant micronutrient deficiencies. This makes it reasonable to continue the practice of regular folic acid supplementation for children with SCD in

this population and the importance of good compliance with folate supplementation should be emphasized as it may not always be optimal.

6.6 CONCLUSIONS

Results in this chapter show that the frequency of the MTHFR C677T genotype in Yemeni children with SCD was not higher than in the general population. In this Yemeni population screening for the MTHFR mutation would be unlikely to identify children at high risk for severe and complicated disease. Plasma tHcy showed no correlation with disease severity or complications and concentrations were decreased and not elevated as expected, this is most probably due to regular folic acid supplementation. Circulatory levels of folate and vitamin B12 and B6 were at the upper normal range in the majority of children, and even more for folate levels, which should raise the question about the proper prophylactic dose of folic acid in children with SCD in general, and in different environmental localities in particular.

CHAPTER SEVEN

SOLUBLE TRANSFERRIN RECEPTOR

7.1 INTRODUCTION

The first report of sTfR detection in plasma showed a higher concentration in patients with iron deficiency and haemolytic anaemias and a lower concentration in aplastic anaemia than controls (Kohgo *et al,* 1987). Bone marrow erythroid precursors are the major source of sTfR. Its measurement in a group of patients with different types of anaemia, including 11 cases with sickle cell anaemia, showed the highest level of sTfR concentration in SS patients (33±17 mg/l), which was six times above that in 84 normal adult controls (5.63±1.42), compared to a lower concentration than controls by approximately 50% in patients with complete bone marrow aplasia (Flowers *et al,* 1989).

Iron deficiency (ID) is the main determinant of sTfR. The concentration of sTfR does not become elevated until functional iron deficiency has developed, and iron deficient erythropoiesis commenced (Skikne *et al,* 1990).

Determination of sTfR is particularly useful in young children with depletion of iron stores whose serum ferritin is usually close to the range for iron deficiency and then sTfR elevation can accurately reflect functional iron deficiency (Skikne *et al,* 1990). A major advantage of sTfR over serum ferritin in the diagnosis of ID is the apparent specificity of its response to changes in iron status and erythropoiesis (Punnonen *et al,* 1997). The production of ferritin and TfR are precisely and reciprocally regulated and the combination of the two parameters provides an accurate evaluation of body iron status (Baynes 1996). Serum ferritin is a useful indicator of the iron storage compartment, while sTfR reflects the functional iron compartment and the ratio between them expressed as TfR/Ferritin ratio or sTfR/log

Ferritin (TfR-F index), was found to be more sensitive in differentiating between iron replete and iron deficient anaemic patients. This ratio reflects the full spectrum of body iron status (Skikne *et al,* 1990, Suominen *et al,* 1998).

sTfR was shown to be a sensitive predictor of non-stainable bone marrow iron in a study of a heterogeneous group of adult patients. Combined with serum ferritin, a high sensitivity of sTfR and high specificity of ferritin was preserved, improving the prediction of bone marrow iron stores (Means *et al,* 1999). Iron status in 521 preschool children (1-6 years) was classified more precisely using the log TfR:F with conversion of values of both sTfR and ferritin to SI units (μg/L), and suggested to be better than either TfR alone or the TfR-Index for defining iron status of children. A log TfR/F cut off of >2.55 identified subjects with iron deficiency and <2.55 those with anaemia of inflammation. With both conditions combined the ratio was <2.55, but increased above >2.55 after resolution of inflammation (Malope *et al,* 2001).

sTfR can also distinguish between patients with IDA and those with the anaemia of chronic disease (ACD) (Punnonen *et al,* 1994). The TfR/log ferritin ratio can discriminate between three groups of patients, those with iron deficiency anaemia (IDA), those with the anaemia of chronic disease (ACD) and those with combined iron deficiency as well as the anaemia of chronic disease (COMBI). The log ratio was superior to both sTfR and ferritin alone and to TfR/ferritin ratio (Punnonen *et al,* 1997). Moreover when compared with bone marrow examination sTfR was the most discriminatory iron test in 20 patients with ACD (75% efficiency) and in 18 patients with rheumatoid arthritis (94% efficiency) (Baillie *et al,* 2003). The lack of elevation of sTfR in cases of ACD may be a result of a cytokine suppression effect on erythropoiesis and erythropoietin production (Beguin 2003).

Another major determinant of sTfR is enhanced erythropoiesis. sTfR correlates with tissue iron receptors and its concentration reflects the intensity of erythropoiesis, increasing in hyperplasia and decreasing with hypoplasia of bone marrow (Huebers *et al,* 1990). Ferrokinetic studies of mean erythron transferrin uptake (ETU), which measures the number of iron-bearing transferrin molecules taken up by tissue receptors per unit time, showed a close positive association between plasma levels of sTfR and ferrokinetic measurements reflecting the rate of erythropoiesis (Huebers *et al,* 1990). In haemolytic disease both serum ferritin and sTfR are elevated, and if iron deficiency is excluded, the serum receptors provide a quantitative measure of total erythropoiesis and erythroid mass expansion (Cook *et al,* 1993). sTfR was also elevated in conditions where red cells are not released efficiently into the circulation from bone marrow such as in thalassaemia, megaloblastic anaemia associated with vitamin B12 or folic acid deficiency and myelodysplatic syndromes (Ahluwalia 1998). Ineffective erythropoiesis is also demonstrated in severe SCD (Wu *et al,* 2005).

The level of sTfR was reported to be high in SCD cases (Flowers *et al,* 1989, Huebers *et al,* 1990). In SS adults sTfR was in the high range (5.6-107.2 mg/L), with levels of serum ferritin from 22-318µg/L, with no sex differences. sTfR concentration was negatively correlated with Hb concentration but was not related to the level of Hb F (Serjeant *et al,* 1996). Conversely in Jamaican children with SCD the level of sTfR was elevated and significantly correlated with both Hb and Hb F concentrations, with no change during infectious episodes or painful crises (Singhal *et al,* 1993a).

Despite lack of standardization and differences in quantitative techniques used for measuring sTfR between various studies, the direction and magnitude of

changes in sTfR concentration across various clinical studies were comparable (Ahluwalia 1998). Currently, establishing international reference values with standardization of the assay are a high priority (Skikne 2008).

There is limited information on sTfR in children with SCD and in Yemeni patients no data is available. This study aimed to determine concentration of sTfR in Yemeni children with SCD and to examine associations with clinical and related laboratory findings.

7.2 OBJECTIVES

- To assess the sTfR concentrations in children with SCD in relation to disease severity category and complications.
- To assess the concentration of sTfR in relation to plasma ferritin and markers of inflammation.

7.3 METHODS

Clinical data was obtained by direct interview and disease severity assessed using a severity index as described in Chapter Three. Haematological investigations used the standard methods described in Chapter Three.

sTfR concentration was measured by ELISA immunoassay (IDEA sTfR IEMA, Orion Diagnostics Oy, Espoo, Finland). The calibrators, controls and patient samples were incubated in microtitration wells coated with monoclonal anti-human sTfR antibody. After a thorough wash of unbound antigens, monoclonal anti-human sTfR antibody was added to the wells and incubated with the immobilized antibody-sTfR complex. Following a second wash step substrate solution was added. The enzymatic reaction was then stopped by addition of acidic solution. The plate was read on an optical plate reader. Patient samples were diluted accordingly until results within the detection rate of the assay were obtained. The final concentration of sTfR

was obtained by multiplication using the dilution factor. The reference ranges were: 0.5–4 years, 1.6–4.0mg/L; 4–10 years, 1.5–3.7mg/L; 10–16 years, 1.4–3.4mg/L, calculated from the population described by Suominen *et al*, (2001)

Ferritin was assayed by ELISA method (Fortress Diagnostics Ltd, Antrim, UK). Diluted sample, control, and calibrator were added to coated wells and mixed with anti-ferritin monoclonal antibody. Immune complex is formed and is immobilized on coated wells. Following a second wash step, the antigen-antibody complex in each well is detected with specific anti-ferritin enzyme labelled antibodies. After removal of the unbound conjugate, the strips were incubated with the substrate solution. The enzymatic reaction was stopped by the addition of acidic solution. Colour change developed in proportion to the amount of immune-complex bound to the wells of the strips and represents the concentration of ferritin in the sample. The reference range for children (6 months to 16 years) is 10-160µg/L.

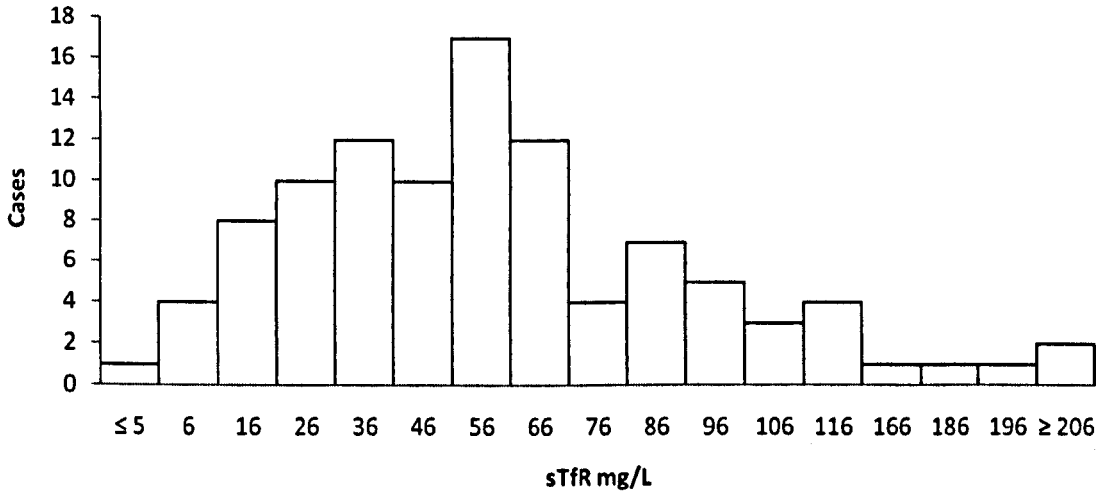
The sTfR-ferritin index was calculated as the ratio of sTfR concentration in µg/ml over the log of ferritin concentration in µg/L as previously described (Punnonen *et al*, 1997).

Concentration of CRP and SAA was determined by ELISA as described in Chapter Three.

7.4 RESULTS

The level of sTfR was determined in 102 children with SCD, (56 males) (Figure 7.1). Due to the skewed distribution the median and IQR was a more informative measure of the central tendency than the mean. The median and [IQR] of plasma sTfR among the study group were 58.5 mg/L [38-81 mg/L].

Figure 7. 1 Case distribution of sTfR



Males had higher mean levels than females (median [IQR], 62 mg/L [44.5-90.5 mg/L] versus 55.5 mg/l [30-67mg/L] (p=0.067) (Table 7.1)

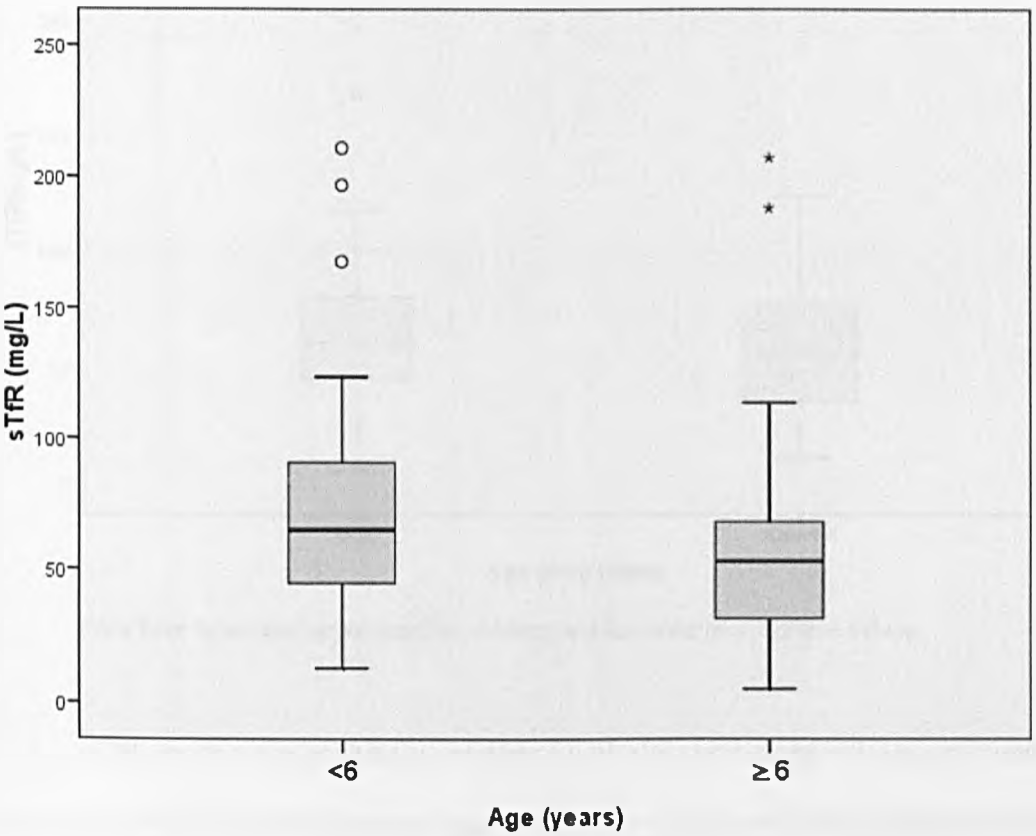
Table 7. 1 Sex distribution of sTfR levels (mg/L)

	No.	Median	IQR	*P value*
Male	46	62.0	44.5-90.5	0.067
Female	56	55.5	30.0-67.0	
All	102	58.5	38.0-81.0	

Age-specific values were assessed by comparison of sTfR levels in children below and above 6 years. The median [IQR] value was 65 mg/L [45-91mg/L] for

younger children and 53 mg/L [31.5-68 mg/L] for those older than six years (p= 0.028) (Figure 7.2).

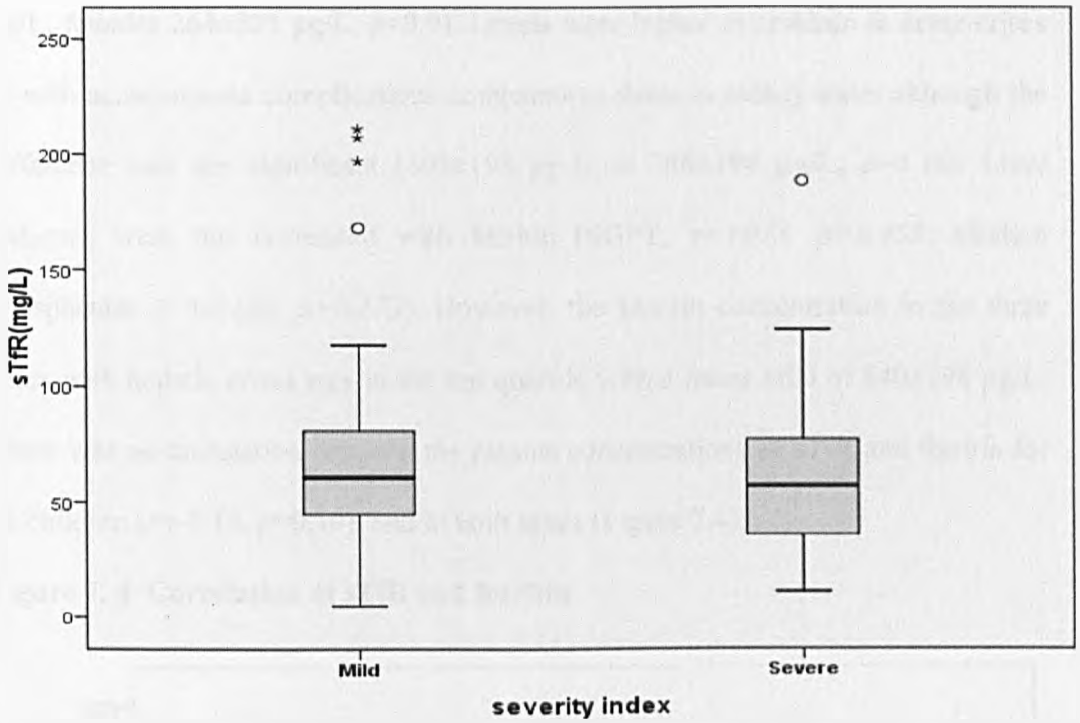
Figure 7. 2 sTfR levels in children below and above 6 years of age



Error bars: lower and upper quartiles, midline: median, asterisks: extreme values

There was no difference between mean sTfR level comparing children with mild or severe disease categories, (mean± SD, 72.6±51 mg/L vs 60.3±33 mg/L, p=0.14) (Figure 7.3). Similarly sTfR was not related to the frequency of clinical events, painful crises, hospitalization or blood transfusion requirements. The values of sTfR in 69 children in steady state condition did not differ from those of 33 children in acute crises or with acute disease complications, (median [IQR] 61 mg/L [45-86 mg/L] versus 46 mg/L [28-69 mg/L], (p=0.079).

Figure 7. 3 sTfR levels by severity category



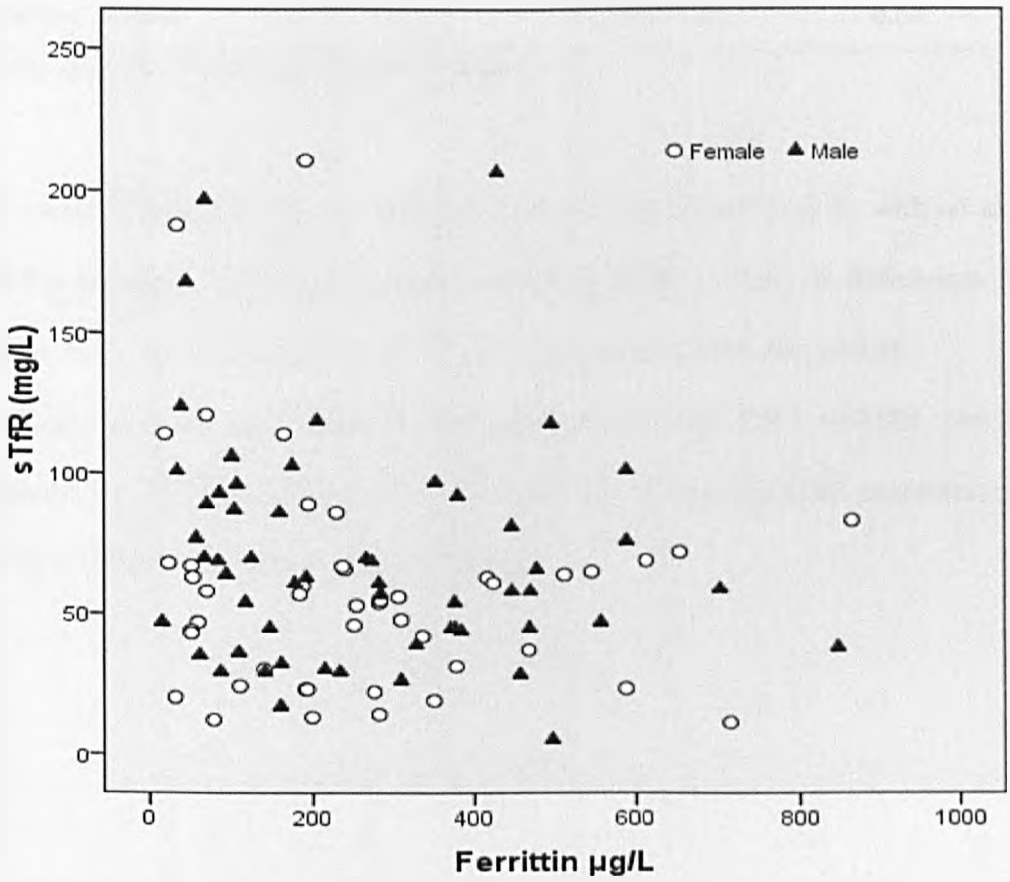
Error bars: lower and upper quartiles, midline: median, asterisks: extreme values

sTfR levels were positively correlated with the percentage reticulocyte count (*r=+0.31, p*=0.002), size of splenic enlargement (*r=+0.20, p*=0.044), and negatively correlated with Hb concentration (*r=-0.28, p*=0.004). There was no association with Hb F (*r=-0.032. p=0.75*), although there was a trend to increasing sTfR values with decreasing Hb F.

Plasma ferritin was assayed for all 102 children, none of whom had received chronic transfusion. Mean (SD) ferritin was 265±196 µg/L, range 15-866 µg/L. Ferritin concentration was within the reference range for 36.3% of children (mean ±SD, 78.9±39.9 µg/L), and above the reference range for 63.7% (mean ±SD, 371±168.8 µg/L). The ferritin level was <30 µg/L in only three children, between 30-70 µg/L in 17 children, and 70-100 µg/L in 5 children, and no child had a ferritin below 12µg/L. Ferritin was correlated with Hb levels (*r=+0.2, p=0.04*), but not with

age (*r=+0.02, p=0.84*), with no sex difference between mean values (males 266±193 µg/L, females 264±203 µg/L, p=0.9). Levels were higher in children in acute crises or with acute disease complications compared to those in steady state, although the difference was not significant (305±198 µg/L vs 246±194 µg/L, p=0.16). Liver enzymes were not correlated with ferritin (SGPT, *r=+073, p=0.465*; alkaline phosphatase *r=+0.138, p=0.173*). However, the ferritin concentration in the three cases with hepatic crises was in the top quartile with a mean ±SD of 640±198 µg/L. There was no correlation between the plasma concentrations of sTfR and ferritin for all children (r=-0.16, p=0.10), and in both sexes (Figure 7.4).

Figure 7. 4 Correlation of sTfR and ferritin



There were no differences in sTfR, ferritin or the sTfR-Ferritin index between those with no history of transfusion (27 children) and those receiving sporadic transfusions (75 children) (Table 7.2). Mean age was similar, with the mean Hb concentration significantly lower in the transfused group.

Table 7. 2 sTfR and ferritin levels by transfusion status

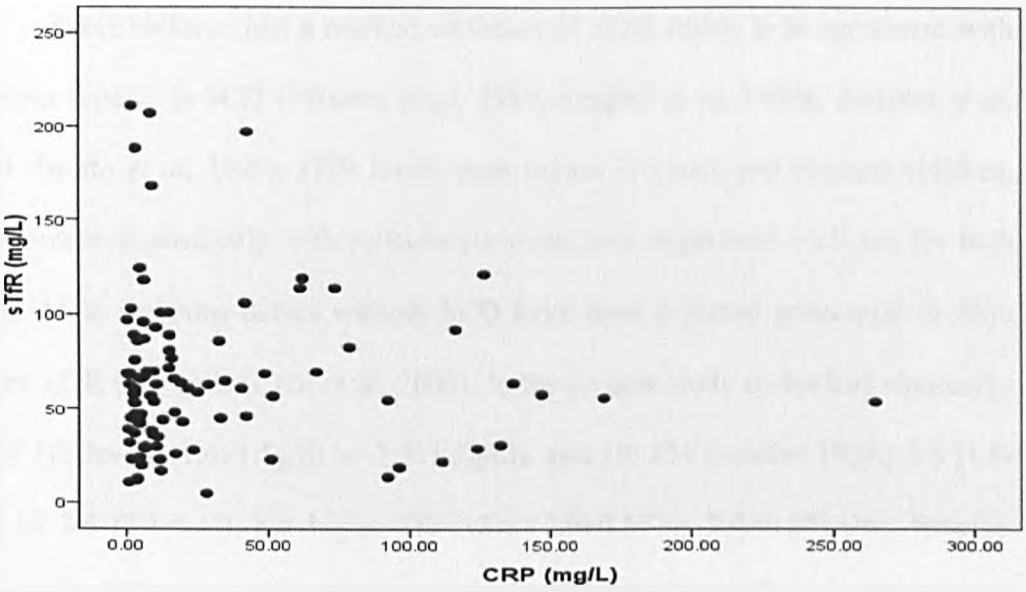
	Non-transfused n = 27	**Transfused n = 75**	***P value***
Age (yrs)	7.1 ± 5	7.3 ± 4.4	0.85
Hb (g/dl)	8.3 ± 1.4	7.6 ± 1.5	0.03
Ferritin (µg/L)*	215 [86-284]	234 [104-384]	0.56
sTfR (mg/L)*	61 [45-70]	58 [37-87]	0.96
sTfR/log ferritin*	27 [17-36]	24 [15-39]	0.68

Values: mean ±SD,* median [interquartile range]

The median [IQR] for the TfR: log ferritin ratio was 25 [16.5-38.0], with no sex differences (male 27 [17-42.3], female 24 [12.8-28.8], $p=0.1$), or differences in relation to the disease severity (mild 27 [19.5-42], severe 24[14-36], p=0.2)

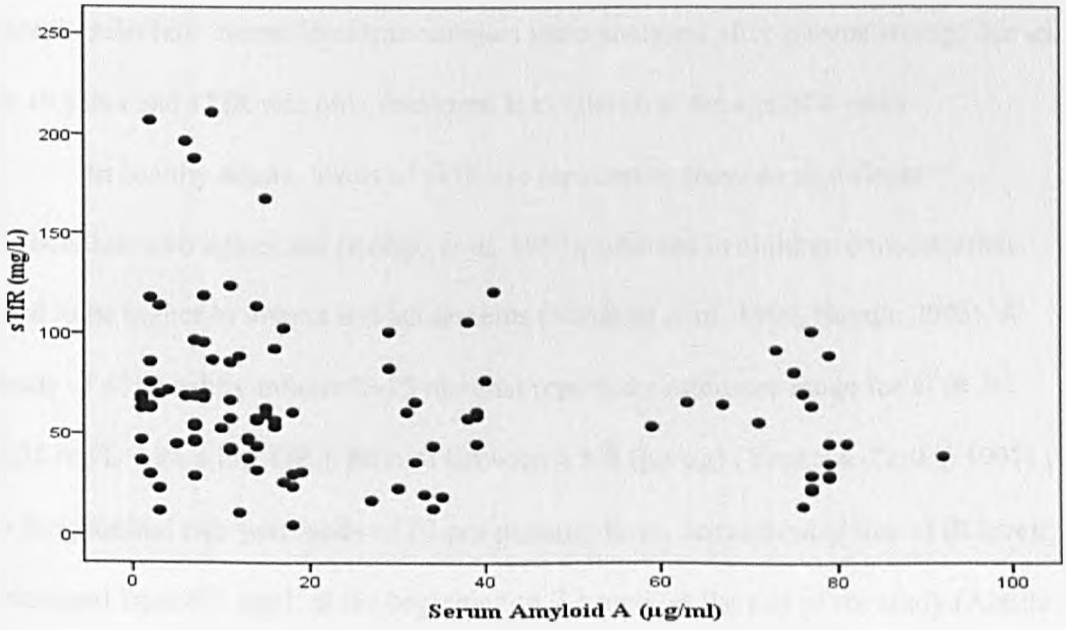
There was no correlation between CRP concentration and sTfR (r=+0.024, p=0.8) (Figure 7.5), CRP and ferritin ($r=+0.003$, p=0.98), or between CRP concentration and the sTfR-ferritin index (r=+0.005, p=0.9).

Figure 7. 5 Correlation of sTfR and CRP



For serum amyloid A (SAA) inverse negative correlation with sTfR was observed (r=-0.24, p=0.015) (Figure 7.6). There was no correlation between SAA and ferritin (*r=0.133, p=0.18*), or between SAA and the sTfR-ferritin ratio (r=-0.14, p=0.16).

Figure 7. 6 Correlation of sTfR and SAA



7.5 DISCUSSION

These children had a marked elevation of sTfR which is in agreement with previous reports in SCD (Flowers *et al,* 1989, Singhal *et al,* 1993a, Serjeant *et al,* 1996, Grotto *et al,* 1999). sTfR levels were higher in males and younger children, and correlated positively with reticulocyte count and negatively with Hb for both sexes. Male newborn babies without SCD have been reported previously to have higher sTfR than girls (Choi *et al,* 2000). In the present study males had marginally lower Hb levels (7.6±1.5g/dl vs 7.9±1.5g/dl), and Hb F% (median [IQR] 2.8 [1.8-4.8] vs 3.6 [2.8-5.4]), but higher Hb A2% (3.0±0.27 vs 2.8±0.37) than females. These sex differences could relate to the sex differences in sTfR concentration and reflect differences in erythropoiesis. In 182 SS Jamaican children sTfR was significantly elevated, (mean ±SD 38.3mg/L, range12.4-83.9mg/L) compared to AA controls (6.7mg/L, range 1.9-12.4mg/L), was higher in males, and correlated negatively with Hb, MCV and with Hb F in both sexes (Singhal *et al,* 1993a). This is similar to the present findings except that sTfR in the Yemen sample was much higher. The reasons for this difference could be related to assay methodology and sample selection. Some Jamaican samples were analysed after plasma storage for up to 10 years and sTfR was only measured for children at the age of 8 years.

In healthy adults, levels of sTfR are reported to show no significant association with age or sex (Kohgo *et al,* 1987), whereas in children concentrations tend to be higher in infants and adolescents (Virtanen *et al,* 1999, Beguin 2003). A study of 485 healthy infants (9-15 months) reported a reference range for sTfR 3-6.55 mg/L with a log TfR:F ratio of between 4.5-8 (μg/μg) (Yeung & Zlotkin 1997). A longitudinal two year study of 60 pre-pubertal boys, demonstrated that sTfR levels increased from 6.9 mg/L at the beginning to 7.2 mg/L at the end of the study (Anttila

et al, 1997). An age related decrease in sTfR concentration was observed in a study of 301 healthy children from between the age of 6 months to 16 years when adult levels were reached (Suominen *et al,* 2001). Estimation of age dependent intervals for sTfR for 183 children showed a higher level for children than adults, with peak values between 6 months-6 years (Kratovil *et al,* 2007). Paediatric reference values for both sTfR and the TfR-ferritin index in 436 apparently healthy children were recently published (Ooi *et al,* 2009).

7.5.1 sTfR and erythropoiesis

Correlation of sTfR with the degree of anaemia is based on the conclusion that sTfR is a reliable indicator of bone marrow activity and the degree of erythropoietic expansion (Singhal *et al,* 1993a, Grotto *et al,* 1999). When iron deficiency is excluded, the level of sTfR provides a quantitative measure of total erythropoiesis that is more sensitive and less invasive than bone marrow examination (Cook *et al,* 1993). In SCD there is an overall increase of haematopoiesis and both plasma and bone marrow sTfR concentrations were elevated and strongly correlated (Dallalio *et al,* 2007). A positive correlation between sTfR and serum erythropoietin and a negative correlation with RBC count and Hb level has been reported in individuals with SCD (Duits *et al,* 2003). A negative correlation with Hb was also observed in the present study. Levels of haematopoiesis are linked to levels of Hb F as SCD patients with low Hb F show higher rates of erythropoiesis with higher sTfR compared to individuals with higher levels of Hb F (Croizat & Nagel 1999). Yet in the present study no correlation with Hb F was observed which was similar to the report of Serjeant et al, (1996) from Jamaica.

The lack of correlation of sTfR with disease severity and complications was in agreement with previous studies (Singhal *et al,* 1993a), and consistent with the

role of sTfR as an indicator of bone marrow erythroid activity. The mean concentration of sTfR in SS children did not differ in those with a variety of clinical complications, or during crises (Singhal *et al,* 1993a). sTfR is considered a good indicator of the haemolytic rate in both effective and ineffective erythropoiesis and a better indicator than the reticulocyte count which increases only with effective erythropoiesis (Ho *et al,* 2003). A decreased haemolytic rate following regular blood transfusion, hydroxyurea therapy or splenectomy may lead to decline of sTfR levels (Singhal *et al,* 1993a, Tancabelic *et al,* 1999, Loukopoulos *et al,* 2000). In the present study there was no correlation between the number of blood transfusions and level of sTfR, and no difference in mean sTfR between those with or without a history of transfusion. This was not unexpected as no patient was receiving regular transfusion, which should reduce the rate of erythropoiesis and as a consequence the level of sTfR. A study of 19 chronically transfused SS patients compared with 31 non-transfused cases, showed sTfR elevation of about five-fold in non-transfused patients and two-fold in the transfused patients compared to controls, and with no difference between reticulocyte counts (Tancabelic *et al,* 1999). This suggests that erythroid marrow activity is still greater than normal even with regular transfusion.

Hydroxyurea therapy decreases ETU and red cell iron utilization in SS patients, an effect suggested to result from increased red cell survival and decreased haemolysis rather than bone marrow suppression (Ballas *et al,* 1999). sTfR has been reported to decrease after hydroxyurea treatment (Loukopoulos *et al,* 2000). Splenomegaly was common in these Yemeni children and was positively correlated with concentration of sTfR, suggesting some cases have hypersplenism with enhanced erythroid activity. Hypersplenism has been associated previously with

increased sTfR and following splenectomy sTfR levels returned to levels of SS children in a steady state without this complication (Singhal *et al,* 1993a).

Several studies have shown that sTfR is not influenced by the acute phase response resulting from inflammation or infection (Ferguson *et al,* 1992, Pettersson *et al,* 1994, Asobayire *et al,* 2001). In children with upper respiratory infections without anaemia sTfR levels were similar to those in healthy controls, although a lower sTfR:F index was observed, probably due to increased serum ferritin secondary to the acute phase response (Dimitriou *et al,* 2000). In the present sample there was no correlation between sTfR and the clinical condition of patients or with markers of inflammation, except for a weak negative and non-significant correlation with SAA. There was no correlation of SAA with the sTfR-ferritin index. The sTfR-ferritin index is considered largely independent of inflammatory status (Ooi *et al,* 2009).

7.5.2 Iron deficiency (ID), ferritin and inflammation

Iron is an essential element required for adequate erythroid function. Since there is no effective means of iron excretion iron homeostasis is regulated tightly through regulation of absorption of dietary content most probably under the control of hepcidin (Munoz *et al,* 2009). Inflammatory conditions can lead to dysregulation of iron homeostasis through iron diversion to the reticuloendothelial system limiting its availability for haem synthesis, with impaired erythroid proliferation and a blunted erythropoietin response complicating the diagnosis of ID (Weiss & Goodnough 2005). Assessment of ID is problematic even in normal children due to difficulty in interpretation of iron indices in a period of changing physiological and metabolic activities and due to the impact of infection (Aggett *et al,* 2002).

SCD is an inflammatory condition and accurate indicators of ID are difficult to define due to alteration of iron indices by a number of non-iron related factors.

Although SCD individuals may be considered at risk of iron overload due to increased iron absorption, iron re-utilization and chronic transfusion, ID is not an uncommon finding (Vichinsky *et al,* 1981, Davies *et al,* 1983). A high rate of ID is particularly common among children living in developing countries where iron deficiency anaemia is highly prevalent due to poor diets and with low bioavailability (Jeyakumar *et al,* 1987, Mohanty *et al,* 2008). Depletion of iron stores diagnosed by bone marrow examination has been reported in a high percentage of SCD children (36%-50%) from India and Nigeria (Nagaraj Rao & Sur 1980, Oluboyede *et al,* 1981, Okeahialam & Obi 1982). Inadequate intake, increased requirements and increased losses from recurrent epistaxis, haematuria and peptic ulceration may be predisposing factors (Davies *et al,* 1983). Sickle cell patients excreted almost 10 times the amount of iron in urine than normal, regardless of the serum iron level, history of transfusion or age, with similar mean iron urinary concentrations in children and adults (Washington & Boggs 1975). ID in SCD could be beneficial and possibly ameliorate sickling by decreasing MCHC, reducing haemolysis and prolonging red cell life span (Lincoln *et al,* 1973, Castro *et al,* 1994b), reducing painful crises (Bouchair *et al,* 2000), which could be precipitated by iron therapy (Haddy & Castro 1982). Evidence for the clinical benefits of iron deficiency is minimal and may be limited to adult patients (Koduri 2003). ID in children is implicated in poor growth and intellectual impairments (Oski 1993). In a growing child with SCD, iron requirements and iron losses are increased and they are at risk of both growth and neurocognitive impairments imposed by the disease itself and compounded by concomitant iron deficiency.

