Red cell membrane abnormalities in Hereditary Spherocytosis patients of KwaZulu - Natal

by

Roshini Bridgemohan

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DECLARATION

This study represents original work by the author and has not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

The author carried out the research described in this dissertation in the Haematology and Chemical Pathology Laboratories of King Edward VIII Hospital Durban and the Department of Molecular Medicine and Haematology at the University of the Witwatersrand Medical School in Johannesburg, under the supervision of Professor V B Jogessar and Professor T L Coetzer.

The work done for this study has not been submitted previously to this or any other University.

R Bridgemohan

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- My co - supervisor Professor T.L. Coetzer from the Department of Molecular Medicine and Haematology at Witwatersrand Medical School in Johannesburg, for her constructive criticisms of this study, her supervision and most important for granting me the permission to work in her laboratory for performing red cell membrane analysis.

- To Mr K. Naidoo, the medical scientist in the Department of Molecular Medicine and Haematology at the Witwatersrand Medical School, Johannesburg for his support and guidance; for preparing the red cell membranes of all the samples and for assisting me in the red cell membrane analysis for four patients when it was impossible for me to be in Johannesburg.
- My subjects, many of whom have travelled long distances to the Haematology Laboratory at King Edward VIII Hospital to be participants in this study, without whom this study would not have been possible.
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TABLE OF CONTENTS

PAGE

CHAPTER INDEX

PAGE

LIST OF TABLES AND FIGURES

FIGURES PAGE

LIST OF ABBREVIATIONS

AHG	-	Anti Human Globulin
BETSH	-	Betamercaptoethanol
Bis	-	N, N, Methylenebisacrylamide
EDTA	-	Ethylenediamine Tetra Acetic Acid
EQA	-	External Quality Assurance
F	-	Female
HS	-	Hereditary Spherocytosis
LDH	-	Lactate Dehydrogenase
LGB	-	Lower Gel Buffer
M	-	Male
MCF	-	Mean Cell Fragility
MCH	-	Mean Cell Haemoglobin
MCHC	-	Mean Cell Haemoglobin Concentration
MCV	-	Mean Cell Volume
NPV	-	Negative Predictive Value
PAGE	-	Polyacrylamide Gel Electrophoresis
PMSF	-	Phenylmethylsulfonyl Fluoride
PPV	-	Positive Predictive Value
RBC	-	Red Blood Cell
RDW	-	Red Cell Distribution Width
SDS	-	Sodium Dodecyl Sulfate
TEMED	-	N, N, N, N - Tetramethylethylenediamine
UGB	-	Upper Gel Buffer
RPM	-	Revolutions per minute

ETHICS

Approval to conduct this study was granted by the Ethics Committee of the University of KwaZulu - Natal.

ABSTRACT

Hereditary Spherocytosis (HS) is a common inherited haemolytic anaemia with variable clinical expression. Fifty subjects with HS from KwaZulu-Natal were studied with the aim of providing further information on the protein abnormalities of the red blood cell (RBC) membrane and their relationship with clinical presentations.

Haematological and biochemical tests were performed by routine procedures. Mean Corpuscular Haemoglobin Concentration (MCHC) in the HS group was 35.1g /dl. This was significantly higher than in normal control subjects (33.6g /dl) (p value < 0.001); indicating its usefulness for the screening of HS. Mean Red Cell Distribution Width (RDW) was also significantly higher in subjects with HS ($p<0.001$); thus providing an additional screening tool.

Erythrocyte membrane proteins from 21 subjects were analysed by SDS - polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli and Fairbanks methods. The most common abnormality was a deficiency of band 3 (10 subjects), followed by a combined spectrin and ankyrin deficiency in five subjects. One subject had increased band 6 and in five cases no abnormality was detected. A decreased ratio of protein 4.1a / 4.1b on the Laemmli SDS PAGE correlated with

an increased reticulocyte count. The degree of haemolysis and clinical findings did not correlate with the type of red cell membrane protein defect.

In this study red cell membrane analysis did not contribute further to the initial laboratory diagnosis. In addition it did not influence clinical management. The presence of red cell membrane abnormalities, either single or multiple, did not correlate with disease severity. Red cell membrane analysis, however, will play an important role for future management such as gene therapy. Red cell membrane analysis is also useful as a research tool to determine the underlying molecular defect and to assess racial or ethnic differences. It is also of value as a differential diagnostic tool in cases where the clinical and laboratory findings are not conclusive for HS.

CHAPTER 1

INTRODUCTION

1.1Clinical features of HS

Hereditary Spherocytosis (HS) is a common inherited haemolytic anaemia with a prevalence in the northern European population of 1 in 5000.[1] HS is not uncommon in the KwaZulu-Natal population. The red cell membrane defects, however, have not been previously studied in KwaZulu-Natal. The clinical features of HS are anaemia, jaundice and splenomegaly. The presence of spherocytes and an increase in the red cell osmotic fragility are the hallmarks of this disease, which has a favourable response to splenectomy. Subjects with band 3 deficiency shows "pincered" red blood cells. (Figure 1)

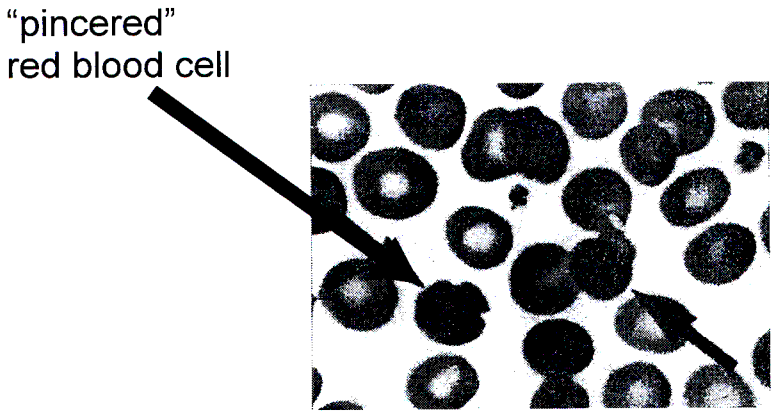


Figure 1 Peripheral smear showing "pincered red blood cells"

This disease is heterogeneous in terms of its clinical presentation, membrane defects and molecular basis. The severity of this disease varies greatly from asymptomatic subjects or patients with slight haemolysis (predominantly with autosomal dominant inheritance) to very severe haemolysis in homozygous and autosomal recessive disorders.[2, 3]

1.2 Structure of the red blood cell membrane of HS

The erythrocyte membrane comprises a lipid bilayer that is supported by the red blood cell skeleton. The red cell membrane and its skeleton provide the erythrocyte the flexibility, durability and tensile strength to undergo large deformations during repeated passages through narrow microcirculatory channels and the spleen during its 4 months lifespan. The lipid 3 bilayer contains numerous proteins including the anion transporter band 3.

The underlying skeleton contains spectrin, actin and protein 4.1. At the interface between these two portions, ankyrin, protein 4.2 and other proteins are found. All proteins are interconnected. The common pathological mechanism in HS is the instability of the membrane with loss of membrane fragments which leads to spherocytosis.

An understanding of the aetiology and pathophysiology of haemolytic states caused by defects of the red cell membrane requires knowledge of its structural organisation.[4] (Figure 2).

