

**ASSESSMENT OF SOME BIOCHEMICAL MARKERS OF
NUTRITIONAL STATUS AMONG ADULT SICKLE CELL
ANAEMIA PATIENTS IN STEADY STATE IN ZARIA**

BY

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May, 2015

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BY

Mohammed MANU, MBBS (ABU) 2003

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May, 2015

DECLARATION BY THE CANDIDATE

I declare that this Dissertation entitled: '**Assessment of some Biochemical Markers of Nutritional Status among Sick Cell Anaemia Patients in steady state in Zaria**' was carried out by me in the Departments of Chemical Pathology and Haematology, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project was previously presented for another degree or diploma at this or any other institution.

Mohammed MANU.....

Name of student	Signature	Date
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CERTIFICATION

This Dissertation entitled: **ASSESSMENT OF SOME BIOCHEMICAL MARKERS OF NUTRITIONAL STATUS AMONG SICKLE CELL ANAEMIA PATIENTS IN STEADY STATE IN ZARIA**, by MANU MOHAMMED met the regulations governing the award of degree of masters in Chemical Pathology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Sickle cell anaemia is a chronic haemolytic state which is characterized by hypercatabolism that could predispose to malnutrition. The low socio economic background of our patients may worsen their health status. The study was aimed at assessing some biochemical markers of nutritional status among adult sickle cell anaemia patients in steady state in Zaria. It is a cross-sectional descriptive study which was conducted in the Departments of Chemical Pathology and Haematology, Ahmadu Bello University Teaching Hospital, Shika, Zaria, Kaduna State. Some biochemical markers of nutritional status of 60 adult SCA patients in steady state and 60 healthy non SCA controls were assessed. Mean ($\pm$ SEM) serum vitamin D concentration was significantly lower in SCA patients 14.55 ± 1.49 ug/L compared with controls 25.87 ± 4.29 ug/L ($p < 0.05$). Mean ($\pm$ SEM) serum calcium concentration was significantly lower in SCA patients 2.20 ± 0.06 mmol/L compared with controls 2.33 ± 0.04 mmol/L ($p < 0.05$). Mean ($\pm$ SEM) serum albumin concentration was significantly lower in SCA patients 33.40 ± 1.66 g/L compared with controls 40.13 ± 0.85 g/L ($p < 0.05$). Mean ($\pm$ SEM) serum inorganic phosphate concentration was significantly higher in SCA patients 2.00 ± 0.13 mmol/L compared with controls 1.26 ± 0.08 mmol/L ($p < 0.05$). The mean ($\pm$ SEM) BMI Kg/m^2 was significantly lower in SCA patients 21.37 ± 0.35 Kg/m^2 compared with controls 23.27 Kg/m^2 ($p < 0.05$). The study also showed no correlations between BMI and the biochemical markers measured in both SCA patients and controls.

In conclusion, mean sera concentrations of vitamin D, calcium and albumin among adult SCA patients in steady state were low compared with controls, so also BMI. The mean serum concentration of inorganic phosphate however, was significantly higher in SCA than in the control subjects. There were no correlations between BMI and biochemical markers measured in the study population. Therefore SCA patients in steady state are more malnourished when compared to the controls.

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ABBREVIATIONS AND SYMBOLS

AA	Atomic Absorption
AAS	Atomic Absorption spectroscopy
ABU	Ahmadu Bello University
ABUTH	Ahmadu Bello University Teaching Hospital
ACS	Acute chest syndrome
ALB	Albumin
ARDs	Acute Respiratory syndromes
ATP	Adenosine triphosphate
AVN	Avascular necrosis
BCG	Bromocresol Green
BMD	Bone mineral density
BMI	Body mass index
BMT	Bone marrow transplant
CA	Cellulose acetate
Ca ²⁺	Calcium ions
cAMP	Cyclic Adenosine monophosphate
CDC	Centre for disease control
cGMP	Cyclic Guanosine monophosphate
CLSI	Clinical Laboratory and Standards Institute
cm/s	centimetre per second
CPBA	competitive protein binding assay
CPC	CresolphthaleinComplexone
DBP	Diastolic blood pressure

DNA	Deoxynucleic acid
EDTA	Ethylenediaminetetraacetic acid
eg.	Example
etc.	etcetera
EIA	Enzyme immunoabsorbant
ELISA	Enzyme-linked ImmunoSorbent assay
et al	And others
FGF	Fibroblast growth factor
Fig.	Figure
Gc-globin	Group-specific component globulin
GFR	Glomerular filtration rate
GIT	Gastrointestinal tract
Hb	Haemoglobin
HbA	Haemoglobin A
HbF	Haemoglobin F
Hb ^{Philadelphia}	Haemoglobin Philadelphia
HbS	Haemoglobin S
HbS Lep B	Haemoglobin S Lepore
HbS O Arab	Haemoglobin S O Arab
HbSC	Haemoglobin SC
HbSD	Haemoglobin SD
HbSD Punjab	Haemoglobin SD Punjab
HbSE	HaemoglobinSE
HbSS	Haemoglobin SS
HIV	Human immune deficiency virus
HPLC	High performance liquid chromatography
ICMA	Immunochemiluminescence assay

ID-MS	isotope dilution-mass spectrometry
IEF	Isoelectric focusing
IL	Illinois
Invitro	Outside body
Invivo	Inside body
ISEs	Ion selective electron
IU	International unit
KDa	kilodalton
Kg	kilogramme
Kg/m ²	Kilogramme per metre square
Km	Kilometre
LCMS	liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenasem
mg/dl	milligramme per deciltre
ml	milliliter
MAPK	mitogen-activated protein kinase
mmHg	Millimetre of mercury
mmol/L	millimole per litre
mol	mole
n	Sample size
NADP	nicotinamide-adenine dinucleotide phosphate
ng/ml	nanogramme per millilitre
nm	nanometre
nmol	nanomole
NO	Nitric oxide
NSAID	Non steroidal anti-inflammatory drug
NW	Normal weight

0 ^c	Degree Celsius
P	Probability
PCR	Polymerase chain reaction
pH	-log of hydrogen ion concentration
PO ₃ ²⁺	Phosphate ions
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor- κ B ligand,
RBC	Red blood cell
RCTs	Randomized controlled trials
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rpm	revolution per minutes
RRA	Radioreceptor Assay
SBP	Systolic blood pressure
SCA	Sickle cell anaemia
SCD	Sickle cell disease
SEM	Standard error of mean
SNPs	Single nucleotide polymorphisms
SPSS	Statistical Package for the Social Science
TAMV	Time-average mean velocity
u	micro
ug/L	microgramme per litre
umol/L	micromol per litre
UV	Ultra violet
UVB	Ultra violet B
VCAM -1	vascular cell adhesion molecule 1

VD2	Vitamin D 2
VD3	Vitamin D 3
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VTD	Vitamin D
VTDD	Vitamin D deficiency
WBC	White blood count
WHO	Whole Health Organization
α	Alpha
γ	Gamma
δ	Delta
α^-	Alpha thalassaemia gene (minor)
α^+	Alpha thalassemia gene (major)
β	Beta
β^-	Beta thalassemia gene (minor)
β^+	Beta thalassemia gene (major)
%	Percent
$\pm$	Plus or minus
$\leq$	Equal to or less than
$\geq$	Equal to greater than
$<$	Less than
$>$	Greater than
$\approx$	Approximately

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Sickle cell anemia (SCA) is a genetic disease that results from the substitution of valine for glutamic acid in the β -globin chain of the hemoglobin molecule (Pauling and Itano, 1949). The consequence of this amino acid substitution is the formation of hemoglobin S (HbS). Under low oxygen tension and/or conditions of acidosis, HbS precipitates and forms polymerized crystals called tactoids (hemoglobin polymers), which distort the red blood cells (Nelson and Cox, 2005; Ganong, 2003). The resulting sickle-shaped red cells lose their pliability and cannot navigate the small capillaries, become sticky, and adhere to the small veins, small arteries, and other blood vessels causing vaso-occlusion (Aster, 2005; Bunn and Forget, 1977). In addition, red blood cells homozygous for HbS (HbSS) are susceptible to premature destruction, with a red blood cell life span of 8–25 days as compared to 100–120 days for normal red blood cells (Solankiet *al*, 1988).

In the last few decades, studies have documented the presence of micro- and macronutrient deficiency among individuals with SCA and their possible association with immunologic, nutritional and growth abnormalities (Heymanet *al*.,1985; Gray *et al*., 1992; Serjeant *et al*., 2001). Patients with sickle cell anemia (SCA) have low bone mass compared to healthy subjects (Lalet *al*, 2006). Low bone mass in these patients is apparent even after adjusting for age, height, pubertal development, and lean body mass,

suggesting that the deficits cannot be fully explained by short stature, delayed puberty, or altered body composition. Chronic haemolytic anemia and the resulting erythroblastic hyperplasia may contribute to bone demineralization in SCA (Serjeant and Serjeant, 2001). Furthermore, reduced physical activity, decreased circulating growth hormone, vitamin D deficiency and poor dietary intake of bone-forming nutrients are likely contributing factors (Buisonet *al*, 2004). Suboptimal peak bone mass acquisition in childhood may contribute to the development of osteoporosis in later life (Heanyet *al*, 2002).

Studies using direct measure of nutritional status (Enwonwu and Lu, 1991; Gray *et al.*, 1992; Kennedy *et al.*, 2001), indirect assessment of nutritional status (Henderson *et al.*, 2005), and application of nutritional supplementation (Prasad and Cossack, 1984, Heymanet *al.*, 1985), have established the association between SCA and the presence of nutritional deficiency among patients with the disease. These studies showed that although intake might be sufficient when measured against the recommended daily dietary allowance for age and sex, it is still insufficient for the individual with SCA due to the increased nutritional demand imposed by the disease. The result was the manifestation of malnutrition-like features (Prasad, 1997; Al-Saqladiet *al*, 2008; Hyacinth, *et al*, 2010).

1.2 STATEMENT OF THE PROBLEM

Nutritional deficiencies have not been given serious consideration in the assessment of SCA patients in this environment. It will be essential for physicians to adopt a nutritional approach as a part of the management modality for SCA in the light of

the fact that more than two-thirds of the patients with SCA live in areas with low socioeconomic status and have little to no means of accessing the current methods of management. Therefore the present study is aimed at assessing some biochemical markers of nutritional status in this group of patients.

1.3 JUSTIFICATION OF THE STUDY

The assessment of nutritional status in SCA patients could improve in the management of their conditions.

1.4 AIM AND OBJECTIVES OF THE STUDY

Aim

To assess some biochemical markers of nutritional status among adult SCA patients in steady state in Zaria.

Objectives

The objectives of this study were as follows:

1. To determine serum levels of vitamin D, calcium, phosphate and albumin in adult SCA patients in steady state in Zaria and HbAA controls.
2. To compare the serum levels of vitamin D, calcium, phosphate and albumin obtained from adult SCA patients in steady state and apparently healthy non SCA HbAA controls.
3. To determine the anthropometric parameters of adult SCA patients in steady state and HbAA controls.
4. To correlate the anthropometric parameters with serum levels of vitamin D, calcium, phosphate and albumin obtained from the study population.

1.5 RESEARCH HYPOTHESIS

H₀: Sick cell anaemia does not affect nutritional status (Null hypothesis).

H₁: Sick cell anaemia affects nutritional status (Alternate hypothesis).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 SICKLE CELL ANAEMIA

2.1.1 HISTORY OF SICKLE CELL ANAEMIA

Sickle cell was first observed in the blood of a 20 year old student in 1900 by James Herrick, a Chicago physician. He described a young medical student from Grenada in the West Indies in a paper titled “Peculiar-elongated and sickle shaped red blood corpuscles in a case of severe anaemia.” This was followed by other findings and case reports from other places (Victoria *et al*, 1975).