Low serum ferritin is considered the most specific screening test for ID, but normal ferritin level does not exclude this deficiency (Vichinsky *et al,* 1981, Adekile

et al, 1985). Serum ferritin level is either normal or elevated in the majority of SCD individuals who have received no or sporadic transfusion and whether in a steady state or during crises, which is usually higher (Hussain *et al,* 1978, Buffone *et al,* 1980, Russo-Mancuso *et al,* 1992, Stettler *et al,* 2001, Kordes *et al,* 2007). In some cases despite a normal or high serum ferritin, there is a complete absence of iron in bone marrow stains suggesting iron transport defect and compartmentalization (Peterson *et al,* 1975, Natta *et al,* 1985). Plasma ferritin was a poor biomarker of iron overload and was not correlated with iron on liver biopsy in children with SCD receiving chronic transfusion (Harmatz *et al,* 2000).

The raised ferritin values in SCD are most likely secondary to chronic inflammation and iron diversion toward retention into the reticuloendothelial system (Walter *et al,* 2009). The cut-off concentration of less than 12μg/L for diagnosis of ID is considered to be too low in the presence of inflammation (Witte 1991). Vichinsky et al., (1981) adopted a higher ferritin cut-off value (25μg/L) in assessment of ID in children with SCD, and which was derived from previous studies in chronic inflammatory conditions. They also recognized the limitation of ferritin in diagnosis of ID and considered the response to iron therapy as confirmatory. Serum ferritin <30μg/L was considered diagnostic for ID in SCD, with high specificity (98.7%), but low sensitivity (32%) (Rao *et al,* 1984). In a recent review, a level of 30μg/L was suggested to be better predictor of bone marrow iron in a condition without clinical evidence of infection or inflammation and a level of 30-100μg/L for anaemia with inflammation or chronic disease (Weiss & Goodnough 2005). In the present study 19.6% and 24.5% of children had ferritin concentrations less than 70 and 100μg/L respectively. Using different cut-offs for sTfR, ferritin and their ratio produced different results as shown in Table 7.3. Log sTfR/ferritin of >2.55 as

suggested by Malope et al, (2001) captured 96% (24/25) of children who had plasma ferritin concentrations < 100 μg/L. Should these cut-off values be applied to cases with SCD then the prevalence of ID would be much higher than that reported in several previous studies.

Table 7.3 Different cut-offs for ferritin, sTfR and their ratio by Hb level

Cut-offs		Hb <7.8g/dl* n=47 (%)	Hb ≥7.8* n=55 (%)	All n=102 (%)
Ferritin	30 μg/L[1]	2 (1.96)	1(0.98)	3 (2.94)
	100 μg/L[1]	14 (13.7)	11(10.78)	25 (24.5)
	273 μg/L[2]	28 (27.45)	30 (29.4)	58 (56.9)
sTfR	4 mg/L[3]	0 (0)	0 (0)	0 (0)
	15.2 mg/L[2]	1 (0.98)	3(2.94)	4 (3.9)
TfR-F index	5.3[2]	0 (0)	2 (1.96)	2 (1.96)
log (TfR/F)	2.55[4]	16 (15.7)	20 (19.6)	36 (35.3)

Hb: below and above median value, 1: (Weiss & Goodnough 2005), 2: (Phiri *et al,* 2009), 3: kit manufacturer, 4: (Malope *et al,* 2001).

Ferritin as a positive acute phase reactant can increase in infection, inflammation and liver cell damage, affecting its value in assessment of iron status in these situations (Cook *et al,* 1993). To overcome this problem, nomograms for CRP (or erythrocyte sedimentation rate) intended for quantitative adjustments of serum ferritin concentrations have been proposed. There are discrepancies with these, as ferritin and CRP combination did not improve predictive values for bone marrow iron (Coenen *et al,* 1991, Baumann Kurer *et al,* 1995).

The elevated plasma ferritin values found in this study were similar to many previous reports (Hussain *et al,* 1978, Buffone *et al,* 1980, Vichinsky *et al,* 1981, Russo-Mancuso *et al,* 1992, Stettler *et al,* 2001, Kordes *et al,* 2007). In the present analysis ferritin was normal in 36.3% and high for 63.7% of children. A study of 104

non-transfused SS African American children in a steady state showed 26% were normal and 74% had high ferritin levels (Stettler *et al,* 2001). Ferritin was also high in all 34 non-transfused SCD American children, and associated sonographic liver susceptometry scanning showed a normal liver iron concentration in the majority with a low concentration in 21% (Kordes *et al,* 2007).

The lack of correlation between ferritin and age and number of transfusions was in agreement with other reports (Buffone *et al,* 1980, Adekile *et al,* 1985). Liver cell damage may contribute to the raised level of ferritin. Although higher values were observed in cases with hepatic crises, no correlation was found between ferritin and liver enzymes indicating that liver injury was not the main cause of the raised ferritin values, as previously reported (Brownell *et al,* 1986).

Concentrations of sTfR did not differ comparing SS patients with and without ID classified by ferritin concentration <12μg/l, suggesting sTfR is an inappropriate biomarker for the assessment of ID in these cases (Singhal *et al,* 1993a). In SCD, discordance between ferritin and sTfR as markers of ID might be explained by the effects of hypererythropoiesis and inflammation. Serum ferritin increases as part of the acute phase response, whereas sTfR increases in response to enhanced erythropoiesis. Furthermore inflammatory status is associated with elevation of cytokines which could suppress erythropoiesis and erythropoietin production and consequently sTfR. Hypoxia is an additional factor which increases sTfR levels, either secondary to stimulation of erythropoietin production or directly by up-regulation of TfR through the hypoxia-inducible factor-1α (HIF-1α) pathway (Tacchini *et al,* 1999). Children with SCD may experience hypoxia due to anaemia, respiratory complications and chronic haemoglobin oxygen desaturation (Quinn & Ahmad 2005). Hepcidin a key regulator of iron absorption and recycling induced by

anaemia and hypoxia and suppressed by erythropoiesis and inflammation was reported to be in the low normal range in SCD (Ezeh *et al,* 2005, Kroot *et al,* 2009). The influence of iron status, the rate of erythropoiesis, inflammation, and hypoxia in SCD are complex and difficult to quantify without comprehensive assessment of several biomarkers. Iron metabolism and assessment of iron status with validation of suitable serum ferritin cut-off values in SCD children requires further investigation.

A limitation of this study is that the data do not provide sufficiently detailed information on how sTfR concentrations, or its ratio with ferritin, correlate with iron deficiency as no other iron biomarkers were assessed and bone marrow studies were not possible. It is difficult to determine what proportion of the sTfR response was related to erythropoiesis or to concomitant iron deficiency, and the same conclusion can be made for interpretation of ferritin in relation to inflammation.

7.6 CONCLUSIONS

A marked elevation of sTfR was demonstrated in Yemeni children with SCD. The levels were higher in younger children and correlated with the degree of anaemia and erythropoiesis. There was a significant correlation of sTfR with splenomegaly, which may indicate hypersplenism in some cases. Serial longitudinal measurement of sTfR in these children would help clarify this association. The concentration of sTfR did not differ by disease severity or in relation to disease complications. There was no correlation between sTfR and inflammatory markers. Ferritin level was either normal or elevated and not correlated with age or history of transfusion. Use of different cut-off values similar to that have been proposed in inflammatory diseases should be evaluated. Improving diagnosis of iron deficiency in these children and the balance between the risks and benefits of iron administration should be further evaluated, especially in children.

CHAPTER EIGHT

ACUTE PHASE REACTANTS

8.1 INTRODUCTION

Inflammation is a local response to inflammatory stimuli such as infection or tissue injury, and if these stimuli are severe this can produce systemic changes referred to as the acute phase response (Kushner & Rzewnicki 1994). Acute-phase responses are a systemic pathophysiological phenomenon, characterized by changes in concentration of plasma proteins known as acute phase proteins and associated with multiple physiological and biochemical alterations (Gabay & Kushner 1999). The plasma levels of acute-phase proteins may change by at least 25% during inflammatory diseases with an increase (positive acute phase protein e.g. complement proteins, C-reactive protein, serum amyloid A, α1-acid glycoprotein, fibrinogen) or decrease (negative acute-phase protein e.g. albumen, transferrin, transthyretin) in concentration (Morley & Kushner 1982).

Inflammation is a damaging process to vascular endothelial cells, basement membrane and matrix components leading to leakage of plasma proteins and micro-vascular haemorrhage contributing to organ dysfunction (Lentsch & Ward 2000). In sickle cell disease vaso-occlusive episodes resulting from impediment in the blood microcirculation and tissue ischemia can induce these local inflammatory processes with monocyte and macrophage infiltration. These cells produce several inflammatory mediators which provoke and modulate the acute phase response both in the steady state situation and during painful crises (Stuart 1993).

Inflammation in SCD is evident by activation of endothelial cells, blood cells, the coagulation system as well as the acute phase response (Hebbel 2004). Recurrent episodes of localized tissue ischemia and re-perfusion injury can provoke local

inflammation with repeated stimulation of cellular and humoral inflammatory mediators, leading to low-grade chronic inflammation (Platt 2000). This results in persistent elevation of some inflammatory markers such as human CRP which exacerbates ischemic infarction in areas of pre-existing tissue damage. This is likely to occur via a complement dependent mechanism which is potentially preventable with specific drug inhibitors of CRP binding to exposed ligands of damaged cells thereby reducing complement activation by CRP (Pepys *et al,* 2006, Pepys 2008). It is recognized that HU therapy diminishes production of some inflammatory mediators and up-regulates other protective mechanisms including plasma anti-inflammatory IL-10 and IL-10 neutrophil gene expression which were positively and significantly correlated with levels of Hb F (Lanaro *et al,* 2009).

Understanding inflammation in SCD is important as it relates to pathogenesis and certain markers may be useful predictive factors for sickle cell crisis. The aim of the present analysis was to examine the role of the APR in relation to disease severity in order to consider the potential utility of this assessment in terms of disease management and prognosis.

8.2 OBJECTIVES

- To quantify the acute phase proteins C-reactive protein and serum amyloid A in children with SCD both in the steady state and during acute disease complications.
- To determine the association of these acute phase reactants with disease severity.

8.3 METHODS

Measurement of both C-reactive protein and serum amyloid A was by ELISA.

C-reactive protein (CRP): Diluted standard sera and patient samples were incubated with microtitre strips coated with anti-CRP antibody. During this incubation step CRP in the samples are bound specifically to the wells. After removal of unbound serum proteins by a washing procedure, the antigen-antibody complex is detected with specific conjugated antibodies. Following removal of unbound conjugate, the strips were incubated with substrate solution. Absorbance values were determined based on the colour change developed which was in proportion to the amount of immune-complex bound to the wells of the strips. The final CRP concentration was read from the standard and corrected for the dilution factor

Serum Amyloid A (SAA): Standards and samples were added to microtitre strips pre-coated with monoclonal SAA specific antibodies. SAA present in samples is bound specifically to wells and becomes immobilized. After removal of unbound serum proteins by a washing procedure, antibody specific for SAA was added to each well to "sandwich" the immobilized SAA. All wells were washed thoroughly and substrate solution added. The colour change occurred in proportion to the amount of immune-complex bound to the wells of the strips and absorbance values were measured on a plate reader. The final concentration of SAA was read from the standard curve with correction for dilution factor.

Hb, leukocyte differential count, ferritin and plasma albumin were analysed following standard procedures as described in Chapter Three.

Disease severity assessment, definition of steady state, acute disease complications and clinical events was that described in Chapter Three

8.4 RESULTS

A total of 102 children were analysed according to their clinical status. Their haematological and APR measurements are summarized in Table 8.1. Sixty nine children were in a steady state and 33 had acute disease complication.

Table 8. 1 Clinical and laboratory data of children in steady state or with acute disease complication

Category	Steady state n=69	Acute disease complication, n=33	*P value†*
Mild (n=37)	26	11	0.82
Severe (n=65)	43	22	
Age (yrs)	7.8±1.4	5.9±4.3	0.04
Hb g/dl	7.9±1.4	7.4±1.7	0.10
PMN%	37.3±12.8	39.3±11.6	0.44
CRP mg/L	6.3 [3.0-20.8]	17.4 [7.6-56.5]	0.025
SAA mg/L	13.0 [7.0-30.0]	32.0 [8.5-76.5]	<0.001
Ferritin µg/L	195 [74-364]	282 [125-463]	0.16
Albumin gm/L	4±0.65	3.7±0.5	0.09

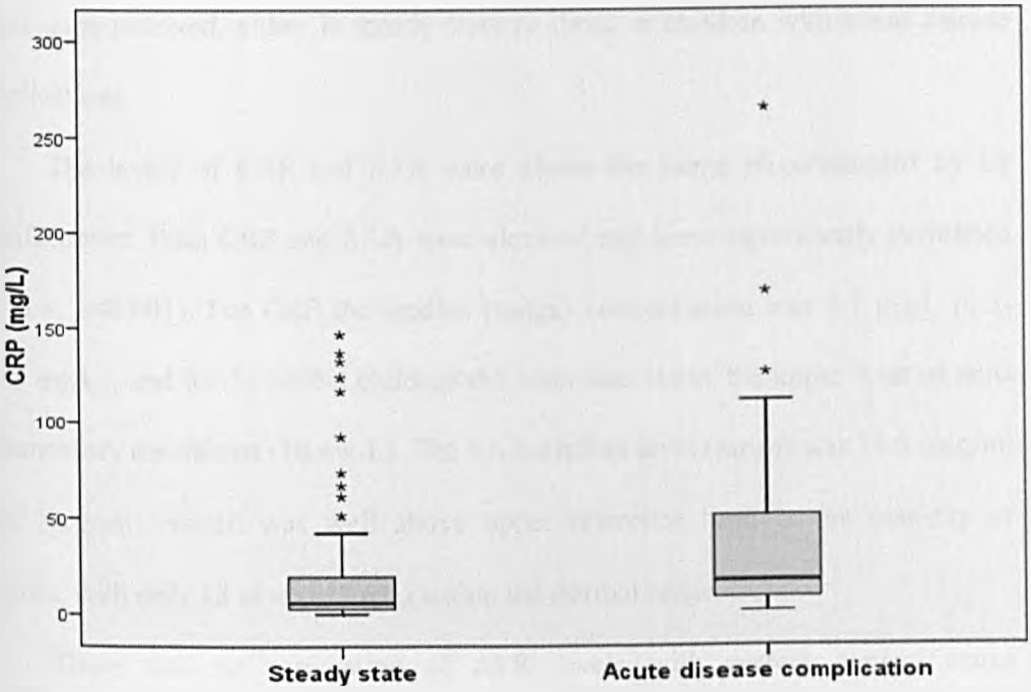
Values: mean ±SD, or median [IQR]

†Chi square between mild and severe group, t-test for difference between means, Mann-Whitney for difference between medians

Comparing these two groups of children there were no differences in the proportion of mild or severe disease, or sex differences although children with acute disease complications were younger. The concentration of Hb and the percentage of PMN cells did not differ between the two groups. Mean concentration values for both CRP and SAA were significantly higher in children with acute disease complications (Figure 8.1 and 8.2). Plasma albumin was below 3.4g/dl in 21 (20.6%)

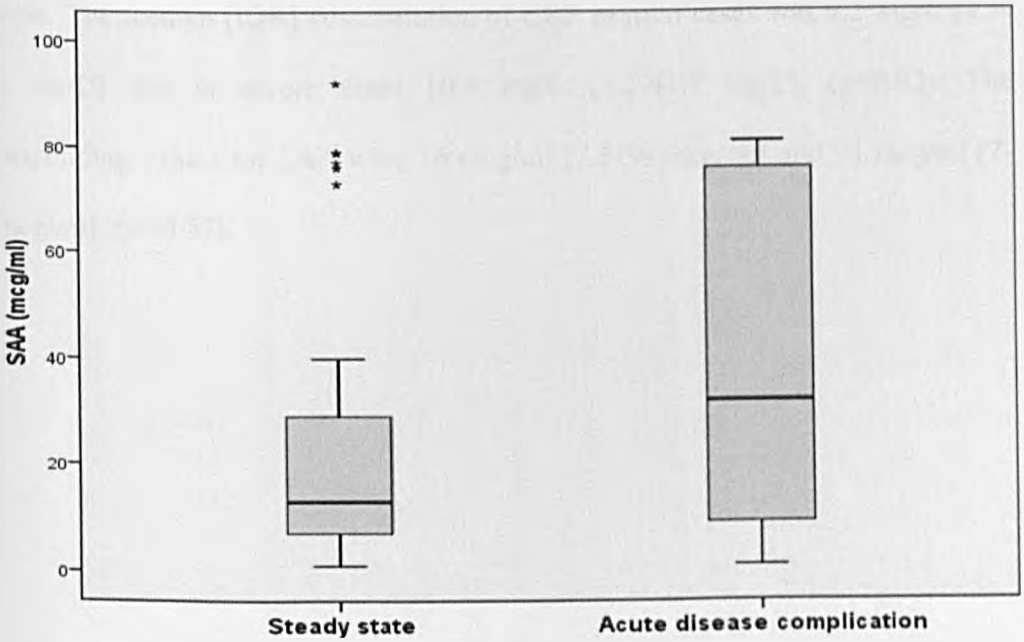
children and its mean concentration was lower in children with acute disease complications (p=0.09).

Figure 8. 1 Boxplot of CRP in steady state or acute disease complications



Error bars: lower and upper quartiles, midline: median, asterisks: extreme values

Figure 8. 2 Boxplot of SAA in steady state or acute disease complications



Error bars: lower and upper quartiles, midline: median, asterisks: extreme values

The plasma ferritin was higher in children with acute disease complications, although this difference was not significant. There was no correlation between plasma ferritin level and past history of blood transfusion or the number of transfusions received, either in steady state or those in children with acute disease complications.

The levels of CRP and SAA were above the range recommended by kit manufacturers. Both CRP and SAA were elevated and were significantly correlated ($r = 0.4$, $p<0.001$). For CRP the median (range) concentration was 9.7 mg/L (0.3-264.6 mg/L), and for 50 (49%) children the level was above the upper limit of non-inflammatory conditions (10 mg/L). The SAA median level (range) was 14.5 mcg/ml (1-92 mcg/ml), which was well above upper reference limit in the majority of children, with only 18 cases (17.6%) within the normal range.

There was no association of APR levels with current clinical status (symptomatic, asymptomatic, hospitalized, non-hospitalized), or individual clinical variables including painful crisis, hospitalization, blood transfusion or infection episode. The median [IQR] concentration of CRP in mild cases was 9.2 mg/L [4.5-23.4 mg/L] and in severe cases 10.6 mg/L [3.2-41.9 mg/L], (p=0.92). The corresponding values for SAA were 16 mcg/ml [7.5-39 mcg/ml] and 14 mcg/ml [7-38 mcg/ml], (p=0.57).

8.5 DISCUSSION

8.5.1 Main findings in comparison with previous studies

These results confirmed that the acute phase reactants CRP and SAA were significantly elevated in children with SCD, which is consistent with previous studies (Akinola *et al,* 1992, Hedo *et al,* 1993, Singhal *et al,* 1993c, Bourantas *et al,* 1998, Moore *et al,* 1998). The concentration of CRP and SAA were well above the normal reference range for the majority of cases who were in a steady state and were significantly more elevated during acute disease complications. These estimates were point elevations although serial measurements of CRP, alpha-1-acid glycoprotein (AGP), and fibrinogen have all been shown to be evaluated in SCD (n=20) patients from Birmingham (UK), when in steady state or during crises (Akinola *et al,* 1992). Patients with frequent crises consistently have higher steady state APR, although elevation of CRP, a fast-reacting acute phase protein compared to the slower-responding proteins AGP and fibrinogen is consistent with repeated minor sub-clinical episodes of vascular occlusion and associated inflammation occurring during steady state but insufficient to produce pain.

The hypothesis that APR may be helpful in detection of early changes in the prodromal phase of a sickle cell crisis lead to a study by the same group designed to monitor APR in both prodromal and established phases of painful crises (Akinola *et al,* 1992, Stuart 1993). Increased CRP and SAA concentrations occurred early in crises and sometimes within the prodromal phase. Rapidly resolving crises (within 24 hours of hospitalization) showed minor and transient rises of these reactants compared to responses in children requiring treatment for four days or more, or who had very high values during steady state (Stuart *et al,* 1994). It was concluded that CRP and SAA were of potential value for monitoring crisis and subsequent resolution. A Jamaican study of SS (n=143) and SC (n=35) children reported raised

CRP and SAA concentrations in SS to a greater extent than SC disease or normal controls, and there was clear evidence of raised values of both proteins in 18% of SS patients in steady state (Singhal *et al,* 1993c). Conversely Becton et al., (1989), who assessed plasma CRP, C3 and α1-antitrypsin in SS patients (n=75) at routine and non-routine visits (infection or pain episodes), found no significant alterations in plasma concentrations in asymptomatic individuals who had a mean value within the normal range. Values were markedly increased in patients with crises and were higher in the presence of bacterial infection.

Hypoalbuminaemia, defined as plasma albumin less than 3.4g/dl, is a marker of disease severity and associated with poor clinical outcome in acutely ill adults patients (Vincent *et al,* 2003), or children (Horowitz & Tai 2007). In the present study hypoalbuminaemia occurred in 20.6% of children with a lower mean concentration in those with acute disease complications. Plasma albumin decreases during the acute phase response probably due to diminished hepatic synthesis and diversion of protein production to those required for host defence (Ceciliani *et al,* 2002). Redistribution of plasma albumin probably occurs following interstitial changes secondary to the acute phase reaction and is accompanied by minimal changes in capillary wall oncotic gradient (Franch-Arcas 2001).

8.5.2 Pathophysiology and clinical relevance

SCD is considered an inflammatory disease associated with recurrent tissue ischemia, infarction and re-perfusion injury, which provoke a series of inflammatory mediators leading to chronic activation of inflammatory processes and which probably contribute to vascular injury (Platt 2000). Inflammation is apparent as there is an increase in basal leukocyte count, abnormal activation of granulocytes, monocytes, and vascular endothelial cells which are associated with increased levels

of multiple inflammatory markers. This occurs during both symptom free intervals and is intensified during crises (Moore *et al,* 1996, Hebbel 2004).

Acute phase proteins are rapidly up-regulated, and produced principally in hepatocytes, under the control of inflammatory cytokines, including interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which originate at the site of injured tissue (Gabay & Kushner 1999). Although, acute elevation of these reactant proteins may have beneficial effects in terms of homeostasis and minimizing tissue damage caused by microvascular infarctions, chronic elevations may nevertheless be deleterious (O'Brien & Chait 2006). In SCD these proteins could have mediator effects on the vascular inflammatory process resulting in a vicious circle of vascular occlusion and inflammation (Belcher *et al,* 2000). The inflammatory cytokines elevated in steady state and crisis are derived from activated vascular endothelium (IL-1, IL-6, IL-8, TNF-α), from activated platelets, and from monocytes and microphages (IL-1, TNF-α) and this is accompanied by up-regulation and expression of numerous adhesion molecules (Pathare *et al,* 2003, Brittain & Parise 2007). IL-6, the main stimulator of acute-phase proteins, has been reported to be significantly higher in crisis than in the steady state situation (Bourantas *et al,* 1998, Makis *et al,* 2000b, Pathare *et al,* 2004). However, measurement of these cytokines in plasma is not straightforward because of their short plasma half-lives and the presence of blocking factors (Gabay & Kushner 1999).

CRP and SAA are the major human acute phase reactants (Steel & Whitehead 1994), and are the most sensitive acute phase proteins reflecting inflammatory activity. This is based on their low basal concentrations, rapid response following an inflammatory stimulus (increasing within 6-10 hours, peaking around 48 hours), the large incremental change and their short half life (van Leeuwen & van Rijswijk

1994). Concentrations of CRP in the non-inflammatory state range from less than 50 μg/L to10 mg/L, but following an acute-phase stimulus values may increase to above 500 mg/L, which is 10,000-fold the normal level (Pepys & Hirschfield 2003). Plasma concentrations of SAA normally range between 1-5mg/L, but during an inflammatory reaction reach levels 500-2000 fold higher (Malle & De Beer 1996).

CRP and SAA have been implicated in the prediction of morbidity and outcome in chronic disease states (Jylhava *et al,* 2009, Simic-Ogrizovic *et al,* 2009). Minor elevation of CRP levels (3–10 mg/L) may indicate only mild degrees of tissue stress or injury, even when no obvious inflammatory response is clinically apparent, and a predictive value is described for both diseased and healthy individuals (Kushner *et al,* 2006). Persistent high concentration of SAA may predispose to the development of secondary amyloidosis, a complication of chronic inflammation presented mostly as nephropathy (Gillmore *et al,* 2001). Secondary amyloidosis has been reported in SCD (Simsek *et al,* 2006).

Levels of APR are influenced by both clinical and genetic factors. Twin studies suggest that 52% of baseline values for CRP and 59% for SAA could be attributable to genetic factors (MacGregor *et al,* 2004). Multiple studies have demonstrated that several common polymorphisms of the CRP gene are important determinants of baseline circulating CRP concentrations, and this could explain some of the phenotypic inter-individual variability (Hage & Szalai 2009). The distribution of CRP haplotypes in SCD patients, and their influence on plasma levels and disease expression require further investigation.

8.5.3 APR and disease severity

The association of the APR with clinical severity assessment has been described in only a few studies. Hedo et al., (1993) reported serum concentrations of seven acute phase reactants in 73 Nigerian SS patients, mean age ±SD (21.5±5.4 years). All patients were at steady state with varying degrees of disease severity. They were grouped into three severity grades according to the number of pain crisis, the degree of anaemia and the development of specific complications. A significant elevation of CRP, and α1-antitrypsin with reduction in serum transferrin level was reported. In regression analysis only CRP and transferrin correlated significantly with the severity index. Using the same SI derived from Hedo et al., (1993), 35 patients with sickle/β-thalassaemia were evaluated by Makkis et al., (2006)., who reported a correlation between SI and high sensitivity CRP (hsCRP) (r=0.64, p=0.01). Serum VCAM-1 levels also were increased during crisis above values for steady state and control subjects.

In a retrospective study hsCRP was determined in SS (n=26) and SC (n=17) adult patients from Curacao at steady state and compared to matched African American blood bank donors. Elevated levels of CRP were observed in patients compared to controls, but when correlated with disease severity (assessed by frequency of clinical events: painful crises, stroke, ACS in the previous three years) no significant association between CRP and severity was detected (Schnog *et al*, 2004). In SS children (n=34) the degree of severity of painful crisis was related to acute CRP level graded by an episode severity index (Etienne-Julan *et al*, 2004). IL-8 and VCAM-1 were also raised at time of crises.

The present study showed no association between CRP or SAA and disease severity. The degree of severity of the painful crisis rather than the frequency might

be more influential on the APR response. Even though CRP and SAA generally reflect the extent and activity of the underlying inflammatory disease their levels showed a wide variation even in individuals with an apparently similar degree of disease activity (MacGregor *et al,* 2004). This may reflect genetic differences. The study of Hedo et al., (1993) was carried on adult patients at steady state and used a different scoring system to the present study. The participants reported by Makis et al., (2006) were children, nevertheless the study was limited to cases with double heterozygous SCD (sickle β^0 thalassaemia and β^+ thalassaemia) and no SS cases were included. Since there is no rigorous definition of steady state and the balance between steady state and crisis is fragile, some children might be evaluated while they were in the peak of sub-clinical crisis and others in the resolution phase.

It has been reported that a large proportion of SCD children with painful crisis were treated at home and children seemed to tolerate higher pain intensity levels than adults before they take medications for pain (Gil *et al,* 2000). Furthermore, patients managed at home for their painful crises have shown considerable degrees of variation in their acute phase response, ranging from a minimal rise in CRP and SAA to high values similar to those in children admitted to hospital (Stuart *et al,* 1994). A further source of variation is the differences in study design and population settings. Single specimen collection may be affected by the time of sample collection, and for those admitted to the hospital would vary depending on the child's condition when admitted (i.e. early or delayed admission). CRP and SAA elevation are not specific markers and their ability to differentiate between inflammation associated with SCD and other factors (for example concomitant infection) may be complicated, particularly in a low resource setting as in the Yemen, where malaria and other infections are prevalent. In a study from

Tanzania the median CRP concentration was twice that of the Western European children in one quarter of apparently healthy children, reflecting their higher morbidity burden (Hurt *et al,* 1994).

Accumulating evidence suggests that inflammation plays a critical role in the pathophysiology of SCD, and affected individuals may experience chronic low grade inflammation. The contribution of the inflammatory process to vascular injury and the development of chronic organ dysfunction, a common SCD complication, require more detailed longitudinal studies. These cohort studies are necessary in order to further identify the regulation of inflammatory and anti-inflammatory factors in relation to clinical severity as well as in relation to prognosis of SCD. Such studies might provide a basis for therapeutic use of anti-inflammatory agents in these children. The interaction and modification of inflammatory responses with the child's nutritional status should also be studied.

8.6 CONCLUSIONS

CRP and SAA, the two major positive and rapid responding reactants, were elevated well above the reference values for the majority of SCD children in steady state, and to greater extent in those with crises or acute disease complications. Hypoalbuminaemia was present in approximately 20% and mean plasma albumin concentration was marginally lower in cases with acute disease complications. The study was unable to demonstrate associations between CRP or SAA with the Severity Index or with individual clinical variables.

CHAPTER NINE

GROWTH ASSESSMENT

9.1 INTRODUCTION

Physical growth is known to be impaired in SCD and affected children are usually lighter and shorter than their healthy counterparts. The prevalence of moderate underweight American children with SCD was 41%, and 25% for severe undernutrition (Warrier *et al,* 1994), with a prevalence of wasting of 11% (Henderson *et al,* 1994). In Ghanaian children and adolescents with SCD stunting was reported in 44% and almost all were underweight, irrespective of height (Konotey-Ahulu 1996). Causes of growth retardation in SCD are complex and multiple factors are likely to contribute, such as the haematological and cardiovascular state, social factors, endocrine function, metabolic and nutritional status (Serjeant 2001). The growth rate in these children is inversely related to the degree of anaemia and is likely to be associated with deficiency of specific nutrients as well as low dietary intakes of essential nutrients, decreased absorption and increased losses or utilisation (Enwonwu 1988, Wethers 1989).

Although growth failure and under-nutrition are common, the underlying mechanisms have not been well studied. The precise role of intrinsic or extrinsic factors is unclear in relation to inadequate food intake or increased demands associated with higher energy expenditure and requirements. External and internal factors are likely to be acting together to a different degree against a variable genetic, environmental and socio-economic background. Inadequate intake can result from anorexia and reduction in energy intake during acute disease complications and hospitalization, and subsequently remain low for weeks following discharge (Malinauskas *et al,* 2000, Fung *et al,* 2001b).

Increased resting energy expenditure (REE) was reported in patients with SCD and contributing factors may include increased protein turnover, hypererythropoiesis, cardiac work overload and chronic low grade inflammation (Salman *et al,* 1996, Barden *et al,* 2000, Hibbert *et al,* 2005, Akohoue *et al,* 2007).

Growth impairment is also related to endocrine dysfunction and micronutrient deficiencies. A detailed review summarising the evidence related to poor growth and under-nutrition in children with SCD with regard to anthropometric status, disease severity, body composition and metabolism, micronutrient deficiency and endocrine dysfunction was completed in preparation for this research (Appendix G).

Growth monitoring is an important tool in the assessment of health and adequate nutrition and in chronic clinical conditions growth status can be a sign of disease activity and severity and could be used as a marker of efficacy of medical and nutritional interventions (Gokhale & Kirschner 2003).

There is scarce information about growth and nutritional status from the Arabian Peninsula and from Middle Eastern countries and in the Yemen such data are not available. This analysis therefore was undertaken to describe the growth and nutritional status of children with SCD from Yemen and to assess the correlation of growth parameters with disease severity, haematological and biochemical findings.

9.2 OBJECTIVES

- To assess growth and nutritional status in comparison to international reference values.
- To compare anthropometric findings with disease specific growth data.
- To correlate growth status with disease severity, haematological and biochemical parameters.

9.3 METHODS

Procedures of anthropometric measurements are described in Chapter Three. After explanation to the guardian and assurance of the child, all measurements were taken and recorded by the researcher assisted by the attendant nurse for correct positioning of the child with length/height measurements. The parents were requested to remove their child's shoes or other footwear, with the child in light clothing. Length was measured to the nearest 0.1 cm for children less than 2 years and height (nearest 0.1 cm) for older children. Weight measurement was recorded to the nearest 0.1 kg. The MUAC was measured at the mid-point between the acromion and olecranon process, on a relaxed hanging left arm (nearest 0.1 cm). Measurements were taken in duplicate and mean values were obtained. BMI was calculated using the standard formula: weight (kg)/ height (m^2). Height for age (HAZ), weight for age (WAZ), weight-for-height (WHZ), MUAC and BMI z-scores were computed from the recently published World Health Organization (WHO) reference values (WHO Multicentre Growth Reference Study Group, 2006-2007). The SPSS syntax file (igrowup.sps) provided in the WHO website was used for this purpose. Stunting (indicating long term malnutrition and poor health), wasting (indicating recent or current weight loss) and underweight (indicating stunting and/or wasting) were defined as HAZ, WHZ, and WAZ <-2SD from the reference median, respectively. These indicators were considered as severe if the values were <-3SD. WHZ was not calculated for children above 10 years of age due to variability in this index based on pubertal status, BMI z-score was used to evaluate wasting in this age group using <-2SD and <-3SD z score cut-offs.

For anthropometric comparative assessment the International Growth Reference Values available for the World Health Organization (WHO) standards

were used. Local growth reference data for the Yemeni population were not available and the feasibility of collecting control growth data for a suitable healthy population sample was beyond the scope and budget of this study. Anthropometric data for children under 5 years was compared with that obtained from a local Yemeni population who were surveyed in 1997 (Sunil 2009), and with a Jamaican SCD specific reference values.(Thomas *et al,* 2000).

Severity assessment, haematological and biochemical analysis were completed as described in Chapter Three.

9.4 RESULTS

Anthropometric measurements of weight, height, MUAC and BMI were obtained for all 102 children. Figure 9.1 and 9.2 show weight and height plots by child age and sex.