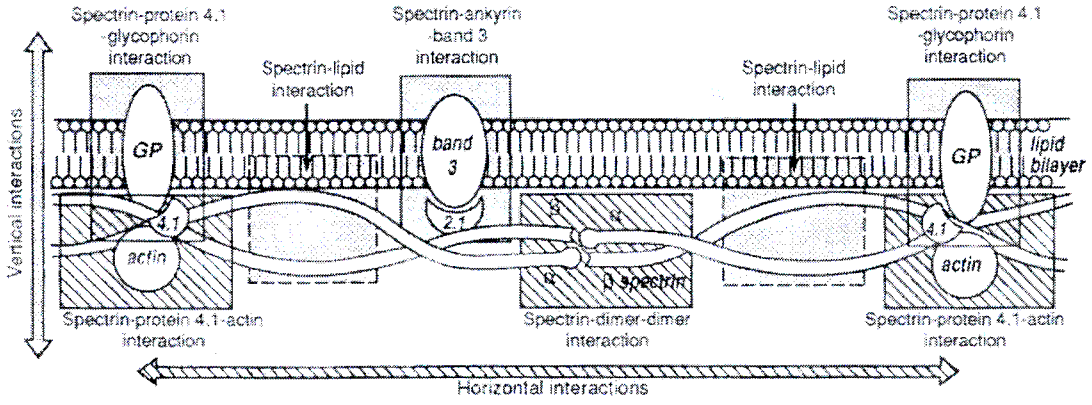


Figure 2. Schematic diagram illustrating the assembly of the membrane proteins.

The vertical interactions of the membrane protein-lipid association are perpendicular to the plane of the membrane and involve:

- spectrin - ankyrin - band 3 interaction
- spectrin - protein 4.1- glycophorin C connection
- weak interactions between spectrin and the negatively charged lipids of the inner half of the membrane lipid bilayer.

The horizontal protein contacts are important in the maintenance of the structural integrity of the cell, accounting for the high tensile strength of the erythrocyte.[5]

1.3 Membrane proteins

All major proteins of the red cell are now well defined in terms of their function and structure, including a complete amino acid sequence.

Membrane proteins can be solubilised in sodium dodecyl sulfate (SDS) and separated by polyacrylamide gel electrophoresis (SDS-PAGE). [6]

These proteins can be divided into two groups:

a) Integral proteins, which are embedded in the lipid bilayer

b) Peripheral proteins of the sub membrane skeleton

Because of their high solubility in non-ionic detergents, the integral proteins are extracted from the membrane, leaving behind the insoluble membrane skeletons.[5]

In contrast, the peripheral proteins, spectrin, ankyrin, actin, protein 4.1, 4.2 and other minor proteins can be extracted from the membrane by aqueous solutions. The red cell membrane skeleton underlies the lipid bilayer and is a loosely knit two dimensional protein network consisting mainly of the structural proteins, α and β spectrin, actin and protein 4.1.[5]

The skeleton is linked to the lipid bilayer by interactions with the integral membrane proteins band 3 and glycophorin C. Near the centre of spectrin tetramers, β spectrin is linked to ankyrin, the protein that connects spectrin to the major trans- membrane protein, the anion transporter (band 3). At the distal ends of spectrin tetramers, spectrin binds to the membrane via linkage to protein 4.1, which binds to the transmembrane glycoprotein, glycophorin C.

These vertical protein-protein, protein-lipid bilayer connections are critical in the stabilisation of the lipid bilayer, precluding its loss from the cells.

1.3.1 Major membrane proteins

The major membrane proteins are listed as follows:

a) SPECTRIN

Spectrin is the most abundant and the largest protein of the skeleton. It consists of two chains α and β, both of which are largely composed of homologous 106-amino acid repetitive segments.[7] Important functional domains of spectrin are the ankyrin - binding sites, located in β spectrin in the 15th repetitive segment, and the tail region of the heterodimers, where spectrin binds to oligomeric actin with the aid of protein 4.1.

The function of spectrin is to maintain cellular shape, regulate the lateral mobility of the integral membrane proteins and provide structural support for the lipid bilayer.

b) ANKYRIN (Band 2.1)

Ankyrin is the major connecting protein that links the membrane skeleton to the bilayer.[8] Disruption of these linkages significantly decreases membrane stability.

Ankyrin is an asymmetric polar protein that contains three functional domains:

(i) a positively charged segment - where ankyrin binds to the cytoplasmic segment of the anion transporter

(ii) a neutral domain - where the protein binds with high affinity to β spectrin, thereby anchoring spectrin to the membrane.

(iii) a regulatory domain - which modulates the interaction of ankyrin with both spectrin and band 3 protein.[8]

c) BAND 3

Red cell band 3, the major integral membrane protein, is the anion exchanger.[9] This anion exchanger is a major trans -membrane glycoprotein that transports anions across the cell membrane. This protein also serves as binding site for ankyrin, thereby anchoring the spectrin based skeleton to the lipid bilayer membrane.[9] It also provides a binding site for proteins 4.1 and 4.2, several glycolytic enzymes, haemoglobin and hemichromes.

The principal transport function of the anion transporter is to carry carbon dioxide from the tissues to the lungs: following hydration of the poorly soluble carbon dioxide to carbonic acid by the erythrocyte carbonic anhydrase, the bicarbonate anion is released from the cells into plasma in exchange for the chloride anion.[9] The two functions of the protein, i.e. the binding of the skeleton and anions transport, are mediated by two distinct structural domains. The 40 - kD N - terminal ankyrin binding domain is located in the cytoplasm, while the C - terminal 55 - kD anion transport portion spans the membrane as much as 13 to 14 times, with the C - terminus of the protein exposed to the cytoplasm. Band 3 is glycosylated at a single external site (Asn-642). Heterogeneity of the N - glycan chains of the individual band 3

molecules accounts for its diffuse migration on SDS- PAGE.

In the normal red cell membrane, band 3 protein is present in the form of dimers, tetramers and larger oligomers.[10] Only the tetramer / oligomer species are associated with the skeleton: the remaining dimeric band 3 proteins are readily extractable by detergents under mild conditions.

d) PROTEIN 4.1

The 4.1 protein has two important functional domains.[11]

- the spectrin binding domain which promotes the binding of spectrin to actin.
- the amino terminal domain binds to glycophorin C and the negatively charged lipids of the inner hemi-leaflet of the lipid bilayer, thereby attaching the distal ends of spectrin tetramers to the membrane.

Protein 4.1 is a phosphoprotein that has four structural domains Viz. 30kDa, 16kDa, 10kDa and 22 to 24kDa. The primary role of 4.1 is the linkage of the spectrin - actin membrane skeleton to the lipid bilayer by facilitating complex formation between spectrin - actin, the cytoplasmic domain of band 3 and p 55 / Glycophorin C. On Laemmli SDS gels, protein 4.1 is separated into two isoforms,

4.1a and 4.1b. The latter isoform is predominant in reticulocytes and is converted into the 4.1a isoform by deamidation of Asn 502.[12]

e) PROTEIN 4.2

Protein 4.2 is associated with several membrane proteins, including the cytoplasmic domain of the anion transporter, ankyrin and protein 4.1 but its function is unknown.

f) ACTIN

Erythrocyte β actin is located in the junctional complexes of the hexagonal skeletal lattice, where about 12 actins form one junctional complex. Dematin, previously referred to as band 4.9, binds to actin and induces bundling of actin filaments. This activity is independent of spectrin and the 4.1 protein but is completely abolished by dematin phosphorylation.