2.1.2 EPIDEMIOLOGY OF SICKLE CELL DISEASE

The sickle cell trait is widespread throughout Africa with low frequencies (<1%–2%) in the north and south of the continent and high but variable frequencies throughout much of equatorial Africa (Akinyanju, 1989). Sickle cell disease also occurs in other parts of the world (Konotey-Ahulu, 1974; Akinyanju, 1989). Statistics from World Health organization (WHO) showed that haemoglobinopathies as a whole are the commonest inherited disorders throughout the world with over 200 million carriers (WHO, 1996). Over 70% are found in Africa with sickling disorders accounting for a significant proportion (WHO, 1996).

Characterization of the DNA structure flanking the β -globin locus of HbS suggests that the mutation has arisen on at least three independent occasions in the African continent, referred to as β -globin haplotypes and named after the areas where they were first described: Benin, Senegal and Central African Republic or Bantu (Pagnier *et al*, 1984; Nagel *et al*, 1985; Chebloune *et al*, 1988). The HbC trait is believed to be a relatively recent mutation limited to West Africa where it occurs at high frequencies (>20%) in central Ghana and Burkina Faso, in only 2% in Nigeria and does not occur except in peoples of West African origin, in East and Central Africa (Akinyanju, 1989).

Nigeria is the most populous country in West Africa with a population of over 178 million and annual growth rate of 2.82 % (Waldometers.info, 2014). The prevalence of the disorder varies between 2 and 3 % (Esan, 1975; Akinyanju, 1989). The magnitude of the problem in this country is therefore enormous in the setting of growing number of surviving sickle cell disease patients in a worsening economy (Akinyanju, 1989).

2.1.3 PATHOPHYSIOLOGY OF SICKLE CELL ANAEMIA

Normal haemoglobin is made up of two α -chains and two β - chains forming a tetramer. The α -chains are produced from the α -globin gene located on chromosome 16 while the β -globin chains are produced from the β -globin gene on chromosome 11.

Sickle-cell anaemia is caused by a point mutation in the β -globin chain of haemoglobin, causing the hydrophilic amino acid glutamic acid to be replaced with the hydrophobic amino acid valine at the sixth position (Serjeant, 2001). GAG-GTG. The β -globin gene is located on chromosome 11. The association of two wild-type α -globin subunits with two mutant β -globin subunits forms haemoglobin S (HbS). Under low-oxygen conditions (being at high altitude, for example), the absence of a polar amino acid at position six of the β -globin chain promotes the non-covalent polymerization (aggregation) of haemoglobin, which distorts red blood cells into a sickle shape and decreases their elasticity (fig.2.1). In deoxygenated state haemoglobin S is relatively insoluble and forms needle like tactoids which distort the shape of erythrocyte, producing the characteristic sickle cell deformity. The elongated multipointed and rigid sickled erythrocytes cause an increase in viscosity and obstruct the blood flow in vessels forming the basis of vasoocclusive phenomenon (Serjeant, 2001). In addition the sickled

erythrocytes are mechanically more fragile phagocytosed more readily and are destroyed more rapidly in the circulating blood compared to non-sickled cells. This forms the basis of the anaemia and haemolysis in this disease even in steady state. Healthy red blood cells typically live 100–120 days, but sickle cells only survive 8–25 days (Solankiet *al*, 1988).



Figure 2.1 picture showing sickled RBC (fotosearch.com)

2.1.4 CLINICAL FEATURES

2.1.4.1 General Features

The clinical manifestations of the disease are quite variable and include repeated painful vaso-occlusive, haemolytic, aplastic episodes and sequestration crises. Complications affect various organs and systems mainly skeletal, genito-urinary, gastrointestinal, spleen, hepato-biliary, cardiopulmonary and central nervous system (Edinb, 1998). Neonatal screening and the introduction of prophylactic penicillin in early childhood has reduced mortality to less than 2% by 10 years of age (Bonds, 2005). These changes in life expectancy have shifted the spectrum of clinical problems to an increased focus on chronic organ dysfunction in developed countries.

2.1.4.2 Painful Crises

The hallmark clinical manifestation of SCA is the acute vaso-occlusive event or painful episode. This unique type of pain can start as early as 6 months of age, recur unpredictably over a lifetime, and require treatment with opioids (Smith *et al*, 2005). In children, painful crises often present as dactylitis of the hands and feet ('hand-foot syndrome') and may result in premature closure of the affected epiphysis, leading to shortened deformed bones (Serjeant *et al*, 1994). Painful events are the top cause of emergency room visits and hospitalizations (Ballas 2005; Ballas and Lusardi, 2005), and are also a measure of disease severity and predictor of early death in adults (Steinberg, 2005). Sickle pain can be the prodrome of a serious and potential fatal complication of SCD in some patients (Ballas, 2005).

2.1.4.3 Infections

There are several abnormalities of the immune system in persons with SCA. The early loss of splenic function, either as a result of splenic sequestration and subsequent splenectomy or as a result of 'auto-splenectomy', is a major component in conferring increased susceptibility to encapsulated organisms such as *Strep pneumoniae* and *Salmonella spp.*, and leading to, pneumococcal sepsis especially in children under the age of 3 years. Before the advent of prophylactic penicillin administration, children with SCA were 30-600 times more prone to developing invasive pneumococcal disease, manifesting as septicemia or meningitis (Halasa *et al*, 2007). There are also abnormalities of leucocytes function, complement, immunoglobulins and cell-mediated immunity (Serjeant and Serjeant, 2001). White cell count is known to be a marker of severity in SCD and it is well known that part of the pathogenesis of vascular occlusion in SCD is due to increased leukocyte adherence to its endothelium (Okpala, 2004).

2.1.5 EFFECTS ON MAJOR ORGAN SYSTEMS

2.1.5.1 The Respiratory System

Pulmonary complications are one of the commonest types of complications in SCD. They are also a major cause of acute morbidity and mortality in SCD (Thomas *et al*, 1982; Gray *et al*, 1991; Platt *et al*, 1994). Acute chest syndrome (ACS) consists of a combination of signs and symptoms including dyspnoea, chest pain, fever, cough, multifocal pulmonary infiltrates on the chest radiograph, and a raised white cell count. It is a form of lung injury that can progress to adult respiratory distress syndrome (ARDS).

2.1.5.2 The Cardiovascular system

Blood pressure is typically low in persons with SCA, and in fact what is a 'normal' blood pressure in non-SCD persons may be clinically quite high for those with the disease (Grell *et al.*, 1981; Homi *et al.*, 1993; Pegelow *et al.*, 1997). Various theories for this have been forwarded including defects in renal tubular sodium and water conservation, lowered peripheral vascular resistance and stimulation of renal prostaglandin synthesis (Homi *et al.*, 1993).

2.1.5.3 The liver and spleen

Acute splenic sequestration crisis is a life-threatening complication of homozygous SCD. It is rare in adults due to progressive splenic fibrosis as a result of repeated infarctions and occurs mainly in infants and young children aged less than 8 years (Solanki *et al.*, 1986) and 30% are under the age of 5 years (Emond *et al.*, 1985). It may also occur in patients who have not developed splenic fibrosis and adult patients with sickle cell thalassaemia and sickle cell-haemoglobin C (Hb-SC) and also in patients with high levels of foetal haemoglobin, Hb-F (Serjeant, 1970).

2.1.5.4 The Musculoskeletal System

The painful crisis that has been described already can affect any part of the body, especially the extremities, back or chest. Its severity, location and duration may vary within groups of patients and within each individual. Moreover, the frequency and severity of each pain crisis may change as a person grows from childhood to adulthood, with the break point being the late teens (Serjeant and Serjeant, 2001). The frequency of

the painful crises denotes the gravity of the sickling disorder and three or more crises annually indicate severe disease (Ballas, 2005).

2.1.5.5 The Nervous System

Cerebrovascular accidents occur in 8 to 17% of patients with SS disease, cerebral infarction being more common in children and intracranial haemorrhage commoner in adults. The overall incidence of first overt infarction in HbSS patients by age 20 years is 11% and by age 45 years is 24% (Wong and Powars, 2005).

Neurocognitive dysfunction has been shown to exist in children with SCD, with behavioral problems, low self-esteem and disturbances in body image perception being more evident in them (Brown *et al*, 1993). Developmental processes may be a little slower in children with SCD as well, and the presence of clinically silent brain damage has been documented (Serjeant and Serjeant, 2001). Silent infarcts have been reported to signal future cerebrovascular events.

2.1.5.6 The Genito-Urinary system

Priapism is a painful failure of detumescence which could be due to excess release of contractile neurotransmitters, obstruction of draining venules, malfunction of the intrinsic detumescence mechanism, or long-lasting relaxation of intracavernosal smooth muscle (Serjeant and Serjeant, 2001). Low-flow priapism is more common, and is associated with a reduction in venous outflow, hypoxia, acidosis, stasis and tissue ischaemia.

Renal involvement, known as sickle nephropathy, similar to that seen in diabetic nephropathy is common in sickle cell disease (Ataga and Orringer, 2000), and at older ages is a major cause of illness and death (Thomas *et al.*, 1982). Microalbuminuria (MA) has been reported in 6.2% of paediatric patients with HbSS genotype, but 10% in teenagers with HbSS (Wigfall *et al.*, 2000). In adults, the prevalence rises to 17-49% of those with HbSS genotype (Sesso *et al.*, 1998). The clinical relevance and the pathogenic mechanism(s) underlying MA in sickle cell anaemia are unclear but increased glomerular filtration rate (GFR) and renal plasma flow (RPF) are noted in early years in individuals with SCD Schmitt *et al.*, 1998; (Allon, 1990). It has been proposed that this hyperfiltration leads to gradual sclerosis of the glomerular capillaries and predisposes to renal insufficiency in these patients (Schmitt *et al.*, 1998; Ataga and Orringer, 2000).

2.1.6 SICKLE CELL ANAEMIA AND NUTRITION

Sickle cell anaemia patients require nutrients for effective physiological activities in the body. Such nutrients could be in form of macro and micronutrients. Insufficient macronutrients intake, demonstrated by clinical improvement following dietary intervention was reported by Hayman *et al.*, in 1985. The results showed that protein and energy supplement could improve clinical status and growth in SCA patients, whereas vitamins and minerals supplementations alone did not change clinical status or growth. Several studies have also investigated the role of micromolecules in the pathogenesis of sickle cell disease, by measuring static circulating levels and/or effects observed from dietary supplementation studies (Prasad and Zafralla 1984; Prasad, 1998).

2.1.6.1 Sickle cell anemia and undernutrition

Past studies have shown and documented that SCA leads to a state of undernutrition and poor growth. Most of the studies have linked hypermetabolism with undernutrition in SCA. Simply put, hypermetabolism is a state of increased caloric demand with a high rate of catabolism (nutrient breakdown) compared with anabolism (nutrient buildup). In SCA, however, there is a shift toward increased catabolism, leading to increased nutrient demand. Some studies have documented hypermetabolic states among children with SCA (Borelet *et al.*, 1998; Hibbert *et al.*, 2006).

Hibbert and colleagues (2006) reported that increased myocardial energy demand, along with increased production of pro-inflammatory cytokines, was associated with increased resting energy expenditure (REE), a surrogate marker of a state of hypermetabolism. Other investigators supported their findings with the observation that, although individuals with SCA may consume diet deemed adequate for a person without the disease, it might be insufficient to maintain normal body function and metabolism, as reflected by delayed growth, slowed maturation, and low weight/height for age (Henderson *et al.*, 1994; Leonard *et al.*, 1998). The shortened life span of sickle red blood cells compared with normal red blood cells (Solankiet *al.*, 1988) explains this finding. The increased destruction of these cells creates a need for increased erythropoiesis which leads to increased protein turnover and thus increased energy demand (Buchowskiet *al.*, 2001; Hibbertet *al.*, 2006). Increased haemolysis results in decreased red cell count and anemia. As a compensatory mechanism to maintain tissue oxygenation, the heart rate is increased, leading to increased myocardial energy demand (Hibbertet *al.*, 2006), with the

net effect of an increase in myocardial energy requirement and thus total energy requirement.