Figure 9. 1 Weight for age plot for all children and by sex

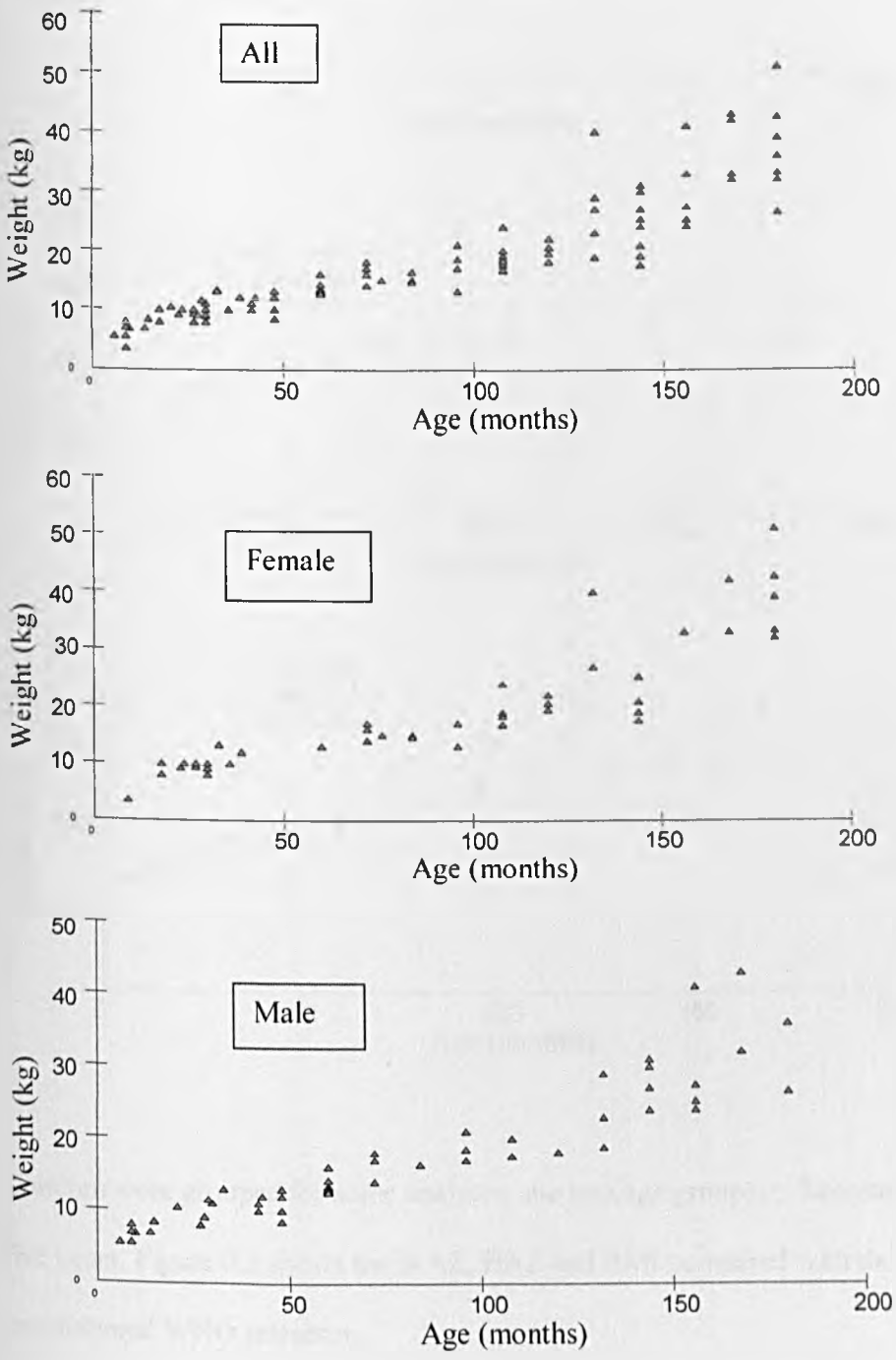
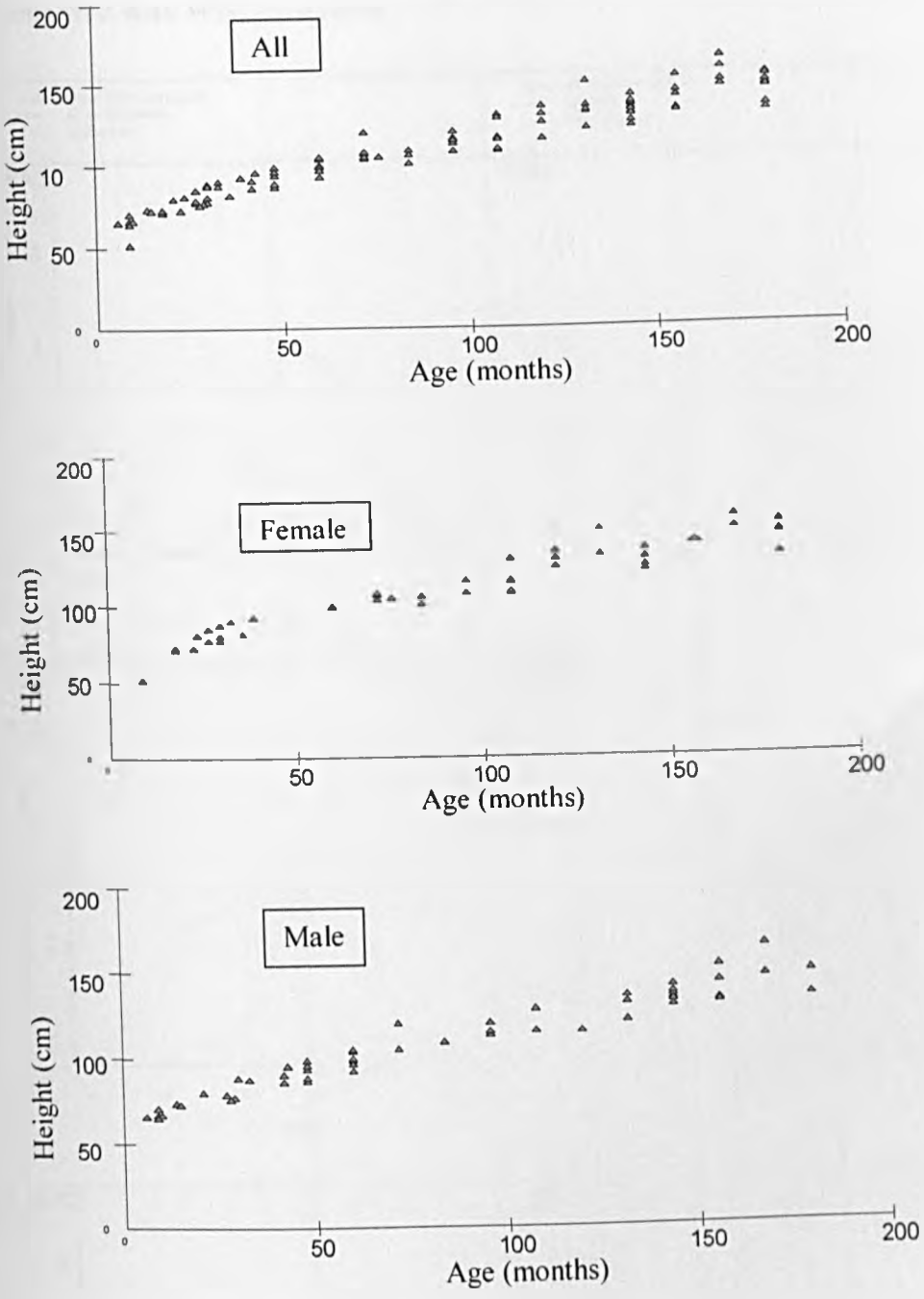
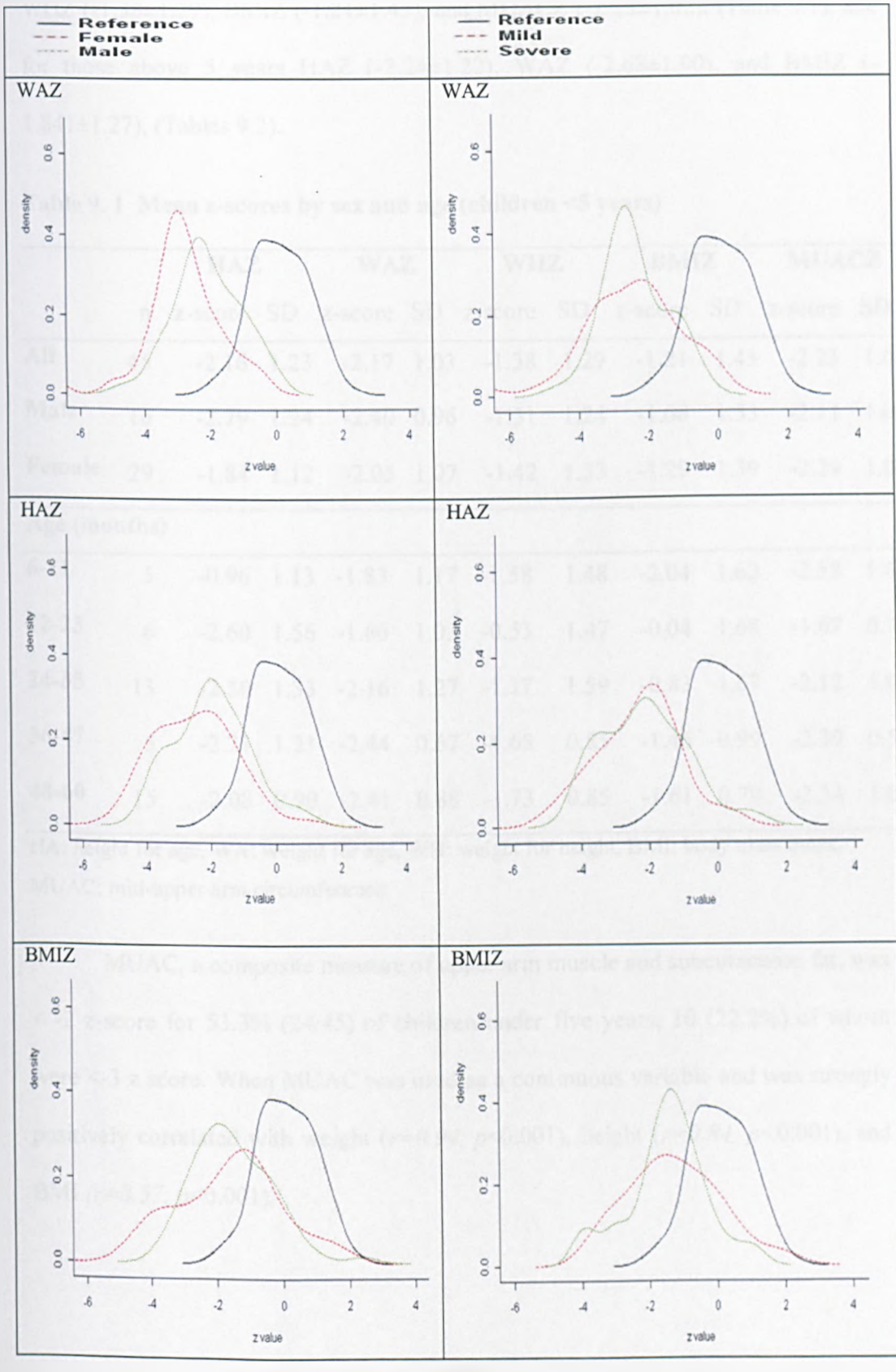


Figure 9. 2 Height for age plot for all children and by sex



Children were grouped for some analyses into two age groups, ≤ five years and > five years. Figure 9.3 shows the WAZ, HAZ and BMI compared with the international WHO reference.

Figure 9. 3 Distribution of WAZ, HAZ, and BMIZ by sex and severity compared with WHO reference



Most children were found to have z-scores less than zero (the reference population mean). For children ≤ 5 years HAZ (-2.16±1.23), WAZ (-2.17±1.03), WHZ (-1.38±1.29), BMIZ (-1.21±1.43), and MUACZ (-2.23±1.02), (Table 9.1), and for those above 5 years HAZ (-2.24±1.22), WAZ (-2.68±1.00), and BMIZ (-1.841±1.27), (Tables 9.2).

Table 9. 1 Mean z-scores by sex and age (children <5 years)

		HAZ		WAZ		WHZ		BMIZ		MUACZ	
	n	z-score	SD	z-score	SD	z-score	SD	z-score	SD	z-score	SD
All	45	-2.16	1.23	-2.17	1.03	-1.38	1.29	-1.21	1.43	-2.23	1.02
Male	16	-2.79	1.24	-2.40	0.96	-1.31	1.24	-1.08	1.53	-2.11	1.01
Female	29	-1.84	1.12	-2.05	1.07	-1.42	1.33	-1.29	1.39	-2.29	1.03
Age (months)											
6-11	5	-0.96	1.13	-1.83	1.17	-1.58	1.48	-2.04	1.62	-2.58	1.42
12-23	6	-2.60	1.56	-1.66	1.03	-0.53	1.47	-0.04	1.68	-1.67	0.79
24-35	13	-2.50	1.33	-2.16	1.27	-1.17	1.59	-0.83	1.67	-2.12	1.07
36-47	5	-2.21	1.21	-2.44	0.67	-1.68	0.85	-1.46	0.99	-2.39	0.54
48-60	15	-2.08	0.90	-2.41	0.88	-1.73	0.85	-1.61	0.79	-2.34	1.01

HA: height for age, WA: weight for age, WH: weight for height, BMI: body mass index, MUAC: mid-upper arm circumference.

MUAC, a composite measure of upper arm muscle and subcutaneous fat, was < -2 z-score for 53.3% (24/45) of children under five years, 10 (22.2%) of whom were <-3 z score. When MUAC was used as a continuous variable and was strongly positively correlated with weight ($r=0.94$, $p<0.001$), height ($r=0.84$, $p<0.001$), and BMI ($r=0.57$, $p<0.001$).

Table 9.2 Mean z-scores by sex and age (children >5 years)

		HAZ		WAZ		BMIZ	
	n	z-score	SD	z-score	SD	z-score	SD
All	57	-2.24	1.22	-2.68	1.00	-1.84	1.27
Male	30	-2.36	1.23	-2.98	0.94	-1.90	1.55
Female	27	-2.11	1.21	-2.19	0.94	-1.78	0.89
Age (years)							
6	7	-1.63	1.19	-1.98	0.87	-1.34	0.89
7	3	-3.02	0.88	-3.01	0.74	-1.27	0.47
8	5	-2.17	0.82	-2.78	1.42	-2.10	1.48
9	7	-2.53	1.43	-2.91	0.97	-1.69	0.57
10	4	-1.84	1.39	-3.10	0.56	-3.12	0.88
11	5	-1.60	1.61	-	-	-1.26	1.38
12	10	-2.64	0.72	-	-	-2.54	1.42
13	5	-2.38	1.25	-	-	-1.86	1.21
14	4	-1.01	1.20	-	-	-1.85	0.68
15	7	-3.01	1.09	-	-	-1.23	1.84

HAZ: height for age Z score, WAZ: weight for age Z score, BMIZ: body mass index Z score.

The difference in mean values between male and female children was significant for HAZ ($p=0.01$), in children under five years, but not for WAZ, WHZ, BMI and MUAC, or for all anthropometric indicators for children older than five years (all $p>0.05$). There were no differences between these two age groups for any anthropometric indicators except for BMIZ. The mean BMI z-score was significantly lower in older children (>5 years) (mean ±SD >5 yrs, -1.84±1.27 vs <5 yrs -1.21±1.43, $p=0.022$). A BMI score of <-2 z-score was found in 36 (35.3%) children,

two thirds of whom were above five years. Weight and height for age z-scores were reduced across all age groups, but the frequency of stunting and wasting was higher in children older than five years (stunting, ≤ 5 yrs, 48.9%, vs >5 yrs, 57.9%; p>0.05) wasting 26.7% vs 42.1%, p=0.025) (Table 9.3 and 9.4).

Table 9. 3 Prevalence of stunting, underweight and wasting in children < 5 years by sex and age

		Stunting		Underweight		Wasting	
	n	n (%)	CI	n (%)	CI	n (%)	CI
All	45	22 (48.9)	(34.1-65.9)	27 (60.0)	(45.8 - 76.9)	12 (26.7)	(12.6-40.7)
Male	16	10 (62.5)	(39.5-93.9)	11 (68.8)	(47.6 - 99.0)	4 (25.0)	(0.7-49.3)
Female	29	12 (41.4)	(21.7-61.0)	16 (55.2)	(35.3 - 75.0)	8 (27.6)	(9.6-45.6)
Age (months)							
6-11	5	1 (20.0)	(0.0-65.1)	2 (40.0)	(0.0-92.9)	2 (40.0)	(0.0-79.4)
12-23	6	3 (50.0)	(1.7-98.3)	3 (50.0)	(1.7-98.3)	1 (16.7)	(0.0-54.8)
24-35	13	7 (53.8)	(22.9-84.8)	8 (61.5)	(31.2-91.8)	4 (30.8)	(1.8-59.7)
36-47	5	2 (40.0)	(0.0-92.9)	3 (60.0)	(7.1-1.0)	1 (20.0)	(0.0-65.1)
48-60	15	9 (60.0)	(31.9-88.1)	11 (73.3)	(47.6-99.0)	4 (26.7)	(1.0-52.4)

CI: 95% confidence interval

Table 9.4 Prevalence of stunting and wasting in children >5 years by sex and age

		Stunted		Wasted	
	n	n (%)	95% CI	n (%)	95% CI
All	57	33 (57.9)	(44.2 - 71.6)	24 (42.1)	(28.4 - 55.8)
Male	30	19 (63.3)	(59.0 - 100)	13 (43.3)	(23.9 - 62.7)
Female	27	14 (51.9)	(31.2 - 72.6)	11 (40.7)	(20.4 - 61.1)
Age (years)					
6	7	2 (28.6)	(0.0 - 69.2)	2 (28.6)	(0.0 - 69.2)
7	3	3 (100.0)	(83.3 – 100)	0 (0.0)	(0.0 - 16.7)
8	5	2 (40.0)	(0.0 - 92.9)	2 (40.0)	(0.0 - 92.9)
9	7	5 (71.4)	(30.8 - 100)	1(14.3)	(0.0 - 47.4)
10	4	2 (50.0)	(0.0 - 100)	3 (75.0)	(20.1- 100.0)
11	5	2 (40.0)	(0.0 - 92.9)	2 (40.0)	(0.0 - 92.9)
12	10	8 (80.0)	(50.2 - 100)	6 (60.0)	(24.6 - 95.4)
13	5	3 (60.0)	(7.1 - 100)	3(60.0)	(7.1- 100.0)
14	4	0 (0.0)	(0.0 - 12.5)	2 (50.0)	(0.0 - 100.0)
15	7	6 (85.7)	(52.6 - 100)	3 (42.9)	(0.0 - 86.7)

There were no significant differences in the prevalence of moderate and severe undernutrition between male and female children (Table 9.5). Approximately 50% (28/55) of stunted, 42% (15/36) of wasted and 43% (20/47) of underweight children had severe malnutrition with z-score less than minus three.

Comparing anthropometric data in these children with SCD to a reference survey population from Yemen significant differences were observed in WA% and WH% growth indicators (Table 9.6), with increased growth deficits in affected children.

The weighted average for these differences across age strata was significant only for WH% (Mantel-Haenszel weighted OR =2.84, 95% CI limits 1.39-5.99, p=0.0028).

Table 9. 5 Sex-specific prevalence of moderate and severe growth deficits

Indicator	All		Male		Female†	
	n	(%)	n	(%)	n	(%)
WAZ* (<-2 z score) (moderate and severe underweight)	47	(66.2)	24	(33.8)	23	(32.4)
WAZ* (<-3 z score) (severe underweight)	20	(28.2)	13	(18.3)	7	(9.9)
HAZ(<-2 z score) (moderate and severe stunting)	55	(53.9)	29	(28.4)	26	(25.5)
HAZ (<-3 z score) (severe stunting)	28	(27.5)	16	(15.7)	12	(11.8)
WHZ (<-2 z score) (moderate and severe wasting)	36	(35.3)	17	(16.7)	19	(18.6)
WHZ (<-3 z score) (severe wasting)	15	(14.7)	10	(9.8)	5	(4.9)

*calculated for 71 children ≤ 10years, †sex comparison all $p>0.05$

Table 9. 6 Prevalence of undernutrition (<-2 z-score, children <5 years) compared to population survey data, Yemen 1997 (Sunil 2009)

Age	SCD	Survey	HA%		WA%		WH%	
(months)	(n)	(n)	SCD	Survey	SCD	Survey	SCD	Survey
6-11	(5)	(1040)	20.0	33.1	40.0	41.8	40.0	18.9
12-23	(6)	(1540)	50.0	60.8	50.0	54.8	16.7	19.2
24-35	(13)	(1430)	53.8	58.0	61.5	53.4	30.8	10.4
36-47	(5)	(1297)	40.0	62.2	60.0	51.0	20.0	9.0
48-60	(15)	(1270)	60.0	64.7	73.3	50.8	26.7	8.3

Weight and height measurements were also plotted on a Jamaican SCD specific growth reference which showed that 14.7% and 25.5% of Yemeni children were less than the Jamaican third percentile for weight and height respectively and 24.5% and 7.8% were above the 50th percentile (Table 9.7).

Table 9. 7 Weight and height compared to Jamaican SCD growth reference data (Thomas *et al,* 2000)

	Weight			Height		
Jamaican percentile	Female n=46	Male n=56	All n=102	Female n=46	Male n=56	All n=102
<3rd	8 (7.8)	7(6.8)	15 (14.7)	15 (14.7)	11 (10.8)	26 (25.5)
3rd - 50th	28 (27.5)	34 (33.3)	62 (60.8)	27 (26.5)	41 (40.2)	68 (66.7)
>50th	10 (9.8)	15 (14.7)	25 (24.5)	4 (3.9)	4 (3.9)	8 (7.8)

Brackets: percentage

There was no correlation between Severity Index with any of the growth indicators used in this study.

To assess the likelihood of an association between growth deficits and factors assumed to affect growth status, univariate and logistic regression analyses were used. The adjusted odds ratios were estimated with 95% confidence intervals. Variables included in the model were those with $p<0.2$ identified by univariate analysis, and potential confounders empirically considered to influence growth. These were: gender, age, age at first presentation, Hb concentration, reticulocyte count, Hb F%, serum albumin, SAA, CRP serum ferritin, sTfR, alkaline phosphatase and severity score. They were selected for regression analysis using a backward multiple logistic regression model. The dependent variables were coded 1 if the value fell below -2 z score and otherwise coded 0. This implies that if the variable receives a value of 1 then the child had growth failure. Univariate analysis showed significant

associations of WHZ with Hb F%, reticulocyte count, age at presentation, sTfR and severity score. These factors lost significance in multivariate analysis. Significant associations were the sex of the child for height/age; the child's age and alkaline phosphatase concentration for weight/age; the child's age for weight/height; and child age, and serum amyloid A for BMI, which showed an inverse association with serum albumin (Table 9.8).

Table 9. 8 Multivariate logistic regression analysis for factors associated with growth deficit †

Indicator	Variable	OR	95%CI	*P value*	AOR	95% CI	*P value*
Height/Age (HAZ)	Female	-	-	-	-	-	‡
	Male	2.32	1.04-5.19	0.04	2.939	1.19-7.23	0.02
Weight/Age (WAZ)	Age	1.02	1.01-1.04	0.11	1.031	1.01-1.04	<0.001
	ALP	0.99	0.99-1.00	0.17	0.995	0.99-1.00	0.04
Weight/Height (WHZ)	Age	1.05	1.03-1.07	0.000	1.058	1.03-1.08	<0.001
	Hb F%	0.81	0.68-0.95	0.014	1.065	0.87-1.29	0.52
	Retics %*	0.87	0.78-0.97	0.016	0.873	0.72-1.04	0.14
	Age AFP**	1.07	1.00-1.13	0.025	1.032	0.94-1.3	0.49
	sTfR	0.98	0.97-0.99	0.037	1.00	0.98-1.01	0.95
	Severity	1.16	0.99-1.34	0.052	1.059	0.80-1.40	0.69
Body mass index (BMIZ)	Albumin	0.55	0.28-1.09	0.09	0.455	0.21-0.95	0.04
	SAA	1.01	1.00-1.03	0.05	1.07	1.00-1.03	0.05
	Age	1.00	1.00-1.01	0.06	1.008	1.00-1.01	0.05

† Variables included in the model were: gender, age, age at first presentation, Hb concentration, reticulocyte count, Hb F%, serum albumin, SAA, CRP, serum ferritin, sTfR, alkaline phosphatase and severity score.
‡Reference group, * Retics: reticulocyte count%, ** Age AFP: age at first presentation, AOR: adjusted odds ratio, ALP: alkaline phosphatase, sTfR: soluble transferrin receptor, SAA: serum amyloid A.

9.5 DISCUSSION

The results of this study confirm findings from previous reports from many countries showing growth impairment of children with SCD (Phebus *et al,* 1984, Platt *et al,* 1984, Ebomoyi *et al,* 1989, Zago *et al,* 1992, Soliman *et al,* 1999, Thomas *et al,* 2000, Al-Saqladi *et al,* 2008). The growth status of these Yemeni children was comparable to some reports from developing countries (Oyedeji 1991, Athale & Chintu 1994, Jaiyesimi *et al,* 2002), but showed much greater deficits than reported for children from developed countries (Mann 1981, Caruso-Nicoletti *et al,* 1992, Henderson *et al,* 1994, Williams *et al,* 1997, Zemel *et al,* 2007, Mitchell *et al,* 2009).

Mean weight and height z-scores in almost all children were at least 1 SD below that of the WHO reference. The new international growth standards recommended by the WHO were used as local growth reference data were not available. The WHO values are based on samples taken from six selected countries from different geographical regions (Brazil, Ghana, India, Norway, Oman and the United States) and include healthy breastfed infants receiving high quality complementary diets. This is in contrast to the previous NCHS standards which were developed from North American samples of mostly artificially-fed children who were less representative for international comparisons (de Onis *et al,* 2007).

Using of international growth references are widely practiced in child growth monitoring worldwide and about 68% of countries used the National Centre for Health Statistics (NCHS)/WHO reference in 2004, while local standards were mostly used in the European countries (de Onis *et al,* 2004). The new WHO standards were developed to replace the NCHS/WHO international growth reference and have been recommended for use with individual children and in child population assessment (Yang & de Onis 2008). They have been well-received and by 2008 more than 135

countries were reported to have completed the transition or to be in the process of implementing the new standards (IASC 2008). In certain clinical circumstance, such as in SCD, in order to assess the impact of disease severity on child growth, with adequate control for the effects of socioeconomic and genetic factors, then a selection of healthy siblings living in the same environmental would be preferable for comparison. These should not replace growth reference standards such as those generated by WHO, as these international standards are intended to show the expected growth of children under the best possible conditions as every child has a right for full potentiality for optimal physical growth.

There has been a paucity of growth studies on SCD from the Arabian Peninsula and the current study is the first to report those growth data from Yemen. Early reports from Eastern Saudi Arabia, where the disease is generally mild, showed no differences in serial height and weight measurements for 14 male and 7 female children during the first 2 years of life compared with matched controls (Perrine *et al,* 1981). Although in younger children the growth deficit may be less marked as disease episodes have had limited time to affect growth. For this reason growth deficits are more likely to occur in older children. In a recent study of 97 Omani children (90 SS, 7 S β^0 thalassaemia), weight was below the NCHS 5th percentile in 68%, compared with 28% for age and sex matched controls. When these data were plotted against the Jamaican sickle cell reference values, 14% were less than the 3rd percentile, 65% were between the 3^{rd}-50^{th} percentiles, and 21% above the 50^{th} percentile (Jaiyesimi *et al,* 2002). These results were comparable to the Yemeni sample with corresponding estimates of 14.7%, 60.8% and 24.5% respectively. For height about a quarter of Yemeni children were below the third percentile and only 7.8% above the 50^{th} percentile. The Omani study did not report height data and this

is the first study to compare growth in Arabian children with SCD with the well established Jamaican reference data.

In this sample the mean z- scores for HAZ (-2.2±1.23), WAZ (-2.4±1.02), and WHZ (-1.38±1.3), were lower than those reported for 73 SS Brazilian children (<8 years) using NCHS references, (HAZ-0.64±0.94, WAZ -0.82±1.08, WHZ-0.49±1) (Silva & Viana 2002). A longitudinal study of 148 American children (birth-18 years) evaluated annually for 4 years, reported growth failure (defined as $\leq 5^{th}$ percentile) in 26% (weight), 22% (height) and 24% (BMI) (Zemel *et al,* 2007), which compared to 8.2%, 9.6% and 1.4% for the Brazilian sample (Silva & Viana 2002). The higher prevalence of stunting (54%), wasting (35%) and underweight (47%) in this study was most likely explained by the high background prevalence of childhood malnutrition in the general population of Yemen as reported in the most recent National Survey (Sunil 2009). In this survey for children less than five years, the prevalence of stunting, wasting and underweight in the coastal region (the area from where SCD sample was obtained) were 42%, 20.1% and 46% respectively, which are less than the corresponding percentage deficits in the study children with SCD. The greater weight deficit is consistent with the high prevalence of wasting in these children which corresponds with the general description of sickle cell patients as having a thin phenotype. MUAC was strongly correlated with other anthropometric indices but most prominently with weight (r=0.94), which is consistent with its measurement of muscle and fat mass. Previous studies of body composition and REE in patients with SCD have reported marked reduction of body fat and fat-free mass indicating a global deficit of energy and protein stores associated with elevated protein turnover and higher energy consumption (Singhal *et al,* 1993b, Borel *et al,* 1998, Barden *et al,* 2002). These alterations suggest a state of

metabolic imbalance between demands and expenditure which in the long-term leads to shortage of substrate and eventually growth impairment (Singhal *et al,* 1997). The deficit in BMI of <- 2 z-score occurred in 35% of children, but it was greater in older children than younger children (42.1% vs 26.7%) indicating the cumulative long-term deficit.

Children with SCD have been reported to have normal birthweight and length with growth restriction usually commencing between 6 months and 2 years of age (Kramer *et al,* 1980, Stevens *et al,* 1986, Thomas *et al,* 2000). However, analysis of a contemporary sample from USA has shown that with high food consumption and presumable optimal health care some individuals may become overweight during adolescence, especially females, although most of those children who were underweight in childhood remained underweight throughout adolescence (Mitchell *et al,* 2009).

Delayed sexual maturation and pubertal development is frequently associated with growth restriction in SCD, and in females, weight was found to be the dominant determinant factor for age at menarche (Platt *et al,* 1984, Serjeant *et al,* 2001b). Although, sexual maturity staging was not assessed in the present study, among the 12 females aged 12-15 years, four had commenced menarche at age 13-14 years, and of five who were 15 years, two had still not experienced menarche. Catch up in height may occur during puberty especially in females (Zemel *et al,* 2007).

Inadequate food intake can result from anorexia which occurs prior to and at the time of crises or hospitalization and may continue for many weeks after discharge (Malinauskas *et al,* 2000, Fung *et al,* 2001b). Anorexia and decreased food intake could relate to the effects of cytokines acting directly on the brain, modulating gastrointestinal activity and stimulating production of hormones such as leptin and

cholecystokinin which modulate feeding behaviour (Buchanan & Johnson 2007). It is well recognized that SCD is a chronic inflammatory condition and associated with excessive production of inflammatory cytokines (Makis *et al,* 2000a, Brittain & Parise 2007). In the present study the inflammatory markers CRP and SAA were elevated in all children in steady state and to a greater event during acute illness and in the regression analysis SAA was significantly association with the BMI growth deficit. Vaso-occlusive crises and episodes of infection could increase energy expenditure (Singhal *et al,* 1993c). A strong association between CRP and REE has been described, and using stepwise regression analysis, CRP was an important predictor of increased energy expenditure which might indicate a link between inflammation and a hyper-metabolic state in SCD (Hibbert *et al,* 2005). Increased REE might be related to erythroid hyperactivity and accelerated red cell turnover owing to the short life span of sickled red blood cells. Low Hb levels and chronic anaemia are associated with a hyperdynamic circulation and deterioration of cardiopulmonary function. This increases workload and consequently the demand for calories and nutrients. In the univariate analysis wasting was associated with reticulocyte count, sTfR and severity score which may indicate a relationship between growth failure, disease severity and hypererythropoiesis.

Therapeutic measures to reduce disease severity or its complications might lead to improved nutritional status and growth. Regular transfusion over a 2-year period in the STOP trial lead to significant improvement in height, weight and BMI, with growth *z*-scores approaching normal (Wang *et al,* 2005). Splenectomy in SS children resulted in a significant reduction in whole body protein turnover and acceleration of linear growth (Badaloo *et al,* 1991, Singhal *et al,* 1995). Therapy with hydroxyurea has been reported to decrease REE and improve growth (Fung *et al,*

2001a, Hankins *et al,* 2008). In the present study a marginally higher prevalence for growth deficit was observed in children in the severe category, but there were no significant correlations between SI score and anthropometric parameters, which is consistent with other reports (Pellegrini-Braga *et al,* 1995, Singhal *et al,* 1996, Zemel *et al,* 2007). It is possible that the sample size was not sufficient to detect such effects, or that the clinical events affected growth for only a short duration leading to growth faltering which might then be followed by catch-up of growth. These fluctuations could only be captured by longitudinal growth monitoring. Another possibility is that undefined factors impacting growth parameters are not included in the severity scoring.

There was no association between growth and anaemia severity, which was similar to a Jamaican study which reported no significant relationship between haemoglobin concentration, reticulocyte count or irreversibly sickled cells (ISCs) and anthropometric measurements. In that study correlation with disease severity, (measured by the number of hospital admissions), showed no significant association with growth parameters, although a trend towards lower mean weight was observed in patients who were admitted more often (Lowry *et al,* 1977). In pre-pubertal Jamaican children, levels of Hb and Hb F decreased with increasing frequency of hospitalisation, although levels were positively associated with height and weight only in males (Singhal *et al,* 1996). In the present study stunting was associated with male gender, and wasting and underweight with child age. This is in contrast to an American study which found Hb and Hb F concentrations longitudinally associated with height, weight and BMI in females, but not males (Zemel *et al,* 2007). The regression analysis in the Yemeni sample showed male gender to be significantly associated with stunting and wasting, whereas underweight and BMIZ were

associated with increasing child age. The reverse association of growth deficit with plasma albumin and alkaline phosphatase is of interest. Albumin can be low due to malnutrition or as part of negative acute phase response as discussed in Chapter Eight. Alkaline phosphatase is a biochemical marker of bone formation and its level may be related to growth impairment or associated with zinc deficiency (an important element for the enzyme function) and frequently reported in patients with SCD (Fung *et al,* 2008). However, measurement of the bone specific isoform is better than the total enzyme assay. Zinc deficiency was not assessed in this sample.

Serial measurements with longitudinal follow up would be preferable to these cross-sectional assessments and allow measurement of growth rates which would be a better indicator of functional growth, and would identify more clearly intermittent periods of growth failure or catch-up growth. Pubertal staging and sexual maturity status were not assessed, and self-assessment of sexual maturity could have been requested, although the present sample was too small to assess the growth effects of delayed puberty related to disease activity.

9.6 CONCLUSIONS

Growth and nutritional status are severely impaired in children with SCD from Yemen. There was a high prevalence of stunting, wasting and underweight. Growth deficits were associated with age, male gender and inflammatory markers (SAA). The analysis was unable to identify an association between anaemia severity with growth deficit.

Growth monitoring with appropriate nutritional support should be considered as part of the comprehensive care package of children with SCD and should be promoted more widely. There has been limited evaluation of potential nutritional interventions or supplementation aimed at improving the nutritional status and growth of these children. Randomised trials of nutritional interventions in infancy and early childhood are required and are now possible with increasing availability of neonatal screening and early identification of children with this haemoglobinopathy.

CHAPTER TEN

BETA-GLOBIN GENE CLUSTER HAPLOTYPES

10.1 INTRODUCTION

The human β-globin gene is located on the short arm of chromosome 11 in band p15.5. It is organized in a cluster spanning 70 kb from upstream 5′ toward 3′ region and includes an embryonic ε-gene, two fetal genes (Gγ and Aγ), the pseudo β-gene (ψβ) and two adult genes (δ and β) (Grosveld *et al,* 1993). The DNA sequence near to the mutation site in the β^S globin gene was found to be polymorphic and in linkage disequilibrium. Five common distinct haplotypes were identified using specific patterns of restriction endonuclease sites. Four of these are African haplotypes (Benin, Bantu, Senegal, and Cameroon) and are numbered 19, 20, 17, and 3 respectively (Ogedegbe 2007). The first three were classified according to the geographical region and ethnic group in which they originated, but have been expanded to cross this ethnic group of origin (Pagnier *et al,* 1984). The fourth haplotype was identified among the Eton ethnic group living south of Yaoundé, in Cameroon (Lapoumeroulie *et al,* 1992). The fifth, is the Arab-Indian haplotype, numbered 31, and was discovered in the Eastern Oasis of Saudi Arabia and among the tribal population of Central India (Wainscoat *et al,* 1985, Kulozik *et al,* 1986).

Analysis of the β^S polymorphisms is of genetic and anthropologic interest but may also relate to disease severity and drug responses (Nagel *et al,* 1985, Ogedegbe 2007). The Bantu haplotype has been associated with more severe disease and a high incidence of organ damage, conversely the Senegal haplotype was associated with milder disease and Benin haplotype with intermediate disease severity (Powars *et al,* 1990b). The Arab-Indian haplotype has been associated with milder disease, although painful crises, osteomyelitis and avasular necrosis of the femoral head

remain common in patients with this haplotype (Padmos *et al,* 1991). Modified phenotypic expression in the Arab-Indian and Senegal haplotypes has been attributed to their higher Hb F levels. This is associated with the C→T mutation at position –158 XmnI in the Gγ-globin gene promoter which appears to influence Gγ expression and Hb F production (Labie *et al,* 1985, Miller *et al,* 1987). In the presence of this mutation and chronic haemolytic stress the ratio between Gγ:Aγ in SS patients is maintained at the newborn levels of 70:30 (or 60:40) instead of a reversed ratio of 40:60 as occurs in normal individuals by 3-4 months of age. This might be implicated in the high Gγ gene expression and explain the higher Hb F expression as a delay in haemoglobin switch in sickle cell patients (Nagel 1991).