1.3.2 Common membrane protein defects in HS

HS is characterised by a defect of vertical interactions, leading to uncoupling of the lipid bilayer from the skeleton and a release of skeleton free microvesicles.[4] (Figure 3)

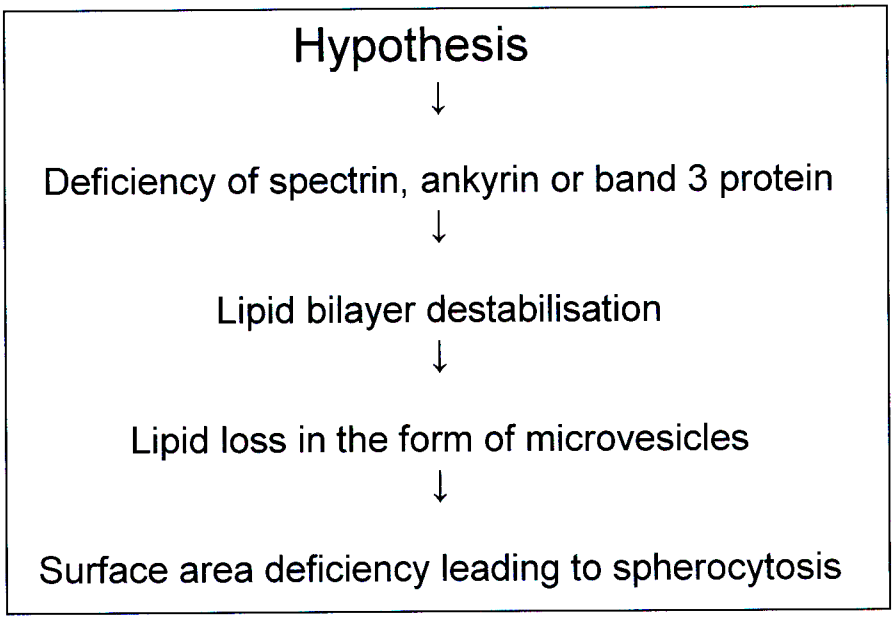


Figure 3. Schematic diagram illustrating the principal molecular defects in Hereditary Spherocytosis.

The underlying molecular defects in HS are heterogenous and several genetic loci have been implicated. In vast majority of cases the abnormalities are quantitative with decreased amounts of the membrane proteins involved in vertical interactions between the bilayer and the skeleton.

Research indicates that the phenotype of HS in humans can be caused by mutations in the genes of α and β spectrin.[13,14] ankyrin[15, 16;] band 3[17] or protein 4.2.[18] The most frequent defect is a combined ankyrin and spectrin deficiency, accounting for almost half of the cases of HS in European and United States populations.[19] In contrast, in South Africa, the most common abnormality is a decreased amount of band 3. The underlying gene defects are point mutations at the two hotspot codons, 490 and 760, as well as a novel E90K mutation, designated band 3 Cape Town (Coetzer et al, Bracher et al) [20]

In southern African blacks, spectrin deficiency is found in vast majority of the cases, but the α or β spectrum mutations have not yet been elucidated. [21]

Another cause of HS is ankyrin deficiency involving chromosome 15 containing the anchoring gene. In some of the cases the defect cannot be identified.[22]

CHAPTER 2

AIM AND OBJECTIVES

2.1 Aim

To study 50 HS patients with the aim of providing further information on abnormalities of red cell membrane proteins and their relationship to the clinical and laboratory aspects of the disease.

2.2 Objectives

1. To evaluate the usefulness of red blood cell indices as an index for screening of HS.

2. To assess the degree of haemolysis in the study population by determining their reticulocyte count; bilirubin and lactate dehydrogenase (LDH) levels.

3. To assess and compare the clinical severity with the number of spherocytes / microspherocytes present on the peripheral smear in patients with HS.

4. To ascertain the co - existence of other haematological conditions viz. haematochromatosis; iron deficiency; vitamin B12 and folate deficiencies and haemoglobinopathies.

5. To identify the red cell membrane protein defects in HS.

6. To correlate the red cell membrane abnormalities with the clinical aspects of the disease.

7. To consider whether the SDS - PAGE analysis of the red cell membrane is of crucial importance and its limitations for a complete evaluation of patients with HS.

CHAPTER 3

SUBJECTS AND SPECIMEN COLLECTION

3.1 Subjects

Fifty unrelated subjects (17 Indians, 14 Whites and 19 Blacks) with HS from Kwa- Zulu Natal were studied. The Indian subjects were descendant of immigrants from various regions of the Indian sub continent. All the black subjects were of Zulu descent and living in KwaZulu Natal. The ancestral origin of the white subjects was not determined.

The diagnosis of HS was confirmed by clinical and laboratory assessment; the presence of spherocytes on the peripheral blood smear; an increased pre - and post - incubated osmotic fragility test; a negative direct anti globulin test (Coombs) and a positive family history. The clinical assessment in all instances was performed by Professor V.B.Jogessar, Department of Haematology, Mandela School of Medicine, University of KwaZulu- Natal. Where this was not possible clinical records were obtained.

3.2 Specimen collection

After obtaining informed consent from all adult patients and normal controls and from parents/ legal custodians in the case of paediatric patients, the following venous blood specimens were collected:

- 3.5 ml in EDTA (supplied by Greiner bio-one)
- 3ml in non- anticoagulant tube
- 6ml in acid citrate dextrose (ACD) solution. This specimen was kept on ice.

The specimen collected in EDTA was analysed for full blood count, reticulocyte count, direct antiglobulin test, haemoglobin electrophoresis, pre - and post - incubated osmotic fragility test.
The clotted blood was analysed for liver function tests, serum vitamin B12 and folate assays and iron studies (serum ferritin, serum transferrin and serum iron.)
The specimen collected in ACD and kept on ice was immediately couriered by air to Professor T.L. Coetzer, The Department of Molecular Medicine and Haematology, Wits Medical School, Parktown, Johannesburg. The red blood cell membranes were prepared by Mr Kuben Naidoo and stored at - 20°C. These specimens were processed by the author at a later date.

CHAPTER 4

METHODS AND MATERIALS

This study was conducted from January 2003 to May 2005. Within 3 hours of specimen collection the following tests were performed:

4.1 Full Blood Count

An automated cell counter, Beckman Coulter Model Maxm (Beckman-Coulter Electronics, Hialeah, Florida), calibrated against normal and abnormal controls, was used to generate haematological parameters and red cell indices. These controls serve as an Internal Quality Control Program. The Thistle Quality controls were processed for the External Quality Control Program. This instrument is based on VCS technology using three measurements: individual cell volume, high frequency conductivity and laser light scatter. Blood smears for white cell differential counts and red cell morphology were made on all specimens. All the peripheral smears were stained using Romanowsky stains (Automated Wescor stainer).

4.2 Reticulocyte count

Reticulocyte counts were performed in all subjects. The EDTA specimen was processed in the automated cell analyser Advia 120. (Bayer Diagnostics, Tarrytown, NY). The reticulocyte indices were measured with the automated flow cytometer. Reticulocytes were stained using the dye oxazine 750. Approximately 20,000 red blood cells were counted for each reticulocyte determination.

The reticulocyte channel of the Advia 120 was calibrated with controls supplied by the manufacturer. This serves as the Internal Quality Control Program for the Reticulocyte count. For External Quality Control Program, Thistle EQA was used.

4.3 Direct antiglobulin test (Coombs test)

Anti Human Globulin Polyspecific (Ortho Clinical Diagnostics Inc. Raritan, NJ, USA) is used in antiglobulin tests to demonstrate the presence or absence of gamma globulin (antibodies) on the surface of the red blood cells. The Direct antiglobulin test reveals the in *vivo* red cell sensitisation, either by auto - antibodies or by allo-antibodies.