As stated above, the patient with SCA is in a state where catabolism exceeds anabolism, resulting in an energy requirement that exceeds the apparently adequate nutrient intake in the absence of SCA. This hypermetabolism has been documented as increased REE, which is a measure of the energy consumption of an individual at rest. Studies showed that individuals with SCA have a higher REE compared with age- and sex-matched healthy controls (Barden *et al.*, 2000). Hypermetabolism may lead to undernutrition because it may cause a diversion of nutrients for growth and other required body functions to support the increased requirement for red cell production, increased myocardial energy demand due to increased heart rate (a compensatory mechanism for anemia), and propagation of the state of chronic subclinical inflammation reported among SCA patients (Hibbert *et al.*, 2006; Akohoue *et al.*, 2007).

Reduced dietary intake also exists in SCA patients, and that the state of undernutrition is due wholly or in part to reduced dietary and energy intake (Fung *et al.*, 2001), with the adequacy of dietary intake decreasing as the individual gets older (Kawchaket *et al.*, 2007). This hypothesis has not gained popularity as expected because other studies have reported little to no difference between the dietary and energy intake of children and adults with SCA compared with healthy non-SCA controls (Singha *et al.*, 2002). Despite this, some investigators have posited that repeated ill health and hospitalization might affect the frequency of dietary and energy intake (Malinauskas *et al.*, 2000; Hyacinth *et al.*, 2010).

High levels of interleukin-6, has been documented to be associated with appetite suppression and, by extension, decreased dietary intake in cancer patients (Rich *et al.*, 2004; van Lettowet *al.*, 2005). Elevated levels of interleukin 6 have also been reported in SCA patients (Hibbertet *al.*, 2005). It is believed that the elevated Interleukin-6 levels observed in SCA patients might act to suppress their appetite, reducing dietary and energy intake. In theory, SCA produces a form of protein energy malnutrition not as a result to poor intake, but to increased energy demand (Hibbertet *al.*, 2005).

2.1.6.2 Effect of infections on nutrition in SCA

Infections occur as a complication of SCA, due to low serum immunoglobulin M levels, impaired opsonization, and slow activation of alternative complement pathway which increases susceptibility to other common infectious agents, such as *Mycoplasma pneumoniae*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Escherichia coli* (Lesourd and Mazari, 1997).

Many studies in the last decade using dietary supplement in patients with SCA have reported improved growth and a decrease in the incidence of infection among the patients receiving these supplements (Salman *et al.*, 1996; Baoet *al.*, 2008).

2.1.6.3 Implication for Growth and Maturation Abnormality

Growth and maturation is affected by SCA, resulting in growth retardation (Henderson *et al.*, 1994) and slowed maturity (Serjeant *et al.*, 2001). Children with SCA attain maturational milestones such as menarche or adrenarche late compared to their age- and sex-matched peers without SCA (Zemelet *al.*, 2007). Their growth is also compromised as a result of diversion of nutrient from buildup of tissues, to principally for replenishing haemolyzed red blood cells and also provision of energy for increased

myocardial demand, also the provision for increased energy demand in a background of inflammation (Hibbert *et al.*, 2006). Studies using dietary supplementation in humans with SCA have reported various benefits of nutritional supplementation (Williams *et al.*, 2004).

2.1.6.4 Nutritional Intervention in Management of SCA

Several attempts have been and are being made to correct the nutritional deficiency in SCA in order to improve growth, body composition, vascular and immune functions (Zemelet *et al.*, 2002; Kaulet *et al.*, 2008; Peranzoniet *et al.*, 2008). The great majority of these interventions have been single-nutrient supplementation, with mixed results (Hyacinth *et al.*, 2010). A few have utilized a combination of nutrients, with really impressive results even with a small sample size (Heyman *et al.*, 1985). Some researchers believe that an approach that utilizes a combination of nutrients (macro- and micronutrients) is likely to produce the best results (Hyacinth *et al.*, 2010). These investigators suggested that because nutrient utilization in the body is a multistep process, with different nutrients feeding into the process at various points, insufficient quantities of one component of this multistep process could have a deleterious effect on the entire body (Chan *et al.*, 1999).

Currently, there are few ongoing pilot clinical trials with the aim of identifying nutritional approaches to managing SCA, using a combination of macro and micro nutrients (Hibbert *et al.*, 2011) to provide additional calories in a low-bulk but high-calorie format that is appealing to children. Some approaches used single macro or micro nutrients or a combination of micro nutrients. Osunkwo *et al.*, (2012), reported an increase in serum vitamin D and vitamin D precursor levels among the supplemented group

compared with placebo. Additionally, they observed a decrease in the number of pain days and an increase in the quality-of-life scores among the supplement compared with the placebo group.

2.1.7 DIAGNOSIS OF SCD

The presence of haemoglobinopathies may be diagnosed from clinical history and physical examination, combined with relatively simple haematological tests (Hendrickse, 1960).

2.1.7.1 Laboratory Diagnosis

Sickle cell disease can be diagnosed in newborns, and older persons. The various laboratory methods are sickling test, solubility test, haemoglobin electrophoresis, isoelectric focusing, high-performance liquid chromatography or DNA analysis. In general, these tests have comparable accuracy. The testing method should be selected on the basis of local availability and cost.

2.1.7.1.1 Haemoglobin sickling test

This is demonstrated in thin wet film of blood sealed with petroleum jelly. If haemoglobin S is present, the red cells lose their smooth round shape and become sickled. This change may be hastened by the addition of the reducing agents such as sodium dithionate or metabisulphate.

2.1.7.1.2 Haemoglobin solubility test

The relative insolubility of deoxygenated haemoglobin S in solutions of high molarity using concentrated phosphate buffers, lysing and reducing agents. With these

reagents haemolysates with haemoglobin S becomes cloudy and those without haemoglobin S remain clear.

2.1.7.1.3 Haemoglobin electrophoresis

Hemoglobin electrophoresis is used as a screening test to identify variant and abnormal haemoglobin, including haemoglobin A₁ (HbA₁), haemoglobin A₂ (HbA₂), haemoglobin F (HbF; foetal haemoglobin), haemoglobin C (HbC) and haemoglobin S (HbS). Alkaline and/or citrate agar electrophoresis is the commonly used method. Separation of haemoglobin is based on variable rates of migration of charged haemoglobin molecules in an electrical field. Haemoglobin is a tetramer with two pairs of globin chains, each containing an identical haeme group. Normal adult haemoglobin (HbA) has two α - and two β -globin chains ($\alpha_2\beta_2$). Foetal haemoglobin (HbF) has two α - and two γ -globin chains ($\alpha_2\gamma_2$). Minor adult haemoglobin (HbA₂) is made of two α - and two δ -globin chains ($\alpha_2\delta_2$).

Haemoglobin electrophoresis has been superseded by more rapid, sensitive and quantitative methods of haemoglobin separation (Rioux *et al*, 1997). Cellulose acetate (CA) electrophoresis at pH 8.2-8.6 can be used to resolve common variants such as HbS and HbC, but it cannot be used to distinguish between HbS and HbD^{Punjab} and HbG^{Philadelphia}. Citrate agar electrophoresis at pH 6.0-6.2 provides better resolution for different haemoglobin variants.

2.1.7.1.4 Isoelectric focusing

Isoelectric focusing (IEF) is a technique for separating different molecules by differences in their isoelectric point (pI). In most cases, the diagnosis of a haemoglobin

disorders can be made using complementary protein chemistry methods such as isoelectric focusing (IEF) and HPLC. IEF on agarose gels separates haemoglobin (Hb) fractions and variants based on their isoelectric points. Hb A and Hb F are clearly resolved by this method. Hb C can also be distinguished from Hb E and Hb O and Hb S from Hb D and Hb G (Campbell *et al*, 1999).

2.1.7.1.5 High performance liquid chromatography

High-performance liquid chromatography (HPLC) is a method that allows the detection of abnormal Hb quickly and precisely, using a small sample amount (Ou and Rognerud, 2001; Old, 2003). Ion exchange HPLC allows the quantification of Hb A₂, Hb F, Hb A, Hb S and Hb C, and screening for Hb variants, thereby being an important method for the investigation of haemoglobinopathies in routine laboratories (Fisher *et al*, 1997; Clarke and Higgins, 2000; Joutovsky *et al*, 2004). The determination of the percentage of an Hb variant and its retention time often allow a more precise identification of the mutant than do electrophoresis methods (Joutovsky *et al*, 2004).

2.1.7.1.6 DNA analysis

After presumptive identification of haemoglobinopathies and thalassemia syndromes and particularly for purposes of genetic counseling, defining the mutation or deletion present may be required. Several molecular techniques are available.

DNA from white blood cells, amniocytes or chorionic tissue may be utilized for diagnosis of various α - and β -globin chain abnormalities. Typically, deletional mutations causing α -thalassemia syndromes and some rare β -thalassemias are diagnosed using

Southern blot hybridization of particular restriction enzyme digests to labeled complementary gene probes.

PCR techniques using allele-specific probes after globin gene amplification, allele-specific primers or deletion-dependent amplification with flanking primers are used in definition of known globin chain mutations/deletions, including those for Hb S, E, D, and O, and several β -thalassemias (Vaysal and Huisman, 1994). For unknown mutations, several PCR-based methods, including denaturing gradient gel electrophoresis and single-strand conformation polymorphism analysis, as well as sequencing of the amplified globin gene DNA may be used (Newton *et al*, 1989).

2.1.8 SAMPLES USED IN ANTENATAL DIAGNOSIS

2.1.8.1 Foetal blood sampling

Isoelectric focusing (IEF), high performance liquid chromatography (HPLC) or variations of these techniques can detect sickle haemoglobin in foetal blood (Blouquit *et al*, 1982). When a sample contains solely fetal blood, the heterozygous condition reveals a major fraction of HbF (80-90%) and equal amounts of HbA and HbS that are easily identified. In contrast, homozygous normal and homozygous HbS fetuses will display either HbA or HbS only respectively. When a mixture of placental and maternal blood is present, the latter red cells (and reticulocytes) can be eliminated by selective haemolysis (Alter *et al*, 1979). The remaining cells can be used for globin biosynthetic studies.

2.1.9.2 Chorionic villi or amniotic cell DNA

DNA extraction: Maternal decidua contamination of trophoblastic tissue must be avoided categorically. Failure to do so can produce catastrophic errors.

Identification of the B^S mutation at the DNA level. Early work used a *Hpa I* restriction fragment length polymorphism (RFLP) to distinguish beta^A-globin and beta^S-globin genes (Kan and Dozy, 1978). The current method of choice employs the polymerase chain reaction (PCR) to amplify the mutated region (CCT GAG GAG to CCT GTG GAG, codons 5-7) for diagnosis.

2.1.9 TREATMENT OF SICKLE CELL ANAEMIA

The treatment goal for sickle cell anaemia aims to relieve pain, prevent infections and manage complications. Bone marrow transplantation is the only potential for cure, but it is used in only a small number of cases because only few patients are able to find compatible donors and also the high cost. Blood transfusions are given when indicated in various situations.

2.1.9.1 Drug treatments

Drug treatment for sickle cell anaemia includes antibiotics and analgesics. Antibiotics, usually penicillin are commonly given to infants and young children, as well as adults to help prevent infections. Pain relief medications ranging from nonprescription nonsteroidal anti-inflammatory drugs (NSAIDs) to opioids are given to control pain.

Hydroxyurea (Droxia) is prescribed for patients with moderate-to-severe sickle cell disease to help reduce the frequency of pain episodes and acute chest syndrome.

2.1.9.1.1 Hydroxyurea

Hydroxyurea is an inhibitor of ribonucleotide reductase that had been in use for many years in the treatment of myeloproliferative disorders. It is an orally available drug that is relatively well tolerated and simple to use. After the demonstration of its ability to induce Hb F production in baboons, hydroxyurea was tested in a number of small clinical trials in adults with SCD (Rodgers *et al*, 1990; Charache *et al*, 1992). A larger study showed a marked decrease in the frequency of painful crises and acute chest syndrome and a reduction in transfusion requirements and hospitalizations in adults with moderate to severe SCD after hydroxyurea treatment (Charache *et al*, 1995).