The regulation of the β-globin gene cluster is complex. It is mediated in part by DNA sequences of regulatory elements, the locus control region (LCR), which is located 6 to 20 kb upstream of the β globin gene and defined by five major DNase I hypersensitive sites (HS-I to HS-V) (Stamatoyannopoulos 2005). Mutations in the LCR sequences would have important effects on β-globin gene expression and consequently on the amount of Hb produced.

Several factors have been assumed to affect Hb F levels, including age, sex, α-globin gene number, haplotype polymorphism and the X-linked F-cell production (FCP) locus (Chang *et al,* 1995). Approximately 50% of Hb F variation is unexplained, although β^S haplotypes are considered the second most important factor after the FCP locus, and accounting for about 14% of this variation (Chang *et al,* 1997). The exact mechanism and extent of haplotype effects on Hb F levels, haematological characteristics, and clinical course of SCD are not fully elucidated.

The clinical manifestations of the SCD in the Arabian Peninsula can be grouped into two distinct forms, a usually mild disease occurring in Eastern area

populations and a severe disease phenotype in Western areas. This clinical variation coincides with the distribution of β^S cluster haplotypes, as the Arab-Indian haplotype is the major genotype in Eastern Saudi (El-Hazmi 1990), Kuwait (Adekile 2001) and Bahrain (Al-Arrayed *et al,* 2003), whereas the Benin haplotype is predominant in the North and South Western provinces of Saudi Arabia. In Oman and the United Arab Emirates the Benin, Bantu and Arab-India haplotypes co-exist, although the later comprises more than 50% of cases (El-Kalla & Baysal 1998, Daar *et al,* 2000).

In Yemen, neither the frequency nor the origin of β^S haplotypes have been investigated. This study was undertaken to analyse for the first time the background of β^S globin gene cluster haplotypes in Yemeni children with sickle cell disease, and to assess their association with haematological and clinical profiles.

10.2 OBJECTIVES

The objectives of this analysis were to:

- Determine the type and frequency of β^S cluster haplotypes.
- Assess the association of β^S haplotypes with disease severity.
- Assess the association of β^S haplotypes with haematological profiles.

10.3 METHODS

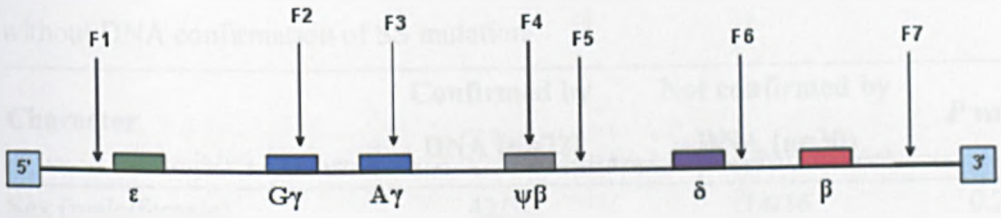
The SS genotype in these 72 children was confirmed by direct sequencing of the beta-globin gene exon1 region in the laboratory of Human and Clinical Genetics in Leiden, the Netherland according to standard procedures using the ABI PRISM™ 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM® Big Dye Terminators v2.0 Cycle Sequencing Kit according to the instructions of the manufacturer.

Haplotype determination

Haplotype analysis of the beta-globin gene cluster has been performed according to the strategy described previously (Sutton *et al,* 1989, Varawalla *et al,* 1992). PCR amplified regions are subjected to restriction enzyme analysis to study the absence or presence of restriction recognition sites for HincII, HindIII and HinfI as shown in figure 1. However, instead of restriction enzyme analysis, a high resolution melting curve analysis (HRMCA) was used to determine the beta-globin gene cluster haplotype. It is a closed-tube method that was developed to detect sequence variations such as SNPs, small insertions and deletions. PCR products are melted in a specialized instrument which monitors fluorescence. A fluorescent dye that emits light in the presence of double stranded DNA (LC Green plus) is added to the reaction and as the temperature increases, the fluorescence decreases; this produces a characteristic melting curve (Wittwer *et al,* 2003, Liew *et al,* 2004, Zhou *et al,* 2004). Since the base sequence of the PCR product determines its melting behaviour, the melting curve will change if the sequence is altered. MCA is able to identify these differences (Montgomery *et al,* 2007). It is possible to analyze the melting behaviour of the whole PCR product in order to scan for mutations or to genotype known sequence variations by adding an unlabeled oligonucleotide probe.

This probe, which functions optimally when the length is below 200 nucleotides, increases specificity of the melting reaction as it decreases the size of the product that is melted. The PCR is performed asymmetric so that the strand to which the probe anneals is produced in excess. The probe is designed to anneal to either the wild type or the mutant allele and is blocked at its 3'-end with a phosphate group to prevent probe extension during PCR; the characteristic melting curve identifies the genotype of each sample (Zhou *et al,* 2004, Montgomery *et al,* 2007).

Figure10. 1: Schematic representation of the β-globin gene cluster



The arrows indicate the locations of the 7 different fragments that contain the 7 polymorphic restriction enzyme sites for HincII (F1, F4 and F5), HindIII (F2 and F3) and HinfI (F6 and F7). The F1 to F7 regions were amplified and investigated by MCA for the presence or absence of the 10 SNP's which alter the restriction enzyme recognition site.

10.4 RESULTS

Results were obtained for 72 SS children including 44 (61%) males. Mean age (±SD) was 6.8±4.4 years. Two thirds (66.7%) were in steady state at the time of blood collection. They were moderately anaemic with a mean Hb (± SD) of 7.7±1.4 g/dl. The level of Hb F (%) varied widely, with a median [IQR] of 3.0 [1.8-4.9].

There were no differences in demographic or basic laboratory characteristics of children with DNA confirmed to be homozygous for SS condition and those without DNA confirmation (Table 10.1).

Table 10. 1 Demographic and basic laboratory characteristics of children with and without DNA confirmation of SS mutation

Character	**Confirmed by DNA (n=72)**	**Not confirmed by DNA (n=30)**	***P value***
Sex (male/female)	42/30	14/16	0.28
Age, years	6.79 ± 4.4	8.29 ± 4.7	0.13
Steady state/ ADC	48/24	21/9	0.74
Hb, g/L	7.74 ± 1.4	7.82 ± 1.7	0.79
WBC, 1000s	9.3 [6.9-13.7]	8.6 [4.9-11.8]	0.07
Reticulocyte, %	3.9 [1.8-7.0]	3.0 [1.5-5.4]	0.16
Hb F, %	3.2 [1.8-5.4]	3.3 [1.9-4.9]	0.70
Hb A2, %	2.9 ± 0.35	2.9 ± 3.5	0.70

Values: mean ±SD or median [interquartile range]; ACD: acute disease complication

The β- gene cluster haplotypes of 144 β^S chromosomes came from 72 children. All cases were homozygous SS confirmed by DNA sequencing. Haplotyping was determined according to the presence (+) or absence (-) of the seven SNPs corresponding to the polymorphic restriction endonuclease sites (Figure

1) in order to be able to compare these data with the literature. Because no siblings were available the phase of the SNP haplotypes could only be determined with certainty in the homozygotes for the same haplotype, as was the case for the majority of subjects studied carrying the Benin haplotype. Also unknown haplotype C was deduced from 2 individuals homozygous for all SNP's. The other haplotypes were deduced by looking at the minimal difference from the most abundant Benin haplotype. The atypical Benin-like haplotype would appear as Benin when investigated according to the traditional restriction enzyme analysis method but melting curve analysis revealed two sequence differences in fragment F2 carrying a G instead of a T in the unknown Benin-like haplotype at positions 5231296 and 5231293 (numbering according to HBB region reference sequence in UCSC Genome Browser on Human March 2006 Assembly) affecting the same recognition site for HindIII. The β-globin cluster haplotypes identified are shown in table 10.2. The Benin haplotype (- - - - + - +) had a frequency of 82.6%, the Bantu (- + - - - - +) of 5.6% and the atypical haplotypes 11.8%. Atypical haplotypes were composed of atypical C (+ - - - - - +) 6.9%, atypical D (+ - - - - + +) 3.5%, atypical E (+ - - - + - +) 0.75% and atypical Benin-like (- - - - + - +) 0.75%. No Senegal, Cameroon or Arab-Indian haplotypes were identified. Forty nine children were homozygous for the Benin haplotype (68%) which was heterozygous with the Bantu haplotype in 8 children (11%) and with atypical haplotypes in 13 children (18%). Two children (3%) were homozygous for the atypical haplotype C, although no child had combinations of atypical haplotypes.

Table 10. 2 Frequency of β^S haplotypes in Yemeni children

Haplotype	5'ε *Hind* II	Gγ *Hind* III	Aγ *Hind* III	3'ψβ *Hind* II	5'β *Hind* II	3'β *Hinf* I	3'β *Hinf* I	Number of chromosomes	%
Benin	-	-	-	-	+	-	+	119	82.6
Bantu	-	+	-	-	-	-	+	8	5.6
Atypical C	+	-	-	-	-	-	+	10	6.9
Atypical D	+	-	-	-	-	+	+	5	3.5
Atypical E	+	-	-	-	+	-	+	1	0.75
Benin-like	-	-	-	-	+	-	+	1	0.75
Atypical sub-total								17	11.8
All								144	100

Haematological data for Benin, Bantu and atypical haplotypes are summarized in Table 10.3.

Table 10. 3 Haematological data in Benin, Bantu and atypical haplotypes

	Benin (n=50)	**Bantu (n=8)**	**Atypical (n=17)**	***P value*‡**
Hb gm/dl*	7.6 ± 1.5	7.9 ± 0.6	7.9 ± 1.4	0.70
Reticulocytes %†	4.5 [2.3-9.0]	2.9 [1.1-5.3]	3.0 [1.5-6.1]	0.16
Hb F %†	3.0 [1.7-5.0]	3.3 [0.5-6.2]	4.5 [2.8-5.4]	0.42
HbA2%*	3.0 ± 0.34	3.0 ± 0.27	3.0 ± 0.37	0.86

*mean± SD, † median [interquartile range], ‡ analysis of variance

There were no significant differences in haematological parameters between the three haplotypes. Based on the Severity Index (SI) 47 (65.3%) children had severe disease and 25 (34.7%) mild disease, and the haplotype distribution for these cases is shown Table 10.4 in relation to disease severity.

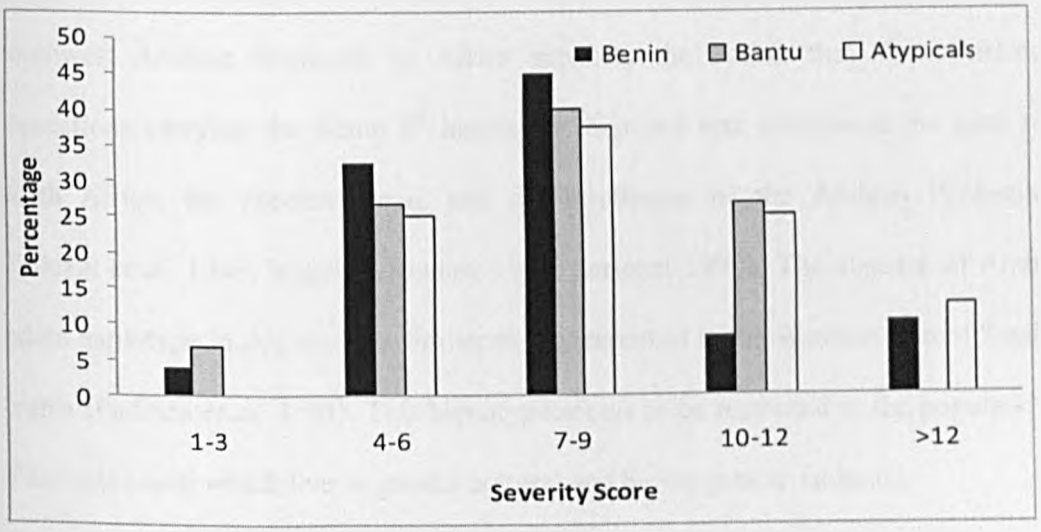
Table 10. 4 Haplotypes distribution by disease severity

Severity category	Benin n (%)	Bantu n (%)	Atypical n (%)	All n (%)
Mild	18 (36.7)	2 (25)	5 (33.3)	25 (34.7) *
Severe	31 (63.3)	6 (75)	10 (66.7)	47 (65.3)
All	49 (68.1)	8 (11.1)	15 (20.8)	72 (100)

*$p = 0.8$

There were 31 (63.3%) children with the Benin haplotype, 6 (75%) with Bantu and 10 (66.7%) with atypical haplotypes in the severe category, compared to 18 (36.7%), 2 (25%) and 5 (33.3%) children respectively in the mild category. The haplotype distribution did not differ between mild and severe disease categories (p=0.8). The mean (±SD) Severity Indices for Benin, atypical and Bantu haplotypes were 7.5 ± 3.3, 7.7±2.3, and 9.3±3.4 respectively (p=0.36). The distributions of severity scores for the different haplotypes are illustrated in figure 10.2.

Figure 10. 2 Haplotypes by severity score



10.5 DISCUSSION

This study showed that Benin was the predominant haplotype in Yemen, which is in agreement with the distribution of this haplotype for contiguous areas in the South Western region of Saudi Arabia and Mediterranean Basin (Table 10.5).

Haplotype analysis is a useful tool for reconstruction of mutation history, as mutations tend to pass to subsequent generations with the same haplotype of origin (Flint *et al,* 1998). The association of specific haplotypes with a geographically distinct population has provided critical information on whether the β^S mutation arose indigenously or spread by gene flow. The high frequency of the Benin haplotype in this study suggests that the β^S mutation originated from the Benin region and was introduced to Yemen by gene flow during the trans-African slave trading and by migration including some individuals from Central Africa origin, but none from the distant Senegal type, and that no migration has apparently come from East Arabia or Asia. It is also selectively maintained by malaria which is transmitted in most of the coastal plain and valleys of Yemen. The geographical proximity of the Southwest Arabian Peninsula to Africa supports the notion that West African populations carrying the Benin β^S haplotype migrated and introduced the gene to North Africa, the Mediterranean, and the Southwest of the Arabian Peninsula (Kulozik *et al,* 1986, Nagel & Ranney 1990, Serjeant 1990). The absence of Arab-Indian haplotype in this study is similar to that reported in the Western area of Saudi Arabia (Padmos *et al,* 1991). This haplotype seems to be restricted to the population of Eastern Saudi which live in greater cultural and hence genetic isolation.

The occurrence of atypical haplotypes in 11.8% of chromosomes in this study is similar to the 11.9% frequency reported from Venezuela (Vivenes De Lugo *et al,* 2003), but less than the 14.4% reported from Iran (Rahimi *et al,* 2006).

Table 10. 5 Distribution of β^S Benin haplotype in the Western Arabian Peninsula and Mediterranean

Country	Number of chromosomes	Benin haplotype (%)	Other haplotypes* (%)	Atypical haplotypes (%)	Reference
Yemen	144	82.6	5.6	11.8	This study
Western Saudi	142	68.3	31.7	-	(El-Hazmi 1990)
Jordan	20	80.0	20.0	-	(El-Hazmi *et al*, 1999)
Lebanon	100	73.0	27.0	0	(Inati *et al*, 2003)
Syria	18	66.7	33.3	-	(El-Hazmi *et al*, 1999)
Palestine	118	88.1	5.9	5.9	(Samarah *et al*, 2009)
Turkey	136	91.9	0.7	7.4	(Aluoch *et al*, 1986)
Egypt	28	100	0	0	(El-Hazmi *et al*, 1999)
Tunisia	66	94.0	0	6.0	(Abbes *et al*, 1991)
Algeria	20	100	0	0	(Pagnier *et al*, 1984)
Greece	82	96.4	0	3.6	(Boussiou *et al*, 1991)
Sicily	64	100	0	0	(Schiliro *et al*, 1992)

*Including: Bantu, Senegal, Cameroon and Arab-Indian haplotypes. (-) Data not provided.

β^S atypical haplotypes mostly occur between the range of 5–10% of chromosomes, with over 20 atypical haplotypes so far identified (Steinberg *et al,* 1998, Romana *et al,* 2000, Zago *et al,* 2000). They occur in variable frequency in different populations. Atypical C (+ - - - - - +), is frequent in Saudi Arabia (9.7%) (Flint *et al,* 1998)., while atypical E (+ - - - + - +) and Benin-like (- - - - + - +) are both rare. The unknown Benin-like is probably not derived from Benin, because the base pair differences effect the same Benin restriction enzyme recognition sites and according to traditional method of haplotype analysis, this is indistinguishable from the regular Benin haplotype.

Atypical haplotypes have been commonly associated with Senegal or Bantu haplotypes and less frequently with Benin, and they were suggested to represent a variation of the Bantu haplotype (Srinivas *et al,* 1988). But recent evidence showed that a mechanism of point mutation and cross-over recombination can occur in the Benin β^S haplotype. Diverse genetic mechanisms have been implicated such as punctual substitutions, gene conversions and simple or double cross-over between two typical β^S haplotypes, or more frequently between a typical β^S haplotype and a different β^A associated haplotype (Romana *et al,* 2000, Zago *et al,* 2000). Heterogeneity of the β^S chromosomes bearing the Benin haplotype at the molecular level has been demonstrated in the Mediterranean populations, where this haplotype is predominant (Patrinos *et al,* 2005).

In central Africa populations where the Benin haplotype occurs in almost 100% of β^S genes and in 60% of β^A genes recombination events would be expected to produce typical rather than atypical haplotypes. In a situation where the typical β^A haplotype of a normal population was uncommon, then the atypical β^S haplotype would be expected to be frequent (Steinberg *et al,* 1998). It is probable that

recombination with the Benin type from β^A chromosomes does not occur, due to their rarity and this is consistent with these results from the Yemen showing high atypical haplotypes in a non-African population. Benin haplotype recombination would occur when the Benin is the predominant single haplotype independent of β^S and β^A haplotype diversity (Zago *et al,* 2001). However, the frequency data for the haplotype patterns in the β^A gene for the general population in Yemen is not available, and the high frequency of the atypical β^S haplotype in this study suggests that this haplotype is less frequent in the normal population. Further research is required to confirm this hypothesis.

The regulatory elements in LCR play an important role in globin gene expression by acting as major enhancers and activators of downstream globin genes (Stamatoyannopoulos 2005). LCR is also involved in developmental control of globin genes, by interaction with γ globin during fetal life and β gene in adults resulting in restriction of γ- globin expression. Mutation in these sequences could affect γ-globin gene expression and Hb F production. In the Benin haplotype, which is known to be associated with low production of Hb F, a mutation in the LCR region resulting from cross-over with the Senegal haplotype in the HS-2 site, lead to high Hb F levels and high Gγ values in affected individuals (Adekile *et al,* 1992, Oner *et al,* 1992). In Yemeni sickle cell patients the absence of Arab-Indian and Senegal haplotypes corresponded with low Hb F levels and very low prevalence of the -158 (C-T) Gγ-Globin Gene XmnI Polymorphism, the two characteristic findings related to these haplotypes (El-Hazmi & Warsy 2000, Al-Nood *et al,* 2004). The predominance of the Benin haplotype, which is known to be associated with low Hb F concentration, could explain the lack of a difference in Hb F levels between the typical and atypical haplotypes, which is consistent with previous reports (Steinberg

et al, 1998). Other factors which contribute to Hb F variation such as X-linked F-cell production, α-globin gene number, gender and age (Chang *et al,* 1995), require further investigation in this population.

Establishing the link between β^S haplotypes and phenotypic expression is important because haplotype characteristics have been associated with different degrees of disease severity. Due to the predominance of the Benin haplotype in this sample, there was insufficient variation to allow adequate comparison of haplotype association with disease severity, and comparison of SI scores for the different haplotypes showed no significant differences. The assumption could be made that as SCD in Yemeni children is associated with the Benin haplotype and is of African origin then disease characteristics would be similar to Africans. Yet, the presence of the mild clinical form in about a third of cases, and a high frequency of splenomegaly, low rate of skin ulcerations and high variability of Hb F suggests that β^S haplotype and Hb F are not the only disease modulators in this community and other genetic and environmental determinants are likely to exist, a view supported by other investigators (Acquaye *et al,* 1985, El-Hazmi 1990, Inati *et al,* 2003). However, the high frequency of α-thalassaemia reported in Yemeni SCD patients (El-Hazmi & Warsy 1999a) could be influential, although the clinical effects of this remain to be evaluated.

Extended analysis of the β^S globin gene cluster showed it as a dynamic region and that recombination in atypical haplotypes occurred in typical β^S haplotypes (Zago *et al,* 2001). The use of new biotechnology demonstrated that high-density SNP mapping across the β-locus, assisted by Haploview analysis, could define β^S haplotypes more accurately than traditional RFLP analysis alone (Liu *et al,* 2009).

This approach may help to elucidate other genetic determinants modulating clinical diversity in SCD.

10.6 CONCLUSIONS

β^S cluster haplotypes were determined for the first time in Yemeni SCD children. The most predominant is the Benin haplotype, which is prevalent in the different Mediterranean countries, followed by Bantu and atypical haplotypes C, D and E. This finding is consistent with the notion that the β^S mutation originated from the Benin region and was introduced to Yemen by gene flow. The higher rate of atypical haplotypes is an interesting finding, which could be related to the low frequency of Benin haplotype in the general population. The spectrum of clinical manifestation and variable severity in this sample could not be explained by association with β^S polymorphism. Other determinants must exist and further research will be required to identify them.

CHAPTER ELEVEN

GENERAL DISCUSSION

11.1 INTRODUCTION

This chapter summarises the main findings related to SCD in children from Yemen, who were attending the Out-Patients or admitted to Al-Wahda General Teaching Hospital in Aden. The clinical characteristics and complications in these children are compared to studies from neighbouring and other countries. Disease severity assessed by a Severity Index classifies two groups of patients, with mild or severe disease and differences between these two clinical categories are discussed. Growth status was assessed and values compared to normal and disease specific references. Haematological and biochemical characteristics were assessed in relation to genetic polymorphisms in the folate metabolic pathway and in β^S globin gene cluster haplotype. The significance of these findings and practical implications are considered and appropriate recommendations emphasized. The study limitations are highlighted and gaps in knowledge are discussed with future research implications.

11.2 CLINCAL ASPECTS

11.2.1 Age and presenting symptoms

The present study described for the first time the clinical pattern of SCD among Yemeni children. Although, this was a hospital-based study more than two thirds of children were symptom free and considered in a "steady state" at the time of inclusion and 17% reported no previous hospital admission.

The finding that two thirds of children were symptomatic by the time of their first birthday probably reflects the severity of SCD in this population. This pattern was comparable to that reported in Western and South-Western Saudi Arabia, where

the disease is characteristically severe (Mulik *et al,* 1991, Padmos *et al,* 1991, El-Hazmi 1992c).

11.2.2 Clinical features and complications

Painful crisis was the most common symptom and a leading cause of hospitalization. A small proportion of children (10%) had no history of painful crisis whereas 25% reported 3 or more painful episodes per year, but they accounted for 67.7% of all episodes. Dactylitis (hand-foot syndrome) which is often the first presenting symptom lead to a diagnosis of SCD in more than half of these children. The pattern of dactylitis was comparable to that reported from Western Saudi Arabia, India and Jamaica (Stevens *et al,* 1981, Kar *et al,* 1986, Mulik *et al,* 1991). Frequency of dactylitis may be an indicator of disease severity as it is rarely observed in patients from Eastern Saudi Arabia where SCD is generally of a milder variant (Padmos *et al,* 1991). Infants who have dactylitis by the first year of life are more likely to develop an adverse outcome at a later age (Miller *et al,* 2000, Powars *et al,* 2005, Foucan *et al,* 2006).

The prevalence of splenomegaly (40%) was similar to that reported from Saudi Arabia (El-Hazmi 1992a). Spleen enlargement decreases significantly with age and correlates with degree of anaemia and reticulocyte count. An enlarged spleen may have reduced function "functional asplenia" (Pearson *et al,* 1969), and the earlier the splenomegaly the earlier the dysfunction (Rogers *et al,* 1978). Splenic dysfunction is considered the most important predisposing factor for severe and recurrent bacterial infection, especially pneumococcal infection (Rogers *et al,* 1978, William & Corazza 2007). In view of this the importance of vaccination in these children should emphasized as pneumococcal vaccine is not currently included within the National Immunization Programme.

It was unexpected that sequestration crises were not encountered in this sample, although a similar finding has been reported from Nigeria (Juwah *et al,* 2004). Sequestration crisis is a common cause of death in young children and parental education on its recognition can dramatically improve early intervention leading to reduced case fatality (Emond *et al,* 1985). History of sudden death associated with pallor and collapse during normal activity in an apparently asymptomatic child raises the possibility of acute sequestration as a cause of death outside hospital. Such incidents may have occurred possibly before the diagnosis of the underlying SCD was obtained (Jenkins *et al,* 1960). Instruction of parents on spleen palpation and recognition of sudden pallor in young children should increase detection rates for sequestration episodes.

ACS is a frequently recognized complication and occurs in up to 50% of SCD patients (Leong & Stark 1998). The rate of 16.7% ACS for this sample was comparable to that of 22% reported from Oman (Jaiyesimi *et al,* 2002). The most affected age group was 2-5 years with a male predominance, which is similar to age and gender patterns in other studies (Castro *et al,* 1994a, Vichinsky *et al,* 1997, Vichinsky *et al,* 2000, Al-Dabbous 2002, Al-Hawsawi 2004, Alabdulaali 2007). It is likely that improved diagnosis using appropriate case definition and a higher suspicion index would lead to better recognition of cases with ACS.

One of the devastating complications of SCD is the development of stroke. It was observed in 4.9 % of children and during the first 10 years of age. This prevalence and affected age group are consistent with other reports and the prevalence estimate was very close to that of 4.01% reported for the Cooperative Study of Sickle Cell Disease by Ohene-Frempong et al., (1998). Overt stroke can be diagnosed clinically with no difficulty, but the diagnosis of silent infarction is more

complex and was not achievable in our situation due to lack of neuro-imaging facilities. Silent infarction in SCD is a common occurrence, affecting up to one third of children (Steen *et al,* 2003). It attracts great awareness due to its link with cognitive impairment and increased risk of overt stroke (Armstrong *et al,* 1996, Miller *et al,* 2001). Stroke prophylaxis programmes using transcranial Doppler and chronic transfusion are established in many developed countries, but in the Yemen there are insufficient resources and inadequate security of blood transfusion safety which hampers the development of this type of programme.

Sickle cell hepatic crises can be mild and self limited or severe and often fatal (Ahn *et al,* 2005). Among five children with hepatic crises, three were admitted during the study period and presented with deep jaundice, hepatomegaly, and normal or mildly elevated liver enzymes, with negative serology for viral hepatitis A and B. All children recovered slowly with supportive therapy. An acute liver insult arising from sickling phenomenon is usually self limited, whereas chronic liver damage is usually associated with viral infection (Teixeira *et al,* 2002). Incidence of viral hepatitis in SCD children is reported not to be higher than amongst the general population (Soliman *et al,* 1995), although, the prevalence of viral hepatitis in SCD patients from Yemen remains to be determined.

Gall stone frequency in SCD increases with age and availability of improved diagnostic facilities. The 5% prevalence of gall stones in this study is probably an underestimate in comparison with other reports (Al-Salem *et al,* 1996, Haider *et al,* 1998). This indicates the importance of abdominal sonography screening for biliary complications in paediatric populations.

Nocturnal enuresis was a common finding and this complication needs to be adequately addressed in children with SCD. Its causes are not fully explained, but are

likely to be multifactorial in origin (Readett *et al,* 1990a). It can be responsive to antidiuretic therapy and other conventional interventions which are used successfully in the management of enuresis in the general population (Figueroa *et al,* 1995). Few care givers use empirically supported therapy or seek professional health advice (Barakat *et al,* 2001), with many using inappropriate and even detrimental interventions such as fluid restriction (Jordan *et al,* 2005). Enuresis can be persistent into adolescence resulting in later psychological and emotional consequences (Jordan *et al,* 2005, Field *et al,* 2008). The pathogenesis of enuresis in children with SCD requires further study including evaluation of interventions aimed at improving the care of these children and assessing their effectiveness.

Few studies have reported epistaxis as a complication of SCD, although this can be serious in some patients (Konotey-Ahulu 1965, Seeler 1972, Davies *et al,* 1983). Epistaxis is not infrequent in Yemeni children, and it is well described by some authors (Kasili & Bwibo 1982, Kar & Devi 1997, Mouele *et al,* 1999). The underling mechanisms of epistaxis remains to be elucidated, but may include both local vascular and systemic haemostatic changes. A further bleeding complication observed in this study and which is infrequently reported (Kaine 1983), is the occurrence of gum bleeding. This may result from vitamin C deficiency which has been reported in SS patients (Chiu *et al,* 1990). Inadequate intakes, increased utilization or urinary excretion of ascorbate would be contributing factors (Westerman *et al,* 2000). The clinical importance of vitamin C status and micronutrient deficiencies in relation to SCD morbidity are important priorities for research as little emphasis has been given to their evaluation in previous research.

The association of SCD with protection from malaria is well established (Nagel 1990, Aidoo *et al,* 2002, Allison 2002) . Although the question arises why

should heterozygotes have some degree of protection which is not observed in homozygotes? Severe anaemia and painful crises are reported in homozygous cases following malaria infection (Fleming 1989, Juwah *et al,* 2004). Because malaria is a significant cause of morbidity and mortality in SCD, administration of appropriate antimalarial prophylaxis is required for those travelling to or living under malaria endemic conditions (Ambe *et al,* 2001, Glikman *et al,* 2007). In Yemen malaria epidemiology varies considerably between areas due to geographic, climatic and seasonal factors, with a higher malaria prevalence in the coastal plains and valleys and a lower prevalence or an absence of transmission in high altitude and desert areas (WHO 2002). In view of this variable endemicity with unstable transmission antimalarial chemoprophylaxis in children with SCD in Yemen should be investigated taking into account these epidemiological differences. Currently there are no clear guidelines or recommendations and practice is dictated by the attending medical practitioner.

Local facilities for isolation of viral and bacterial pathogens are very limited and of poor quality. Improvement in laboratory support facilities with adequate quality control procedures are important steps required in order to improve diagnosis of infectious complications. Penicillin prophylaxis and enhanced immunization of children with SCD should be emphasized, including addition of pneumococcal vaccines to the immunization schedule.

11.2.3 Consanguinity

The high consanguinity rate for first cousin marriages in patient's families reflects the frequency of related marriage in the general population of Yemen (Gunaid *et al,* 2004). A proportion of SCD cases could be attributable to consanguinity and reduction in risk is partly dependent on addressing this specific

problem (Rajab & Patton 1997). Reduction of related marriage is difficult and complex influenced by the socio-cultural acceptability for such changes. For example, in Saudi Arabia a premarital testing programme was not successful in decreasing the number of high risk marriages, with about 88-90% of known high risk couples completing their marriages despite the incompatibilities status (Alhamdan *et al,* 2007). A multilevel approach including health education, effective genetic counselling, earlier time of testing, and integration of both premarital and neonatal screening programmes is required in order to improve the rate of success in these high risk couples.

11.2.4 Mortality

The accurate estimation of the mortality rate for children with SCD requires accurate case registration through neonatal screening as well as active surveillance with accurate death registration. The low mortality observed in these Yemeni children does not reflect the overall mortality in this community. In view of late detection and inadequate death registrations the family history revealed that 18% of investigated families had experienced a SCD related death, a figure which approximates the 18-26% mortality reported for the well described Jamaican cohort (Serjeant & Serjeant 1993). Mortality risk has greatly decreased in developed countries, whereas estimates from sub-Saharan Africa suggest that as many as half of SCD children have died before five years of age (WHO 2006). This high rate is amenable to reduction by about 50% or more, and in one study from Nigeria mortality declined from 20.7% in 1988 to 0.6% in 1995. This reduction can be achieved through the implementation of comprehensive care programmes as well as improving specialist services which is attainable even in limited resource settings (Rahimy *et al,* 2003, Akinyanju *et al,* 2005).

11.2.5 Severity assessment

The analysis of severity assessment has demonstrated that about two thirds of children had experienced severe disease manifestations. This is the first time that an evaluation of disease severity has been undertaken in Yemen and verifies the diversity of presentation and the grouping of children into two distinctive clinical forms. The existence of this heterogeneity in expression in individuals living in the same area of the South-Western Governorates and broadly sharing the same environmental conditions is a stimulus for further analysis in order to elaborate the role of various genetic and environmental determinants

Comparing the severity profiles with other studies which used the same SI scores showed that Yemeni children were similar to patients from South Western Saudi Arabia (El-Hazmi *et al,* 1993), but had higher severity scores than Senegalese patients (Diop *et al,* 1999). Both mild and severe clinical forms of homozygous SCD have been reported from Saudi Arabia (El Mouzan *et al,* 1989, Padmos *et al,* 1991), and other Arabian Peninsula countries (El-Kalla & Baysal 1998, Jaiyesimi *et al,* 2002). This phenotypic heterogeneity can be explained at least in part by the differences in Hb F level and β^S globin polymorphism. The Arab-Indian haplotype is associated with high Hb F levels and milder disease, compared to African haplotypes which have lower Hb F levels and often more severe disease (El-Hazmi 1993, Daar *et al,* 2000). A further modulating factor is the co-inheritance of α- thalassaemia, which was reported to be prevalent in Yemeni SCD children (El-Hazmi & Warsy 1999a). The clinical effects and contribution of α-thalassaemia in modifying phenotypic expression in this population requires evaluation.

The findings of this study of significant differences in SI scores in relation to age and gender are of interest. Females had a significantly higher rate of painful

crisis than males, and this is consistent with previous reports of higher incidence of painful crisis in females during the first decade of life, and after fifteen years painful crises were sharply increased in males (Platt *et al,* 1991, Gill *et al,* 1995). These findings have been attributed to haematological factors (Hb F and F cells) and hormonal factors such as ovarian oestrogen with its effect on NO bioavailability (Hayes *et al,* 1985, Reiter & Gladwin 2003). The findings indicate that gender and age-adjusted analyses are preferable than data pooling.

Despite the low mean Hb F concentrations observed in this study, Hb F was still a significant determinant of severity. Small increments in Hb F levels may have an ameliorating effect on painful crises, which are a core component of the severity scoring system (Platt *et al,* 1991). Hb F level is also related to XmnI mutation with higher concentrations in the presence of the mutation (Thein & Menzel 2009). This mutation has been reported to be very low in Yemeni children with SCD and similar in frequency to those from South-Western Saudi Arabia and other Arab countries, where severe SCD has been encountered (El-Hazmi & Warsy 2000). However, the relationship between Hb F levels and disease severity is not straightforward as not all cases with high Hb F have mild disease, or conversely those with low levels not experiencing severe disease (Donaldson *et al,* 2001, El-Hazmi & Warsy 2001, Inati *et al,* 2003).

The significant difference in mean polymorphonuclear leukocyte counts between children in mild and severe categories corresponded with the importance of raised PMNs as a predictor of increased risk for early mortality, ACS and stroke (Platt *et al,* 1994, Miller *et al,* 2000), and probably with frequency of painful events (Benkerrou *et al,* 2002). The clinical benefit caused by myelosuppressive effects of

hydroxyurea has been attributed to reduction of leukocyte cells, which often occurs before Hb F levels increase (Charache *et al,* 1996).

It was of interest that a higher proportion of malaria infections were documented in children in the severe group. They may be more symptomatic, have different haematological and inflammatory responses, or their splenic and/or immune dysfunction may impair the ability to clear malaria parasites. Malaria may influence disease severity indirectly through induction of crisis or increased blood transfusion requirements or hospitalization. This finding needs more detailed investigation with preferably longitudinal studies as the association is likely to be influenced by level of malaria endemicity.

11.2.6 Growth assessment

There are few studies on growth assessment of children with SCD from the Arabian Peninsula and the current study is the first to report growth data from Yemen. The results showed that the growth of Yemeni children was comparable to reports from developing countries (Oyedeji 1991, Athale & Chintu 1994, Jaiyesimi *et al,* 2002), and much worse than that reported from developed countries (Mann 1981, Caruso-Nicoletti *et al,* 1992, Henderson *et al,* 1994, Williams *et al,* 1997, Zemel *et al,* 2007, Mitchell *et al,* 2009). Mean weight and height z-scores for these children were at least 1 SD below the WHO reference, and lower than reported from other studies on SCD (Silva & Viana 2002). When data was compared with a Jamaican disease specific weight reference 14.7% were less than the 3rd percentile, 60.8% were between the 3rd-50th percentiles and 24.5% above the 50th percentile. These results were comparable to findings in Omani children (Jaiyesimi *et al,* 2002). For height about a quarter of these children were below (<3rd percentile) the Jamaican reference curves (Thomas *et al,* 2000).