A 5% suspension in normal saline of the patient's red blood cells was prepared. Two drops of this suspension was placed in a glass tube and washed three times with 0.9% sodium chloride solution. The same wash procedure was used for the negative and positive controls. Two drops of broad spectrum Anti – Human Globulin (AHG) was added to the patient's washed red blood cells, as well as to the negative and positive controls.

The preparation was centrifuged at 3,500 r.p.m. in a DADE IMMUFUGE and examined for red cell agglutination. The absence of red cell agglutination determined a negative direct antiglobulin test result.

Sensitised red cells (coated with IgG and complement) were used as positive control. O Rh Negative red cells were used as negative control. The South African National Blood Services supplied both these controls.

4.4 Osmotic Fragility Test:

Pre - incubated test:

Within 5 hours of collection of the sample the pre - incubated osmotic fragility test was carried out. This test is essentially a measure of the surface - to - volume ratio of the cell. This test which is performed on fresh red blood cells is also a measure of

the proportion of cells that have undergone splenic conditioning. Small volumes (50ul) of well-mixed blood were added to 5 mls of buffered saline solutions of varying concentrations. When red blood cells are placed in a series of graded hypotonic sodium chloride solutions, water rapidly enters the cells and osmotic equilibrium is achieved. The cells swell, become spherical, and eventually a critical volume is reached at which point the cell bursts and the cellular content, haemoglobin, is released. The fraction of red cells lysed at each saline concentration was measured in a spectrophotometer. The test was carried out at room temperature. In this study the patients' red blood cells were placed in a series of graded hypotonic salt solutions i.e. of varying concentrations from 0.1% to 1.0% sodium chloride solutions and incubated at room temperature for 30 minutes to ensure complete lysis. The suspensions were then centrifuged at 5000 r.p.m. and the optical density was measured at a wavelength setting of 540nm using the 1% sodium chloride solution as blank. The percentage lysis at each sodium chloride concentration was calculated and plotted against the sodium chloride concentration of each tube. A normal control was set up and processed similarly.

The concentration of saline causing 50% lysis i.e. median corpuscular fragility (MCF) was recorded.

Post incubated Osmotic Fragility Test:

Fresh EDTA blood for both the patient and normal control were incubated at 37°C for 24 hours and the test was carried out the same as for the pre - incubated osmotic fragility test. An osmotic fragility curve was drawn using the calculations as for the pre - incubated osmotic fragility test.

4.5 Serum Vitamin B12 and Folate Assay

This assay is based on chemiluminescence method.

Chemiluminescence occurs when a chemical reaction causes a susceptible molecule to enter an unstable excited state and then return to a stable form with the emission of light.

Specimens were collected in non-anticoagulant tubes and the sera were separated. The serum was processed in the Beckman Access that is calibrated with normal and abnormal controls and whose performance is quality controlled by processing both internal control (manufacturer) and external controls (Thistle).

4.6 Serum Ferritin

The method used for determining ferritin was the Immage® Immunochemistry systems by Beckman Coulter. Ferritin (FER) reagent, is used in conjunction with the IMMAGE® Immunochemistry Systems and the Ferritin Calibrator, intended for the quantitative determination of ferritin in human serum by turbidimetric immunoassay. The IMMAGE FER reagent is based on the highly sensitive Near Infrared Particle Immunoassay (NIPIA) rate methodology. The anti- ferritin coated particle (AFCP), binds to ferritin molecules (FER) in the sample resulting in the formation of insoluble aggregates causing turbidity. The turbidity is measured using the near infrared light source and detector. The rate of particle formation is directly proportional to the concentration of ferritin in the sample.

The Internal Quality Control used for this assay is Liquichek level 1,2 and 3 supplied by Biorad Laboratories, Ca, USA. External Quality Controls from the Royal College of Pathologists, Australia were used.

4.7 Serum LDH, Bilirubin, Transferrin and Iron

The multi - channel SYNCHRON LX® Systems Beckman Coulter LX20 was used to process specimens for LDH, bilirubin, transferrin and iron levels. Lactate Dehydrogenase (LD-P) Reagent is used to measure the lactate dehydrogenase activity by an enzymatic tare method. In the reaction. LD-P catalyzes the reversible reduction of pyruvate to L- lactate with the concurrent oxidation of reduced ß – nicotinamide adenine dinucleotide (NADH) to ß- nicotinamide adenine dinucleotide (NAD).

Total Bilirubin Reagent is used to measure the total bilirubin concentration by a timed endpoint Diazo method. In the reaction, the bilirubin reacts with the diazo reagent on the presence of caffeine, benzoate, and acetate as accelerators to form azobilirubin.

Transferrin Reagent is used to measure the transferrin concentration by a turbidimetric method. In the reaction, transferrin combines with specific antibody to form insoluble antigen- antibody complexes.

Transferrin in serum is completely saturated by adding excess ferric ion in the form of ferric chloride. Any iron not bound to transferrin is absorbed by aluminium oxide in the column. Iron bound transferrin in the supernatant is measured by the Total Iron

Binding Capacity Reagent (IBCT). IBCT is used to measure the iron concentration by a timed - endpoint method.

This multi-channel Beckman Coulter LX20 is calibrated with Beckman Coulter Tri- level controls Decision level 1,2 and 3. For EQA, the Australian Royal College of Pathologists controls are used.

4.8 Haemoglobin Electrophoresis

Haemoglobin electrophoresis, using the Beckman Paragon haemoglobin electrophoresis system was used to screen for haemoglobin variants on all subjects (pH of buffer: 8.6 alkaline).

If abnormal haemoglobins were found, electrophoresis using an Acid buffer (pH = 6.0) was performed using the Beckman Paragon Acid Kit.

Known abnormal controls as supplied by Beckman Coulter were used.

The principle of electrophoresis is based upon the fact that haemoglobins, when placed in an electric field will migrate towards one of the electrode poles. The Paragon kit provides for the electrophoretic separation of haemoglobins in an alkaline buffered agarose gel.

After electrophoresis the haemoglobins in the gel are immobilised in a fixative solution and the gel is dried to a film. The haemoglobin pattern is visualised by staining the film with Paragon Blue Stain Solution (protein specific stain). This pattern is quantitated by Beckman- Coulter Model CDS-200 Computing Densitometer System.

Alkaline agarose electrophoresis effectively resolves normal haemoglobin (A and F) and major variants (S and C), but cannot distinguish the minor Variants G and D, E and O from Hb S and Hb C respectively. Electrophoresis at acid pH (6.0) not only resolves normal and major haemoglobins, but also allows Hb G and D to be distinguished from Hb S, and Hb E, Hb O, and Hb C to be resolved from one another.

4.9 Polyacrylamide gel electrophoresis - SDS - PAGE

The polypeptides of the human erythrocyte membrane were analysed by polyacrylamide gel electrophoresis. Polyacrylamide gels are formed by the polymerisation of acrylamide monomers into linear chains and the linking of these chains with bis. Polymerisation is catalysed by ammonium persulphate and stabilised by TEMED. The concentration of acrylamide and the ratio of acrylamide to bis determines the pore size of the resultant

three dimensional network and hence its sieving effect on proteins of different sizes.

4.9.1 Red cell membrane preparation

For logistical reasons the red cell membranes were prepared by the scientist, Mr K.Naidoo, at the Department of Molecular Medicine and Haematology at Witwatersrand Medical School in Johannesburg.