It was originally proposed that hydroxyurea may elevate Hb F levels by accelerating erythroid differentiation in the bone marrow, leading to the appearance of “foetal-like” cells in the peripheral blood (Stamatoyannopoulos, 2005). More recent studies have shown that hydroxyurea generates nitric oxide (NO) *in vivo*, which results in the activation of the NO/cGMP signaling pathway and the upregulation of γ -globin gene expression in patients with SCD (Cokic *et al*, 2003).

2.1.9.2 Transfusion

Blood transfusions are often critical for treating sickle cell disease. Transfusions may be used either as treatment for specific episodes or as chronic transfusion therapy to prevent life-threatening complications. Ongoing transfusions can also help improve height and weight in children with sickle cell disease.

2.1.9.3 Bone Marrow or Stem Cell Transplantation

The idea of replacing the bone marrow, which is the source of the defective sickle cells, with bone marrow that produces normal RBC is an intuitive therapeutic approach in SCD. However, for many years, such an approach was considered too risky for a nonmalignant disorder such as SCD, since the mortality of the procedure itself was around 20%. The reduction in mortality following bone marrow transplantation (BMT), resulting from recent advances in immunosuppressive therapy and supportive care and the fact that long-term survival of patients with β -thalassemia after BMT was shown to be greater than 90 % (Lucarelliet *al*, 1991), resulted in renewed interest in this therapy for SCD. Clinical trials that were conducted in children with SCD in Europe and the US showed greater than 90% long-term survival (Vermeylenet *al*, 1993; Bernaudinet *al*, 1993; Walters et *al*, 1996). A major limitation in the use of BMT for the treatment of SCD is the fact that a matched sibling donor is available to less than 15% of patients who are suitable candidates for transplantation (Walters et *al*, 1996).

2.2 METHODS OF ASSESSING NUTRITION STATUS

Nutritional screening is the process of identifying characteristics known to be associated with nutrition problems. Its purpose is to identify populations, sub-groups or individuals who are malnourished or at nutritional risk. The availability of methods for the nutritional therapy of hospital patients has caused clinicians to realize the need for reliable methods to select patients for such treatment (Kalantar-Zadehet *al*, 2003). In the early years, following the introduction of the techniques of parenteral nutrition and enteral nutrition, the decision to use those therapies was mostly a clinical one. More recently attempts have been made to employ methods of nutritional assessment to select patients for therapy on the basis that they are ‘malnourished’.

Methods for the study of the nutritional status of populations are well established and have been available (Jensen *et al*, 2012). The information needed for an assessment of the nutritional status of populations falls into four main groups: Clinical observation and examination, anthropometric measurements, biochemical measurements and dietary history.

2.2.1 Clinical observation and examination.

This includes an assessment of the patient’s general condition and close examination of skin, hair, and teeth. This includes an assessment for pallor, clinical assessment of body fat stores, wasting of muscle mass, edema, skin rash, thinning of hair, and evidence of specific nutritional deficiencies. Examples of specific signs include the flag sign or the loss of hair colour associated with a period of malnutrition, followed by recovery with a return of normal hair color and texture to normal.

2.2.2 Anthropometric measurements.

Anthropometric measurements used in nutritional assessment include a measured weight, length or height, and head circumference and these measurements are followed over time to assess short- and long-term growth and nutritional status. For children with chronic disease, a midarm circumference (MAC) and triceps skinfold (TSF) thickness are also part of the assessment to determine body fat and protein stores.

2.2.3 Dietary history.

The dietary history is an essential component of the nutritional assessment. The dietary history provides information not only on the amount and quality of food consumed, but also on the eating patterns and behaviors of the family. This part of the nutritional assessment also provides information on the number of meals, snacks, and beverages consumed; special foods eaten by the child and family; vitamin and mineral supplements ingested regularly; food allergies; intolerances; and unusual feeding behaviors.

This study employed the use of biochemical and physical methods in the nutritional assessment of SCA patients.

2.2.4 Biochemical methods

Nutritional information can be obtained from plasma, serum, urine, stool, hair, and nail samples. The latter two are rarely used clinically. Depending on the underlying medical condition and related nutritional problems from the history and physical

examination, a focused laboratory assessment inform of biochemical measurements may be obtained.

Different biochemical methods of assessment of nutritional status can provide data that may reflect a person's recent dietary intake or long-term intake, or it may not reflect intake at all. Many variables can affect nutrient levels in body fluids. These include activity level, disease condition, stress, age, gender, and the status of other nutrients. There is no one definitive value that correctly identifies an actual deficiency state and it is not always clear if a deficiency state is due to that nutrient or some other metabolic alteration that can cause an abnormal level. A good test should be sensitive to changes in status of a nutrient and be minimally influenced by other factors.

There are many biochemical substances that can be used to assess nutritional status in SCA patients such as concentrations of serum iron, transferrin, plasma albumin and pre albumin, vitamin D, calcium, phosphate, zinc and copper. In this study vitamin D, calcium, phosphate and albumin were used to assess the nutrition status of SCA patients in steady state.

2.3 VITAMIN D

Vitamin D is a fat-soluble vitamin that regulates calcium homeostasis and is vital for bone health (Holick 2004). While it can also be obtained from dietary sources or supplements, vitamin D₃ (cholecalciferol) is synthesized in the human skin from 7-dehydrocholesterol upon exposure to ultraviolet-B (UVB) radiation from sunlight. Vitamin D₂ (ergocalciferol) is a vitamin D analog photosynthesized in plants, mushrooms, and yeasts; vitamin D₂ is also sometimes used in vitamin D food fortification (Bikle 2014). When vitamin D₃ in skin is inadequate due to insufficient exposure to UVB radiation, oral intake of vitamin D is necessary to meet vitamin D requirements.

2.3.1 Vitamin D metabolism

Cholecalciferol and ergocalciferol are biologically inactive precursors of vitamin D and must be converted to biologically active forms in the liver and kidneys. Indeed, following dietary intake or synthesis in the epidermis of skin after UVB exposure, both forms of vitamin D enter the circulation and are transported to the liver by the vitamin D-binding protein (and to a lesser extent by albumin). In hepatocytes (liver cells), vitamin D is hydroxylated to form 25-hydroxyvitamin D. Exposure to sunlight or dietary intake of vitamin D increases serum levels of 25-hydroxyvitamin D (fig.2.2). The 25-hydroxyvitamin D constitutes the major circulating form of vitamin D, and the sum of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ levels in serum is used as an indicator of vitamin D nutritional status (Volmeret *al*, 2015). The renal 25-hydroxyvitamin D-1 α -hydroxylase enzyme (also known as CYP27B1) eventually catalyzes a second

hydroxylation that converts 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D (calcitriol).

The production of 1,25-dihydroxyvitamin D in the kidneys is regulated by several factors, including serum phosphorus, calcium, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23), and 1,25-dihydroxyvitamin D itself. While the kidney is the main source of 1α -hydroxylase activity, extra-renal production of 1,25-dihydroxyvitamin D has also been demonstrated in a variety of tissues, including skin, parathyroid gland, breast, colon, prostate, as well as cells of the immune system and bone cells (Bikle 2004). Most of the physiological effects of vitamin D in the body are related to the activity of 1,25-dihydroxyvitamin D (Holick 2003).

2.3.2. Mechanisms of action

Most, if not all, actions of vitamin D are mediated through a nuclear transcription factor known as the vitamin D receptor (VDR) (Sutton and McDonald 2003). Upon entering the nucleus of a cell, 1,25-dihydroxyvitamin D binds to the VDR and recruits another nuclear receptor known as retinoic acid X receptor (RXR). In the presence of 1,25-dihydroxyvitamin D, the VDR/RXR complex binds small sequences of DNA known as vitamin D response elements (VDREs) and initiates a cascade of molecular interactions that modulate the transcription of specific genes. Thousands of VDREs have been identified throughout the genome, and VDR activation by 1,25-dihydroxyvitamin D is thought to directly and/or indirectly regulate 100 to 1,250 genes (Groberet *al*, 2013).

2.3.3 Functions of vitamin D

The functions of vitamin D are known to affect the followings:

Calcium balance

Maintenance of serum calcium levels within a narrow range is vital for normal functioning of the nervous system, as well as for bone growth and maintenance of bone density. Vitamin D is essential for the efficient utilization of calcium by the body (Holick 2004). The parathyroid glands sense serum calcium levels and secrete parathyroid hormone (PTH) if calcium levels decrease below normal (Figure 2.2). Elevations in PTH stimulate the activity of the 25-hydroxyvitamin D₃-1 α -hydroxylase enzyme in the kidney, resulting in increased production of 1,25-dihydroxyvitamin D. Higher levels of 1,25-dihydroxyvitamin D result in VDR activation and changes in gene expression that normalize serum calcium by (1) increasing the intestinal absorption of dietary calcium, (2) increasing the reabsorption of calcium filtered by the kidneys, and (3) mobilizing calcium from bone when there is insufficient dietary calcium to maintain normal serum calcium levels (Deluca 2004).

Phosphorus balance

The regulations of calcium and phosphorus homeostasis are closely related and the calciotropic hormones PTH and 1,25-dihydroxyvitamin D can also control serum phosphorus. Specifically, 1,25-dihydroxyvitamin D increases D increases intestinal phosphorus absorption by stimulating the expression of a sodium-phosphate co-transporter in the small intestine while PTH increases urinary excretion of phosphorus by reducing reabsorption in the kidney (figure 2.3). It is not yet clear whether

1,25-hydroxyvitamin D can directly regulate renal phosphorus transport. The phosphaturic hormone fibroblast growth factor 23 (FGF-23), secreted by osteoblasts (bone-forming cells), limits the production of 1,25-hydroxyvitamin D by inhibiting 25-hydroxyvitamin D-1 α -hydroxylase (Fukumoto 2014).

It is well known that modest increases in serum phosphate levels significantly affect red-cell metabolism, increased red-cell metabolism, increased 2,3 di-phosphoglycerate (DPG) levels, and cause decreased affinity of oxygen for hemoglobin, i.e., a rightward shift of the hemoglobin-oxygen dissociation curve. Such changes could have significant effects on these patients since a rightward shift in the hemoglobin-oxygen dissociation curve would be associated with decreased oxygen saturation and an increased sickling of red cells, as well as shortening of the delay time for hemoglobin polymerization (Kontessiset *al*, 1992).

Cell differentiation

Cells that are dividing rapidly are said to be proliferating. Differentiation results in the specialization of cells for specific functions. In general, differentiation of cells leads to a decrease in proliferation. While cellular proliferation is essential for growth and wound healing, uncontrolled proliferation of cells with certain mutations may lead to cancer. The active form of vitamin D, 1,25-dihydroxyvitamin D inhibits proliferation and stimulates the differentiation of cells through binding to the VDR (Holick 2004).

Immunity

Acting through the VDR, 1,25-dihydroxyvitamin D is a potent immune system modulator. The VDR is expressed by most cells of the immune system, including

regulatory T cells and antigen-presenting cells, such as dendritic cells and macrophages (Lin and White, 2004). Under specific circumstances, monocytes, macrophages, and T cells can express the 25-hydroxyvitamin D₃-1 α -hydroxylase enzyme and produce 1,25-dihydroxyvitamin D, which acts locally to regulate the immune response (Smolders *et al*, 2011, Edfeldt *et al*, 2010). There is considerable scientific evidence that 1,25-dihydroxyvitamin D has a variety of effects on immune system function, which may enhance innate immunity and inhibit the development of autoimmunity (Dranow, 2011). Conversely, vitamin D deficiency may compromise the integrity of the immune system and lead to inappropriate immune responses.

Insulin secretion

The VDR is expressed by insulin-secreting cells of the pancreas, and the results of animal studies suggest that 1,25-dihydroxyvitamin D plays a role in insulin secretion under conditions of increased insulin demand (Boulonet *et al*, 1999; Zeitze *et al*, 2003). Cross-sectional and prospective studies suggest that insufficient vitamin D levels may have an adverse effect on insulin secretion and glucose tolerance in type 2 diabetes (noninsulin-dependent diabetes mellitus) (Heer and Egert 2015).