The high prevalence of stunting (54%), wasting (35%) and underweight (47%) is most likely explained by the high background prevalence of childhood malnutrition in the general population of Yemen (Sunil 2009). Although many children (≤ 5 years) with SCD were clearly malnourished, more marked differences might be expected in older children as growth deficits would tend to increase with age.

In regression analysis growth deficits were significantly associated with male sex and with increasing child age. Most previous growth studies did not comment on the sex of participants and have analyzed pooled data. However in some studies greater growth deficits have been reported in males (Phebus *et al,* 1984, Silva & Viana 2002, Zemel *et al,* 2007), although not in others (Stevens *et al,* 1986). The reason for this sex difference in growth is unclear, but might be related to variation in the degree of anaemia, Hb F level, energy intakes and hormonal changes between males and females, especially at the time of puberty (Phebus *et al,* 1984, Stevens *et al,* 1986, Modebe & Ifenu 1993, Singhal *et al,* 1996, Silva & Viana 2002).

Children with SCD have normal birthweight and length with growth restriction usually commencing between 6 months and 2 years of age (Kramer *et al,* 1980, Stevens *et al,* 1986, Thomas *et al,* 2000). The pattern of increased growth deficit with age observed in the present study indicating cumulative long-term growth deficit and was consistent with previous reports (Kramer *et al,* 1980, Pellegrini-Braga *et al,* 1995, Thuilliez *et al,* 1996).

In the present study the inflammatory markers CRP and SAA were elevated in all children and in the regression analysis SAA was significantly association with low BMIZ. The association of reticulocyte count, sTfR and severity score in the univariate analysis with wasting may indicate a relationship between growth failure

and disease activity and a hyper-metabolic state (Hibbert *et al,* 2005). There were no significant correlations between SI score and anthropometric parameters, which is consistent with other studies (Lowry *et al,* 1977, Singhal *et al,* 1996, Zemel *et al,* 2007).

There was a significant inverse association of growth deficit with plasma albumin and alkaline phosphatase in regression analysis. Reduced albumin concentration can be low due to malnutrition or reflect a negative acute phase response. Alkaline phosphatase is a biochemical marker of bone formation and its level may be related to growth impairment and delayed skeletal maturation or to an associated zinc deficiency which is frequently reported in SCD (Fung *et al,* 2008).

11.3 BIOCHEMICAL PROFILES

11.3.1 sTfR

The marked elevation of sTfR in children with SCD demonstrated in this study was in agreement with several other reports (Flowers *et al,* 1989, Singhal *et al,* 1993a, Serjeant *et al,* 1996, Grotto *et al,* 1999). sTfR was higher in males and in younger children. Males had relatively lower levels of Hb and Hb F concentrations which could explain this gender difference. sTfR concentration is normally high in children, decreases with age, peaking at 6 months-6 years, and reached adult levels by 16 years (Virtanen *et al,* 1999, Suominen *et al,* 2001, Kratovil *et al,* 2007). sTfR concentrations are reported to be correlated positively with the reticulocyte count and negatively with Hb levels for both sexes, although not with Hb F (Singhal *et al,* 1993a, Serjeant *et al,* 1996).

In SCD there is an overall increase in hematopoietic activity. Positive correlations between sTfR and serum erythropoietin and negative correlations with

RBC count and Hb level have been reported (Duits *et al,* 2003).The significant correlation of sTfR with the degree of anaemia in this analyses supports the assumption that sTfR is a reliable indicator of bone marrow expansion and erythropoietic activity (Cook *et al,* 1993, Singhal *et al,* 1993a, Grotto *et al,* 1999).

In the Yemeni children sTfR correlated positively with reticulocyte count and negatively with Hb concentration. These are parameters which are important indicators for increased haemolysis (Ho *et al,* 2003). A suppression of haemolysis and erythropoiesis with regular blood transfusion, hydroxyurea therapy or splenectomy has been associated with significant decline in sTfR levels (Singhal *et al,* 1993a, Ballas *et al,* 1999, Tancabelic *et al,* 1999, Loukopoulos *et al,* 2000). Children in this study only received transfusion sporadically, therefore a correlation between sTfR and transfusion frequency would not be expected.

The present findings of a lack of correlation between sTfR with various clinical complications, disease severity or with biomarkers of inflammation, is consistent with many studies which show the same lack of association (Ferguson *et al,* 1992, Singhal *et al,* 1993a, Pettersson *et al,* 1994, Dimitriou *et al,* 2000, Asobayire *et al,* 2001). The sTfR-ferritin index may be more useful in diagnosis of iron deficiency than sTfR, transferrin or ferritin alone in the presence of inflammation (Ooi *et al,* 2009).

Iron deficiency in SCD children living in developing countries is reported to be common (Nagaraj Rao & Sur 1980, Oluboyede *et al,* 1981, Okeahialam & Obi 1982, Jeyakumar *et al,* 1987, Mohanty *et al,* 2008), although in this sample, normal or elevated serum ferritin were observed which were similar to those reported for 104 non-transfused SS African American children (Stettler *et al,* 2001). There was no child with plasma ferritin below12µg/L, which is the reference cut-off value for

diagnosis of iron deficiency. A single parameter such as ferritin for assessment of iron status in SCD is unlikely to be sufficient, as alteration of ferritin concentration may be caused by inflammation. An adjusted cut-off for serum ferritin has been proposed to allow for this (Witte 1991). Furthermore, sTfR has been reported to be elevated in SCD and cannot discriminate between patients with or without iron deficiency (Singhal *et al,* 1993a). This situation complicates the assessment of iron status using iron biomarkers in children with SCD. Bone marrow examination is unrealistic in most cases. The assessment of iron status, therefore, remains problematic. Hepcidin, a recently discovered marker of erythropoiesis, may be useful for complementing iron status assessments and further research is needed to determine its utility as an iron biomarker in these children.

11.3.2 APR

SCD is an inflammatory condition, and the findings of this study confirmed that both CRP and SAA, two major human acute phase reactants, are elevated in children with SCD, which is in agreement with many previous reports (Akinola *et al,* 1992, Hedo *et al,* 1993, Singhal *et al,* 1993c, Stuart *et al,* 1994, Bourantas *et al,* 1998, Moore *et al,* 1998). The presence of inflammation in SCD is evident through increase activated leukocytes and endothelial cells and elevated inflammatory markers during symptom free intervals and which are intensified during crises (Moore *et al,* 1996, Hebbel 2004). The acute phase proteins are up-regulated by inflammatory cytokines (IL-1,TNF-α, IL-6) produced at the site of injured tissue (Gabay & Kushner 1999). These inflammatory cytokines are elevated in SCD during the steady state and more markedly during crisis (Bourantas *et al,* 1998, Makis *et al,* 2000b, Pathare *et al,* 2003, Pathare *et al,* 2004, Brittain & Parise 2007). The

persistent elevation of APR responses could have long-term deleterious effects (Belcher *et al,* 2000, O'Brien & Chait 2006).

In SCD both CRP and SAA are affected by Hb genotype and levels are higher in SS than SC or normal AA controls (Singhal *et al,* 1993c). Heritability is an important determinant and accounted for about 52% of baseline values for CRP and 59% for SAA (MacGregor *et al,* 2004). CRP genetic polymorphisms have been identified and also could explain some inter-individual variability (Hage & Szalai 2009). The distribution of CRP haplotypes in SCD patients and their influence on plasma levels and clinical diversity warrants further investigation.

The results of studies which have reported associations between inflammatory markers and the clinical severity in SCD are controversial. Some investigators have found positive correlations (Hedo *et al,* 1993, Makis *et al,* 2006), whereas others found no association (Schnog *et al,* 2004), as in the present study. APR in children has been shown in one study to be correlated well with the degree of severity of painful crisis (Etienne-Julan *et al,* 2004). What is unclear is the extent to which raised APR responses preceded and predispose to crises, or are a consequence of crises. It is more likely that the degree of severity of painful episodes is the primary influence on APR concentrations than the converse. Other possible sources of variation in APR include difference in study design, severity scoring system, age of participants and population setting.

Improvement in understanding of the inflammatory process and its contribution to vascular injury and chronic organ damage could provide a basis for use of anti-inflammatory as a therapeutic option.

11.4 GENETIC ASPECTS

11.4.1 MTHFR and homocysteine metabolism

The frequency of MTHFR C677T in Yemeni children is described for the first time in this study. The prevalence of the MTHFR C677T genotype was 12.7% with an allele frequency of 7.4%. This is similar to prevalence estimates among SCD patients from numerous reports in the neighbouring countries and elsewhere (Zimmerman & Ware 1998, Cumming *et al,* 1999, Romana *et al,* 2002, Al-Absi *et al,* 2006). The MTHFR TT frequency was not higher than in the Yemeni general population (2% vs. 2.2%) (Schneider *et al,* 1998), and is similar to several other reports (Balasa *et al,* 1999, Adekile *et al,* 2001, Romana *et al,* 2002, Fawaz *et al,* 2004, Al-Absi *et al,* 2006) . The allele frequency was lower than for the general population (7.4% vs 17.4%), and was comparable to the (9% vs.15%) reported by Romana et al., (2002) in Guadeloupian population. The lack of an association in this analysis between MTHFR C677T genotype and SI, or with individual clinical complications is in agreement with other studies (Andrade *et al,* 1998, Zimmerman & Ware 1998, Balasa *et al,* 1999, Cumming *et al,* 1999, Driscoll & Prauner 1999, Adekile *et al,* 2001, Balasa *et al,* 2002, Romana *et al,* 2002). In all reported studies there was only a small number of individuals with the TT genotype (range 0-8 cases), limiting the statistical power for an analysis in relation to this genotype.

The low tHcy level in this analysis was expected as all participants received daily folate and mostly on a regular basis. The high folate intake would normalize the plasma tHcy concentration and blunt any effect of a MTHFR polymorphism on tHcy. Administration of folic acid decreased tHcy concentration by 53% in children with SCD, and with normal plasma folate levels and an absence of folate deficiency (van

der Dijs *et al,* 1998). The high values of whole blood folate in the present study were most likely a result of good patient compliance with folic acid supplementation.

Although, the homozygous TT genotype has been associated with lower folate and higher tHcy concentration, the two children with homozygous TT in this study had normal tHcy levels. The number was insufficient to detect any effect of this mutation on tHcy or on disease severity or complications in the present sample.

Folate requirement in children with SCD may be higher than in the general population (Kennedy *et al,* 2001, Segal *et al,* 2004), and in a developing country like Yemen, where malnutrition is highly prevalent, it is a reasonable approach to provide regular folic acid supplementation for these children.

The study raises several questions related to folate status and Hcy in SCD. These include:

- Which level of tHcy is associated with minimal risk?
- Should tHcy reduction be considered irrespective of baseline folate status?
- Can the tHcy level be used as an indicator for adequate folate intake?
- What other determinants of tHcy in SCD patients should be considered?
- What is the optimal daily requirement for folic acid and related vitamins in relation to population prevalence for deficiencies of these nutrients?
- Should folate requirements be individualized in SCD, for example in relation to rate of haemolysis, MTHFR genotype, age, or growth status?
- Are there any long-term side effects of high folate intake?
- What is the role of 5-MTHF as a direct endothelial function modulator?
- What is the most suitable measurement method for the assessment of folate status in SCD?

- Is there accumulation of intracellular non-functionally active folate species as a result of persistent high circulatory folate, and what are their effects?

11.4.2 β^S haplotypes

The pattern of β^S globin gene cluster haplotypes in Yemeni sickle cell patients has not been previously characterised. Data provided by this study showed the Benin haplotype to be predominant, a finding in concurrence with the β^S Benin haplotype distribution in the Arabian Peninsula and in the countries of the Mediterranean Basin.

The dominant frequency of the Benin haplotype in this study suggests that the β^S mutation originated from the Benin region and was introduced to Yemen and other parts of the region by gene flow during the slave trade route movements and migration (Kulozik *et al,* 1986, Nagel & Ranney 1990, Serjeant 1990). This is supported by the strong historical and geographical relationship between Yemen and Africa.

Atypical haplotypes in the β^S gene are estimated to occur with frequency between 5–10%, (Steinberg *et al,* 1998, Zago *et al,* 2000). In this analysis it occurred in 11.8%, which was similar to the 11.9% reported from Venezuela (Vivenes De Lugo *et al,* 2003), but less than the 14.4% reported from South Iran (Rahimi *et al,* 2006). It was assumed that atypical β^S haplotype would be high if the typical haplotype in β^A of a normal population was not common, particularly in a population where Benin is the predominant single haplotype (Steinberg *et al,* 1998, Zago *et al,* 2001). The high frequency of atypical β^S haplotype in this study suggests that this haplotype is less frequent in the general population of Yemen. However, it would be of interest to investigate the distribution of β^S haplotype in the general population which could provide further support to the current findings.

In contrast to the Arab-Indian haplotype, which was not detected in this sample, the Benin and Bantu haplotypes are usually associated with low production of Hb F and absence of the -158 XmnI mutation. An association between β^S haplotypes and disease severity in this study could not be established. The likely explanation is that there was insufficient variability allowing the documentation of such a relationship, due to Benin haplotype predominance and the small number of Bantu with complete absence of the AI haplotype. Although, Yemeni children bearing the Benin haplotypes were in the majority and the overall disease profile was similar to African reports, nevertheless there are some characteristics which were different to Africans, such as the considerable clinical diversity, a high frequency of splenomegaly, low rate of skin ulcerations and variable Hb F concentrations. This may suggest that the β^S haplotype is not the only disease modulator. Other genetic determinants such as double heterozygous, SNPs affecting Hb F levels (e.g. BCL11A) and those associated with increased risk of specific complications require further investigation.

11.5 LIMITATIONS

As this was a cross-sectional study, clinical assessment and laboratory data were collected at a single time-point and therefore did not provide information about longitudinal changes in the course of the disease. As a hospital-based study ascertainment bias could lead to the inclusion of a greater number of symptomatic than asymptomatic patients. Data was collected retrospectively which could lead to a variable degree of recall bias for clinical events, and parents and children may have better recall for more severe symptoms. Information about vaccination coverage was not obtained and this is important as vaccine uptake should be reinforced in these children. Data on quality and sources of genetic counselling provided to parents was

not collected, and also information on the type and method of malaria prophylaxis. For each child a single reading of the laboratory assay was obtained and duplicate measurements would have provided improved quality assessments. Lack of effective laboratory facilities in the Yemen, including facilities for blood culture restricted the accurate diagnosis of bacterial or viral acute febrile illnesses, limiting the etiological classification of infection episodes. The accurate diagnosis of other double heterozygous sickle cell conditions and Hb genotype was limited as alkaline electrophoresis was the primary analytic method and acid electrophoresis, IEF, HPLC or PCR were not available. The same limitation was extended to the accuracy of the quantitative estimation of Hb variants. Investigation for family carrier state especially for diagnosis of $S\beta^0$ thalassaemia was also not undertaken, and this condition is only excluded by the low HbA2 level ($\leq$3.5%). Blood count was measured manually without reporting of MCV, MCH and MCHC as automatic blood count instruments were not available, and these indices were not completed routinely. Subjective variability was minimised as reading of all blood counts was carried by the same haematology technician. Only plasma ferritin was measured for the assessment of iron status and measurements of additional iron biomarkers would be more informative. Financial restrictions on support for the study limited a more comprehensive assessment of iron biomarkers.

Although the main objectives of this study were achieved, it was beyond the time limits and budget available to cover other aspects of this multisystem disease, particularly in the absence of previous patient information from this area. Future research should be more specific and focus on defined problems, preferably with inclusion of appropriate controls which is one of the shortcomings of the present study. Extra-ordinary efforts are needed to undertake research work in this complex

and unpaved road for scientific activity in Yemen. Many unusual circumstances arose due to the unique situation in Yemen which are briefly outlined in the following section.

Unique situation in Yemen:

Yemen is facing multiple complicated problems and challenges which are not only restricted to the political arena and security issues that threaten peace and social stability, but also involve almost all aspects of life in this country. Economically, Yemen is the poorest country in the region with rapid population growth and a high prevalence of malnutrition. Nearly half of the population live below the line of poverty, and the unemployment rate is high. Oil production and revenue have declined in recent years. The agricultural sector is in recession and the majority of food is imported. Qat consumption is a wide-spread habit and has damaging effects on health, as well as on the family budget. Qat is often grown in preference of food corps. Water supplies are insecure with a current severe shortage and potentially threatening a crisis situation. About 50% of population has no sustainable access to improved water sources or sanitation services. These problems are all associated with resource mismanagement, imbalanced distribution, governmental corruption, a high rate of illiteracy and strong tribal traditions, which entangled together, hinder real progress and development.

The health system is embedded in this situation and was considered to be in crisis a decade ago. A plan had been proposed for health sector reform but has not materialised and the countries condition continues to deteriorate. With the low share of health allocation in the national budget, consumers shoulder approximately 50% of the total health care cost. Overall health coverage is approximately 50%, but this is below one third in many rural areas. Heath services are not only inadequate and

underutilized but are of poor quality. There is a profound weakness of infrastructure, severe shortage of health manpower, deficiency of medical supplies and chronic under-funding, with a wide gap between needs and resources.

Laboratory services are below minimum standards, with deficiency of basic laboratory instruments, absence of regular maintenance and an irregular supply of consumables. Blood transfusion services are based mainly on volunteer blood donation and sometimes through donation campaigns with minimal screening and cross-matching. Microbiological diagnosis and isolation of causative agents is unavailable for most health facilities including referral hospitals and virology diagnostics and isolation is almost absent. There is over-prescription of drugs, and almost all drugs can be sold over the counter, with a high percentage of smuggled drugs and in the absence of quality control or monitoring. Unregulated and unsupervised private medical practice is increasing, not as a complementary system but as a competitor for public health services, with high cost and often poor quality. Profound and fundamental reforms are needed to achieve progress and to impact on the care of children with SCD.

11.6 CONCLUSIONS

This study has provided essential descriptive data about the clinical features, severity and complications, and the biochemical and genetic characteristics of SCD in Yemeni children. The data has practical implications and can be used to guide initiatives related to improving the comprehensive care of these children. The introduction of a screening programme and establishing a longitudinal cohort study from birth would better characterise the natural history of SCD in this population and promote an improved preventive approach. This would help substantially in determining the magnitude and extent of SCD in this population and promote effective control measures, and reduce the disease burden on families, on health care services, and on the society as a whole.

The study demonstrated that SCD is a serious health problem affecting children in Yemen from early in life, where affected individuals are exposed to a number of serious clinical events and complications. This information is clinically relevant and has important implications for future development of management protocols and guidelines. The majority of children became symptomatic in the first two years, mostly with dactylitis. Painful crisis was more frequent in older children. Some age and gender differences in clinical manifestations were observed. Malaria infection was frequent and induced crisis in some cases. The overall disease profile and complications were similar to those reported in children from the western part of Saudi Arabia and in Africans. There was a high prevalence of consanguinity among families, reflecting the high rate of related marriage in this society and inadequate education about the increased risks of hereditary diseases such as SCD with consanguineous marriage.

Variation in clinical expression was established using a Severity Index to classify mild and severe forms of SCD in these children. Analysis of severity assessment showed associations with age, gender and Hb F level. The concentration of Hb F was generally low, with wide variation but correlated inversely with disease severity. Malaria infection was more frequent in the severe category, indicating a link between malaria risk and morbidity profiles in this endemic area. The SI was used successfully in both symptomatic and asymptomatic children at time of enrolment, and allowed objective clinical comparison with similar studies from other geographical locations.

The low frequency of the MTHFR C677T genotype in Yemeni children with SCD, and absence of correlations with clinical complications and severity may indicate that screening for the MTHFR mutation would be unlikely to identify children at high risk for severe and complicated disease in this population. Plasma tHcy was not elevated and showed no correlation with disease severity or complications, and circulatory folate was high which most probably reflected good compliance with regular folic acid supplementation.

The marked elevation of sTfR probably reflected a high level of haemolysis and hypererythroid activity. The concentration of sTfR did not differ by clinical condition, disease severity or in relation to complications. The significant correlation of sTfR with splenomegaly may indicate a variable degree of hypersplenism in some cases. These cases require serial measurements and prolonged follow-up may show they would benefit from splenectomy if hypersplenism was confirmed. The lack of a correlation between sTfR and inflammatory markers is frequently reported in chronic inflammatory conditions and was also demonstrated in the present study. The association between plasma ferritin and sTfR was distorted influenced by the

magnitude of inflammatory effects and erythroid activity in SCD on ferritin, sTfR and their ratio, which is difficult to quantified under these conditions. The use of these biomarkers and hepcidin as an indicator of iron status requires further research.

The level of inflammatory markers was altered in these children demonstrated by elevated CRP and SAA levels. Their levels were well above the reference range for the majority in the steady state and were significantly higher during crises or with acute disease complications. Plasma albumin concentration was decreased in about one fifth of children and mean concentration was marginally lower in those with crises or acute disease complications. The study was unable to demonstrate associations between CRP or SAA and SI or individual clinical variables.

The Benin haplotype is the most predominant β^S cluster haplotype in Yemeni SCD children, with frequency similar to that in Western Saudi and Mediterranean countries. This finding is consistent with the notion that the β^S mutation originated mainly from the Benin region and was introduced to Yemen by gene flow. The higher rate of atypical haplotypes is an interesting finding, which could be related to the low frequency of the Benin haplotype in β^A in the general population. The spectrum of clinical manifestations and variable severity in these children could not be explained by association with β^S polymorphism, indicating other determinants are likely to be present and require further investigation.

11.7 RECOMMENDATIONS

1. It is a priority to establish a specialized comprehensive care program with a primary role of maintaining periodic evaluation, prevention and timely appropriate treatment for acute disease complications. This should include a more standardised approach to management guidelines.
2. It is important to issue national standard medical care guidelines for the management of patients with SCD. This can provide uniformity in the type and quality of care offered to individual patients and can help in improving case management.
3. Health authorities should encourage the introduction of specialized sickle cell clinics provided with well trained health professionals and equipped with standard laboratory facilities. To improve utilization, health care with basic investigations and treatment should be offered free of charge.
4. It is critical to facilitate the development of a neonatal screening programme for SCD and to monitor its effectiveness and sustainability. Appropriate interventions could be evaluated in clinical randomized trials following neonatal screening.
5. The introduction of the polyvalent pneumococcal vaccine should be promoted with emphasis on improved child immunization. Prompt diagnosis and management of infection with better availability of blood culture is required. Initiation of penicillin prophylaxis at three months of age should be commenced.
6. Effective preventive measures on the control of malaria are required to reduce exposure to malaria in these patients. The use of chemoprophylaxis may need to be evaluated, and introduction of bed nets promoted.

7. Diagnostic facilities, neuro-imaging, TCD, blood transfusion safety and stroke preventive measures should be improved.
8. Hydroxyurea should be considered for patients with severe manifestations. Appropriate guidance on selection criteria, dosage and follow-up arrangements should be made available.
9. A health education programme should be initiated to increase awareness of SCD as an important problem in this population. This should target primary health care workers, professional staff, patients and their families as well as the general public. Emphasis should be given on the importance of screening, continuous medical follow-up, health maintenance strategies and on an accurate local registry programme.
10. Families should be provided with psychological and economic support to cope with the extra-demands of SCD. Promotion of community awareness and participation through support groups and non-governmental organizations should be encouraged.
11. Parents should be taught about their child's nutritional requirements with emphasis on supplemental folic acid and appropriate growth monitoring in order to identify significant delays, as nutritional support may be required and early detection and intervention will lead to growth promotion.
12. Pre-marital testing and effective genetic counselling must be encouraged emphasizing reduction in consanguineous marriage.
13. It is of importance to undertake a comprehensive longitudinal research study to identify the natural history of SCD in Yemeni children and to explore the role of environmental and genetic determinants on phenotypic diversity.

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APPENDICES

APPENDIX A: SCD MANAGEMENT OUTLINES*

Category	Intervention	Comments
Infection	Prophylactic penicillin.	From 3 months to 5 years, indefinitely in recurrent invasive infection or splenectomy.
	Pneumococcal vaccination.	Conjugate pneumococcal 7-valent vaccine (3doses, by 2 years) followed by 23-valent vaccine at 2 years, and 5 yearly thereafter.
	Other prophylaxis	Vaccination for H.influenzae, Hepatitis A and B, Meningococcal (ACWY), and Influenza. Malaria prophylaxis in endemic areas and for travellers.
Anaemia	Red cell transfusion.	Simple top-up transfusion for aplastic or ASS crisis. Prevention of recurrent ASS by transfusions for children less than 2-3 years or splenectomy in older children and those with hypersplenism.
	Prevention of transfusion complications	Phenotypic matching (E,C, and Kell) to prevent transfusion reaction and alloimmunization. Better screening to prevent blood born infections. Chelating therapy: desferrioxamine (parenteral), deferasirox or deferiprone (oral).
Painful crisis	Acute pain relief. Patient controlled analgesia (PCA).	Pain assessment, hydration, adequate analgesia (opioids and non-opioids), adjunctive drugs (anxiolytics, antiemetic, antipruritic and laxatives), frequent monitoring for effectiveness of analgesia, associated complications and hypoxia. PCA should not be used unless there is established protocol and trained nursing and medical staff.
Chronic severe disease	HU, chronic transfusion, BMT	HU effects possibly through increase HbF, RBC hydration, NO generation, decrease neutrophils and cell adhesion. BMT is expensive, age < 16 yrs, limited matched donor and 5-10% mortality.
Acute chest syndrome	Improve hypoxia.	Simple or exchange transfusion, O2 supplementation, optimum pain control and fluid management, corticosteroid and mechanical ventilation in severe cases.
	Prevention of infection.	Antibiotic combination with macrolide to cover mycoplasma, and chlamydial infections.
	Incentive spirometry.	May prevent atelectasis and deterioration. Chest physiotherapy.
	HU, chronic transfusion	For repeated episodes.
Chronic lung injury prevention	Monitoring pulmonary function and oxygen saturation. Screening for pulmonary hypertension.	For early detection of chronic lung disease. Echocardiography, if TRV≥2.5 m/s, consider HU, transfusions, drugs (prostacyclins, sildenafil, and bosentan).

Category	Intervention	Comments
Stroke prevention	Primary prevention.	Screening with TCD from age 2-16 yrs (high risk ≥200 cm/s, conditional 170-200 cm/s), chronic transfusion.
	Secondary prevention.	Regular transfusion to reduce HbS <30%, HU where transfusion is contraindicated or unavailable, BMT.
Maintenance of renal function	ACE inhibitors.	For proteinuria and/or hypertension. Avoid NSAIDs
	Dialysis and renal transplantation.	End stage renal disease.
Haematuria	Maintain diuresis.	Bed rest, adequate fluid, diuretics, urine alkalinisation, transfusion, renal medullary carcinoma must be excluded.
Enuresis	Behavioural changes.	Minimize fluid intake at bedtime, frequent waking and voiding, urine alarm. Overnight oxygen saturation if there is history of snoring or obstructive apnoea, ENT advice.
	Drugs.	Desmopressin.
Priapism	Medical.	Pain relief, hydration, warm baths, ?transfusion, aspiration and corporal irrigation with diluted phenylephrine.
	Surgical.	Shunts (mainly glans-cavernosum, or cavernosaphenous, dorsal veincavernosa), internal artery embolization. Penile prostheses for impotent.
	Prevention of recurrence	Anti-androgens, stilboestrol, oral etilefrine, or pseudoephedrine, sildenafil.
Gallbladder disease	Cholecystectomy.	Laparoscopic operation is preferred for symptomatic or elective surgery
Avascular hip necrosis	Physical therapy	Bed rest, decrease weight bearing in early stage may prevent rapid degeneration.
	Decompression coring procedures.	No satisfactory result with available interventions, due to many failure and postoperative complications.
	Hip replacement surgery.	
Chronic leg ulcers	Pain relief, debridement, hydocolloid dressing, topical antimicrobials, oral zinc, transfusions, ?HU, skin graft.	Prevention of trauma, rest, supporting stock and leg elevation and physiotherapy to prevent ankle fixation.
Psycho-social issues	Psychological and social support.	Cognitive behavioural therapy to learn skills of coping with pain episodes and chronicity. Regular neuropsychological and developmental assessment. Family support.

*Derived from multiple references

APPENDIX B: THERAPEUTIC AND INVESTIGATIONAL AGENTS

Category	Agent	Possible Mechanism
Anti-sickling	Hydroxycarbamide (hydroxurea)	Ribonucleotide inhibitor, cytostatic effects
-Hb F modulation	Decitabine	DNA methyltransferase inhibitor, hypomethylation
	Butyrate (short chain fatty acid)	Histone deacetylase inhibitor
	Erythropoietin	Increases F cells
	Thalidomide,lenalidomide, &	Regulate globin gene transcription, increase F cells
	pomalidomide	Increase γ-globin gene mRNA in cultured human
	Mithramycin and angelicin	erythroid cells
-Through other pathways	Nitric oxide, Arginine	Vasodilatation, increase flow, decrease adhesion
	Purified poloxamer 188 (Flocor)	Anti-adhesion, anti-inflammation
	Pyridyl derivatives of benzaldehyde	Formation of covalent bonds with HbS
	Niprisan (plant extracts)	Inhibit sickling by unknown mechanism
	L-carnitine (used for PHT)	Reduced hemolysis by stabilising cell membrane
Red cell rehydration	Clotrimazole	Gardos channel inhibition
	ICA-17043 (Senicapoc)	Clotrimazole derivative, more potent and less toxic
	Mg pidolate	K:Cl co-transport inhibition
	NS 1652, NS 3623	Chloride movement blockage
Anti-adhesion	RGD peptide anti-adhesion antibodies	Red cell endothelial adhesion
	Anti-von-Willebrand factor	PAF-induced adhesion
	Anti-integrin receptors	Anti-white cell adhesion
	Sulphasalazine	Endothelial activation, inhibitor of NF-kb
Anti-oxidative therapy	Glutamine, N-acetylcysteine	Gulutathione metabolism
	Deferiprone	Chelate membrane iron
Anti-thrombotic	Heparin, acenocoumarol, Aspirin	Decrease thrombin
	N3-fatty acids	Inhibition of platelets activation
Transfusion therapy	Simple transfusion, pheresis	Decrease HbS cells, improve O2 carrier capacity
Transplantation	Allogeneic BMT, non-myeloablative,	Haemopoietc stem cell producing red blood cells
	umbilical cord blood	with normal Hb.
Gene therapy	Viral delivery of β-globin gene	Gene correction or addition of corrective gene

Source: (Vichinsky 2002, Okpala 2006a, Trompeter & Roberts 2009)

APPENDIX C: QUESTIONNAIRE

Serial No. ☐☐☐ Code: ☐☐ ☐☐☐

Age: ☐☐ mos ☐☐ yrs New☐ Repeated ☐ Date____/____/___

Gender: M ☐ F ☐

Address: _____

H/O: hospital admissions- Y ☐ N ☐

NO.	Date	Cause of admission	Duration
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

Blood transfusions-: Y ☐ N ☐ , Times/year.01☐ 02 ☐ 03 ☐ 04 ☐

Painful crises: Y ☐N ☐home treated ☐☐ /year. Hospital treated ☐ ☐ /year

01 ☐02☐03☐04☐ 01☐02☐03☐04 ☐

Age at first presentation: ☐☐ mos

Age at first episode of hand-foot syndrome ☐☐ mos

Sequestration crises: Y☐ N☐ DK☐Times/yr ☐01☐02☐03☐04

Aplastic crises: Y☐N☐,DK☐ Times/yr ☐01☐02☐03☐ 04

Hyperhemolytic crises: Y ☐ N☐DK ☐ ☐ 01 ☐02☐03☐ 04

Acute chest syndrome: Y☐ N ☐ ☐ 01 ☐02☐ 03☐04

Cerebrovascular crises: Y☐N☐ , ☐ times

Stroke: Y ☐ N☐ Hemiparesis☐convulsion☐ aphasia ☐

Eyes complications: Y ☐N☐ loss of vision☐vitreous Hge ☐Retinopathy ☐

Avascular bone Necrosis: Y☐ N ☐ Hip:☐ R/L. shoulder:☐ R/L.

Others:_____

Leg ulcers: Y☐ N ☐acute:☐ recurrent:☐ chronic: ☐

Priapism: Y ☐N ☐Times/Y ☐☐ Minor ☐☐Major ☐☐

Gall stones: Y☐ N ☐,Operated: Y ☐N ☐

Jaundice in the last year: Y ☐ ☐

Enlarged liver in the last year: Y☐N ☐DK☐

Enlarged spleen in the last year: Y☐N☐ DK ☐

Any bleeding tendency: Y☐ N☐ Epistaxis Y☐N☐ Others:_____

Any heart failure: Y☐ N☐ DVT: Y☐ N☐

Any renal disorders: haematuria: Y☐ N☐ Enuresis: Y☐ N☐ polyuria: Y☐ N☐

Infections episodes: times/year 01☐ 02☐ 03☐ 04☐

Respiratory: Y☐ N ☐,URT : ☐☐☐ times/year. LRT: ☐☐ times/year

CNS: Y☐N☐ times/yr☐ Septicaemia: Y☐ N☐times/yr☐☐

Osteomyelitis: Y☐N ☐Site:_____ Septic arthritis: Y☐N ☐ Site:_____ Malaria in the last year: Y☐ N☐ DK☐

Activities:

Do as many activities as siblings do Y☐ N☐

Play outside often along with children of the same age Y☐N ☐

Missed school more than others Y ☐N☐How many weeks in the last year ☐☐

School grade below average Y☐ N ☐

Drugs	Dose	Frequency	Duration
Folic acid			
Multivitamins			
Penicillin			
Others			

Age at menarche: ☐☐Yrs

Family history: Father: diseased ☐ trait ☐ DK ☐

Mother: diseased ☐ trait ☐ DK ☐

Consanguinity: Y☐N ☐

1st ☐2nd ☐ FR ☐

Other sib's: diseased No.☐ Trait No.☐ DK No. ☐

Family death due to SCD: Y☐N ☐age at death: ☐☐ mos ☐☐ yrs

Patient in acute crises: ☐ in steady state: ☐

O/E pallor: Y ☐ N☐conj. ☐ tongue☐ nail ☐ Jaundice: Y ☐ ☐

Facial changes: Y☐ N☐ bossing: Y☐ N☐ prominent upper jaw: Y ☐ N ☐

Weight: ☐☐☐ kg Height: ☐☐☐ cm MUAC: ☐☐cm

Vital signs-RR ☐☐ PR ☐☐ BP ☐☐☐ / ☐☐ TEMP- ☐☐,☐

Respiratory system: URTI: Y ☐N ☐ LRTI: Y☐N ☐

Breathing sounds- normal ☐ increase ☐decrease ☐ absent ☐

Added sounds: crepitations ☐ rhonchi ☐ others:_____ Dullness Y☐N☐

Cardiovascular system:

Heart sounds: normal☐ regular☐ Murmurs: Y☐ N☐Sys☐ Diast☐ HF: Y☐N☐

Abdomen: normal☐ distended☐ soft☐ tender☐ BS: present☐absent☐

Liver: palpable☐☐ cm/bcm soft☐ firm☐ tender☐

Spleen: palpable ☐ ☐cm/bcm soft☐ firm☐ tender☐

Extremities: Joints swollen tender restricted movement

Hip	☐	☐	☐
Knee	☐	☐	☐
Ankle	☐	☐	☐
Shoulder	☐	☐	☐
Phalangeal	☐	☐	☐

Others: _____

Local bone tenderness: Y☐ N☐site:_____

Leg ulcers: Y☐ N☐ site----------

Finger clubbing: Y☐N☐ Hands☐ feet☐ both☐

CNS: Motor deficit Y☐ N☐ type----------

Sensory deficit Y☐ N☐ type----------

Speech deficit Y☐ N☐ type----------

Investigations:

Hb☐☐ gm/dl. Blood group- A☐ B☐ AB☐O☐ RH☐☐

WBC☐☐☐☐☐ pmn☐☐ % lymphocyte☐☐ %mono☐☐ %

Eosino☐☐% Baso☐☐%

Reticulocyte counts☐☐ % ESR☐☐ mm/hr

Blood urea☐☐ Creatinine☐☐

S.electrolytes: K+☐☐Na+☐☐Ca++☐☐Phosphorus☐☐

LFT: total protein☐☐Albumin☐☐Bilirubin T☐☐conj☐☐unconj☐☐

SGPT☐☐ SGOT☐☐alkaline phosphatase☐☐☐

CSF: cells☐☐☐ pmn☐☐ lymph☐☐ protein☐☐ sugar☐☐

Gram-positive☐ negative☐ culture: negative☐ positive☐

Bact:_____

Urine analysis;-casts☐RBC☐WBC☐Protein☐ S.G☐☐☐

Chest x-ray: normal☐ new lung infiltrate☐cardiomegaly

Abdominal US: normal☐ gallstones☐Others_____

Plasma level of:

Homocysteine: ☐☐, ☐ µmol/L) MTHFR-Genotype: CC ☐ CT ☐ TT ☐

Folate: ☐ ☐, ☐ nmol/L

Vitamin B12: ☐ ☐ ☐ pmol/L

Vitamin B6: ☐ ☐ ☐ nmol/L

Follow up of admission cases: DOA:__/__/__ DOD: __/__/__

Cause of admission:_____

Case progression:_____

_____

_____

_____

Hospital treatment:_____

_____

_____

Outcome:

Discharge ☐ Died ☐

Complications at discharge:

_____

APPENDIX D: PARTICIPANT INFORMATION SHEET

Sickle cell disease is an inherited blood disease, transmitted from parents to their children (boys and girls), resulting in an abnormal shaped red blood cell, which produce abnormal haemoglobin. Haemoglobin is the part of blood that carries oxygen from lungs to all other parts of the body. Haemoglobin is controlled by two genes, one from each parent. When a child inherits two sickle genes they have sickle cell disease. If they have a normal gene and a sickle gene they become carrier (sickle cell trait) but are not diseased.