Erythrocyte ghosts were prepared from blood collected in acid citrate dextrose by the hypotonic lysis procedure with the addition of Phenylmethylsulfonyl Fluoride to prevent proteolysis at the lysis step.[23]

The samples and wash solutions were kept on ice at all times. Red blood cells were washed 3 times with cold 0.9% NaCl in a Jouan Centrifuge, 10 minutes each at 2500 rpm at 4°C in 10ml polystrene tubes using a swing- out bucket rotor. The supernatant and "buffy coat" were aspirated and discarded.

The lysis buffer was made up of 3mM $Na_2 HPO_4 2H_2 0$; 0.1mM EDTA; 0.1mM PMSF; MilliQ water. The pH of the buffer is adjusted to 8.0 with the addition of 0.2% NaH_2PO_4.

The washed red cells were transferred to 50ml Beckman centrifuge

tubes. The red blood cells were lysed with 30ml lysis buffer. The tubes were centrifuged for 15 minutes at 15,000 rpm at 4°C in a Beckman J2-21 centrifuge. The resulting deep red supernatants were aspirated, leaving red, translucent pellets of packed ghosts over minute, opaque, cream colored 'buttons'. The tubes were swirled and the tightly packed buttons containing white blood cells at the bottom of the tubes were aspirated. The wash procedure was repeated four times using lysis buffer. After the final wash membranes appeared fluffy white. At the final wash the buffer was aspirated until a 1:1 ratio of membranes to buffer was left.

The membranes were kept on ice and solubilised as follows:

40ul	5x Suspension solution (Tris 50mM, EDTA 5mM, 5% SDS, 25% Sucrose. pH to 8.0 with HCl)
5ul	Sucrose & Dye (2.5% Sucrose; 0.5% Bromophenol Blue)
4ul	βETSH (Betamercaptoethanol) - added in fume cupboard)
150ul	Membranes (added last)

The aliquots were well mixed and boiled immediately for 1 minute and stored at -20 ° C.

4.9.2 Protein determinations

The Coomassie Plus - The Better Bradford ™ Assay Kit. (Supplied by Pierce, Rockford, IL) was used for total protein quantitation. The Protein concentration was measured using bovine serum albumin (BSA) as a standard. The Coomassie Plus Kit is a quick and ready to use coomassie- binding, calorimetric method for total protein concentration. This modification of the well known Bradford method greatly reduces the tendency of coomassie reagents to give non-linear response curves by a formulation that substantially improves linearity for a defined range of protein concentration. In addition, the Coomassie Plus Reagent results in significantly less protein to protein variation than is observed with other Bradford type coomassie formulations.

When coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue. Performing the assay in the test tube is simple: Combine a small amount of protein sample with the assay reagent, mix well, incubate briefly and measure the absorbance at 595nm. Protein concentrations are estimated by reference to absorbances obtained for a series of standard protein dilutions, which are

assayed alongside the unknown samples.

Method

Using 5-ml glass tubes, protein samples were prepared in duplicate using 5ul of membrane. 5ul of 5N NaOH was added. This helps in the solubility of the protein.

Using the standard curve, the protein concentration estimate for each unknown sample is estimated. BSA, which is supplied with the Coomassie Plus ^{TM} kit has a concentration of 2ug/ul.

When generating the standard curve, BSA is added to the Coomassie reagent as follows in duplicate glass tubes:

	Volume of BSA	Amount of protein
Standard 1	1ul	2ug
Standard 2	2ul	4ug
Standard 3	4ul	8ug
Standard 4	6ul	12ug
Standard 5	8ul	16ug

1.5 ml of Coomassie Plus Protein Assay Reagent (component of Product number 23236 by Pierce, Rockford, IL) was added to all the tubes. The tubes were mixed immediately and allowed to stand for 5 minutes.

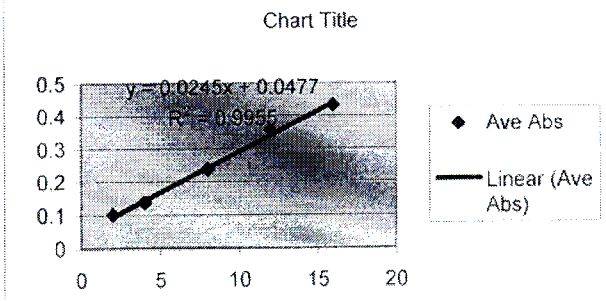
The absorbance at 595nm was determined for each sample using the Beckman DU 65 spectrophotometer within 30 minutes of preparation.

The standard curve was plotted in Microsoft Excel using the line equation as well as the correlation co-efficient (r^2) (Figure 4). From the equation of the line as well as taking the dilution factors when solubilising the membranes into account, the concentration of the membrane proteins were worked out by the program.

(Figure 4)

(Dilution factor for membrane solubilization: 150/199 = 0.75)

Protein Concentration Determination			
Date: 23/06/2004			
Roshni's Samples			
Standard Curve			
ug BSA	Ave Abs	Absorbance (595nm)	
2	0.102	0.093	0.111
4	0.138	0.123	0.153
8	0.2385	0.244	0.233
12	0.3555	0.356	0.355
16	0.4325	0.427	0.438



Patient Samples				
Buffy No.	Absorbance (595nm)		Ave Abs	[Prt] ug/ul
191	0.222	0.225	0.2235	1.076327
193	0.274	0.206	0.24	1.177347
194 (Con)	0.448	0.407	0.4275	2.325306
195	0.107	0.097	0.102	0.332449
196	0.248	0.193	0.2205	1.057959
202	0.154	0.168	0.161	0.693673
205	0.2	0.204	0.202	0.944694
207	0.244	0.241	0.2425	1.192653
208	0.233	0.244	0.2385	1.168163
209	0.231	0.243	0.237	1.15898

Figure 4 Protein determinations and the graph used to determine the concentration of protein in the sample.

The membrane protein concentration was used to determine the aliquot of membrane sample that was required for both the Laemmli and the Fairbanks PAGE.

4.9.3 SDS – Protein Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for all subjects utilising both the discontinuous buffer system of Laemmli with 12% acrylamide [24] and the continuous buffer system of Fairbanks[6], with an exponential gradient of 3.5% - 17.5% acrylamide.

Proteins were stained with Coomassie blue and gels were scanned using a densitometer from Hoefer Scientific Instruments (San Fransciso) which was attached to a computer. The amount of the major membrane proteins was then expressed as a ratio to band 3 e.g. band 4.1 / band 3; band 4.2 / band 3; spectrin / band 3.

The amount of each protein was compared to normal values obtained from normal controls. A decreased spectrin / band 3 ratio indicates a decreased spectrin level on. A decreased band 3 is reflected by the increased ratios of all proteins relative to band 3.

<u>40%Acrylamide with 1.5% Bis:</u>
40g Acrylamide
1.5 g Bis
Make up to 100 ml with MilliQ water

10%SDS:
10g SDS and make up to 100ml with MilliQ water

25% Glycerol:
25 ml Glycerol and make up to 100ml with MilliQ water

10% APS:
0.5g APS and make up to 5ml with MilliQ water

0.5% TEMED:
50ul TEMED and make up to 10ml with MilliQ water.

10x Tris acetate buffer:

48.46 g Tris (0.4M)	Molecular weight	= 121.14
116.41g Sodium Acetate (0.2M)	Molecular weight	= 82.03
7.44 g EDTA (0.02M)	Molecular weight	= 372.24

Make up to 900ml with MilliQ water.
pH to 7.4 with glacial acetic acid.
Adjust to 1 litre with MilliQ water.

1x Tris acetate buffer (Fairbanks Running Buffer):
100ml 10x Tris acetate buffer
895 ml MilliQ water
5 ml 20% SDS

Coomassie Blue Stain:
0.5g Coomassie Blue R-250
250ml Isopropanol
100ml Acetic acid

Make up to 1 litre with MilliQ water

Stir overnight and filter through Whatman's No 1 paper.