Blood pressure regulation

The renin-angiotensin system plays an important role in the regulation of blood pressure (Sheng 2006). Renin is an enzyme that catalyzes the cleavage (splitting) of a small peptide (angiotensin I) from a larger protein (angiotensinogen) produced in the liver. Angiotensin-converting enzyme (ACE) catalyzes the cleavage of angiotensin I to form angiotensin II, a peptide that can increase blood pressure by inducing the

constriction of small arteries and by increasing sodium and water retention. The rate of angiotensin II synthesis is dependent on renin (Sigmund 2002). Research in mice lacking the gene encoding the VDR indicates that 1,25-dihydroxyvitamin D decreases the expression of the gene encoding renin through its interaction with the VDR (Li *et al* 2002). Since inappropriate activation of the renin-angiotensin system can contribute to the development of hypertension, achieving adequate vitamin D levels may be important for decreasing the risk of high blood pressure.

2.3.4 Vitamin D Deficiency

In vitamin D deficiency, calcium absorption cannot be increased enough to satisfy the body's calcium needs (Holick 2003). Consequently, PTH production by the parathyroid glands is increased and calcium is mobilized from the skeleton to maintain normal serum calcium levels—a condition known as secondary hyperparathyroidism. Although it has long been known that severe vitamin D deficiency has serious consequences for bone health, research suggests that less obvious states of vitamin D deficiency are common and increase the risk of osteoporosis and various other health problems.

Severe vitamin D deficiency leads to the following abnormal metabolic conditions

Rickets

In infants and children, severe vitamin D deficiency results in the failure of bone to mineralize. The process of mineralization, which involves the production of crystals of calcium phosphate by bone-forming cells, determines the hardness and strength of bones. Vitamin D deficiency severely affects rapidly growing bones. The growth plates of bones

continue to enlarge, but in the absence of adequate mineralization, weight-bearing limbs (arms and legs) become bowed. In infants, rickets may result in delayed closure of the fontanelles (soft spots) in the skull, and the rib cage may become deformed due to the pulling action of the diaphragm. In severe cases, low serum calcium levels (hypocalcaemia) may cause seizures. Although fortification of food has led to complacency regarding vitamin D deficiency, nutritional rickets is still being reported throughout the world (Wagner and Greer 2008; Goldcareet *al*, 2014).

Osteomalacia

Although adult bones are no longer growing, they are in a constant state of turnover, or "remodeling." In adults with severe vitamin D deficiency, the collagenous bone matrix is preserved, but bone mineral is progressively lost, resulting in a softening of bones (Osteomalacia), bone pain, and increased risk of osteoporosis (Jones and Hansen 2009).

Osteoporosis

Osteoporosis occurs when there is a loss of mineral from bone mainly in the form of calcium as well as architectural loss of normal bone structure. The loss of mineral content of the bone is referred to as a loss of bone mineral density in the bone.

Osteopenia

Osteopenia is diagnosed when there is reduced mineral content in bone, but not as low as to be considered osteoporosis.

Muscle weakness and pain

Vitamin D deficiency causes muscle weakness and pain in children and adults. Muscle pain and weakness were prominent symptoms of vitamin D deficiency in a study of Arab and Danish Muslim women living in Denmark (Bringhurst, *et al*, 2003). In a cross-sectional study of 150 consecutive patients referred to a clinic in Minnesota for the evaluation of persistent, nonspecific musculoskeletal pain, 93% had serum 25-hydroxyvitamin D levels equal to or below 20 ng/mL, with a mean concentration of 12.1 ng/mL, which is indicative of vitamin D insufficiency (Plotnikoff and Quigley 2003). Loss of muscle strength greatly contributes to increased risk of falling and bone fractures, especially in older people (Dendrea *et al* 2010). In addition, long-term vitamin D insufficiency may be a contributing factor to osteoporosis in the elderly.

2.3.5 Risk factors for vitamin D deficiency

Both environmental factors and cultural practices result in variations in vitamin D status:

Environmental conditions: Geographical locations, including latitude and altitudes, and atmospheric conditions (e.g., air pollution, presence of clouds) greatly influence the intensity of UVB radiation that reaches the ground. Seasonal changes also affect the quality and quantity of UVB rays and thus vitamin D production in skin (Cooper *et al* 2011; Webb, *et al.*, 1998).

Concealed clothing style: In a study of 2,032 Middle Eastern women, who wore a headscarf or covered all skin for religious or cultural reasons, 96% had serum 25-hydroxyvitamin D levels less than 20 ng/mL, and 60% had vitamin D levels below 12 ng/mL (Nicholas *et al* 2012). Rickets and osteomalacia are not uncommon in the Middle East and North African regions where children and women cover the majority or all of their skin whenever outside (Bassile *et al* 2013).

Sun safety measures: Sun protection practices, including limiting sun exposure, wearing protective clothing and hats, and applying sunscreens, hinder skin exposure to sunlight and thus result in lower vitamin D₃ production and circulating vitamin D metabolites unless there is adequate oral intake. Of note, the application of sunscreen (2 mg/cm²) with a sun protection factor (SPF) of 10 reduces UVB radiation by 90% (Balk 2011).

Exclusively breast-fed infants: Infants who are exclusively breast-fed and do not receive vitamin D supplementation are at high risk for vitamin D deficiency, particularly if they have dark skin and/or receive little sun exposure (Wagner and Greer 2008). Human milk generally provides 10-80 IU of vitamin D per liter (L), which corresponds to 0.2-1.5 mcg/day (8-60 IU/day) when using an average daily milk intake of 0.75 L (25 oz) (Dawodu and Tsang 2012). The American Academy of Pediatrics recommends that all breast-fed and partially breast-fed infants be given an oral vitamin D supplement of 400 IU/day (Wagner and Greer 2008). Maternal vitamin D supplementation during breast-feeding may contribute to improved vitamin D status of the breast-fed infant, especially in populations with a high prevalence of vitamin D deficiency (Thiele, *et al*, 2013). Older infants and toddlers exclusively fed milk substitutes (e.g., soy-based

formulas) and weaning foods that are not vitamin D fortified are at risk for vitamin D deficiency (Wharton *et al*, 2003).

The efficiency of vitamin D synthesis, absorption, and metabolism also depends on a variety of biological factors:

Skin pigmentation: People with a dark complexion synthesize less vitamin D on exposure to sunlight than those with light-colored skin (Chen *et al* 2007). A national US survey reported average 25-hydroxyvitamin D serum levels of 28 ng/mL, 20 ng/mL, and 14 ng/mL in Caucasian, Mexican American, and African American women aged 20 to 39 years old, respectively (Gindeet *al*, 1988).

Genetic variations: Vitamin D bioavailability varies among individuals and is dependent on the level of circulating vitamin D-binding protein (DBP), a carrier protein that binds 85%-90% of circulating 25-hydroxyvitamin D. The fraction of circulating serum 25-hydroxyvitamin D that is not bound to DBP can be considered readily bioavailable, i.e., more quickly available to act on target tissues. Serum DBP concentrations are influenced by genetic variations (called polymorphisms) in the sequence of the gene coding for DBP. Thus, for a given level of total 25-hydroxyvitamin D, the level of bioavailable 25-hydroxyvitamin D will greatly depend on that of DBP. A polymorphism found to be prevalent in African Americans has been recently associated with a lower level of DBP, suggesting that DBP polymorphisms across different ethnical populations may render the measure of total 25-hydroxyvitamin D sometimes inadequate to reflect vitamin D status (Poweet *al*, 2013).

Older age: The elderly have reduced capacity to synthesize vitamin D in skin when exposed to UVB radiation and are more likely to stay indoors or use sunscreen, which prevents vitamin D synthesis. It has been estimated that across Canada, the US, and Europe, the prevalence of vitamin D deficiency ranges between 20%-100% in free-living elderly (Holick *et al* 2011). Moreover, institutionalized adults who are not supplemented with vitamin D are at extremely high risk of vitamin D deficiency (Harris *et al*, 2008).

Chronic kidney disease (CKD): Vitamin D deficiency in patients with impaired renal function is due to a reduced synthesis of 1,25-dihydroxyvitamin D and an increased loss of 25-hydroxyvitamin D in urine (Allain and Dhesi 2003; Doorenbos *et al*, 2009).

Fat malabsorption syndromes: Vitamin D deficiency is common among people with cystic fibrosis and both cholestatic and non-cholestatic liver diseases due to decreased absorption of dietary vitamin D and impaired conversion of vitamin D to 25-hydroxyvitamin D (Pappa *et al*, 2008).

Inflammatory bowel disease: People with inflammatory bowel disease like Crohn's disease appear to be at increased risk of vitamin D deficiency, especially those who have had small bowel resections (Jahsen *et al*, 2002).

Obesity: Obesity (body mass index ≥ 30 kg/m²) increases the risk of vitamin D deficiency (Arunabhet *et al*, 2003). Once vitamin D is synthesized in the skin or ingested, it can be sequestered in body fat stores, making it less bioavailable to people with higher body fat mass. Vitamin D supplementation trials have shown that obese people reached much lower levels of serum 25-hydroxyvitamin D levels compared to normal weight (BMI <25 kg/m²) participants with equivalent oral dosages (Gallagher *et al.*, 2013).

Magnesium deficiency: Recent findings suggest that high magnesium intakes may reduce the risk of vitamin D insufficiency. Magnesium regulates the activity of critical enzymes in vitamin D metabolism, which would explain how magnesium deficiency negatively affects vitamin D status (Deng *et al*, 2013).

2.3.6 Assessing vitamin D nutritional status

Growing awareness that vitamin D insufficiency has serious health consequences beyond rickets and osteomalacia highlights the need for accurate assessment of vitamin D nutritional status. It is currently agreed that the measurement of total serum 25-hydroxyvitamin D concentration is the best indicator to evaluate vitamin D status. However, high-quality evidence is still needed to ensure that the current cutoff values are optimal to define states of insufficiency and deficiency (Holick *et al* 2011).

Data from supplementation studies indicate that vitamin D intakes of at least 800-1,000 IU/day are required by adults living in temperate latitudes to achieve serum 25-hydroxyvitamin D levels of at least 30 ng/mL (75 nmol/L) (Holick *et al* 2011). Finally, total serum 25-hydroxyvitamin D concentrations may not always adequately reflect vitamin D bioavailability (Powe *et al* 2013) and additional evidence is needed to improve the determination of vitamin D status in different ethnic populations.

2.3.6.1 Vitamin D assay

A complicating factor in the study of vitamin D and its effects has been variability in accuracy and precision of assays for 25(OH)D. Currently, liquid chromatography-mass spectrometry (LC-MS) and high performance liquid chromatography (HPLC) techniques are considered the most accurate and are increasingly available in laboratories worldwide

(Binkley *et al*, 2008; Ross, 2011). However, most literature in the past 20 years has quoted chemiluminescent assays or radioimmunoassays (still the most readily available), which are subject to more errors in observation and interpretation more than LC-MS and HPLC (Ross, 2011).

2.3.6.2.1 Extraction and Deproteinization

Before the advent of commercially available methods, it was common to use two-phase, liquid-liquid partitions with organic solvents and solvent mixtures, including methylene chloride, hexane, diethyl ether, ethanol/chloroform/water, methylene chloride/methanol, hexane/isopropanol and cyclohexane/ethyl acetate. Ethanol and methanol have also been used to free 25(OH)₂D from DBP when 25(OH)₂D is measured.