When the red blood cells of a person with sickle cell disease don't get enough oxygen, these cells assume an abnormal shape (sickle or banana shape) and are easily destroyed. Sickle cells can get stuck in blood vessels and prevent blood from reaching parts of the body. This causes pain and can damage the body internal organs.

Blood test is needed for the diagnosis of a case of sickle cell disease.

If the child nutritional status is altered this might affect the severity of the child's disease, we intend to measure various nutrients and vitamins in the blood to find out how their presence or absence relates to your child's health. Understanding this better could help us to improve the treatment of children with sickle cell disease.

APPENDIX E: CONSENT FORM

Serial NO: ----------
Code: ----------

Dear participant:

This study is aiming to determine some factors correlated to the development and severity of sickle cell disease among children. The results obtained will contribute to the understanding of some aspects of these diseases and help in the identification of possible preventive measures that may help to reduce the severity of these ailments. The gathered data will be used confidentially and only for the research purposes.

We expect no health complications for your child as a result of the use of the procedures in this study. If you agree to participate, a blood sample of large teaspoonful (6ml) will be collected from your child. Also you would be requested to answer a questionnaire, which will take approximately 15-20 minutes. The questionnaire will ask about the recent and past history of your child's illness, some socio-economic information and other facts on familial background. This will be followed by a full clinical examination of your child.

You are completely free not to participate. If you decide not to participate, this will not modify your relationship with the doctors and other health personnel in the hospital and will not affect in any way the care and treatment your child receives.

Signature of the investigator

Signature or mark of participant
(Parent/guardian)

Date: ------- / ------- / ----------

Date: ----- / ---- /---------

APPENDIX F: BLOOD SAMPLE MANAGEMENT FORM

Serial No. __/__/__/__ Code : __/__/__/__/__/__

Age:__/__/ Months___/__/Year Gender: male / female

Date: / / time: /am /pm

Patient fasting: Yes__ No__ Last meal less than 3-4hrs: Yes__No__

Patient's posture: setting __ / supine__

Blood Sample: (Venopuncture)- Quantity of blood: _____ (ml)

Sample placed on ice immediately: Yes__ No__

Sample transfer to the hospital lab. After: _____ (mints)

Sample aliquots: -50µl in Eppendorf tube (MCT) for (MTHFR) test_____

-50µl in MCT+1ml 1% ascorbic acid for folate test_____

-1ml for CBC. _____

Plasma preparation:

Centrifuged after: ____(mints) of collection at_____/rpm. for____mints

Plasma aliquots: -1.5ml in MCT for urea/createnine and LFT _____

*-1.5ml-2ml in MCT for tHcy, vit B12 &B6 (frozen)*_____

Sample collected and *labelling* by: _____ signature: _____

Sample transfer to refrigeration at-70 after: ___/___hrs. Of collection

Sample transfer to LSTM must include:

0.5ml MCT whole blood_____

1.5ml MCT haemolysate_____

2 ml MCT plasma._____

Transfer at: _____ ^{0}C. On date __/__/__ time: __/__

Arrived: date__/__/__ time__/__/__

Investigator signature: _____

APPENDIX G: PUBLICATIONS

Brief Con n unication

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Beta-Globin Gene Cluster Haplotypes in Yemeni Children with Sickle Cell Disease

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The DNA sequence flanking the mutation site in the β^S-globin gene is polymorphic and in linkage disequilibrium. Five common distinct haplotypes have been identified using specific patterns of restriction endonuclease sites. Four of these are African haplotypes (Benin, Bantu, Senegal, and Cameroon) and are named after the geographical region and ethnic group in which they originated [1]. The fifth is the Arab-Indian haplotype, which was discovered in the Eastern Oasis of Saudi Arabia and among the tribal population of Central India [2].

Analysis of the β^S polymorphisms is of genetic and anthropologic interest, but may also relate to disease severity. The Bantu haplotype has been associated with severer disease and a high incidence of organ damage, conversely the Senegal haplotype is associated with milder disease, and the Benin haplotype with intermediate disease severity [3]. The Arab-Indian haplotype has also been associated with a milder disease variant [4]. Modified phenotypic expression in the Arab-Indian and Senegal haplotypes has been attributed to their higher Hb F levels and an association with the C→T mutation at position −158 *Xmn*I in the Gγ-globin gene promoter region. However, the exact mechanism and extent of haplotype effects on Hb F levels, haematological characteristics, and clinical course of sickle cell disease (SCD) have not been fully elucidated.

The clinical manifestations of SCD in the Arabian Peninsula can be grouped into 2 distinct forms, a usually mild disease occurring in the Eastern area populations and a severe disease phenotype in Western areas. This clinical variation coincides with the distribution of β^S cluster haplotypes, as the Arab-Indian haplotype is the major genotype in Eastern Saudi, Kuwait, and Bahrain, whereas the Benin haplotype is predominant in the Northern and South-Western provinces of Saudi Arabia [5–6].

This study was undertaken to analyse for the first time the background of β^S-globin gene cluster haplotypes in Yemeni children with SCD, and to assess their association with haematological and clinical profiles.

The study was conducted at Al-Wahda Hospital, Aden, Yemen. Seventy-two children (<16 years) with Hb SS confirmed by electrophoresis who attended the sickle cell clinic were included. A severity index (SI) was calculated based on frequency of painful crises, hospitalization, blood transfusion, infection, and specific complications during the previous year, and children were grouped into mild SI (≤6) and severe SI (>6) categories as previously described [7]. Standard laboratory methods were employed for all haematological investigations. The SS genotype in these 72 children was confirmed by direct sequencing of the β-globin gene. Haplotype analysis of the

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Table 1. Frequency of β^S haplotypes in Yemeni children

Haplotype	5′ ε *Hind* II	Gγ *Hind* III	Aγ *Hind* III	3′ ψβ *Hind* II	5′ β *Hind* II	3′ β *Hinf* I	3′ β *Hinf* I	Chromosomes	%
Benin	–	–	–	–	+	–	+	119	82.6
Bantu	–	+	–	–	–	–	+	8	5.6
Atypical C	+	–	–	–	–	–	+	10	6.9
Atypical D	+	–	–	–	–	+	+	5	3.5
Atypical E	+	–	–	–	+	–	+	1	0.75
Benin-like	–	–	–	–	+	–	+	1	0.75
Atypical sub-total								17	11.8
All								144	100

Table 2. Haematological data in Benin, Bantu and atypical haplotypes

	Benin (n = 50)	Bantu (n = 8)	Atypical (n = 17)	p value (ANOVA)
Hb, g/dl	7.6 ± 1.5	7.9 ± 0.6	7.9 ± 1.4	0.70
Reticulocytes, %	4.5 (2.3–9.0)	2.9 (1.1–5.3)	3.0 (1.5–6.1)	0.16
Hb F, %	3.0 (1.7–5.0)	3.3 (0.5–6.2)	4.5 (2.8–5.4)	0.42
HbA2%	3.0 ± 0.34	3.0 ± 0.27	3.0 ± 0.37	0.86

Data presented as means ± SD or medians (IQR).

β-globin gene cluster was performed by high-resolution melting curve analysis, as described previously [8], using the LightScanner instrument and software (LightScanner®; Idaho Technologies Inc., USA).

Results were obtained for 72 SS children including 44 (61%) males. Mean age (±SD) was 6.8 ± 4.4 years. Two thirds (66.7%) were in steady state at the time of blood collection. They were moderately anaemic with a mean Hb level of 7.7 ± 1.4 g/dl (±SD). The level of Hb F varied widely, with a median of 3.0% (IQR: 1.8–4.9%).

The β-gene cluster haplotypes of 144 β^S chromosomes came from 72 children. All cases were homozygous SS confirmed by DNA sequencing. Haplotyping was determined according to the presence (+) or absence (–) of the 7 SNPs corresponding to the polymorphic restriction endonuclease sites. The β-globin cluster haplotypes identified are shown in table 1. The Benin haplotype (– – – – + – +) had a frequency of 82.6%, the Bantu (– + – – – – +) of 5.6%, and the atypical haplotypes 11.8%. Atypical haplotypes were composed of atypical C (+ – – – – – +) 6.9%, atypical D (+ – – – – + +) 3.5%, atypical E (+ – – – + – +) 0.75%, and atypical Benin-like (– – – – + – +) 0.75%. No Senegal, Cameroon or Arab-Indian haplotypes were identified. Forty-nine children were homozygous for the Benin haplotype (68%), which was heterozygous with the Bantu haplotype in 8 children (11%) and with atypical haplotypes in 13 children (18%). Two children (3%) were homozygous for the atypical haplotype C, although no child had combinations of atypical haplotypes. Haematological data for Benin, Bantu, and atypical haplotypes are summarized in table 2. There were no significant differences in haematological parameters between the 3 haplotypes. Based on the SI, 47 (65.3%) children had severe disease and 25 (34.7%) mild disease. There were 31 (63.3%) children with the Benin haplotype, 6 (75%) with Bantu and 10 (66.7%) with atypical haplotypes in the severe category, compared to 18 (36.7%), 2 (25%), and 5 (33.3%) children, respectively, in the mild category. The haplotype distribution did not differ between mild and severe disease categories (p = 0.8). The mean (±SD) SI for Benin, atypical and Bantu haplotypes were 7.5 ± 3.3, 7.7 ± 2.3, and 9.3 ± 3.4, respectively (p = 0.36).

This study showed that Benin was the predominant haplotype in Yemen, which is in agreement with the dis-

Table 3. Distribution of β^S Benin haplotype in the Western Arabian Peninsula and Mediterranean

	Chromosomes, n	Benin haplotype, %	Other haplotypes[1], %	Atypical haplotypes, %	Reference No.
Yemen	144	82.6	5.6	11.8	this study
Western Saudi	142	68.3	31.7	–	5
Jordan	20	80.0	20.0	–	16
Lebanon	100	73.0	27.0	0	13
Syria	18	66.7	33.3	–	16
Palestine	118	88.1	5.9	5.9	17
Turkey	136	91.9	0.7	7.4	18
Egypt	28	100	0	0	16
Tunisia	66	94.0	0	6.0	19
Algeria	20	100	0	0	1
Greece	82	96.4	0	3.6	20
Sicily	64	100	0	0	21

Dashes indicate that the data was not provided.
[1] Including Bantu, Senegal, Cameroon and Arab-Indian haplotypes.

tribution of this haplotype for contiguous areas in the South Western region of Saudi Arabia and Mediterranean Basin (table 3).

The association of specific haplotypes with a geographically distinct population has provided critical information on whether the β^S mutation arose indigenously or spread by gene flow. The high frequency of the Benin haplotype in this study suggests that the β^S mutation originated from the Benin region and was introduced to Yemen by gene flow during the trans-African slave trading and by migration including some individuals from Central Africa origin, but none from the distant Senegal type, and that no migration has apparently come from East Arabia or Asia. This mutation is also selectively maintained by malaria which is transmitted in most of the coastal plain and valleys of Yemen. The geographical proximity of the Southwest Arabian Peninsula to Africa supports the notion that West African populations carrying the Benin β^S haplotype migrated and introduced the gene to North Africa, the Mediterranean, and the Southwest of the Arabian Peninsula [2]. The absence of Arab-Indian haplotype in this study is similar to that reported in the Western area of Saudi Arabia [4]. This haplotype seems to be restricted to the population of Eastern Saudis which live in greater cultural and hence genetic isolation.

The occurrence of atypical haplotypes in 11.8% of chromosomes in this study is similar to the 11.9% frequency reported from Venezuela [9]. The atypical Benin-like is probably not derived from Benin, because the base pair differences affect the same Benin restriction enzyme recognition sites and, according to traditional methods of haplotype analysis, this is indistinguishable from the regular Benin haplotype. There is evidence that a mechanism of point mutation and cross-over recombination can occur in the Benin β^S haplotype [10]. In a situation where the typical β^A haplotype of a normal population was uncommon, then the atypical β^S haplotype would be expected to be frequent [11]. It is probable that recombination with the Benin type from β^A chromosomes does not occur due to their rarity, and this is consistent with these results from the Yemen showing high atypical haplotypes in a non-African population. However, the frequency data for the haplotype patterns in the β^A gene for the general population in Yemen are not available, and no controls were investigated in this study but the high frequency of the atypical β^S haplotype suggests that this haplotype is less frequent in the normal population. Further research is required to confirm this hypothesis.

In Yemeni sickle cell patients, the absence of Arab-Indian and Senegal haplotypes corresponded with low Hb F levels and very low prevalence of the -158 (C-T) Gγ-globin gene *Xmn*I polymorphism, the 2 characteristic findings related to these haplotypes [12]. The predominance of the Benin haplotype, which is known to be associated with low Hb F concentration, could explain the lack of a difference in Hb F levels between the typical and atypical haplotypes, which is consistent with previous reports [11]. Due to the predominance of the Benin haplotype in this sample, there was insufficient variation to allow adequate comparison for disease severity, and SI scores for the different haplotypes showed no significant differences. The assumption could be made that as SCD in Yemeni children is associated with the Benin haplotype and is of African origin, then disease characteristics would be similar to Africans. Nevertheless, the presence of the mild clinical form in about a third of cases, and a high frequency of splenomegaly, low rate of skin ulcerations and high variability of Hb F suggests that β^S haplotype and Hb F are not the only disease modulators in this community and other genetic and environmental determinants are likely to exist, a view supported by other investigators [13]. However, the high frequency of α-thalassemia reported in Yemeni SCD patients [14] could be influential, although the clinical effects of this association remains to be evaluated.

The use of new biotechnology demonstrated that high-density SNP mapping across the β-locus, assisted by Haploview analysis, could define β^S haplotypes more accurately than traditional RFLP analysis alone [15]. This approach may help to elucidate other genetic determinants modulating clinical diversity in SCD.

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ORIGINAL ARTICLE

FREQUENCY OF THE MTHFR C677T POLYMORPHISM IN YEMENI CHILDREN WITH SICKLE CELL DISEASE

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□ *The frequency of the methylenetetrahydrofolate reductase enzyme (MTHFR) C677T mutation was determined using polymerase chain reaction (PCR) and with measurement of plasma total homocysteine (tHcy), folate, vitamins B6, B12 and disease severity in 102 SS children from Yemen. The homozygous TT genotype for MTHFR C677T was present in 2% (2/102), and heterozygous CT in 10.8% (11/102), giving an allele frequency of 7.35%. The T allele was not associated with raised plasma tHcy or increased disease severity. The mean [±SD (standard deviation)] tHcy was 2.8 ± 1.7 μmol/L, increased with age and was highest in children >10 years (3.6 ± 2.5 vs. 2.5 ± 1.2 μmol/L, p <0.05). Whole blood folate and plasma vitamin B12 levels were normal or elevated, and 4% had vitamin B6 deficiency. In Yemeni children with sickle cell disease the frequency of the MTHFR C677T mutation was not higher than expected in the general population and was not associated with disease severity.*

Keywords MTHFR, Homocysteine metabolism, Sickle cell disease, Yemen

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INTRODUCTION

Vaso-occlusion plays a central role in the pathophysiology of sickle cell disease. Elevated plasma total homocysteine (tHcy) concentration is a recognized risk factor for vascular and thrombotic disease in the general population (1). Therefore, tHcy may influence the clinical phenotype/severity of sickle cell disease. Raised homocysteine levels have a detrimental effect on endothelial tissues and could contribute to the organ damage observed in sickle cell disease individuals as early as the first year of life (2). Elevated levels of plasma tHcy may be caused by a deficiency of folate or B vitamin co-factors, or by reduced activity of the key enzyme in homocysteine-methionine metabolism: 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) (3). A thermolabile variant with 50–60% reduced activity of this enzyme is caused by a missense point mutation at nucleotide 677 with thymidine replacing cytidine (C677T), resulting in an alanine for valine substitution at position 222 of amino acid sequence (Ala222Val, rs1801133) (4,5). Normal MTHFR activity is crucial for maintenance of the pool of circulating folate and to prevent accumulation of homocysteine (6). The MTHFR C677T allele is associated with decreased blood folate and raised plasma tHcy (7). This mutation has also been reported to increase the risk for neural tube defects and pregnancy complications, and to decrease the risk for colon cancer and for adult acute lymphocytic leukemia (8).

Worldwide prevalence of the MTHFR C677T polymorphism shows extensive geographic variation, with frequency of the homozygous (TT) genotype in Caucasians between 10–30%, and highest prevalence in Mexicans and lowest in Blacks (9). Individuals with this mutation may have higher folate requirements for regulating homocysteine metabolism (7) and with sub-optimal folate intake tHcy concentration is raised by about 50% (10).

Patients with sickle cell disease suffer from chronic hemolysis, leading to accelerated erythropoiesis and increased folate requirements. Sickle cell disease patients with a mutated MTHFR have increased folate requirements with risk of hyperhomocysteinemia if folate requirements are not met. Administration of folic acid can lower plasma tHcy levels in a dose dependent manner (11), supposedly limiting endothelial damage and thrombosis in sickle cell disease (12). Increased plasma homocysteine is reported in children with sickle cell disease (13,14), and in those who develop stroke (15). The MTHFR deficiency has been associated with sickle cell vascular complications, including avascular osteonecrosis (AVN), stroke, retinopathy and acute chest syndrome (ACS) by some (16,17) but not all investigators (18–24).

The aim of this study was to estimate the prevalence of the MTHFR C677T mutation and to determine plasma concentrations of tHcy, folate,

vitamin B12 and B6 in children with sickle cell disease from Yemen, and to assess the association with disease severity.

PATIENTS AND METHODS

The study was conducted at the Al-Wahda General Teaching Hospital, Aden, Yemen, which is the major referral hospital for women and children serving urban and peri-urban areas. For the purpose of the study, the Sickle Cell Clinic was held daily during a six-month period from March to August 2005, in order to improve coverage. Information on the sickle cell disease clinic facility was advertised via local newspapers and health and education authorities.

The study protocol was approved by the Liverpool School of Tropical Medicine Ethical Committee and the National Ethical Committee in Yemen. Written informed consent was obtained from each participant or the parent/guardian of the child.

All symptomatic or asymptomatic children (<16 years) with sickle cell disease were recruited from the clinic or following hospital admission. Diagnosis was based on clinical manifestations, a positive sickling test, and confirmation by hemoglobin (Hb) electrophoresis. The clinical profile of the children enrolled has been previously described (25). Data were collected through a direct interview using a pre-structured questionnaire. A detailed health history was obtained and clinical examination was conducted by one observer (A-W. M. A-S). Data on family history, past medical history and frequency of acute clinical events covering a 1-year retrospective period was collected, including previous hospital admissions, blood transfusions, severity and frequency of crises, infection episodes, ACS, avascular bone necrosis, skin ulcers, gallstones, priapism and urinary disorders. Definitions of clinical events were those proposed by the Sickle Cell Disease Co-operative Study Group (26). One or both parents were interviewed and requested to provide all available medical documents (medical prescriptions, previous investigations, and notes of hospital discharge). Previous admissions were cross-checked against hospital records. Intake of folic acid or multivitamin supplements was recorded.

Clinical severity was assessed on the basis of frequency of painful crises, hospitalization, blood transfusion, episodes of infection and sickle cell-related complications. A severity index (SI) was calculated for each child using the total score for the preceding full year (27,28). A score of <6 disease was considered mild, and >6 severe. This cut-off facilitated comparison with previous studies categorising mild or severe cases (29).

A non-fasting venous blood sample of 5–7 mL was collected into a heparinized tube, at a standardized time (9–11 am), with the child in a sitting position. A fasting blood sample is preferred for homocysteine determination,

although collection after three hours of food intake is satisfactory (30). Blood specimens were centrifuged at room temperature within 5–10 minutes of collection and plasma aliquots stored at −70°C. A whole blood hemolysate was produced using freshly prepared 1% ascorbic acid solution. Biochemical analyses were completed at the Liverpool School of Tropical Medicine, Liverpool, Merseyside, UK, and genetic analysis at the Department of Blood Coagulation, Sanquin Laboratory, Amsterdam, The Netherlands.

Total plasma homocysteine, whole blood folate and plasma vitamin B12 were analyzed by automated methods using the DPC Immulite 2000 Analyzer (DPC-UK, Glyn Rhonwy, Gwynedd, Wales, UK). Analysis of tHcy was performed through a solid-phase, two-site chemiluminescent, immunometric assay (normal reference range in adult 5.0–12 μmol/L), the reference range in children under 15 years was reported to be lower (3–10.6 μmol/L) (31). Whole blood folate and plasma vitamin B 12 were measured by competitive, liquid-phase chemiluminescent assay. The manufacturer's reference values for whole blood folate and vitamin B12 were 43–295 ng/mL, and 174–878 pg/mL, respectively. Plasma pyridoxal-5'-phosphate (PLP) was determined by isocratic high performance liquid chromatography (HPLC) using fluorescence detection. The PLP is fluorescinated and extracted following a single precipitation step. A mobile phase consisting of 97% 25mM K_2HPO_4 pH 3.0: 3% MeCN is used at a flow-rate of 1.75 mL/min. (LDC ConstaMetric III pump; LCD/Milton Roy, Riviera Beach, FL, USA). Detection was accomplished using a fluorescence detector (ThermoSeparation Products FL2000; Thermo Separation Products, Saint Peters, MO, USA) with an excitation wavelength set at 320 nm and an emission wavelength of 415 nm. Prepared samples are compared to a set of plasma calibration standards to quantify the level of PLP. The reference level for vitamin B6 in our laboratory was (5–30 ng/mL).

DNA analysis was performed by restriction fragment length polymorphism (RFLP) after conventional polymerase chain reaction (PCR). The DNA encoding for the MTHFR gene was amplified using the following primers: sense primer 5'-TGA AGG AGA AGG TGT CTG CGG A-3'; antisense primer 5'-AGG ACG GTG CGG TGA GAG TG-3'. The PCR product was incubated with restriction enzyme *Hinf*I and the presence of the MTHFR 677T allele was assessed by gel electrophoresis.

All statistical analyses were carried out with the SPSS version 15 for Windows (SPSS Inc., Chicago, IL, USA). The independent sample *t*-test, χ^2 or Fisher's exact test and Mann-Whitney-U test were used for univariate analysis. Correlations were performed using Pearson's or Spearman's tests. A multivariate analysis was used to assess associations between clinical and biochemical variables, without correction for multiple testing. The level of significance was $p < 0.05$.

RESULTS

Of the 102 sickle cell disease children, 56 were male and 46 female with an overall mean age 7.2 years (range, 6 months to 15 years). Blood samples were obtained from 69 children while in steady state, and from 33 children during acute disease complications, 24 of whom required hospital admission.

The prevalence of homozygous TT of the *MTHFR* 677gene was 2% (2/102), and heterozygous CT 10.8% (11/102), giving an allele frequency of 7.35%. The observed homozygote to heterozygote ratio was consistent with the Hardy-Weinberg equilibrium ($p^2 = 0.76$, $2pq = 0.216$, and $q^2 = 0.04$) with similar allelic frequency between males and females (7.1 *vs.* 7.6%, $p = 0.92$), (Table 1). This ensured that there was panmixia and no selection against or in favor of certain genotypes.

Using the SI, 65 children (63.7%) scored as severe and 37 as mild (36.3%). Table 2 summarizes the association of sickle cell disease severity with the MTHFR genotype and allele frequency. There was no homozygous variant type in mild cases. No significant differences were observed between mild and severe cases in relation to either genotype distribution or allele

TABLE 1 Frequency of the MTHFR C677T Genotype

Genotype	Female ($n = 46$)	Male ($n = 56$)	Total ($n = 102$)	%	p Value
CC	40	49	89	87.2	Ref.[a]
CT	5	6	11	10.8	0.98
TT	1	1	2	2.0	0.88
Total allele frequency	7/92	8/112	15/204	7.3	0.90

CC: normal; CT: heterozygous; TT: homozygous.
[a]Reference group.

TABLE 2 The MTHFR Genotype Distribution and Allele Frequency by Clinical Severity of Sickle Cell Disease

	Genotype frequency, % (n)			Allele frequency, % (n)		
Degree of sickle cell disease	CC[a]	CT[b]	TT[c]	C	T	p Value[d]
Mild	30.4 (31)	5.9 (6)	0.0 (0)	91.9 (68)	8.1 (6)	0.75
Severe	56.9 (58)	4.9 (5)	2.0 (2)	93.0 (121)	7.0 (9)	–
p Value[e]	0.42	0.21	0.43	–	–	–

[a]Homozygous wild type.
[b]Heterozygous variant type.
[c]Homozygous variant type.
[d]Difference between C and T alleles.
[e]Difference between mild and severe.

frequency. There was no association between vascular complications (painful crisis, stroke, ACS) and the MTHFR genotype. A child with AVN and five children with stroke all had the wild MTHFR genotype.

The tHcy plasma concentration was available for 88 subjects; 59 in steady state and 29 with acute disease complications or when admitted to hospital. The mean (±SD) tHcy was 2.8 ± 1.7 µmol/L (range 1.9 to 9.8 µmol/L) and median 1.9 µmol/L. There was no difference in mean concentration between males and females (2.9 ± 1.8 *vs.* 2.7 ± 1.6 µmol/L, $p > 0.05$). The plasma tHcy increased with age and was higher in children older than 10 years compared to younger children (2.5 ± 1.2 *vs.* 3.6 ± 2.5 µmol/L, $p < 0.05$) (Table 3). The mean tHcy plasma concentration during acute disease complications or hospitalization was 2.75 ± 1.44 µmol/L which did not differ from that in the steady state of 2.86 ± 1.89 µmol/L, $p = 0.89$). Twenty-one children had tHcy over the upper inter-quartile range, although no child had hyperhomocysteinemia including three cases with stroke. The mean severity score was significantly increased with increasing tHcy concentrations from the lower quartile (3.8) to upper quartile (5.3), with a mean of 4.6 ± 3.7 µmol/L, $p < 0.001$).

Two children with homozygous TT had normal tHcy levels. There was no correlation between tHcy concentration with MTHFR genotype, disease severity, Hb level, or whole blood folate concentration. The median plasma pyridoxine level was 38 ng/mL (range 3.9–96) and four children had values below the reference range, three of these were scored as severe disease and the one who had the highest tHcy level had a CC genotype. Only one child had a vitamin B12 assay value below the normal range.

TABLE 3 Mean Homocysteine Concentration (µmol/L) in Relation to Demographic and Clinical Characteristics

	$n = 88$[a]	Mean ± SD	p Value
Sex:			
Male	41	2.7 ± 1.6	0.73
Female	47	2.9 ± 1.8	
Age:			
<10 years	62	2.5 ± 1.2	0.04
>10 years	26	3.6 ± 2.5	
Clinical Status:			
Steady state	59	2.9 ± 1.9	0.89
ADC/hospitalization[b]	29	2.8 ± 1.4	
Hemoglobin Concentration:			
<9.0 g/dL	71	2.8 ± 1.8	0.85
>9.0 g/dL	17	2.9 ± 1.7	

[a]Homocysteine analysis for 88 cases.
[b]ADC: acute disease complication.

TABLE 4 Plasma Concentration of Homocysteine, Folate, Vitamins B6 and B12 by MTHFR Genotype and Disease Severity

	MTHFR Genotype			Clinical severity		
	CC ($n = 89$)	CT/TT ($n = 13$)	p Value[a]	Mild ($n = 37$)	Severe ($n = 65$)	p Value[a]
tHcy (μmol/L)	1.9 (1.9–2.7)	1.9 (1.9–5.1)	0.54	1.9 (1.9–4.3)	1.9 (1.9–2.7)	0.96
Folate (ng/mL)[b]	521 (311–823)	605 (344–739)	0.77	437 (311–622)	571 (319–924)	0.15
Vitamin B6 (ng/mL)	38 (31–48)	40 (28–52)	0.75	40 (33–49)	36 (27–47)	0.49
Vitamin B12 (pg/mL)	439 (355–723)	395 (295–970)	0.61	402 (312–804)	460 (363–735)	0.52

Values given are median (inter-quartile range).

[a]Difference between CT/TT and CC genotypes, and between mild and severe (Mann-Whitney U test).

[b]Whole blood folate.

The median level of whole blood folate was 530 ng/mL (range 111–6720), with 22% of cases within the normal range and 78% above this range. Ninety-one children reported regular intake of oral folic acid in a dose of 5–10 mg daily and a few children intermittently used non iron multivitamin supplements There was no significant difference in mean tHcy values between those who had taken folic acid on a regular basis ($n = 81$) and those who had not ($n = 9$) tHcy (2.8 ± 1.8 μmol/L *vs.* 1.9 ± 1.0 μmol/L, $p = 0.14$). In univariate analysis there was no correlation of child age, whole blood folate, plasma vitamin B6, B12, or tHcy with MTHFR genotype (Table 4). Multiple regression analysis showed no association between these variables with MTHFR genotype. With disease severity as a dependent variable, child age had a significant negative association, [adjusted OR (odds ratio) = 0.21, 95% CI (confidence interval) 0.06-0.74, $p = 0.01$].

DISCUSSION

In Yemeni children with sickle cell disease, the MTHFR C677T genotype prevalence was 12.7% with an allele frequency of 7.4%.This is similar to prevalence estimates in sickle cell disease patients from Bahrain 8% (32), Jamaica 8.3% (21), USA 8.6% (18) and Guadeloupe 9% (22). A previous study of 46 subjects from Yemen, showed the CT heterozygous frequency to be 30.4% and homozygous TT to be 2.2%, with an allele frequency of 17.4% (33). The *cis* MTHFR mutation has not been studied in the Yemeni population. In the current study, the MTHFR TT frequency was not higher than in the general Yemeni population (2.0 *vs.* 2.2%), which is consistent with reports from other countries in the Arabian Peninsula (19,32,34), and elsewhere (22,23). The overall allele frequency was lower than that for the

general population (7.4 *vs.* 17.4%), as reported by Romana et al. (22), who observed an allele frequency of 9% in sickle cell disease patients compared to 15% in controls. There was a lack of an association with specific clinical events, suggesting little clinical impact of this gene variant on the course of sickle cell disease. The young age of many of these children with sickle cell disease would have resulted in a disease profile with few severe disease complications that are common in older children.

In this analysis there was no association between the MTHFR C677T genotype and overall sickle cell disease severity, or with individual clinical complications. This is in agreement with other studies which reported no difference in genotype distribution in relation to vascular complications (14,18–24). The number of cases with the TT genotype in all of these studies was small (range 0–8 cases), limiting their statistical power. A single study reported a significant MTHFR association with occurrence of the combined vascular complications (stroke, AVN, retinopathy and ACS), although this was not significant when each complication was considered separately (17). Kutlar et al. (16), compared 45 patients (>15 years) with AVN and 62 patients without, and observed a significant positive association between the MTHFR mutation and AVN (35.6 *vs.* 12.9%, $p = 0.006$). In the present study, only one child (15 years) with the wild MTHFR genotype developed AVN. A larger number of older children would be required to further assess this association.

The mean tHcy was reported to be increased in 46 sickle cell disease Caribbean patients from Curaçao compared to controls, although both cases and controls had comparable levels of plasma folate, vitamin B12 and B6 (13). Administration of folic acid (2–4 mg/day) decreased tHcy concentration by 53%. Elevated tHcy in sickle cell disease was related to pyridoxine deficiency (14). Other studies have reported no difference in plasma tHcy, folate or B12 concentrations between children with sickle cell disease and controls (23,35,36). All participants in this study received folate supplementation which may blunt the effect of MTHFR on tHcy.

Whole blood folate values in this study were higher than the reference range, which probably indicates good patient compliance with their long-term folate supplementation. The presence of a younger red cell population resulting from accelerated erythropoiesis, would increase folate levels as younger red cells have higher folate concentration (37,38). With sub-optimal folate status, plasma tHcy and folate are highly correlated in children with sickle cell disease (13), although this correlation is not maintained when folate status is satisfactory (35,36), as observed in the present study. In individuals with MTHFR C677T, the inverse relation between plasma tHcy and plasma folate was intensified with the number of T alleles (39,40) and homozygous TT has been associated with lower folate level and higher tHcy concentration (7,41). Folic acid supplementation reduced and

often normalized elevated tHcy in individuals with this enzyme defect (10,42,43). The two children with homozygous TT in this study had normal tHcy levels. Folate deficiency and hyperhomocysteinemia were absent in these children, probably reflects their adequate level of compliance with folic acid supplementation. Nevertheless, the importance of good compliance with folate supplementation should be emphasized as it may not always be optimal.

In conclusion, the frequency of the MTHFR C677T genotype in Yemeni children with sickle cell disease was not higher than expected in the general population. In this Yemeni population, screening for the MTHFR mutation would be unlikely to identify children at high risk for severe and complicated disease.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Severity of Sickle Cell Disease in Yemeni Children

Homozygous sickle cell disease (SCD) is characterized by a highly variable clinical course, and marked individual variation in disease severity [1]. Quantitative severity assessment could provide a useful tool for patient selection for therapeutic and preventive interventions [2]. In children, severity assessment is more difficult than adults due to age-related clinical, haematological and immunological changes [3]. We assessed quantitatively SCD severity in Yemeni children. The study was conducted at Al-Wahda Hospital, Aden. Children (<16 years) with confirmed Hb SS by electrophoresis were included, as previously described [4]. A Severity Index (SI) was calculated based on frequency of painful crises, hospitalization, blood transfusion, infection and specific complications, during the previous year. Children were grouped into mild (≤6) and severe (>6) score categories [5].

One hundred and two children (56 males) were enrolled, (mean age 7.2 years). Sixty-five children (63.7%) categorized as severe and 37 (36.3%) as mild. Severe symptoms were more frequent in females, and increased with age from 5 years ($p<0.01$) (Fig. 1). Females had a higher rate of painful crisis (crises/child/year) than males (3.16 vs 2.0, $p<0.05$). Painful crisis, hospital admission and blood transfusions were more frequent in the severe category (all $p<0.001$) (Table 1). Stroke (4.9%) and gallbladder stone (4.9%) occurred only in children with severe disease. Malaria was diagnosed in 21 children (16 in the severe and 5 in the mild group). Percentage of Hb F was lower in severe cases ($p<0.01$). Polymorphonuclear leucocytes, total and direct bilirubin were higher in severe cases (all $p<0.05$). There were no significant differences for Hb, serum total protein, albumin, liver transaminases, BUN and creatinine.