The Fairbanks SDS- PAGE (exponential gradient gel) was set up as follows for the two reservoirs of a gradient mixer:

	Reservoir mixtures	
	(left) 3.5%	(right) 17.5%
40% Acrylamide +1.5% Bis	3.0 ml	3.4ml
10xTris acetate buffer	3.4 ml	0.8ml
10% SDS	0.68ml	0.16ml
MQ water (MilliQ)	26.9ml	1.62ml
25% Glycerol	-	2.0ml
10% Ammonium persulphate (fresh)	0.4 ml	0.02ml
TEMED	11ul	-
0.5% TEMED	-	0.5ml

TEMED and APS were added last. Both taps in the reservoir were closed.

The mixtures were stirred at moderate speed using a magnetic stirrer. The plunger was put 2mm from the surface of 17.5% mixture. The plunger tube was clamped. The plunger is used to create a closed system such that the resulting gel has a uniform gradient with a 17% acrylamide concentration at the bottom of the gel and 3.5% at the top. If the plunger was not used, the solutions would mix and a gel with uniform acrylamide concentration will form.

The peristaltic pump was set at a speed of 5.5. The connecting screw was opened allowing the gel mixtures to mix and the outlet screw was immediately opened. The gel was poured with a 12 combs gel in place and allowed to set for a few hours. The gel electrophoresis system used is from Hoefer Scientific Instruments in San Francisco, USA. The model of the vertical slab unit is SE400. The size of the gel plate is 14 x 18 cm.

20 and 22ug aliquots of each specimen were electrophoresed at 45V for 17 hours in 1x Tris Acetate Buffer with 0.1% SDS. During solubilization, SDS and β-mercaptoethanol were added to the protein before boiling one minute. This ensured that the proteins were reduced and no disulfide linkages were present. The SDS gave the peptides an overall negative charge and hence the proteins were separated according to size. Thus the proteins electrophoresed towards the positive electrode, which is the anode i.e. the proteins moved from the cathode towards the anode. The gel was stained in Coomassie Blue overnight and destained in 10% acetic acid and 10% methanol overnight.

The gel was destained in 10% acetic acid until background was clear.

The Laemmli SDS - PAGE was set up as follows:

30% Acrylamide:
30g Acrylamide and adjust to 100ml with MilliQ water.

Laemmli Running Buffer:
6.06g Tris (0.025M) Molecular weight = 121.1
28.8g Glycine (0.19M) Molecular weight = 75.07
2.0g SDS

Make up to two litres with MilliQ water.

Lower Gel Buffer 1.5 M (Laemmli):
18.17g Tris Molecular weight = 121.4

Make up to 90 ml with MilliQ water
pH to 8.8 with HCl
Adjust to 100ml with MilliQ water

Upper Gel Buffer 0.5M (Laemmli):
6.06g Tris Molecular weight = 121.14

Make up to 90ml with MilliQ water
pH to 6.8 with HCl

Adjust to 100ml with MilliQ water

	12% Resolving gel	**4% Stacking gel**
30% Acrylamide	12ml	1.3ml
1% Bis	3.2ml	1.0ml
4Xresolving buffer-Lower Gel Buffer	7.5 ml	-
4xStack buffer – Upper Gel Buffer	-	2.5ml
10% SDS	0.16ml	0.02ml
MQ(water)	7.0ml	4.9ml
10% APS w/v(made fresh)	0.2ml	0.2ml
TEMED	0.015ml	0.0075ml

The comb with 12 wells was placed in the gel space. Resolving gel was poured and overlayed with water. The gel was left aside to set for 3 hours. The water was decanted. The stacking gel was then poured and left aside for an hour to set.

45ug aliquots of membrane preparation were electrophoresed at 75V for 17 hours. The gel was stained in Coomassie Blue and de-stained as for the Fairbanks gel.

5.0 Statistical methods

Descriptive statistics, Mann-Whittney and the student t- test were the methods used to analyse the data.

All analyses were taken at the 5% level of significance. Statistical significance was defined as the p value less than 0.05.

Descriptive statistics are for summarizing the data and it gives the mean and standard deviations.

Mann - Whittney test:

The Mann - Whittney test is a non - parametric method for analysis. This test is used to check the results obtained with the t - tests.

t - test:

This was used to carry out tests of significance for the means of small number of samples.

CHAPTER 5

RESULTS

5.1 Demography

The demography of the study group is shown in Table I

Table I Demography of the study group

	Males	**Females**	**Total**
Indians	10	7	17
Whites	8	6	14
Blacks	10	9	19
Total	28	22	50

5.2 Red cell parameters

The red cell parameters, obtained for patients with HS were compared with normal controls.

Statistical significance was defined as a p value less than 0.05. Using the t - distribution of the student t - test to determine the significance for the means of small samples the following results were obtained. (Table II)

Table II - Red cell parameters, means and p values

	Subjects (n=50)		**Normal (n=50)**		
Parameters	Mean	Range	Mean	Range	P value
Rbc (M) 10^{12}/l	4.62 S.D. 1.03	2.52-6.37	4.49 S.D 0.53	4.7 - 6.1	0.583
Rbc (F) 10^{12}/l	3.84 S.D. 0.79	2.68-5.23	4.09 S.D. 0.41	3.8-5.2	0.191
Hb (M) g/dl	13.8 S.D. 3.18	6.7-18.8	13.5 S.D. 1.61	14.0-16.0	0.666
Hb (F) g/dl	11.5 S.D. 2.59	7.9 -15.6	12.0 S.D. 0.95	11.5-16.0	0.374
MCV fl	85.98 S.D. 7.43	71.8 - 101.8	87.92 S.D. 4.46	80-94	0.116
MCH pg	29.86 S.D. 2.55	22.5-34.2	29.79 S.D. 1.62	27.0-32.0	0.863
MCHC g/dl	34.92 S.D. 1.75	30.1-37.7	33.75 S.D. 0.96	30.0-35.0	<0.001
RDW %	16.18 S.D. 4.94	11.5-29.3	13.53 S.D. 1.06	11.5-14.5	<0.001

On comparing the HS group vs the normal control for RBC; Hb; MCV and MCH the p value was > 0.05, hence there was no statistical significance with these parameters.

The mean MCHC values in the HS group were higher than that of the normal control group. The mean RDW was increased in the HS group. For MCHC and RDW the p values were <0.001 and this was statistically significant.

Using the student t test for the calculation of the predictive values and cross tabulation of the parameters MCHC; RDW; MCHC and RDW in combination the following results were obtained:

26 subjects with MCHC < 35.0g/dl had HS. All the control subjects had MCHC < 35.0g/dl. This represents a sensitivity of 48.0%. (Confidence interval 33.9% - 62.4%).