SYNTHESIS

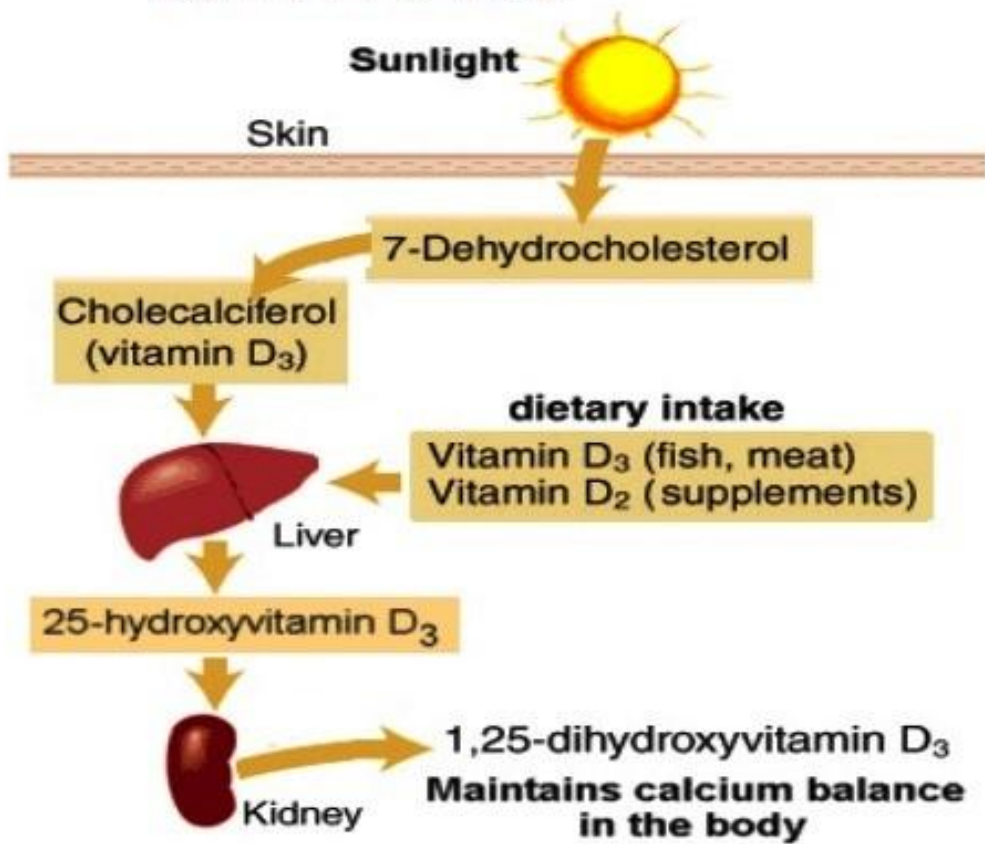


Figure 2.2 Diagram showing VTD synthesis (scientificpsychic.com)

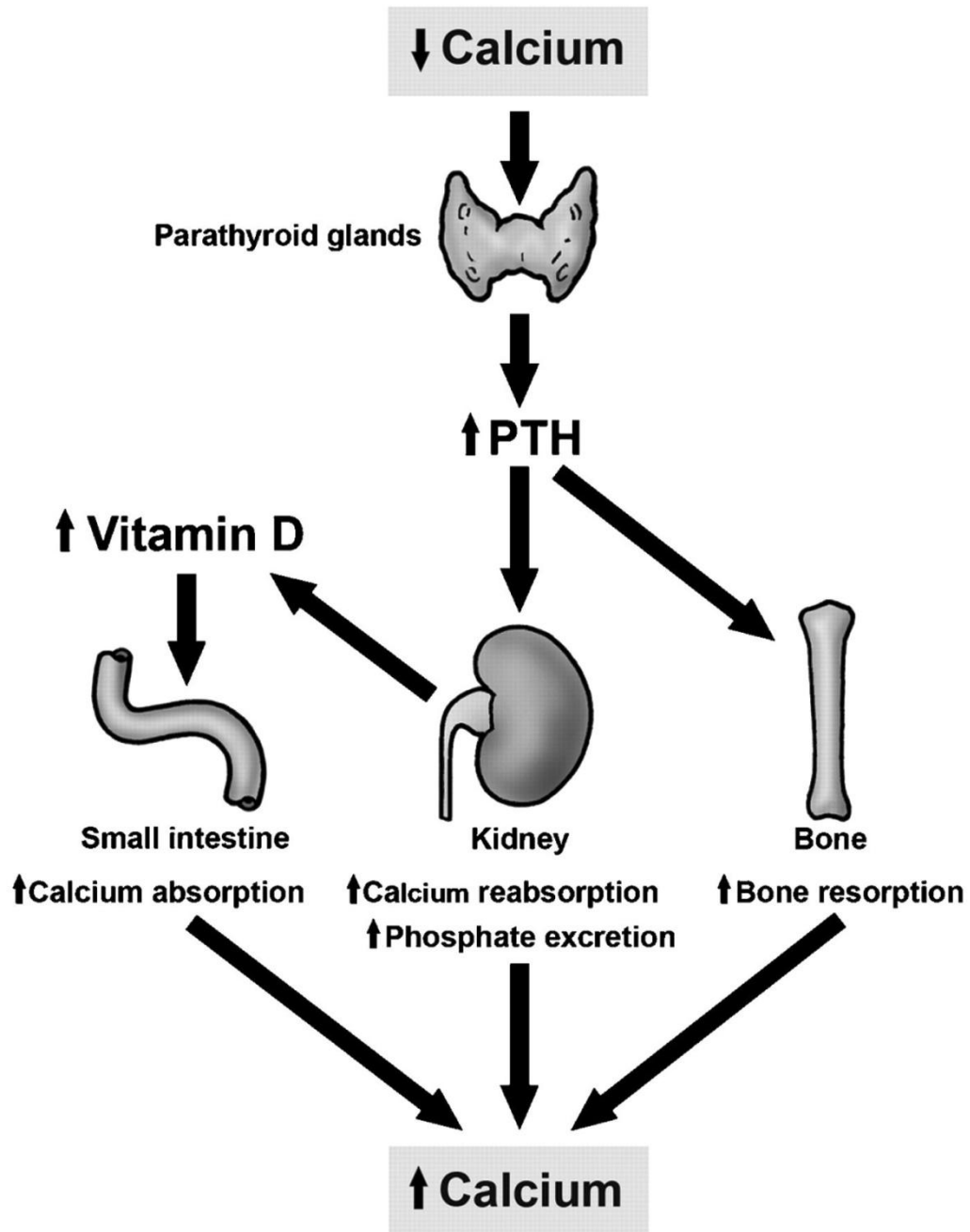


Figure 2.3 Diagram showing calcium and phosphate regulations (scientificpsychic.com)

2.3.6.3 Column Chromatography

Differences in their polarities, related to their numbers of hydroxyl groups have been used to separate vitamin D and its metabolites. With three hydroxyl groups, 1, 25(OH)₂D is more polar than 25(OH)D, with its two hydroxyls, which is more polar than vitamin D, with one hydroxyl group.

2.3.6.4 Measurement of 25-Hydroxyvitamin D

The metabolite is measured by competitive protein binding assay (CPBA), competitive or noncompetitive immunoassay (RIA, EIA, ICMA), ultraviolet (UV) absorption after separation by HPLC or by mass spectrometry after separation by chromatography. Determination by HPLC with UV absorption requires appropriate HPLC equipment, specialized training, and a larger sample. Most clinical laboratories that measure 25(OH)D have chosen the more familiar competitive immunoassays or CPBAs. These assays are relatively easy to perform, use widely available reagent sets and require only a small volume of plasma.

2.3.6.5 Measurement of 1, 25-Dihydroxyvitamin D

The active form of vitamin D, 1,25(OH)₂D circulates at approximately 1/1000 of the concentration of 25(OH)D and at significantly lower concentrations than other dihydroxylated metabolites—facts that together with the extreme hydrophobicity and instability of the compound greatly complicate its determination in serum (Hollis, 2007). The most widely used methods require extraction, chromatography and quantification by radioreceptor assay (RRA) or RIA.

2.3.6.6 Radioreceptor Assay.

Assays using vitamin D receptors from calf thymus or chick intestine have been reported but are rarely used in clinical laboratories.

2.3.6.7 Radioimmunoassay.

The first radioimmunoassay for 1, 25(OH)₂D was introduced in 1978 and required a cumbersome purification. After several assays of similar characteristics, in 1996, another RIA was introduced, with an iodinated reporter and use of an equivalent serum matrix. For this method, sample purification is not needed (Hollis, 2007).

2.3.6.8 Specimen Requirements

Serum is typically used for measuring vitamin D metabolites, although plasma generally is acceptable for assays using extraction and chromatography. Once separated from the clot, serum is relatively stable at both room temperature and 4 °C; specimens should be frozen if the analysis is delayed. Vitamin D metabolites in serum or plasma do not appear to be sensitive to light and do not require special handling in the laboratory.

Reference intervals for vitamin D metabolites are method dependent. A representative one for 1, 25(OH)₂D is: 15 to 60 pg/mL or 36 to 144 pmol/L

2.4 Albumin

In humans, albumin is the most abundant plasma protein, accounting for 55–60% of the measured serum protein (Gosling 1995). It consists of a single polypeptide chain of 585 amino acids with a molecular weight of 66 500 Da. The chain is characterized by having no carbohydrate moiety, a scarcity of tryptophan and methionine residues, and an abundance of charged residues, such as lysine, arginine, glutamic acid and aspartic acid (Peters 1996). The mature, circulating molecule is arranged in a series of α -helices, folded and held by 17 disulphide bridges. The folding creates sub domains of three contiguous α -helices in parallel. A pair of sub domains faces each other to form domains. These can be seen as cylindrical structures with polar outer walls and a hydrophobic central core (Doweiko and Nompleggi 1991).

The serum albumin concentration is a function of its rates of synthesis and degradation and its distribution between the intravascular and extravascular compartments. The total body albumin pool measures about 3.5–5.0 g kg⁻¹ body weight (250–300 g for a healthy 70 kg adult). The plasma compartment holds about 42% of this pool, the rest being in extravascular compartments. Some of this is tissue-bound and is therefore unavailable to the circulation. Each day, 120–145 g of albumin is lost into the extravascular space. Most of this is recovered back into the circulation by lymphatic drainage. Albumin is also lost into the intestinal tract (about 1 g each day), where digestion releases amino acids and peptides, which are reabsorbed. There is minimal urinary loss of albumin in healthy subjects. Of the 70 kg of albumin that passes through the kidneys each day, only a few grams pass through the glomerular membrane. Nearly all of this is reabsorbed, and urinary loss is usually no more than 10–20 mg day⁻¹.

Albumin binds to a surface receptor called albondin, which is widely distributed in many capillary beds, except in the brain (Schinitzer and Oh 1994). Bound albumin enters vesicles within the endothelial cell and is discharged on the interstitial side within 15 s. The rate of transfer is increased with the addition of long-chain fatty acids (LCFAs) to albumin, and with the cationization and glycosylation of the molecule (Peters 1996).

2.4.1 Synthesis of Albumin

In humans, albumin synthesis takes place only in the liver (Lundsgaard-Hansen 1986). Albumin is not stored by the liver but is secreted into the portal circulation as soon as it is manufactured. In healthy young adults, the rate of synthesis is 194 (SD 37) mg kg⁻¹ day⁻¹, or about 12–25 g of albumin per day. The rate of synthesis rate varies with nutritional and disease states. The liver can increase albumin synthesis to only 2–2.7 times normal because most of the liver's synthetic machinery is already devoted to albumin at rest (Peters 1996).

Albumin will be synthesized only in a suitable nutritional, hormonal and osmotic environment. The colloid osmotic pressure (COP) of the interstitial fluid bathing the hepatocytes is the most important regulator of albumin synthesis.

The mRNA concentration available for action on ribosomes is an important factor controlling the rate of albumin synthesis. Trauma and disease processes will affect the mRNA content (Lloyd *et al.* 1987). A reduction in albumin mRNA concentration, caused by a decrease in gene transcription, is seen in the acute-phase reaction mediated by cytokines, mainly interleukin-6 (IL-6) and tumour necrosis factor α (TNF- α) (Brenner *et*

al. 1990). A decrease in gene transcription is also seen in hepatoma cells and in hepatocytes damaged with carbon tetrachloride (Peters 1996).