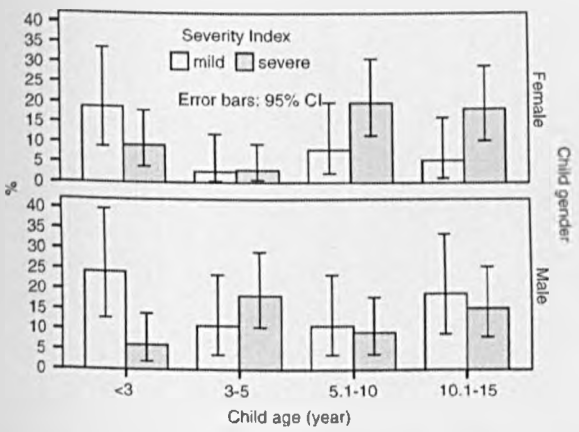

FIG. 1. Child age category by gender and severity index of homozygous SCD.

This is the first reports of two forms of SCD in Yemen. Clinical variation in Saudi patients has been related to different levels of Hb F and the presence of African and Arab-Indian βs haplotypes [6]. In Yemen βs haplotype is unknown, although the diverse clinical manifestations suggest mixed polymorphisms. We used the SI proposed by El-Hazmi [5], which had been validated in previous studies from Saudi Arabia [5, 7–9] and Africa [10]. The average SI in Yemeni children was 7.8, with 63.7% scored as severe, which compares to 73.8% in Saudi Arabia [7], and 51.7% in Senegal [10].

A severe SI was more frequent in females. Previous studies have reported higher incidence of painful

TABLE 1
Clinical events, complications and relevant haematological and biochemical parameters by SI category

Parameter	Mild ($n=37$)	Severe ($n=65$)
Painful crisis***, *n* (%)		
0	7 (18.9)	5 (7.7)
1–2	29 (78.4)	29 (44.6)
≥3	1 (2.7)	31 (47.7)
Hospitalization***, *n* (%)		
0	18 (48.6)	7 (10.8)
1–2	19 (51.4)	53 (81.5)
>3	0 (0)	5 (7.7)
Blood transfusions***, *n* (%)		
0	24 (64.9)	22 (33.9)
1–2	13 (35.1)	37 (56.9)
≥3	0 (0)	6 (9.2)
Acute chest syndrome, *n* (%)	4 (10.8)	10 (15.4)
Stroke, *n* (%)	0 (0)	5 (8.8)
Gallstone, *n* (%)	0 (0)	5 (8.8)
Enuresis, *n* (%)	9 (24.3)	23 (35.4)
Leg ulcer, *n* (%)	0 (0)	5 (7.0)
Gross haematuria, *n* (%)	1 (2.7)	8 (12.3)
Hb (g/dl)	7.9 ± 1.5	7.6 ± 1.4
WBC ($\times 10^9$/l)	10.4 ± 5.6	10.2 ± 5.1
Polymorphs (%)*	34.4 ± 12.5	39.9 ± 12.0
Hb F (%)**	5.8 ± 5.6	3.0 ± 1.9
Total bilirubin (μmol/l)***	25.6 (14.9–35.9)	42.7 (27.4–64.9)
Direct bilirubin (μmol/l)**	13.7 (6.7–22.2)	20.5 (13.7–30.8)

Values are given as *n* (%), mean ± SD, median (Interquartile range, IQR).
*$p<0.05$, **$p<0.01$, ***$p<0.001$ (*t*-test, or Mann–Whitney U test).

crisis in females during the first decade of life [8], and in males after 15 years [9]. The reasons for these age and sex differences remain unclear.

The low mean Hb F% (4.0 ± 3.9) was comparable to reports from Riyadh (3.6 ± 2.9) and Qunfuda (5.0 ± 3.9) in Saudi Arabia [10]. Even with low Hb F% small increments may ameliorate painful crises, a core component of the severity scoring system [9]. There was a higher frequency of malaria in severe SCD. These children will more frequently be symptomatic, and have different haematological and immunological responses.

This report on SCD severity from the Yemen highlights disease variability in this region. The use of SI could facilitate comparison between geographically diverse study groups.

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Growth and nutritional status of children with homozygous sickle cell disease

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Abstract

Background: Poor growth and under-nutrition are common in children with sickle cell disease (SCD). This review summarises evidence of nutritional status in children with SCD in relation to anthropometric status, disease severity, body composition, energy metabolism, micronutrient deficiency and endocrine dysfunction.

Methods: A literature search was conducted on the Medline/PUBMED, SCOPUS, SciELO and LILACS databases to July 2007 using the keywords sickle cell combined with nutrition, anthropometry, growth, height and weight, body mass index, and specific named micronutrients.

Results: Forty-six studies (26 cross-sectional and 20 longitudinal) were included in the final anthropometric analysis. Fourteen of the longitudinal studies were conducted in North America, the Caribbean or Europe, representing 78.8% (2086/2645) of patients. Most studies were observational with wide variations in sample size and selection of reference growth data, which limited comparability. There was a paucity of studies from Africa and the Arabian Peninsula, highlighting a large knowledge gap for low-resource settings. There was a consistent pattern of growth failure among affected children from all geographic areas, with good evidence linking growth failure to endocrine dysfunction, metabolic derangement and specific nutrient deficiencies.

Conclusions: The monitoring of growth and nutritional status in children with SCD is an essential requirement for comprehensive care, facilitating early diagnosis of growth failure and nutritional intervention. Randomised controlled trials are necessary to assess the potential benefits of nutritional interventions in relation to growth, nutritional status and the pathophysiology of the disease.

Introduction

It is generally accepted that homozygous sickle cell disease (SS) impairs physical growth during childhood and early adolescence and that affected children are lighter and shorter than healthy counterparts. Growth retardation in sickle cell disease (SCD) is complex and multiple factors are likely to contribute, such as the haematological and cardiovascular state, social factors, endocrine function and metabolic and nutritional status.[1] Growth rate is inversely related to the degree of anaemia and is likely to be associated with deficiency of specific nutrients as well as low nutrient intake, decreased absorption and increased losses or utilisation.[2,3]

For example, the prevalence of underweight in American children with SCD was

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41% for moderate and 25% for severe under-nutrition[4] with a prevalence of wasting of 11%.[5] Stunting was reported in 44% of Ghanaian children and adolescents and almost all those with SS were underweight, irrespective of height.[6]

Although growth failure and under-nutrition are common, the underlying mechanisms have not been well studied and the precise role of intrinsic or extrinsic factors is unclear in relation to inadequate food intake or increased demands associated with higher energy expenditure and requirements. External and internal factors are likely to act together to a different degree against a variable genetic, environmental and socio-economic background. The aim of this review is to summarise the evidence related to poor growth and under-nutrition in children with SCD with regard to anthropometric status, disease severity, body composition and metabolism, micronutrient deficiency and endocrine dysfunction. An important aspect of these analyses is determining whether phenotype, nutritional deficits or anaemia individually contribute to growth restriction, or whether it is a combination of these factors which is important.

Methods

A literature search using the Medline/PUBMED, SCOPUS, SciELO and LILACS electronic databases for studies published up to July 2007 was conducted. The search terms sickle cell combined with nutrition, anthropometry, growth retardation, height and weight, body mass index (BMI) and specific micronutrients (zinc, iron, vitamins A, B group, C, D, E and folate) were used. Additional articles were identified by checking reference lists of retrieved articles. From a total of 423 published studies, 42 with relevant data (25 cross-sectional and 17 longitudinal) were selected. In addition, data were made available from unpublished studies (one cross-sectional and three longitudinal). The following data were extracted from these studies: age, disease severity, clinical presentation and growth parameters, use of blood transfusion, therapeutic interventions, micronutrient status and other nutritional and endocrine assessments, and haemoglobin genotype. The resulting data were tabulated by geographical location, age, anthropometric characteristics and types of controls.

There are four major genotypes within the definition of SCD: homozygous sickle cell (SS) disease, sickle haemoglobin C (SC) disease, sickle cell β^+ thalassaemia (S β^+ thalassaemia) and sickle cell β^0 thalassaemia (S β^0 thalassaemia).[7] The internationally accepted definition of SCD, two β-globin gene variants at least one of which is the sickle cell gene, is used and the gene variant for the four common genotypes are indicated when known. In this review, the term 'sickle cell anaemia' is used synonymously only for homozygous SS disease, and the majority of studies reviewed relate to this genotype.

Results

Nutritional status and disease severity

Inadequate intake can result from anorexia, a prominent symptom in affected children even in the absence of demonstrable infection, and it often precedes a painful crisis by days or weeks.[8] At the time of hospital admission, energy intake during acute illness is decreased by as much as 44% of the recommended daily amount (RDA) (SD 9%); during follow-up, intake is closer to 90% of RDA.[9] Dietary intakes can be reduced markedly prior to admission and remain sub-optimal for weeks.[10] In a Jamaican study, no significant relationship was demonstrated between haemoglobin concentration, reticulocyte count or irreversibly sickled cells and anthropometric measurements. Correlation with disease severity, measured by the number of

hospital admissions, showed no significant association with growth parameters, although a trend towards lower mean weight was found in patients who were admitted more often.[11] In pre-pubertal Jamaican children, levels of haemoglobin (Hb) and fetal haemoglobin (Hb F) decreased with an increasing number of hospitalisations of both sexes, although levels were positively associated with height and weight only in males.[12]

Vaso-occlusive crises and episodes of infection could increase energy expenditure.[13] A strong association between C-reactive protein and resting energy expenditure has been described, which might indicate a link between inflammation and a hyper-metabolic state in SCD.[14] Increased resting energy expenditure (REE) might relate to erythroid hyperactivity and accelerated red cell turnover owing to the short life span of sickled red blood cells. Low Hb levels and chronic anaemia are associated with hyperdynamic circulation and deterioration of cardiopulmonary function. This increases workload and, consequently, the demand for energy and nutrients.

There is evidence that nutrient supplementation can reduce clinical illness. Supplements given by the nasogastric route to SCD children with growth retardation (weight and height <5th centile) led to a rapid and sustained increase in growth and a reduction of pain crises and episodes of infection.[15] The authors found no lipid malabsorption and a normal histological appearance of the intestinal mucosa and submucosa and concluded that inadequate energy intake was responsible for the growth retardation.

Other therapeutic measures to reduce disease severity or complications (i.e. blood transfusion, splenectomy and hydroxyurea) might lead to improved nutritional status and growth. Children in the Stroke Prevention Trial in Sickle Cell Anaemia (STOP) who received transfusion regularly over a 2-year period demonstrated significant improvement in height, weight and BMI, with growth *Z*-scores approaching normal.[16] Those with homozygous SCD showed a significant reduction in whole body protein turnover (from 8.9 g/kg/d to 6 g/kg/d) after splenectomy, thereby contributing to positive energy balance[17] and acceleration in linear growth.[18] Therapy with hydroxyurea has been reported to decrease REE in treated SS children, suggesting that it might curtail a hypermetabolic state and offer clinically important secondary benefit.[19] In the Hydroxyurea Safety and Organ Toxicity (HUSOFT) extension study, improved growth rates were demonstrated in SS children treated with hydroxyurea. Their increased weight and height resulted in a growth pattern similar to that of children with Hb S β^+ thalassaemia or healthy controls.[20] Studies related to growth, specific micronutrients and disease severity are considered in later sections of this review.

Growth studies

Studies reporting growth of patients with SCD are summarised in Tables 1–6. Adult patients are often described as slender with low weight, relatively tall with long extremities, short trunk, narrow shoulders and hips, with a deep chest and increased anterior-posterior diameter. Many of these changes were found to be less pronounced and inconsistent in children, and some investigators considered this appearance in SCD to be an exaggeration of the normal characteristics of Africans.[21]Affected children were reported to have poor nutrition and their weight was consistently below the median reference values.

North American studies (Table 1). An early study of the growth of 48 American black children with sickle cell anaemia (aged 2–13 yrs) reported that the majority were thin with low weight and height. There was no correlation between growth parameters and the clinical course, arterial oxygen saturation or family childhood weight patterns.[22]

TABLE 1. *North American studies.*

Reference*	Year	Country	*n*	Design	Age (y)	Weight†	Height†	Other assessments	Controls	Comment
Whitten[22]	1961	USA	48	CS	2–13	96% <5th centile	81% <5th centile	Normal span & U/L segment	79 siblings Stuarts norms	No correlation with C/P or family weight pattern
Booker[25]	1964	USA	18	L	0–2	Around –2 SD	–	Deceleration began at age 6 m	Normal blacks *n*=86	Deficit coincides with start of infection and crises
Jimenez[23]	1966	USA	38	CS	8–17	Significantly lower mean	Significantly lower mean	Hypogonadism	Normal black children, *n*=89	Low U/L segment Span >height
McCormack[24]	1976	USA	46	CS	1–17	Significantly lower mean	Significantly lower mean	Low MUAC and calf circumference Bone age retarded	26 AS, standard of local black children, *n*=900	Delayed skeletal maturation in sickle cell trait (AS)
Kramer[26]	1980	Canada	14 10	L	0, 4, 5	Normal at birth, low subsequently	Normal at birth, low subsequently	Muscle mass area and HC not greatly affected	Black term newborn, *n*=71	Growth deficit started at 6 mths of age & increased over time
Luban[27]	1982	USA	55	L	13–18	Significantly below reference	Significantly below reference	Delayed sexual development Bone age retarded	NCHS reference	Hormonal assays normal in majority
Platt[28]	1984	USA	2115	CS	2–25	Significantly below reference	Significantly below reference	Sexual developmental delay	Howard University study of black children, *n*=2632	Growth deficit in SS >S β thalassaemia >SC, delayed menarche related to low weight
Phebus[29]	1984	USA	133	L	1–18	All <50th centile	All <50th centile	Maximum growth velocity after 14 y (F) & 16 y (M)	NCHS reference	Growth deficit by 2 yrs, M>F
Henderson[5]	1994	USA	63	CS	3–18	14% <5th centile	13% <5th centile	25% <5th centile 11% wasting (low wt/ht)	NCHS reference	Impaired growth & puberty in 11–18-yr-olds
Williams[98]	1997	USA	61	CS	2–17	22% <5th centile	19% <5th centile	Inadequate nutritional intake	NCHS reference	59% families below poverty line
Cepeda[30]	2000	USA	30	CS	8–19	Significantly low mean difference by average 12 kg	Significantly low mean difference by average 8 cm	Delayed sexual maturation by average 0.75 Tanner stage	Age, sex, race & socio–economic–matched, *n*=30	No significant difference in self–esteem or body image
Wang[16]	2005	USA	94	L	2–16	WAZ −0.71 score	HAZ −0.51 score	BMI −0.60 *Z*-score	NCHS reference Transfused 53 Standard care 41	Improved growth on long-term transfusion
Zemel[31]	2007	USA	148	L	0–18	26% <5th centile	22% <5th centile	BMI <5th centile in 24%, puberty delayed by 1–2 y	NCHS reference	Puberty affected by impaired growth & haematological status in F

* First author; CS, cross-sectional; L, longitudinal; F, female; M, male; C/P: clinical picture; HC, head circumference; MUAC, mid upper-arm circumference; BMI, body mass index; WAZ, weight-for-age *Z*-score; HAZ, height-for-age *Z*-score; † weight or height-for-age unless otherwise stated.

TABLE 2. *Jamaican studies.*

Reference*	Year	*n*	Design	Age (y)	Weight†	Height†	Other assessments	Controls	Comment
Ashcroft[35]	1972	99	CS	12–21	Mostly >–2SD	Variable	Bone age retarded >–2SD	Jamaican standard & local students, *n*=235	Younger cases shorter, older cases as tall as controls
Lowry[11]	1977	99	CS	2–13	Lower mean at all ages	No significant difference	Haematological parameters not correlated with deficit	Jamaican rural standard, *n*=2765	No correlation with hospital admission rate
Ashcroft[36]	1981	82	L	12–21	All below median	Below median	Menarche delayed by 2.3 y	Jamaican rural standard, *n*=12,934	Height exceeded standard by ages 16 (F) & 18 y (M)
Stevens[37]	1983	64	L	4–6	Significantly lower mean than controls	Significantly lower mean than controls	Low MUAC & short limbs	Normal AA, sex- & age-matched, *n*=123	Standing/sitting height normal
Stevens[32]	1986	455	L	0–9	Significantly lower mean than controls	Significantly lower mean than controls	Sexual & skeletal delay, SC not affected	Age- & sex-matched, *n*=231	Deficit began 2 y earlier in F than in M
Thomas[39]	2000	315	L	0–18	Normal at birth, low subsequently	Normal at birth, low subsequently	Growth catch-up at ages 15 (M) & 18 y (F)	NCHS reference	Growth reference curves produced from data

* First author; CS, cross-sectional; L, longitudinal; F, female; M, male; MUAC, mid upper-arm circumference; AA, normal adult haemoglobin; † weight or height-for-age unless otherwise stated.

TABLE 3. *Latin-American studies.*

Reference*	Year	Country	*n*	Design	Age	Weight†	Height†	Other assessments	Controls	Comment
Souza[40]	1983	Brazil	14	CS	6m–12y	All <10th centile	All <10th centile	Low serum zinc High serum cupper	NCHS reference	No correlation between zinc levels & growth deficit
Britto[42]	1985	Brazil	34	CS	6–20y	Significantly lower mean than controls	No significant difference	Menarche & bone age significantly lower than controls	AA $n=16$	Controls matched by age, race, economic status
Zago[43]	1992	Brazil	125	CS	7m–20y	40% <10th centile	31% <10th centile	Delayed sexual maturation	$n=1041$ & Brazilian standard	Post-pubertal weight deficit
Pellegrini-Braga[44]	1995	Brazil	34	L	0–18y	Significantly lower mean than controls	Significantly lower mean than controls	Growth velocity impairment, bone age delay, low serum zinc & ferritin	Siblings AS $n=9$ Non-siblings AA $n=35$	Growth deficit tends to increase with age. Hypercupraemia
Cipolotti[45]	2000	Brazil	76	CS	9m–20y	Median <50th centile	Median <50th centile	41% < expected parental height	NCHS reference	Father's height obtained from records
Silva[33]	2002	Brazil	100	L	5m–8y	WAZ –0.70 score	HAZ –0.65 score	Low BMI	NCHS reference	Growth deficit in SS >SC & M >F
Gonzáles-Fernández[46]	1992	Cuba	110	CS	4m–17y	No significant difference	No significant difference	No significant difference in bone age	Cuban standard	No significant differences in gestational age or birth weight

*First author; CS: cross-sectional; L: longitudinal; F: female; M: male; AA: normal adult haemoglobin; BMI: body mass index; WAZ: weight-for-age *Z*-score; HAZ: height-for-age *Z*-score; †weight or height for age unless otherwise stated.

TABLE 4. *African studies.*

Reference*	Year	Country	*n*	Design	Age (y)	Weight†	Height†	Other assessments	Controls	Comment
Mpemba-Loufoua[51]	2001	Congo	72	CS	10–18	Significantly lower mean than controls	Not measured	71% of cases no menarche at 14–18y, 10% in controls	AA females *n*=40	Only females included. Sexual maturity delayed in 37%
Mabiala-Babela[50]	2005	Congo	91	CS/L	8–14	Significantly lower mean than controls	Significantly lower mean than controls	Lower BMI, lean body mass, body fat %	AA *n*=95	Body composition decreased more in cases with severe disease
Thuilliez[52]	1996	Gabon	131	L	0–18	26.7% >–2SD	26.7% >–2SD	Pubertal delay in 13% Menarche mean age 15y	African multi-ethnic reference	Growth deficit increased with age
Ebomoyi[47]	1989	Nigeria	719	CS	2–13	All <50th centile	All <50th centile	MUAC <50th centile	Local controls *n*=979 & Harvard standard	SS growth less than controls & standards
Oyedeji[48]	1991	Nigeria	102	CS	9m–17y	All <3rd centile	Around 3rd centile of reference	Symptom frequency & education	Nigerian elites *n*=421	Low school performance & high school absence
Modebe[34]	1993	Nigeria	20	CS	17–35	Significantly lower mean in males	Significantly lower mean in males	Low BMI, MUAC & skin folds in males. Low daily energy intake in males, normal in females	Normal siblings of similar age *n*=15	Gender-related growth difference. Small sample for older group
Oredugba[49]	2002	Nigeria	177	CS	1–18	Around 3rd centile of reference	Around 3rd centile of reference	Low MUAC in 21% with maxillary protrusion & malocclusion.	Normal children *n*=122, local anthropometric reference	72% of cases & controls of low socio-economic status. No significant growth differences
Athale[53]	1994	Zambia	144	CS	10–38	60% <5th centile	53% <5th centile	Delayed sexual maturation. Educational delay & high school drop-out	NCHS reference	Children >10y included. Frequent psychosocial problems

*First author; CS, cross-sectional; L: longitudinal; F, female; M, male; AA, normal adult haemoglobin; MUAC, mid upper-arm circumference; BMI, body mass index; †weight or height for age unless otherwise stated.

TABLE 5. *The Middle East and India.*

Reference*	Year	Country	*n*	Design	Age (y)	Weight†	Height†	Other assessments	Controls	Comment
Soliman[54]	1999	Egypt	182	L	1–20	–	27% <−2 *Z*-score 67% <−1 *Z*-score	Low MUAC, U/L segments, delayed sexual maturation	Normal *n*=200. Constitutional GR *n*=30, GH defect *n*=25	Slow linear growth velocity increased with age, transfusion no effect
Mansour[55]	2003	Iraq	75	CS	18	77% <5th centile	47% <5th centile	BMI <20 in 77%, delayed sexual maturation	Males *n*=75 NCHS reference	All patients male, marked GR in severe disease
Jaiyesimi[56]	2002	Oman	97	CS	10m–12y	68% <5th centile 4% >50th centile	–	Moderate/severe disease in 71%	Age, sex-matched *n*=97 & NCHS reference	Compared with Jamaican reference 14% <3rd & 21% >50th centiles
Perrine[57]	1981	Saudi	21	L	0–3	No significant difference	No significant difference	No developmental delay	USA & Saudi references *n*=21	Mild disease with high Hb F levels
Al-Saqladi	2007	Yemen	102	CS	0.5–15	72% WAZ <−2 *Z*-score	55% HAZ <−2 *Z*-score	52% BMI <−2 *Z*-score. Low MAUC	NCHS reference	Author's unpublished data
Mukherjee[58]	2004	India	58	CS	2–14	Significantly lower mean than controls	Significantly lower mean than controls	Low BMI, MUAC, sitting height, skinfold thickness	Normal AA *n*=86	Arab–Indian haplotype with severe disease

*First author; CS, cross-sectional; L, longitudinal; F, female; M, male; HC, head circumference; MUAC, mid upper-arm circumference; BMI, body mass index; WAZ, weight-for-age *Z*-score; HAZ, height-for-age *Z*-score; GR, growth retardation; GH, growth hormone; AA, normal adult haemoglobin; † weight or height for age unless otherwise stated.

TABLE 6. *European studies.*

Reference*	Year	Country	*n*	Design	Age (y)	Weight†	Height†	Other assessments	Controls	Comment
Caruso-Nicoletti[59]	1992	Italy	76	CS	1–17	16% <3rd centile	80% <50th centile 10.5% <3rd centile	Benin haplotype in majority. Normal level somatomedin C	British reference (whites)	Moderate growth deficit. No difference between SS & βS thalassaemia
Dickerhoff	2007	Germany	341	L	2m–43y	12.6% <3rd centile	17.3% <3rd centile	–	German & Turkish references	Unpublished data
Fijnvandraat	2007	Netherlands	91	L	5–15	Weight/height 2.8% <−2 SD Age 5: 3% Age 10: 2% Age 15: 3%	25% <−2 SD Age 5: 10.6% Age 10: 14.3% Age 15: 50%	–	Dutch reference (whites)	Author's unpublished data
Mann[60]	1981	UK	96	L	3m–19y	–	11–16% <−2 SD	Varied clinical manifestations. Low mortality	British reference (whites)	Ethnic origin: West Indies, Africa, Yemen
Patey[61]	2002	UK	56	CS	3–9	Mean weight *Z*-score 0.32 Mean (AC) *Z*-score 0.93	Mean height *Z*-score 0.28 Mean (AC) *Z*-score 0.59	Mean BMI *Z*-score 0.23 similar to (CC) 0.30 but lower than (AC) 0.82	Caucasian $n=57$ African/Caribbean (AC) $n=63$	Significant difference compared with similar ethnic group
Telfer	2007	UK	180	L	2–15	6.5% <−2 *Z*-score Age 2: 3.7% Age 5: 3% Age 10: 8% Age 15: 11.5%	4.2% <−2 *Z*-score Age 2: 2% Age 5: 1.5% Age 10: 6.5% Age 15: 6.6%	4.2% <−2 *Z*-BMI score	Tanner reference	Unpublished data

*First author; CS: cross-sectional; L: longitudinal; F: female; M: male; HC: head circumference; BMI: body mass index; AC: African/Caribbean; CC: Caucasian; †weight or height for age unless otherwise stated.

Jimenez *et al.*[23] compared 20 SS females with 774 race-matched controls (11–40 yrs). There was delay in onset of menarche and age at first pregnancy, decreased fertility and an increased incidence of abortion and premature delivery. In a separate group of 38 cases in the same study, a low weight, height and upper-to-lower segments ratio was observed compared with 89 control black children of the same age. McCormack *et al.*[24] reported the growth of 46 American black children and adolescents with SS disease. In all age groups (1–17 yrs), they had lower mean height, weight, mid-upper-arm circumference (MUAC), thinner body build and delayed skeletal maturation compared with controls.

Height and weight deficit probably occurs early in life. Booker *et al.*[25] reported weight deceleration starting at about 4–6 months of age, coinciding with the onset of crises and infections and continuing during the 1st 2 years of life. Age-related growth deficit will be difficult to demonstrate accurately with longitudinal birth cohort studies until neonatal screening for haemoglobinopathies becomes more widely available. In a prospective study of 14 Canadian neonates with Hb SS, Kramer *et al.*[26] found no significant differences in birthweight or length compared with controls, indicating an absence of disease effect on fetal growth.[26] During follow-up of ten pairs of these children to 3–6 years of age, a growth deficit was noted from about 6 months of age.

In a 3-year longitudinal study which included 26 boys and 29 girls with sickle cell anaemia (13–18 yrs), there was subnormal weight and height and significant retardation in growth velocity. Skeletal maturation and sexual development were significantly retarded but, with adjustment for bone age and Tanner staging, sexual development was considered appropriate for bone age.[27]

A larger, cross-sectional, multi-centre study was undertaken which included 2115 cases with different sickle cell syndromes (1404 SS and the remainder with SC disease, S β^+ thalassaemia or S β^0 thalassaemia).[28] The mean height and weight of affected subjects were significantly below reference values and the difference became apparent after 7 years of age. Children with Hb SS and S β^0 thalassaemia were consistently smaller and less sexually mature than those with SC disease and S β^+ thalassaemia. Sexual maturation followed the pattern of height and weight, and time of menarche correlated well with weight and age.

Height and weight impairment at all ages and in both sexes compared with published growth reference values was reported in a cohort study of 133 SS American children followed from early childhood to adolescence.[29] The deficit in height and weight had commenced by 2 years, increased with age and was more pronounced in males of all ages. Growth velocity curves for 13 adolescents showed significant delay of pubertal growth. The mean difference in weight and height in a study of 30 SS children (8–19 yrs) paired with matched controls of the same age, sex, race and socio-economic status was a deficit of 12 kg weight and 8 cm height, with a 0.75-year delay in sexual maturation based on Tanner staging.[30] No difference in body image was detected between cases and controls. A recent longitudinal study of 148 SS children showed that the growth deficit for one or more indicators occurred in 84% of subjects, and 26%, 22% and 24% were <5th reference centile for weight, height and BMI, respectively. Puberty was delayed by 1–2 years. Disease severity assessed by hospitalisation, blood transfusion and haematological status was associated with longitudinal growth in females but not in males.[31] The cause for this sex difference is unclear, but other studies have reported similar findings and related it to differences in the level of Hb, Hb F, energy intake and hormonal changes, especially at the time of puberty.[12,29,32–34]

Jamaican studies (Table 2). Ashcroft *et al.*[35] studied growth in 99 adolescents (12–21

yrs) with sickle cell anaemia who had low mean weight and delayed skeletal age (based on hand radiography) compared with normal and sickle cell-trait (AS) controls. Height differences were variable: younger patients were shorter whereas older ones were as tall as controls.

Lowry *et al.*[11] studied 99 SS children (2–13 yrs) and reported a mean value for weight below Jamaican reference values for both sexes, although little difference was observed in height. In their follow-up study of 82 SS children (2–21 yrs), Ashcroft & Serjeant[36] reported that, while the weight deficit persisted, height continued to increase and final height was equal to or better than that of normal subjects. This was presumed to be a result of delayed epiphysial fusion with final height determined by the degree of delay. In a further study, the anthropometric measurements of 64 SS children showed a significant deficit in mean weight, height and MUAC by 4–6 years.[37] Limbs were shorter than those of controls, although the sitting–standing height ratio was normal.

A longitudinal study of children with SS and SC disease, followed from birth to 9 years of age and compared with normal AA controls, showed no birthweight differences for either gender; the weight deficit in the SS children commenced before the end of the 1st year of life.[32] The deficit appeared to be relatively more marked in girls and a similar trend was observed for height. Weight and height velocity deficits increased after the age of 7 years and there was a bone age difference by 5 years with a retardation of 0.4 years in boys and 0.6 years in girls. By the age of 8, this had increased to 1 and 1.3 years in boys and girls, respectively. Children with SC disease showed no growth deficit.[32] The time of the growth spurt was delayed by 1.4% years in 44 homozygous SCD adolescents and normal height was attained by 17.9 years.[38]

Disease-specific growth reference curves for children with homozygous SCD were produced using data obtained from a cohort of 315 children aged 0–18 years by the LMS (lambda-mu-sigma) method which is used to normalise and smooth growth centile curves.[39] Values from the LMS smoothed curves were used to generate centiles expressed at selected ages as standard deviation scores (*Z*-scores) using NCHS growth reference standards. Mean height and weight at birth in both sexes were similar to reference values but fell away subsequently before catching up at around 15 years in girls and 18 years in boys.[39] The applicability of this reference curve to countries other than Jamaica needs to be evaluated.

Latin-American studies (Table 3). In a study of 14 SCD Brazilian children (6 mths–12 yrs), all had growth retardation and weight and height were <10th centile of the NCHS reference.[40] Serum zinc levels were low but not correlated with growth deficit. Low serum zinc was also reported in 18 SS Venezuelan children.[41] In 34 Brazilian SCD patients (6–20 yrs), low weight-for-age but not height-for-age was significantly associated with delayed menarche and bone age.[42] Compared with pubertal matched controls, no difference in levels of serum-follicle stimulating hormone (FSH) or luteinising hormone (LH) before or after LH–FSH stimulation tests was detected. Another Brazilian study of 86 SS patients under 20 years of age reported weight and height <10th centile in 40% and 31% of cases, respectively, and the weight deficit persisted after puberty.[43] In a follow-up of 34 SS Brazilian patients (0–18 yrs), impaired growth velocity increased with age, and reduced weight and height were associated with low serum zinc and ferritin levels.[44] Family height channels were evaluated in 76 SCD children (9 mths–20 yrs) from Brazil and corrected for parental height. Overall, allowing for mid-parental height, 41% were below the expected centile value and did not attain normal height and weight in adulthood.[45] Although the maximum growth velocity occurred later than normal owing to delayed puberty, the magnitude of this spurt did not compensate

for the early growth delay and final size remained below normal. This contrasts with some Jamaican studies[36,38] and the difference might relate to genetic factors governing parental stature. In another group of 73 SS Brazilian children using NCHS reference values, comparison of *Z*-scores for height or weight-for-age and weight-for-height showed that almost 10% of cases were under-nourished (*Z*-score $\leqslant 2$).[33] After 1 year of follow-up, the weight- and height-for-age deficits became significant and were greater in boys. Conversely, Gonzáles *et al.*[46] reported no significant difference in weight, height and bone age in 110 SCD Cuban children less than 17 years of age (74 SS cases) compared with Cuban standards.

African studies (Table 4). Anthropometric values for weight, height and mid-arm circumference of 719 SS Nigerian children were reported to be <50th centile of the Harvard standards, the most marked deficit being weight-for-age.[47] Compared with healthy Nigerian children, 85 SS children (9 mths–17 yrs) showed weight and height below and around the 3rd centile.[48] In a study of 20 adults, anthropometric measurements were lower in males but not in females.[34] This was associated with lower daily energy and macronutrient intake by males than by controls. A further study of 177 Nigerian children and adolescents (1–18 yrs) with SCD reported anthropometric values close to the 3rd centile of reference values with no significant difference between cases and controls except at the age of 18 years.[49] A high prevalence (21%) of maxillary prognathism and malocclusion was reported among cases. However cases and controls were mostly from a lower socioeconomic class, which might explain the lack of significant differences in anthropometric measurements between the groups. Evaluation of body composition in 91 Congolese SS children (8–14 yrs) showed significantly lower mean weight, height, BMI, lean body mass and percentage of body fat than in age-matched AA controls. Alteration in body composition correlated to the frequency of painful and anaemic crises.[50] Delayed sexual maturation was observed in 72 homozygous SCD Congolese girls with delay in the age at thelarche and menarche. Menarche had not occurred by 14–18 years in 71% of these cases compared with 10% of controls.[51] In a study from Gabon, 27% of 131 children with sickle cell anaemia (<18 yrs) had weights and heights <-2 SD compared with African multi-ethnic reference values.[52] In Zambian children with sickle cell anaemia, 60% and 53% were <5th centile for weight and height, respectively, compared with NCHS reference values.[53]

Middle East and India (Table 5). In a group of transfusion-dependent Egyptian children which included 110 cases of SCD, height was <-2 SD in 27%, and 51% showed a growth velocity <-1 SD. MUAC, triceps skinfold thickness and BMI were significantly lower than in controls, and linear growth was delayed increasingly with age.[54] Despite regular blood transfusion, onset of puberty and sexual maturation were delayed. Mean adult height was not attained in 96% of 75 SCD male Iraqi patients who were all 18 yrs of age, and 45% had delayed sexual maturation.[55] In 97 Omani children (90 SS, 7 S β^0 thalassaemia), weights in 68% were below the NCHS 5th centile compared with 28% of age- and sex-matched non-sicklers. When these data were plotted against Jamaican sickle cell reference values, 14% were <3rd centile.[56] Nutritional status in 102 SS Yemeni children (6 mths to 15 yrs) was compared with NCHS reference values. Growth deficit (<-2 *Z*-score) occurred in 72% based on weight-for-height, in 55% based on height-for-age and in 52% based on BMI (A.-W. M. Al-Saqladi, unpublished data). In Saudi Arabian children, there was no significant difference in serial height and weight measurements during the 1st 2 years of life in either 14 male or 7 female patients compared with matched controls from the eastern region of the country where the disease is generally mild.[57]

A study of 58 SS Indian children (2–14 yrs) reported significantly lower anthropometric values for all indicators except the upper/lower segment ratio compared with normal age- and sex-matched controls. Males and females were affected equally.[58]

European studies (Table 6). Moderate growth delay was reported in 76 white Sicilian children (1–17 yrs) with SCD.[59] Weight and height were <3rd centile of reference values for white British children in 16% and 10.5%, respectively. The majority had Benin haplotypes and showed no growth differences compared with β-S thalassaemia.

Mann[60] reported 61 SS patients (3 mths to 19 yrs) in England whose heights were >2 SD below the mean Caucasian reference value. The varied clinical manifestations compared with reports from Jamaica or North America led the author to conclude that variation depended on many factors including climate, endemic infection and the general standard of nutrition and medical care. Comparison of a further 56 SCD British children with controls of Caucasian (CC) or African/Caribbean (AC) origin showed that they were taller but that their weight and BMI were similar to CC controls.[61] Weight and BMI were significantly lower than in AC controls but there was no difference in height. Three unpublished longitudinal studies were identified, preliminary data for which are summarised in Table 6.

Summary. Growth retardation in children with SCD is well established and SS individuals are affected more severely than children with other sickle cell haemoglobinopathies. Growth failure occurs among affected children in all geographical areas, although the relevance and severity vary with location and are most marked in low-resource settings. Children with SCD have normal birthweight and length, with growth restriction commencing between 6 months and 2 years. European children show better growth than those elsewhere, probably indicating better nutrition and quality of care.