The measure of association and the confidence interval for the MCHC shows:

		Confidence interval
Positive Predictive Value	100%	82.8- 100.0%
Negative Predictive Value	65.8%	53.9 - 76.0%

26 subjects with RDW > 14.5% had HS. This represents a sensitivity of 52%.(confidence interval 37.6% - 66.1%)

Sensitivity = 26/50 X 100 %

43 of the 50 normal control group had RDW < 14.5% which represents a specificity of 86%. (confidence interval 72.6% -93.6%)

Specificity = 43/50 X 100%

Gold Standard

	+	-	
+	a	b	a+ b
-	c	d	c+d
	a+ c	b+d	

Sensitivity = a / a+c

Specificity = d / b+d

Positive Predictive Value = a / a + b

Negative Predictive Value = d / c+ d

The measure of association and confidence interval for RDW shows :

		Confidence interval
Positive Predictive Value	78.8%	60.6 – 90.4%
Negative Predictive Value	64.2%	51.5 – 75.3%

On the combination of both the MCHC >35.0 g/dl and RDW >14.5% the following values were obtained :

		Confidence interval
Specificity	100.%	91.1 - 100.0%
Sensitivity	26%	15.1 - 40.6%
Positive Predictive Value	100%	71.7 - 100.0%
Negative Predictive Value	57.5%	46.4 - 67.9%

Sensitivity and specificity are estimates in a sample population (percentages). 95 % confidence interval is a range of values of specificity in the population. Interpretation : we can be 95 % confident that the real specificity in the population ranges from 91.1% to 100% even though our estimate from the sample was 100 %.

Table III - Probabilities of MCHC and RDW to identify patients with HS.

		Sensitivity	Specificity	PPV	NPV
Index	Cut off values	%	%	%	%
MCHC G/dl	>35.0	48.0	100	100	65.8
RDW %	>14.5	52.0	86.0	78.8	64.2
MCHC & RDW	MCHC >35.0 & RDW > 14.5	26.0	100.0	100.0	57.5

Positive Predictive Values and Negative Predictive Values are influenced by the prevalence of HS in this sampling which is higher than those seen in the normal control population. Thus these figures will be higher than those found in the normal population.

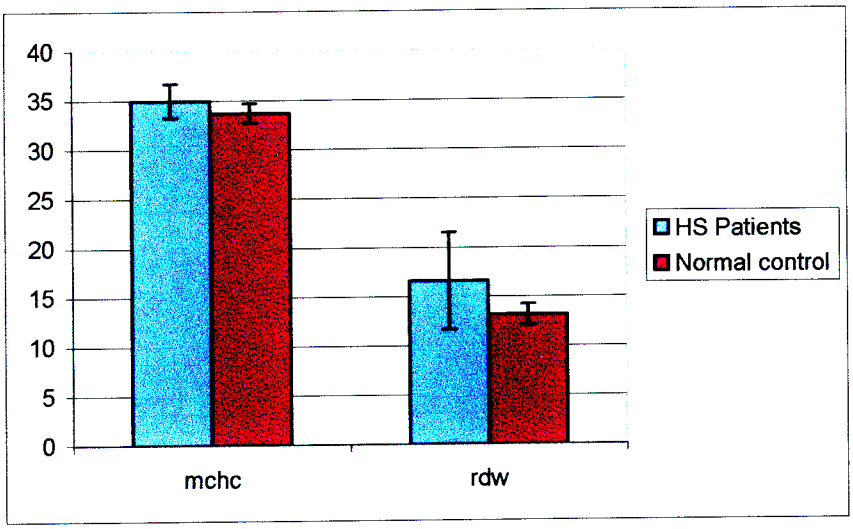


Figure 4 Mean +/- 1 SD MCHC and RDW for HS subjects and Normal controls

5.3 Red cell morphology

All peripheral blood smears showed the presence of spherocytes. The number of spherocytes present varied from 33% to 88% of the total red blood cells. (Figure 6)

Figure 6 Peripheral blood smear with spherocytes

The peripheral smear of two patients showed occasional 'pincered' red cells (Figure 1). Both these patients had band 3 defect.

Table IV - The relationship between the haemoglobin levels, MCF and the percentage of spherocytes present.

Haemoglobin Levels g/dl	Number of subjects	Range of spherocytes %	MCF
6.1 - 10.0	13	30 - 88	↑; ↑↑; ↑↑↑
10.1 – 12.0	8	66 - 80	↑; ↑↑; ↑↑↑
12.1 – 14.0	7	65 - 81	↑; ↑↑; ↑↑↑
>14.0	22	30 - 88	↑; ↑↑; ↑↑↑

There was no correlation between the number of spherocytes present and the haemoglobin in each of the subject. Further there was no correlation between the number of spherocytes present and the median cell fragility.i.e those subjects with high percentage of spherocytes did not always have ↑↑↑ increased median cell fragility.

5.4 Direct Antiglobulin Test (Coombs test)

All 50 patients had negative Direct Antiglobulin Test result. This indicates the absence of an antibody-mediated haemolysis.

5.5 Pre- and post - Incubated Osmotic Fragility Test

The degree of osmotic lysis was assessed subjectively by visual estimation of the degree of deviation of the osmotic fragility graph from the normal in both the pre - and post - incubated specimens. These were assigned as +; ++; +++ (subjective assessment). An objective assessment was done using the Mean Cell Fragility (MCF) assigned as ↑; ↑↑; ↑↑↑.

On comparing the subjective assessments and Mean Cell Fragility (MCF) methods the following information was obtained (Table V):

Table V - Comparison of the Subjective Assessments and Mean Cell Fragility (MCF) methods for Osmotic Lysis.

Pre- incubated osmotic fragility test:

Subjective assessment of degree of lysis	**Number of patients (n=50)**	**MCF Objective**	**MCF Symbol Objective**	**Number of Patients**
Normal (n=50)	-	0.4 - 0.45		-
Osmotic lysis +	12	0.46 – 0.50	↑	9
Osmotic lysis ++	20	0.51 – 0.6	↑↑	39
Osmotic lysis +++	18	0.61 – 0.9	↑↑↑	2

Post – incubated osmotic fragility test:

Subjective assessment of degree of lysis	**Number of patients**	**MCF Objective**	**MCF Symbol Objective**	**Number of Patients**
Normal (n=50)	-	0.46-0.59	-	-
Osmotic lysis +	8	0.6-0.7	↑	17
Osmotic lysis ++	9	0.71-0.8	↑↑	12
Osmotic lysis +++	33	0.81-0.9	↑↑↑	21

The Chi - square method of analysing the data was used to compare both the subjective and objective methods of assessment for the pre - and the post - incubated red cell osmotic fragility graphs.

A p value of <0.001 was obtained on comparing both methods of

assessment of osmotic lysis for the pre-incubated osmotic fragility test. This indicates that there is a high association between the subjective and objective methods of assessment. The subjective method tends to over estimate the extent of red cell osmotic lysis as evidenced in Table V.

A p value of <0.001 was obtained for the post-incubated osmotic fragility test. There is a high association between the subjective and objective methods of assessment. For the post-incubated fragility test the subjective method tends to over estimate the extent of red cell osmotic lysis. (Table V)
Using the defined values as in the objective method of assessment, it clearly differentiated the extent of osmotic lysis as it was guided by specific values read off the osmotic fragility graph. In the subjective method (visual method), there is no clear demarcation to separate the extent of osmotic lysis in subjects. With clearly defined values as in the Mean Cell Fragility method, the degree of lysis can be clearly assessed.
Subject and control examples of pre - and post - incubated osmotic fragility test graphs. : Appendix 1 and Appendix 2.

Table VI: Comparison of Mean Cell Fragility (MCF) values for normal subjects and test subjects for pre - and post- incubated osmotic fragility test at pH 7.4

	NaCl concentration-% Fresh blood	**NaCl concentration-% Blood incubated for 24 hrs at 37°C**
*MCF (50% lysis) normal range	0.4 – 0.45	0.46 – 0.59
MCF (50% lysis) Test samples (n=50)	0.55	0.77

* Mean cell fragility

The average MCF (50% lysis) for the test subjects is greater than the normal MCF ranges for both the pre- and post incubated osmotic fragility test. This result was expected, as all subjects are known HS subjects.