The rate of synthesis depends on nutritional intake, more so than for other hepatic proteins. Fasting reduces albumin production, but specifically omitting protein from the diet causes a greater reduction in synthesis (Peters 1996).

2.4.2 Degradation of Albumin

Total daily albumin degradation in a 70 kg adult is around 14 g day⁻¹ or 5% of daily whole-body protein turnover. Albumin is broken down in most organs of the body. Muscle and skin break down 40–60% of a dose of labeled albumin (Yedgeret *al.*, 1983). The liver, despite its high rate of protein metabolism, degrades 15% or less of the total. The kidneys are responsible for about 10%, while another 10% leaks through the stomach wall into the gastrointestinal tract.

2.4.3 Role of albumin in maintaining microvascular integrity

It is possible that albumin has a role in limiting the leakage from capillary beds during stress-induced increases in capillary permeability (Demlin 1986). Endothelial cells seem to be able to control the permeability properties of the capillary membrane, possibly by altering the nature and distribution of glycoproteins in the vessel wall. Albumin plays a part in this action, though the exact mechanism is not clear. It may involve the strong negative charge on the albumin molecule repelling other negatively charged molecules in the membrane, or it may be a space-occupying function of the albumin molecule that reduces the size of channels. It is likely that only a small amount of albumin is necessary for this function. A direct protective function of albumin is suggested by the observation

that albumin prevents apoptosis in cultured endothelial cells (Traylor and Pearl 1996). Peak protection was seen at physiological concentrations of albumin.

Albumin is the most important source of sulfhydryl groups in the circulation. Nitric oxide (NO) binds to these sulfhydryl groups to form a stable S-nitrosothiol group, and is thus protected from rapid degradation. The effects of albumin on the vasodilatory properties of NO have been studied *in vitro* (Keanyet *al.*, 1993). Albumin slowed the onset and reduced the maximal intensity of the vasodilatory response to NO. It is possible that albumin has a role in the modulation of vascular tone in different vascular beds, though there is no evidence for this.

2.4.4 The prognostic value of serum albumin

Serum albumin appears to be a reliable prognostic indicator in various contexts. A recent review suggests that serum albumin could be an independent predictor of mortality in a wide range of clinical and research settings (Goldwasser and Fieldman 1997). It reports an estimated increase in the odds of death from 24 to 56% for each 2.5 g litre⁻¹ decrease in serum albumin concentration over the range of studies reviewed. Large community-based studies have shown a link between low serum albumin and an increase in morbidity and mortality (Phillips, *et al*, 1989). Albumin concentrations may be a marker for subclinical disease in elderly patients. In studies of hospitalized patients, hypoalbuminaemia is associated with increased length of stay, higher complication rates and higher mortality (Spiegel and Brayer 1994). In one study, a serum albumin concentration of less than 34 g litre⁻¹ was associated with a 30-day mortality rate of

24.6%. This increased to 62% if the serum albumin concentration was 20 g litre⁻¹ or less (Reinhardt *et al.*, 1980).

The prognostic value of serum albumin extends to critically ill patients (Chin *et al.*, 1980). A low serum albumin concentration correlates with increased length of stay in the intensive care unit (ICU) and with complication rates, such as ventilator dependency and the development of new infection (Murray *et al.*, 1988). The daily trend of serum albumin can be a useful tool in predicting the weaning capability of patients needing mechanical ventilation (Sapijaszko *et al.*, 1996).

Some studies reported that serum albumin can be used as a measure of the nutritional state of patients (Ching *et al.*, 1980), while some claim a benefit in supplementing total parenteral nutrition with albumin in terms of reducing in-hospital morbidity (Kaminski and Williams 1990).

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 THE STUDY AREA

This study was conducted in Ahmadu Bello University Teaching Hospital, Shika, Zaria, Kaduna state. Zaria is a heterogeneous city with a population of about 408,198 people (Zaria, 2015). Zaria is located on the plains of Northern Nigeria, 652.6m above the sea level and some 950 km away from the coast (112 031N 7Q 42E). The predominant occupation of the people of Zaria is farming but civil servants and businessmen also form a sizeable part of the population. The predominant religions are Islam and Christianity. Zaria has a tropical climate with temperature range of 15.3-36.2⁰C and rainfall that ranges from 0-816 mm/month. Zaria has two main seasons, a rainy season from April to October and a dry season from November to March (Encyclopedia 2015)

3.2 STUDY POPULATION AND DESIGN

This is a cross sectional descriptive study involving 120 subjects. These consisted of 60 adult SCA patients in steady state and 60 apparently healthy non SCA adults (HbAA) determined from a formula for the calculation of minimum sample size (Oyejide, 1992; Singha, 1996). These were recruited from patients attending sickle cell clinic in Haematology Department and apparently healthy non SCA subjects (controls) recruited from the population of staff and students of ABUTH, Zaria.

3.2.1 Inclusion criteria

1. Subjects with sickle cell anaemia aged between 15 and 60 years of either sex in steady state were selected and recruited for the study.
2. Apparently healthy non SCA individual (HbAA) aged between 15 and 60 years of either sex were also recruited in the study.

3.2.2 Exclusion criteria

1. Sickle cell patients on any drugs known to affect the nutritional status.
2. Sickle cell anaemia patients in acute crises as well as those who had blood transfusion in the last four weeks.
3. Sickle cell anaemia patients with obvious features of malnutrition.
4. Those subjects who declined to give their consent.

3.2.3 Informed Consent

Informed consent for inclusion into the study was obtained from the patients using a standard informed consent protocol (Appendices I and II).

3.2.4 Ethical consideration

Ethical approval for the study was obtained from the Health Research Ethics Committee of Ahmadu Bello University Teaching Hospital (ABUTH), Zaria, in accordance with the declaration of Helsinki as shown in Appendix III.

3.2.5 Sample size determination

The sample size for the study was determined from a standard formula for the calculation of minimum sample size (Oyejide, 1992; Singha, 1996).

Sample size n is given by the formula:

$$n = \frac{Z^2 pq}{d^2}$$

Where n = minimum sample size; Z^2 = value of the standard normal deviate which at 95% confidence level has been found to be 1.96; P = the best estimate of the population prevalence obtained from the literature review, q = (1-p), d^2 = difference between the true population rate and sample that can be tolerated, that is the absolute precision required (in percentage point) on either side of the population.

WHO (2005) reported the prevalence of SCA of 2% in Nigeria. Therefore, at an established prevalence of SCA of 2%, using 4% precision at 95% confidence interval, the minimum sample size, n for the patients with SCA was calculated as follows:

$$\begin{aligned} n &= \frac{(1.96)^2 (0.02) (1-0.02)}{(0.04)^2} \\ &= \frac{3.84 \times 0.02 \times 0.98}{0.0016} \\ &= 47 \end{aligned}$$

The calculated minimum sample size was 47 patients. However, 25% of the calculated sample size was added to improve the precision. Therefore, 60

patients with SCA in a steady state were consecutively recruited for the study. Similarly, 60 apparently non SCA healthy subjects were consecutively recruited as controls.

3.3 SAMPLING TECHNIQUE

Patients who satisfied the inclusion criteria were enrolled into the study as they present to the clinic (convenient sampling). Similarly, apparently healthy (HbAA) controls were recruited at their respective locations. The nature of the study was explained to all consenting subjects and informed consent was obtained. A full history was taken followed by clinical examination, anthropometric measurement and collection of blood specimens.

Anthropometric measurements were carried out on the subjects by measuring their weight and height using weighing balance (Albert, England) and meter rule. Body mass index was calculated using the formula $(\text{Kg})/\text{H}^2 (\text{m})$, where W = weight in kilogram, H =height in meter and the unit is Kg/m^2 . The findings were documented in the profoma (Appendix IV).

3.4 BLOOD SPECIMEN COLLECTION AND PROCESSING

Blood specimens (5ml each) were collected from peripheral vein by antecubital venepuncture from SCA and the control participants. In this case the antecubital fossa was cleaned with methylated spirit and allowed to dry using sterilized 5ml syringe and 22-G needle. This was transferred into a plain bottle and allowed to clot for about 30 minutes. The blood specimens were centrifuged for 5 minutes at 3000 rpm. The sera were separated from the cells and transferred

to serum bottles and analysed immediately. However, samples that could not be analysed on the day of collection due to logistic problems were stored frozen at 20°C until the possible time of analyses not exceeding three days.

3.5 CHEMICALS

The chemicals used for the measurements of vitamin D, calcium, albumin and phosphate, were procured from WKEA medical supplies corp. (China) and AGAPPE diagnostics (Switzerland GmbH). All the chemicals were of analytical grade or higher.

3.6 EQUIPMENT

Hettich universal 32 centrifuge (Germany) was used to separate the blood specimens. Beckman- Coulter DU-520 general purpose UV/VIS spectrophotometer (Germany) was used for the measurement of serum analytes. RAYTO microplate reader C2100 (China) was used for ELISA technique.

3.7 QUALITY CONTROL

Quality control was observed when carrying out the analysis of the samples by using pooled sera in aliquot to ensure that the results obtained are reliable.

3.8 ANALYTICAL METHODS

3.8.1 Measurement of serum Vitamin D

The serum vitamin D concentrations were measured using the method DiaSorin Liaison (2004).

Principle

The principle of serum vitamin D assay was based on immunological technique. The assay was based on a competitive Enzyme-Immuno-Assay (EIA) technique with a selected monoclonal antibody recognizing 25-dihydroxy vitamin D.

Procedure

This involved two steps. During the first step, 25-hydroxyvitamin D was dissociated from its binding protein and bound to the specific solid phase antibody followed by the addition of vitamin D-isoluminol tracer; unbound material was removed with a wash cycle. In the second step, the reagents were added to initiate the chemiluminescent reaction. The light signal was detected by a photomultiplier as relative light units; this measurement was inversely proportional to the concentration of 25-hydroxyvitamin D. By utilizing several different serum references of known antigen values, a dose response curve was generated from which the antigen concentration of an unknown was automatically calculated by the instrument in relation to the calibration curve stored in memory and then printed out.

3.8.2 Measurement of serum calcium

The serum calcium concentrations were measured using the method of Gitelman (1967).

Principle

Calcium O-cresolphthalein complex procedure was based on the reaction of calcium ions O-cresolphthalein complex in an alkaline solution which formed an intense violet coloured complex with maximum absorbance at 578 nm.

Procedure

The working reagent was prepared by mixing diethylamine (calcium dye reagent) and O-cresolphthalein and 8-hydroxyquinoline (calcium base reagent) in equal proportion. Working reagent, 1.0 ml was dispensed into tubes labeled reagent blank, standard and sample. Specimen, 0.01 ml, was added into appropriate tube labeled sample. Standard, 0.01 ml was also added in the tube labeled standard. All the tubes were mixed well. Distilled water was used for reagent blank. The test sample and the standard were allowed to stand for 5 minutes at room temperature. The instrument was blanked at 578 nm using the reagent blank and absorbances were recorded for standard and the test samples. The results of calcium concentrations from the samples were calculated using the formula, concentration of calcium (mmol/L) = $A \text{ of test} / A \text{ standard} \times \text{concentration of standard}$. Where A= absorbance.

Serum calcium concentration was adjusted to that of serum albumin using the formula, corrected calcium = measured calcium (mmol/L) + [40-albumin (g/L)] $\times 0.02$.

3.8.3 Measurement of serum phosphate.

The serum phosphate concentrations were measured using the method of Fiske and Subbarow (1967).

Principle

In the reaction, inorganic phosphorus reacts with ammonium molybdate in an acidic solution and forms a coloured phosphomolybdate complex. The system monitored the change in absorbance at 365 nm at a fixed time interval. This change in absorbance was directly proportional to the concentration of phosphorus in the sample.

Procedure

Phosphorus reagent 1.0 ml was dispensed each into tubes labeled reagent blank, standard and test. Specimens 0.01 mls, from the standard and test were put into appropriate tube and mixed well. Distilled water was used as blank. The standard and test samples were allowed to stand for 1 minute at room temperature. The instrument was adjusted at 340 nm using the reagent blank and absorbances were recorded for standard and the test respectively. The results of the inorganic phosphate concentrations from the samples were calculated using the formula, concentration of inorganic phosphate (mmol/L) = $A \text{ of test} / A \text{ standard} \times \text{concentration of standard}$. Where A= absorbance.