Body composition and energy metabolism

To understand the nutritional needs and interventions required in children with SCD, it is important to know the nature and magnitude of the body compositional deficits. A study of body composition in 36 Afro-American children with homozygous SCD found significantly lower *Z*-scores for weight, height, MUAC or upper arm fat and muscle in affected children.[62] A marked reduction in fat-free mass (FFM) and body fat indicated a global deficit of energy and protein stores, suggesting that nutritional needs were not being met.

Whole body protein turnover and resting metabolic rates are higher in SS adults than in AA controls. Protein turnover is an energy-consuming process which could account for increased energy expenditure. Patients with SCD disease could therefore be in a hyper-metabolic state, requiring higher energy and protein intake to maintain normal function.[63] The resting metabolic rate was found to be 19% higher in homozygous SCD than in AA controls and the difference was not related to the size of lean body mass.[64] When lean body mass or FFM are taken into account, REE per kg of FFM was 25–50% higher than normal.[65] The composition and tissue-specific metabolic rates comprising lean body mass/FFM in SS subjects is likely to differ from those of AA controls.[64,65] Whole body protein breakdown and synthesis was increased by 32% and 38%, respectively,[66] and the energy cost of increased protein synthesis was estimated to be approximately 50% of increased REE.[67] This increased energy expenditure and protein turnover could result from hyperactivity of bone marrow during erythroblastosis secondary to haemolysis and red cell destruction. The imbalance between energy requirements and expenditure would lead to a marginal nutritional state,

contributing to growth impairment that might potentially be corrected by energy supplements. To adapt to this state, there might be a reduction in physical activity. To compensate for their high resting metabolic rate, patients with SCD might try to economise on energy by decreasing physical activity. This mechanism cannot compensate for long-term energy deficiency or the imbalance between metabolic demands and energy consumption which ultimately lead to growth impairment.[68,69]

Pre-albumin, used to assess nutritional status, has been reported to be low in SCD.[70] Urinary loss of amino acids might also contribute to slow growth. One study reported no differences in the concentration of serum total proteins between SCD children and controls, but serum levels of pre-albumin, all essential and most non-essential amino acids were significantly lower with higher urinary concentration of amino acids.[71]

Changes in carbohydrate and lipid metabolism in SCD have been evaluated by measurement of whole body glucose and lipid metabolism in adults. Results showed that these were not significantly affected and the plasma concentration of insulin, glucagon, cortisol, nor-epinephrine and epinephrine were similar in patients and controls.[66] Serum levels of total phospholipids were within the normal range in children with sickle cell anaemia, while docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), total polyunsaturated fatty acids (PUFA)[72] and cholesterol[73,74] were decreased. With an imbalance between n-3 and n-6 long-chain PUFA in erythrocytes and plasma, alterations in the lipid layers of the red-cell wall might be antecedent to red-cell asymmetry, adhesion and aggregation and precede vaso-occlusion.[75]

Plasma concentration of type I procollagen carboxy-terminal propeptide (PICP), the major collagen produced by osteoblasts during bone formation, and urinary excretion of urinary pyridinoline cross-links (PYD) formed from type I collagen during bone resorption have been used as indirect measures of bone turnover. In adolescents with sickle cell anaemia compared with AA controls, these bone marrow resorption and formation markers were increased, suggesting increased protein formation and breakdown in bone marrow. This could relate to elevation in whole body protein turnover and REE in SS patients.[76] Bone mineral density, assessed by dual-energy X-ray in 25 children and adolescents (9–19 yrs) with severe sickle cell anaemia, was found to be reduced in 64%. This was associated with deficient calcium intake and low serum levels of vitamin D.[77]

Glutamine is the most abundant amino acid in humans and is the preferred fuel for rapidly dividing cells such as reticulocytes. Its use in children with sickle cell anaemia was reported to be 47% higher than in controls and to be associated with a 19% increase in REE and a 66% increase in cardiac output. These changes might be attributable to increased haemoglobin synthesis and cardiac workload.[78] Attempts to lower REE using oral glutamine led to a reduction of about 6%, which was greater in children who were underweight. Improved BMI and body fat components indicated that lowering REE by increasing energy intake and glutamine administration could be an effective way of promoting growth in children and adolescents with SCD.[79]

Metabolic studies suggest that children with SCD have a higher resting metabolic rate and REE, which increases their metabolic demands and requirements for protein and energy. Factors which contribute to higher REE include increases in protein turnover, erythropoieses, cardiac workload and underlying inflammation. The child's body composition, nutritional status and clinical condition all influence metabolic rate and nutritional requirements and these need to be well defined in order to understand the potential role of nutritional interventions for improving health.

Endocrine dysfunction and growth retardation

In children with SCD, delayed sexual maturation is frequently associated with growth retardation.[31] Although its contribution to growth deficit is unclear, it might not have a primary endocrine cause.[3] Determination of gonadotropin concentrations in 40 children with sickle cell anaemia (5–16 yrs) showed a significant increase in LH in children aged 5–10 years and normal levels in older children. The levels of LH and FSH were higher in patients than in controls at the same stage of development of secondary sexual characteristics. This suggested a variation in the rate of maturation of the hypothalamic–pituitary gonadotropin axis rather than gonadal hypofunction.[80]

Evaluation of gonadal function in adults with SCD showed that serum testosterone, dihydrotestosterone (DHT) and androstenedione levels were low.[81] High LH and FSH levels were observed before and after stimulation with gonadotropin-releasing hormone, which correlated with testicular size and retarded secondary sexual characteristics. This suggests that gonadal hypofunction is not related to pituitary failure but is consistent with primary gonadal failure. This study also reported reduced erythrocyte and hair zinc concentrations which significantly correlated with androgen status. The influence of chronic zinc deficiency on gonadal growth and function was considered important. Evaluation of the hypothalamic–pituitary axis by administration of gonadotropin-releasing hormone–thyrotropin-releasing hormones has demonstrated higher concentrations of LH, FSH, thyroid stimulatuig hormone and prolactin hormones in male patients than in controls, which suggests a primary gonadal failure in adults[82] and in children with extreme retardation of puberty.[83]

There is also some evidence for partial hypothalamic hypogonadism.[84] Significantly reduced concentrations of testosterone, LH and FSH in adults with SS disease supports gonadal hypofunction secondary to hypopituitarism.[85] Delayed testicular development has been demonstrated in male sicklers, predominantly in boys aged 10–15 years who had delayed puberty but attained normal sexual maturation.[43]

In a longitudinal study of 55 American children with SCD and reduced weight, height and retarded bone age, there was delayed sexual maturation which, though prolonged, progressed in an orderly manner.[27] The average age of menarche in affected girls was 15.4 *vs* 12.6 years in normal girls. In the majority of these children, hormonal assays indicated an intact pituitary–hypothalamic axis with appropriate adrenal and gonadal responses and only patients with marked delay in sexual maturation showed lower gonadal hormones. Age at menarche in Jamaican girls was delayed by 2.4 years in 99 cases with homozygous SS disease, and by 0.5 years in 69 SC cases compared with a mean age of 13 years in AA controls.[86] Weight was found to be the dominant determining factor for age at menarche in cases and controls. The authors considered their findings favoured sub-optimal nutrition as a cause of pubertal delay rather than an endocrine component.[86]

In 80 Saudi patients with sickle cell anaemia, hormonal assay showed normal levels of T3, T4 and growth hormone, low levels of cortisol, testosterone and LH, and variable changes in FSH.[87] These abnormalities occurred more frequently in the patients with severe disease. Studies of thyroid function have shown that blood levels of thyroxine, thyroxine-binding capacity and the free thyroxine index were not significantly different in 90 SS children (1–15 yrs) than in AS and AA controls.[88] Interest in growth hormone dysfunction has motivated a series of studies by Soliman and co-workers who demonstrated abnormalities in the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis.[54,89–92] In a study of 21 pre-pubertal SS children with poor growth (height <10th centile), defective GH secretion and low insulin-like

IGF-1 and IGF binding-protein-3 were demonstrated in 43%, with a reduced response of IGF-1 production to GH injection. The disease severity score was significantly higher in the group with defective GH secretion than in the group with normal GH secretion. The authors presumed there was partial resistance to GH and that these were major causes of slow growth, especially in individuals with severe SCD.[80] Although reduced elements of the GH/IGF-1 axis in SS children have been found, growth velocity shows poor correlation with endocrine assessment of the axis or thyroid function.[93] Other investigators have reported a significant correlation between IGF-1 and height velocity in a sub-group of sicklers with height <25th centile.[94] In an analysis of different β globulin haplotypes, the CAR/CAR haplotype has shown significantly lower mean growth velocity and reduced concentration of IGF-1 compared with BEN/BEN haplotype, leading to the conclusion that delay of growth in SCD was linked to intrinsic factors and disease severity.[93] In a small study of five SCD children with GH deficiency who received GH therapy for ≥3 years, height *Z*-scores improved significantly.[95]

The normal pituitary response to stimulation tests and the conflicting results of hormonal assessment make it difficult to evaluate the role of endocrinal dysfunction in the pathogenesis of growth impairment. Endocrine function is altered in some children with SCD, and hormonal therapy such as GH or IGF-1 might offer therapeutic options.

Micronutrient deficiency

Micronutrient deficiency could be an important contributor to growth impairment in SCD. In an American study of 170 children (aged 2–12 years) with SCD, 22% were <5th centile in height and/or weight,[96] and the serum levels of zinc, retinol, pre-albumin and retinol binding protein were significantly lower in the 40 cases (who were either growth-retarded or normal) than in controls. Despite an adequate dietary intake of energy, protein, zinc and vitamin A, these children with SCD were leaner and lighter with lower red blood-cell zinc and serum vitamin A concentrations, and higher resting energy expenditure than controls.[97] These findings were reflected in a survey of 61 American SS patients and their families on nutrition knowledge and practice. Overall, 90% of participants were familiar with the different food groups but most failed to consume an appropriate amount of different food groups, and 59% had incomes below the poverty level. The authors concluded that inadequate intake of nutrients was contributing to poor child growth in lower socioeconomic families.[98] A recent study evaluated dietary intake by 24-hour recall over four annual visits in 97 American children with homozygous SCD and reported a suboptimal intake of many nutrients across all ages, including vitamins D and E, folate, calcium, magnesium and zinc, with a trend towards poor diet with increasing age, particularly during adolescence.[99]

Folic acid was the first micronutrient deficiency to be associated with SCD and has been reported frequently.[100–103] Folate deficiency and megaloblastic erythropoiesis were observed in about 10% of patients in Nigeria, and therapeutic administration of folic acid resulted in improved height and weight as well as correction of haematological changes.[104] Other investigators have failed to demonstrate a correlation between growth retardation and folate deficiency as folate supplementation produced no change in haematological or growth parameters.[105–108] Routine supplementation in SCD has been questioned, particularly in developed countries where folate requirements could be provided by a fortified food intake.[109] Vitamin B_6 (pyridoxine) deficiency in adults with SCD has also been reported.[110] In children, assessment of vitamin B_6 status by determination of serum concentrations of pyridoxal 5-phosphate

(PLP) (the major co-enzyme of vitamin B_6) showed that 77% were below the reference cut-off, and there were significant positive associations between PLP levels and BMI *Z*-scores, weight and MUAC.[111] Reduced levels of other B vitamins including B_{12}[112] and riboflavin[113] have been reported. Folic acid and vitamins B_6 and B_{12} are important co-factors in metabolism of the sulphur-containing amino acid homocysteine, and deficiencies can lead to hyperhomocysteinaemia. In the general population, raised homocysteine concentrations are linked to increased risk of cardiovascular disease and stroke.[114] Plasma homocysteine is reported to be elevated in adults[115] and children[116,117] with SCD and significantly so when complicated by stroke.[118] Homocysteine levels can be lowered by supplementation with folic acid or vitamins B_6 and B_{12}. In addition to the maintenance of effective erythropoiesis, these micronutrients can prevent tissue accumulation of homocysteine, thus reducing the risk of endothelial damage and thrombosis.[119–121]

Serum vitamin A status was reported as marginal in 66% of American children with SCD and deficient in 17%. BMI *Z*-scores were low, and there were higher rates of hospital admission of vitamin A-deficient patients than of those with normal levels.[122]

Zinc deficiency in SCD occurs at levels suggesting chronic zinc depletion and appears to be associated with chronic haemolysis and hyperzincuria.[123] Growth retardation and hypogonadism were observed in zinc-depleted men, suggesting its contribution to impaired growth and sexual maturation in SCD.[81,124] In 104 American children (0.4–18 yrs), low plasma zinc was reported in 44% of SS cases and, compared with SS cases with normal plasma zinc, was associated with impairment of height, weight, FFM, skeletal growth and sexual and skeletal maturation.[125] Supplements of elemental zinc (10 mg/day) given for 12 months to 20 children with SCD led to improved rates of linear growth but there was no effect on BMI.[126]

Iron deficiency might not be associated with SCD owing to the availability of iron from red cell destruction and increased intestinal iron absorption in response to chronic anaemia.[127] Even so, patients receiving sporadic transfusions do not acquire excessive iron burden during the 1st 2 decades of life.[128] Iron deficiency in SCD is common,[129] particularly among children living in developing countries where iron deficiency anaemia is highly prevalent.[130] Depletion of iron storage diagnosed by bone marrow examination was reported in a high proportion of SCD children (36–50%) in India and Nigeria.[131–133] Iron deficiency was reported in 16% of non-transfused American children diagnosed by their response to iron therapy.[134] This contrasted with a study of 104 non-transfused patients who showed no haematological or biochemical evidence of iron deficiency.[135] A study of Jamaican children followed from birth to 5 years reported low serum iron in patients and controls by 1 year of age, but levels subsequently became normal.[136] However, a recent cross-sectional study of 141 Jamaican SCD children (1–5 yrs) which used several measurements to determine iron status showed that 8.5% of cases were iron-deficient.[137] Although the exact mechanism of iron deficiency in SCD is not clear, the most probable cause is excessive urinary loss secondary to chronic haemolysis.[138]

Iron deficiency in SCD might be beneficial and possibly ameliorate sickling by decreasing MCHC, which reduces haemolysis, thus prolonging red-cell lifespan[139,140] and reducing painful crises[141] (which can be precipitated by iron therapy).[142] Evidence for the clinical benefits of iron deficiency is minimal and is limited because of difficulties in assessing disease severity.[143] Iron deficiency is associated with growth and intellectual impairment[144] and, in a growing child with SCD, iron requirements are increased. Iron-deficient children are at risk of both growth and neurocognitive impairment imposed by the disease and

compounded by iron deficiency. These consequences should be considered before iron supplementation is withheld.

Vitamin E deficiency occurs in SCD,[145] with a high prevalence in children in developing countries.[146,147] Vitamin E has anti-oxidant properties that could protect red cells against oxidative stress and its administration leads to a decrease in the percentage of irreversibly sickled cells, which might alleviate symptoms.[148] Deficiency of vitamins C[149] and D[150] and of minerals such as magnesium[151] and selenium[152] has been reported, although the exact pathophysiological consequences and contribution to growth delay in SCD are unclear. The potential benefits of individual nutrient or multi-micronutrient supplementation remain to be established.

Food substances with anti-oxidant activity, which might protect red cell membranes from oxidative injury, have been used to treat SCD.[153,154] In a small pilot study, oral administration of dietary omega-3 fatty acid, provided as menhaden fish oil containing docosahexanoic acid and eicosapentanoic acid, produced significant reduction in the mean number of painful crises, blood coagulability and platelet adhesion molecule expression.[155] Omega-3 fatty acids are important components of red cell membranes and their blood levels have been correlated with indices of disease severity and haemoglobin concentration in steady-state SCD. This suggests that there are clinical benefits through protection against haemolysis and reduction in vaso-occlusive episodes or ischaemic organ damage.[156] L-arginine is the natural amino acid substrate for the synthesis of nitric oxide, a potent vasodilator that is deficient during sickle cell crises. When administered orally at a dose of 0.1 g/kg three times a day, it led to a significant reduction in pulmonary artery systolic pressure in SCD patients with pulmonary hypertension.[157] This is consistent with vaso-constriction being a significant contributor to vaso-occlusion.[158] Oral supplementation of magnesium pidolate (540 mg/kg/d) has been used to elevate erythrocyte magnesium and prevent potassium loss by inhibition of the K-Cl co-transport system, resulting in improved sickle red-cell hydration and a decrease in the median number of painful days during a 6-month period of magnesium therapy.[159]

Several micronutrient deficiencies have been reported in patients with SCD. Folic acid is widely administered, usually daily, to children with SCD, although the optimal dose is unclear, which relates to uncertainty concerning the daily requirement. Other nutrients such as zinc, glutamine, l-arginine and anti-oxidants might have therapeutic benefits, and their clinical efficacy needs to be determined.

Future Perspectives

Under-nutrition relates to increased morbidity and mortality in all children, and contributes to poor clinical outcome and severity of disease in children with SCD. Despite major advances in understanding the molecular and genetic basis for SCD, there has been little progress towards lessening the obvious nutritional problems faced by these children.[160] There has been limited evaluation of a variety of nutritional interventions that could influence the natural history of SCD.[161] Improving the nutritional status and growth of these children could have a favourable impact on their clinical course and prognosis. Evaluation of a comprehensive clinical care programme in a sub-Saharan Africa setting produced encouraging results and showed that improved growth and reduced disease severity can be attained.[162] There are good opportunities for such programmes with the introduction of neonatal screening, the identification of children with SCD at birth and early interventions using essential health packages.

Growth monitoring with appropriate nutritional support as part of the comprehensive care of children with SCD should be

promoted. If the types of nutritional deficiency are known, then clear nutritional advice and care can be given by health workers to children and their families. This allows the identification of children who do not adhere to nutritional interventions and of high-risk cases. It might facilitate the use of alternative interventions including drugs, hormones or other treatments in specific cases.

Small stature and delayed sexual maturity can carry long-term psychological consequences that affect the ability of the adolescent with SCD to form normal relationships with the opposite sex, leading to low self-esteem and depression.[163] Growth retardation has been associated with impaired mental development and a low intelligence quotient,[164] and nutritional interventions with their potential for improving long-term growth and development could improve prognosis, particularly if commenced in early childhood before growth retardation becomes established. These interventions might lead to reduction in the severity of crises and vascular complications, or episodes of vasoconstriction.

There is little information on the influence of several important genetic polymorphisms on nutritional status in SCD. For example, methylene-tetrahydrofolate reductase deficiency, which is not infrequent in subjects with SCD,[165–168] would influence host folate status and homocysteine metabolism with possible effects on sickle cell vasculopathy. Similarly, glucose-6-phosphate dehydrogenase deficiency could affect severity of haemolysis in sickle cell anaemia, although some studies of this genotype have shown little additive effect.[169] Pooled data from studies of different haplotype profiles need to be interpreted carefully, taking these various factors into consideration.

In order to assess the benefits for child growth and the reduction of disease severity, randomised trials of nutritional interventions in infancy and early childhood combined with appropriate health care packages are required. There are few studies from Africa and the Arabian Peninsula and increased efforts are required to address this disparity, particularly in low-resource settings.

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Clinical profile of sickle cell disease in Yemeni children

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Abstract

The clinical spectrum of sickle cell disease (SCD) in the Arabian Peninsula varies widely. This is the first report in Yemeni children.

Methods: A hospital-based, cross-sectional study was undertaken in Al-Wahada Teaching Hospital in Aden of children under 16 years with homozygous (SS) SCD.

Results: Fifty-six (55%) were males. There were clinical manifestations in 20% by the age of 6 months and in 67%, 88% and 92% by 1, 2 and 3 years, respectively. Dactylitis (hand-foot syndrome) was the most common presenting symptom and occurred in 54% of cases, followed by acute respiratory infections and other acute febrile illnesses. The main causes of hospitalisation were painful crisis (36%), anaemic crisis (16%) and acute chest syndrome (11%). Hepatomegaly was detected in 72% and splenomegaly in 40%. Cerebrovascular accident, cholelithiasis, hepatic crisis and leg ulcers each occurred in about 5% of patients. There was first- and second-degree consanguinity in 31% and 16%, respectively, of patients' families.

Conclusion: SCD is a serious problem, affecting children in Yemen from an early age. Disease course and severity were similar to that in Africans and American blacks and some reports from western Saudi Arabia. A screening programme linked to comprehensive medical care and genetic counselling is required to improve management and quality of life.

Introduction

Sickle cell disease (SCD) is an inherited blood disorder that results from a point mutation in a single DNA base pair that leads to substitution of valine for glutamate in position 6 of the β-chain and production of abnormal sickle haemoglobin (HbS). It is the most common globin gene disorder in the world and about 250,000 children per year are born with the disease.[1] The clinical picture is characterised by haemolytic anaemia and intermittent episodes of vascular occlusion, with acute and chronic pain and variable organ damage.[2]

Clinical manifestations are markedly heterogeneous and the disease pattern and severity vary widely between individuals in different socio-economic and geographical locations.[3] Although factors modulating phenotypic expression of SCD such as HbF levels, co-inheritance of α-thalassemia and β^s globin cluster haplotypes are well known, other potential genetic modifiers and several single nucleotide polymorphisms (SNPS) that may affect cell adhesion, thrombosis and inflammation are involved.[4]

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Many acute and chronic complications occur. Acute complications such as fulminant bacterial infection and splenic sequestration crises are life-threatening. Chronic haemolysis leads to the formation of pigmented gall stones and chronic organ damage contributes to cerebrovasculopathy, pulmonary and renal damage, leg ulcers and avascular osteonecrosis.[5]

SCD is common in the Arabian Peninsula.[6] The frequency of the β^s gene in Yemen was estimated to be up to 0.04, with an expected birth incidence of 20/10,000/year.[7] This is the first clinical report of SCD in Yemeni children.

Subjects and Methods

This descriptive study was conducted at Al-Wahda General Teaching Hospital, Aden Governorate, Yemen, which is the major referral hospital for women and children in urban and peri-urban areas. For the purpose of this study, the sickle cell clinic was held daily during the 6-month period from March to August 2005. The public was given information about the SCD clinic via local newspapers and the health and education authorities.

The sample included all symptomatic or asymptomatic children with SCD under 16 years of age who had been diagnosed by a positive sickling test, confirmed by SS haemoglobin electrophoresis. Subjects were recruited from the clinic or following hospital admission. A detailed history, clinical examination and review of case records was completed by one observer (AA) using a pre-structured questionnaire. Data on family history, past medical history and frequency of acute clinical events during the previous 3 years were collected. Information on previous hospital admissions, blood transfusions, severity and frequency of crises (vaso-occlusive, splenic sequestration, aplastic, hyperhaemolytic and cerebrovascular) and other complications such as infections, acute chest syndrome, avascular bone necrosis, skin ulcers, gall stones, priapism and urinary disorders were recorded. Definitions of clinical events were those proposed by the SCD co-operative study group.[8] Information was collected from one or both parents who were asked to bring all available medical documents (doctors' prescriptions, previous investigation papers and notes of hospital discharge) to the clinic. Information on previous admissions was cross-checked against hospital admission records. Clinical severity was assessed according to the frequency of pain crises, hospitalisation, blood transfusion, episodes of infection and sickle cell-related complications, as previously described.[9]

Blood investigations

Standard laboratory methods were employed to evaluate all haematological and biochemical tests.[10] Radiography, ultrasound and CT scans were performed when indicated. The study protocol was approved by The Liverpool School of Tropical Medicine's Ethics Committee and the National Ethical Committee, Yemen. Informed consent was obtained from the child's parent or guardian.

Statistical analysis

Statistical analysis employed the SPSS-V14 statistical package. The independent sample *t*-test, χ^2 and Fisher exact tests were used as appropriate. Univariate and multivariate analyses were used to assess associations between clinical and biochemical variables.

Results

A total of 102 SS children were evaluated, 56 males and 46 females. Mean age was 7.2 years (range 6 months to 15 years). More than one-third (38%) were below 5 years of age (Table 1). All patients were Yemeni and the majority (96%) were from Aden and

TABLE 1. *Age and gender distribution.*

Age (yrs)	Female, n=46 (%)	Male, n=56 (%)	Total, n=102 (%)
<1	1 (2.2)	5 (8.9)	6 (5.9)
1–5	15 (32.6)	24 (42.9)	39 (38.2)
6–10	16 (34.8)	10 (17.9)	26 (25.5)
11–15	14 (30.4)	17 (30.4)	31 (30.4)

Lahj, the adjacent governorate. Mean age at diagnosis was 17 months (range 2 months to 7 years). One child presented at 2 months of age and another had no symptoms until 7 years of age. Initial clinical manifestations were present in 20% by 6 months of age and in 67%, 88% and 92% by the age of 1, 2 and 3 years, respectively (Table 2). All those who presented in the 1st 6 months had at least one affected sibling and the family was already alerted to the disease.

The most frequent initial symptom was dactylitis (hand-foot syndrome, Tables 3 and 4) and it was observed in 54% of children, usually in the 1st 2 years of life. Acute respiratory infections (15%) and other acute febrile illnesses (11%) were the second and third most common presenting symptoms, respectively, followed by anaemia (8.8%) and painful crises (10%). Eighty-five patients (83.3%) had been admitted to hospital previously. There was a history of two hospital admissions in 32 patients, three or more in 18, with a total of 159 admissions, giving a ratio of 1.55 admission episodes per child. Seventeen patients gave no history of hospital admission.

Table 5 summarises the main reasons for hospitalisation. Painful crises were predominant (36%), followed by anaemic crises (16%). Among those with acute respiratory symptoms, 11% fulfilled the definition of acute chest syndrome by the presence of a new pulmonary infiltrate on chest

TABLE 2. *Comparison of age at first diagnosis of sickle cell disease in this study with data from Saudi Arabia.*

Age	Yemen %, n=102	S. Arabia[17] %, n=99
3–5 mths	7.8	6.0
6–9 mths	35.3	18.0
10–12 mths	24.5	9.0
13–36 mths	24.5	40.0
4–6 yrs	6.9	21.0
7–10 yrs	1.0	4.0
10 yrs	-	1.0

TABLE 3. *Frequency of presenting features.*

Feature	No. of cases (%)
Hand-foot syndrome	55 (53.9)
Acute respiratory infection	15 (14.7)
Acute febrile illness	11 (10.8)
Painful crisis	11 (10.8)
Anaemia	9 (8.8)
Malaria	1 (1.0)
Total	102 (100.0)

TABLE 4. *Dactylitis as an initial symptom by age compared with Jamaica.*

Age, mths	Yemen, n* (%)	Jamaica,[22] n* (%)
0–5	5/7 (71.4)	8/16 (50.0)
6–11	24/39 (61.5)	40/79 (50.6)
12–23	15/31 (48.4	39/84 (46.6)
24–35	9/15 (60.0)	12/43 (28.0)
36–47	0/2 (0)	2/17 (11.7)
≥48	2/8 (25.0)	1/17 (5.8)
Total	55/102 (53.9)	102/256 (39.8)

* Denominator: sample size.

radiograph. Blood transfusion was required on 160 occasions and 75 patients (73.5%) had a history of previous blood transfusion, 41% had received two-to-five transfusions and only four patients had received more than five transfusions.

Jaundice was detected in 20%. Haemolytic bony changes of the face such as frontal bossing and prominent upper jaw were observed in 28% of children. A cardiac murmur was present in 34 patients (33.3%) and, owing to the presence of a murmur and joint pains, one case had been diagnosed and treated as rheumatic heart disease before the final diagnosis was established. Two children developed cardiac failure. The liver was palpable in 74 cases (72.5%) and enlarged >2 cm in 42 (57%). The spleen was enlarged in 41 patients (40%).

Complications related to SCD are summarised in Table 6. Seventeen children had acute chest syndrome and overt cerebrovascular accident occurred in five. Gall bladder stones were identified in five patients but none required cholecystectomy. Five patients developed hepatic crisis, three of whom were admitted during the study period. During the hepatic crisis, these patients manifested with abdominal pain, deep jaundice and hepatomegaly. Mean (SD) bilirubin level was 543.78 (331.74) μmol/L, with the conjugated moiety >50% [mean (SD) 371.07 (198.36) μmol/L]. The mean (SD) haemoglobin level in jaundiced patients was 8.8 (1.5) g/dl, thus excluding severe haemolysis as the cause of hyperbilirubinaemia. All patients improved with supportive therapy and simple transfusion. Nocturnal enuresis in children ≥6 years of age occurred in 43.9% (25/57), 13 boys and 12 girls. Seven patients had priapism but of a minor type. There was a history of epistaxis in 15 (14.7%) patients and gum bleeding in three. Malaria confirmed by microscopy was diagnosed in 21 (20%) patients in the year before the study and malaria infection was responsible for hospitalisation on 13 occasions.

The mean (SD) haemoglobin level was 7.7 (1.5) g/dl. Only seven patients had a Hb level >10 g/dl, with 49% being in the range 6–8 g/dl. Four patients had severe anaemia with Hb <5 g/dl. Clinical severity assessment found that 56% of patients had severe disease and 44% mild disease with less frequent complications.

The study group involved 86 families. Five patients had one affected sibling, four had two affected siblings and one patient had three affected siblings who were included in the study. The marriage was consanguineous in 62.8% of parents, 31.4%

TABLE 5. *Reasons for admission.*

Diagnosis	No. of admissions Total=159 (%)
Pain crisis	57 (35.9)
Anaemic crisis	25 (15.8)
Acute chest syndrome	17 (10.7)
Malaria	13 (8.2)
Lower respiratory tract infection	12 (7.6)
Hand-foot syndrome	6 (3.8)
Cerebrovascular accident	5 (3.2)
Hepatic crisis	5 (3.2)
Diarrhoea	5 (3.2)
Typhoid fever	2 (1.3)
Cardiac failure	2 (1.3)
Osteomyelitis	1 (0.6)
Meningitis	1 (0/6)
Other*	8 (5.0)

* Includes acute febrile illnesses, epistaxis and dengue fever.

TABLE 6. *Complications of sickle cell disease.*

Complications	*n* (%)
Acute chest syndrome	14 (13.7)
Cerebrovascular accident	5 (4.9)
Gall bladder stone	5 (4.9)
Hepatic crisis	5 (4.9)
Leg ulcer	5 (4.9)
Priapism	7 (12.5)
Epistaxis	15 (14.7)
Haematuria	9 (8.8)
Avascular necrosis	1 (1.0)
Enuresis (n=57)*	25 (43.9)

* Only those aged ≥6 years.

being first cousins, 16.3% second cousins and 15% distant relatives.

There was one death during the study, a 12-year-old girl with severe dengue fever at the onset of an outbreak in Yemen. Information on SCD mortality obtained by family history showed that 18% of the families had had an SCD-related death, and more than 75% of these deaths had occurred during the 1st 15 years of life.

Discussion

Most reports of SCD in the Arabian Peninsula are from Saudi Arabia[11,12] and other Gulf countries including Oman,[13] Kuwait,[14] the United Arab Emirates[15] and Bahrain,[16] but information on SCD in the Yemen is lacking. This study aimed to define the clinical pattern of SCD in Yemeni children who were mainly from the south-west of the country. Although the study was hospital-based, we tried to include all children with SCD within our catchment area by notifying all known affected individuals and opening the clinic daily, free of charge. We were able to recruit 69 patients who were symptom-free in a 'steady state' condition and 17 without previous hospital admission.

Although there is no neonatal screening programme for diagnosing SCD in Yemen, the majority of affected individuals (92.1%) had presented and been diagnosed before 3 years of age. This early presentation might reflect the severity of SCD in Yemeni children which is comparable with that in western and south-western Saudi Arabia[17] where the disease is generally considered to be severe and 73% of children were diagnosed before the age of 3 years (Table 2). Initial symptoms of SCD were recognised in 50% of American children by 1 year of age and in nearly all patients by 5–6 years.[18] In a Jamaican cohort, about one-third of children developed symptoms by the age of 1 year and more than 90% by 6 years.[19] In this study, 67.6% of children had become symptomatic by 1 year and 99% by 6 years of age.

Pain crisis was the most common and troublesome symptom. It occurred in almost 90% of subjects and was the most common reason for hospitalisation in this study and elsewhere.[20] Overall, 41% of patients had three-to-ten episodes and only 9.8% had no pain crisis. Dactylitis (hand-foot syndrome) is an early manifestation of vascular obstruction in the bone marrow of small distal bones in young children and led to the diagnosis of SCD in 53.9% of children in the study. This is similar to findings in Saudi Arabia (55–66%),[17] India (52%)[21] and Jamaica (45%)[22] (Table 4) but higher than in American blacks (11%).[23] This variation could be attributed to patient selection and heterogeneity of disease severity. For example, dactylitis was found to be very rare in eastern Saudi Arabia where it is generally considered to be mild.[11] Infants who developed dactylitis in the 1st year of life were more likely to have an adverse outcome and the relative risk for severe disease with a history of dactylitis is estimated to be 2.6.[24] Dactylitis is also considered to be an initial manifestation of vasculopathy and carries a significant risk for development of cerebrovascular accident in older children.[25]

Splenomegaly was detected in 40% of patients, comparable with the 32–77% reported from Saudi Arabia.[9] The spleen is a site of red cell destruction and if this function is exaggerated (hypersplenism), which happens in some sickle cell patients, then splenectomy might be helpful and should lead to haematological and clinical improvement.[26] However, spleen size is not a reliable indicator of function, and reticulo-endothelial dysfunction occurs in SCD without greatly enlarged spleens owing to 'functional asplenia'.[27] A palpable spleen at or before 1 year of age has been reported to be a risk factor for severe and recurrent bacterial infection, especially pneumococcal infection as a consequence of functional asplenia.[28] In 72 Omani children aged

between 5 and 12 years, splenomegaly was found in 55% of cases, and only 30% of SS individuals had normal spleen function and 40% had severe asplenia with complete absence of splenic visualisation on scintigraphy.[29] It would be interesting to assess spleen function in Yemeni children with this high rate of splenomegaly and variable disease severity.

Despite the high prevalence of splenomegaly in our patients, sequestration crises were not encountered, similar to a report from Nigeria.[30] However, a family history of a sickle cell-related death with sudden pallor and collapse during normal activity in an apparently asymptomatic child raised the possibility of acute sequestration crisis as a cause of death at home. Parents might not be aware of the manifestations of a sequestration crisis and unable to quickly transfer their child to hospital. Instructing parents about spleen palpation, the manifestations of a sequestration crisis and to immediately report any change in spleen size, especially if associated with pallor, is important for early diagnosis and management and could save many children's lives.[31]

Clinically overt stroke occurred in five patients (4.9%), which is consistent with various other reports (3.3–11%).[32] Because neuro-imaging facilities were limited, it was not possible to diagnose silent infarction. For prevention of stroke, a prophylactic programme using transcranial Doppler (TCD) and repeated transfusions is required. A frequent complication was the greater prevalence of nocturnal enuresis in our SCD patients (43.9%) than in the normal population.[33] Males and females were affected equally. This is comparable with data from Nigeria where 49.5% of sicklers had enuresis at 6–15 years of age but with no gender difference.[34] This contrasts with a Jamaican study which reported enuresis to be more common in males than in females (52% *vs* 38%).[35]

Although the heterozygous state of SCD offers relative protection against malaria infection, malaria is still a major cause of morbidity and mortality in sickle cell patients living in malaria-endemic areas.[36] Malaria is endemic in Yemen and a history of malaria in the previous 12 months was reported for 20% of patients. A sickle cell anaemic crisis had been triggered by malaria in some of these cases. It is not clear why patients with homozygous SS with high levels of HbS are not protected from malaria to a greater degree than in the heterozygous state, or why low-level parasitaemia can result in severe manifestations of malaria in homozygous cases.[37]

A limitation of this study is that it was hospital-based and ascertainment bias could lead to the inclusion of more symptomatic than asymptomatic patients. However, every effort was made to obtain adequate information on cases from parents/guardians supported by documentary evidence, but this does not exclude recall bias for clinical events. Lack of effective laboratory facilities restricted the accurate diagnosis of bacterial or viral acute febrile illnesses.

Early diagnosis and management through the implementation of a neonatal screening programme in Yemen combined with comprehensive medical care is required to ensure appropriate and effective early management. The high rate of consanguinity requires improved parental education and pre-marital testing with genetic counselling.

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