3.8.4 Measurement of serum albumin.

The serum albumin concentrations were measured using the method of Bartholomew and Delaney (1967).

Principle;

The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3', 5, 5'-tetrabromo-m-cresol sulphonaphthalein (bromocresol green, BCG). The albumin-BCG complex absorbed maximally at 578nm and the absorbance was directly proportional to the concentration of albumin in the sample.

Procedure

Three test tubes were labeled blank, standard and sample. Albumin standard, 0.01ml of was dispensed into the test tube labeled standard, 0.01ml of sample was put into test tube labeled sample and 0.01 ml of distilled water was put into test tube labeled blank. To all the three test tubes, 3.0mls each of BCG reagent was added, mixed and incubated for 5 minutes at 20-25⁰C. The absorbances of the sample and of the standard against the reagent blank were measured at 630 nm. The results of the albumin concentrations in the samples were calculated using the formula below:

Albumin concentration in g/L= A of test/ A standard × concentration of standard. Where A= absorbance.

3.9 STATISTICAL ANALYSIS:

The data obtained were analyzed using Statistical Package for the Social Science 16.0 (SPSS 16.0) for windows (SPSS Inc. Chicago, IL, USA). Serum concentrations of vitamin D, calcium, albumin, phosphate and BMI obtained from sickle cell anaemia patients were compared with those of apparently healthy individuals using two tailed Student's *t* –test. Correlation between BMI and biochemical markers of nutritional status was carried out using Pearson's regression coefficient. A *p*-value of equal to or less than 0.05 ($p \leq 0.05$) was considered as significant.

CHAPTER FOUR

4.0 RESULTS

4.1 The anthropometric characteristics of the study population

The anthropometric characteristics of the study population are shown in Table 4.1. The mean weight, height and BMI of SCA patients were significantly lower compared with the control group ($p<0.05$).

4.2 Biochemical markers of nutritional status (mean $\pm$ SEM) in the study population

The results of serum vitamin D, calcium, phosphate and albumin from steady state adults SCA patients and non SCA control subjects are shown in Table 4.2. The table shows that, the mean serum vitamin D, calcium and albumin concentrations were significantly lower in SCA patients compared with controls ($p<0.05$). The mean serum inorganic phosphate concentration was significantly higher in SCA patients compared with controls ($p<0.05$).

Table 4.1: Anthropometric characteristics of the study population (Mean $\pm$ SEM)

Subjects	n	Age (years)	Weight (Kg)	Height (meter)	BMI (Kg/m ²)
----------	---	-------------	-------------	----------------	--------------------------

SCA	60	24.03±0.66	53.33±1.32	1.57±0.01	21.37±0.35
Control	60	28.03±0.84	64.05±2.0	1.65±0.01	23.27±0.82
p-value		0.012	0.025	0.00	0.03

Table 4.2: Some biochemical markers of nutritional status (mean±SEM) in the study population

Subjects	n	VTD(ug/L)	Ca ²⁺ (mmol/L)	PO ₃ ²⁺ (mmol/L)	ALB(g/L)
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SCA	60	14.55±1.49	2.20 ±0.06	2.00±0.13	33.40±1.66
Control	60	25.87±4.29	2.33±0.04	1.26±0.08	40,13±0.85
p-value		0.014	0.010	0.00	0.01

n=sample size, VTD= vitamin D, Ca^{2+} =calcium ion, PO_3^{2+} =Phosphate ion, ALB= albumin.

4.3 Correlation between BMI and some biochemical markers of nutritional status among the study population

Tables 4.3 and 4.4 show correlations between BMI and each of serum vitamin D, calcium, albumin and phosphate concentrations in steady state adult

SCA patients and controls respectively, the results show no significant correlations.

Table 4.3: Correlation between BMI and some biochemical markers of nutritional status among adult SCA patients in steady state

	VTD(ug/L)	Ca ²⁺ (mmol/L)	PO ₃ ²⁺ (mmol/L)	ALB(g/L)
r-value	0.110	-0.237	-0.153	0.195
p-value	0.401	0.68	0.242	0.136
correlation	NSC	NSC	NSC	NSC

NSC= Non significant correlation

Table 4.4: Correlation between BMI and some biochemical markers of nutritional status among control

	VTD (ug/L)	Ca ²⁺ (mmol/L)	PO ₃ ²⁺ (mmol/L)	ALB (g/L)
r-value	-0.129	0.056	0.140	0.04
p-value	0.325	0.675	0.287	0.978
correlation	NSC	NSC	NSC	NSC

NSC= Non significant correlation

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

The nutritional status of SCA patients was assessed in this study and it was found that SCA negatively affects nutritional status of the patients even in steady state. This was demonstrated by the findings of low anthropometric parameters and equally low serum levels of vitamin D, calcium and albumin with the exception of phosphorus. The low findings of anthropometric parameters and the above named biochemical markers of nutritional status could be due to repeated crises, increased catabolism and poor utilization of the available nutrients.

The findings of decreased anthropometric parameters including BMI, in SCA patients is consistent with the findings of previous studies (Ashcroft and Serjeant, 1981; Mukherjee and Gangakhedkar, 2004; Nandarwar and Kamdi, 2013). Ashcroft and Serjeant (1981) reported low weight deficit but height continued to increase and final height was equal to or better than the normal subjects. They concluded that this was presumed to be as a result of delayed

epiphysial fusion with final height is determine by the degree of delay. While Mukherjee and Gangakhedkar (2004) attributed that the low anthropometric characteristics in SCA could be due to in adequate food intake because of poor appetite. Nandarwar and Kamdi (2013) in their study concluded that the growth of sickle cell anaemia patients was significantly affected by the disease process,

The finding of low serum vitamin D in this study is consistent with the findings of Rovner *et al*, (2007), Goodman *et al*, (2010), Arlet *et al*, (2013) and Tayo *et al*, (2014). Rovner *et al*, (2007) in their conclusion reported that decreased intake, intestinal malabsorption and increased catabolism were responsible for low vitamin D in SCA patients compared to controls. Arlet *et al*, (2013), concluded that vitamin D deficiency is a key feature in sickle cell-bone disease, while Tayo *et al*, (2014), hypothesized that metabolic demand on liver in SCD may have reduced the capacity of the liver to synthesized vitamin D binding protein.

The low calcium concentrations found among the patients of this study is in agreement with the findings of Mohammed *et al*, (1993), Temiye *et al*, (2005) and Qari (2010). In these studies the reduction in serum calcium levels in SCA patients were attributed to the impairment of the digestive and the absorptive function. It can also be due to decreased albumin intake as found out in this study.

The decreased in mean serum albumin could be attributed to decreased intake or increased catabolism. This finding agreed with that of Yusuf *et al*, (2014). However, other studies, found no significant differences in mean serum

albumin concentrations compared with controls (Akuyamet *al*, 2014, Yahya, 2012, van Dijset *al* 1997).

In concordance with other studies (Temiyet *al*, 2005; Smith *et al*, 2007; Qari, 2010), a significant increase in serum phosphate levels were also found in patients of this study which was attributed to the altered renal handling of phosphate. All the studies concluded that the reasons for increased in serum inorganic phosphate concentration in SCA patients, could be due to the increased in tubular reabsorption of phosphate, haemolysis or retention due to kidney involvement. It can also be due to reciprocal relationship with phosphorus.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

It was concluded from the findings of the present study that:

1. There were low mean serum concentrations of vitamin D, calcium and albumin with high inorganic phosphate among SCA patients compared with controls.
2. The mean BMI of SCA patients in steady state were significantly lower compared with controls.
3. No correlations between BMI and the measured biochemical analytes

6.2 RECOMMENDATIONS

It could be recommended from the findings of the present study that:

1. A routine evaluation of vitamin D, calcium, phosphorus and albumin could be considered in the management of SCA patients.
2. There may be a need for introduction of calcium and vitamin D supplementation in the management of SCA patients.

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APPENDIX I

PATIENT INFORMATION SHEET TO PARTICIPATE IN THE STUDY

Serial No.

Hospital No.

Age

My name is Mohammed Manu, a Lecturer and MSc student in the Department of Chemical Pathology.

1. This study is to assess some biochemical markers of nutritional status among adult SCA patients in steady state in Zaria and the procedure involves blood collection.
2. The tests will be of no cost to you.
3. The results from the study will be treated with strict confidentiality.
4. You are free to participate or decline in this study without any consequence.
5. The study will hopefully improve your present health status and that of others in the future.

If you desire to participate in this study kindly, sign the attached form.

Thank you.

Mohammed Manu

Phone contact: 08034469850

Email address: manumohd@yahoo.com

APPENDIX II

INFORMED WRITTEN CONSENT

I.....of.....
agreed to participate in this study, ‘‘The assessment of some biochemical markers of nutritional status among adult SCA patients in steady state in Zaria. ’The full procedure and the probable benefits of the tests were fully explained to me. I was informed that a sample of my blood will be collected for the tests at no cost to me, If I so wish, the test results will be communicated to me in confidence. I made this consent willingly without being subjected to any pressure.

Participant Name.....Signature.....

Witness Name.....Signature.....

Researcher’s Name.....Signature.....

Researcher’s GSM no.....

APPENDIX III

ETHICAL CERTIFICATE



HEALTH RESEARCH ETHICS COMMITTEE

AHMADU BELLO UNIVERSITY TEACHING HOSPITAL

SHIKA - ZARIA, NIGERIA.

E-mail: abuth@yahoo.com

Website: www.abuth.org

Chairman of Board: Chief, Shuaib Oyedokun Afolabi Fnil
Chief Medical Director: Prof. Lawal Khalid, MBBS, FMCS, FWACS, FRCS(ED) mni
Chairman, Medical Advisory Committee: Prof. Abdullahi Mohammed, MBBS, FWACP, FICS
Director of Administration: Barr. Ishak Bello, LL.B, BL., LL.M, PGDM, AHAN, FCAI

Our Ref: ABUTH/HREC/TRG /36

Date: 16th Sept, 2014

Your Ref: _____

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

Serum Vitamin D Levels in Relation to Calcium, Phosphate and Albumin in Adult Sickle Cell Anaemia patients in Steady State in Zaria.

ABUTH Ethics Committee assigned number:	-	ABUTHZ/HREC/K68/2014
Name of the principal investigator:	-	Dr. Mohammed Manu.
Address of the Principal Investigator:	-	Dept of Chemical Pathology ABUTH Zaria
Date of receipt of valid application:	-	20/4/ 2014
Date of meeting when final determination on ethical approval was made:	-	6 th & 7 th April, 2014

This is to inform you that the research described in the submitted protocol, the consent forms and other participant information materials have been reviewed and **given full approval by the Health Research Ethics Committee.**

Please note: this approval dates from **17th September, 2014– 17th September, 2015**

No participant recruitment into this research may be conducted outside these dates.

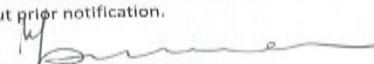
All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: <http://www.nhrec.net>.

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.


Prof. A. I. Mamman

APPENDIX IV

QUESTIONNAIRE FOR THE STUDY OF ASSESSMENT OF SOME BIOCHEMICAL MARKERS OF NUTRITIONAL STATUS AMONG ADULT SCA PATIENTS IN STEADY STATE IN ZARIA.

1. Hospital no.....
2. Address.....
3. Ethnic Group.....
4. Religion.....
5. Age(years).....
6. Sex.....
7. Highest educational level.....
8. Occupation : Civil servant (), Student (), Home maker (), Unemployed (),
Business (), Others ()
9. Drug history.....
10. Weight (kg).....
11. Height (m).....
12. BMI(Kg/m²).....
13. BP.....
14. Laboratory investigations.....
Vitamin D (ug/L)....., Calcium (mmol/L)..... Phosphate (mmol/L).....,
Albumin (g/L).....,