5.6 Serum Vitamin B12 and Folate assay

The Vitamin B12 and Folate levels for all the subjects were within the normal range.

Serum Vitamin B12 levels for the subjects ranged from

222 - 793 pg/ml. (Normal range: 160 – 970 pg/ml).

Serum Folate levels for the subjects were normal and ranged from

1.6 – 13.1ng/ml. (Normal Range: 1.5 - 16.9 ng /ml).

5.7 Serum Ferritin

Five Black females, two Black males and one white female had decreased serum ferritin levels ranging from < 5.0 - 19.4 ng/ml. (Normal range 20-300 ng/ml)

5.8 Serum LDH, Bilirubin and Reticulocyte count

Table VII: The Range and Mean values for Serum LDH, Bilirubin and Reticulocyte counts.

	Number of patients	**%**	**Range**	**Mean**	**Normal Reference Range**	**Normal reference mean**
LDH mmol/l – normal – increased	 14 36	 28 72	 359-497 512-1626	 435 716	 266-500 mmol/l	383
Bilirubin – Normal – increased	 15 35	 30 70	 7-17 19-227	 12 44	 0-17 mmol/l	8.5
Reticulocyte – normal – increased	 10 40	 20 80	 1.4-2.0 2.1- 40.0	 1.8 8.0	 0.2-2.0 %	1.1

25 subjects (50%) had an increase in all three of the above parameters.

12 subjects (24%) had an increase in two of the above parameters.

Two subjects (4%) had normal reticulocyte count (1.4%; 1.8%) with normal bilirubin and LDH levels.

Three subjects (6%) had normal haemoglobin, normal bilirubin, normal reticulocyte count with increased LDH levels.

Three subjects (6%) had normal haemoglobin, normal reticulocyte count, normal LDH levels with increased bilirubin levels.

One subject (2%) had normal bilirubin, normal reticulocyte count with increased LDH and a decreased haemoglobin level.

Four subjects (8%) had normal haemoglobin levels and slightly raised reticulocyte count with normal bilirubin and LDH levels. (Table VII)

Table VIII : Subjects with slightly raised reticulocyte count and normal haemoglobin levels

Reticulocyte count %	**Haemoglobin levels g/ dl**
2.1	15.2
2.7	13.9
2.4	14.1
2.2	17.0

5.9 Haemoglobin Electrophoresis

Forty-nine subjects had normal haemoglobin electrophoretic patterns.

One subject, a 31year old Indian female had an abnormal band on alkali haemoglobin electrophoresis. Acid haemoglobin electrophoresis confirmed heterozygous sickle haemoglobin (Hb S : 41.7%)

5.10 POLYACYRLAMIDE GEL ELECTROPHORESIS SDS-PAGE

Laemmli SDS- PAGE

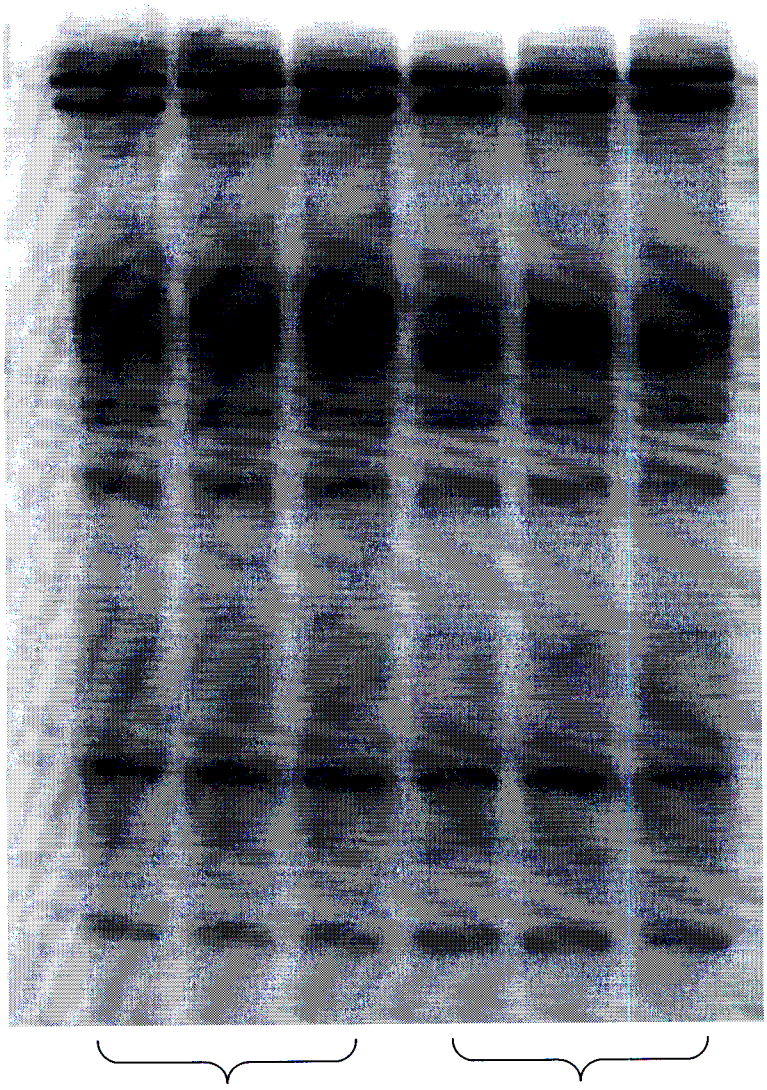


Figure 7 SDS- polyacrylamide (Laemmli) gel stained with Coomassie blue.

The above (Figure 7) is an example of a Laemmli SDS - polyacrylamide gel. The first three lanes are the Control specimen with 45ug, 47ug, 50ug of protein. The next three lanes are for a

subject with 45ug, 47ug, and 50ug of protein. It can be observed that this subject has decreased band 3 as well as decreased protein 4.1b which indicates a high reticulocyte count.

The Laemmli gel resolves protein 4.1 into two isoforms 4.1a and 4.1b. The latter isoform is predominant in reticulocytes. This electrophoretic separation system does not resolve spectrin and ankyrin (protein 2.1).

The following ratios were calculated from the values obtained from the scanning the Laemmli gel. (Table IX)

Table IX: Ratios obtained from scanning the Laemmli gel

	4.1a / 4.1b	4.1 / 4.2	4.1+ 4.2 / b3	4.1 / b3	4.2 / b3
N=	22	23	23	23	23
X	1.51	1.19	0.31	0.17	0.14
X ± 1SD	1.51±0.12	1.19± 0.11	0.31±0.03	0.17±0.02	0.14± 0.02
Normal Range	1.11-1.64	0.98- 1.38	0.26-0.36	0.14- 0.21	0.12-0.20

b3 = Protein band 3
4.1 = Protein band 4.1
4.2 = Protein band 4.2

For the 21 subjects that were processed by Laemmli SDS -PAGE: 11 subjects showed a decreased 4.1a / 4.1b ratio which correlated with an increased reticulocyte count. Nine subjects showed a normal 4.1a / 4.1b ratio which correlated with a normal reticulocyte count.

One subject had a 4.1a / 4.1b ratio of 1.17, which is at the lower limit of the normal ratio and had a raised reticulocyte count of 3.6%. In this study the reticulocyte count was processed immediately on collection of the specimen. However, due to the logistics and the time taken for the specimen to reach the Department of Molecular Medicine in Johannesburg, the red cell membrane preparation was performed at least three days post sample collection. This probably has contributed to the poor correlation of the results in the above case.

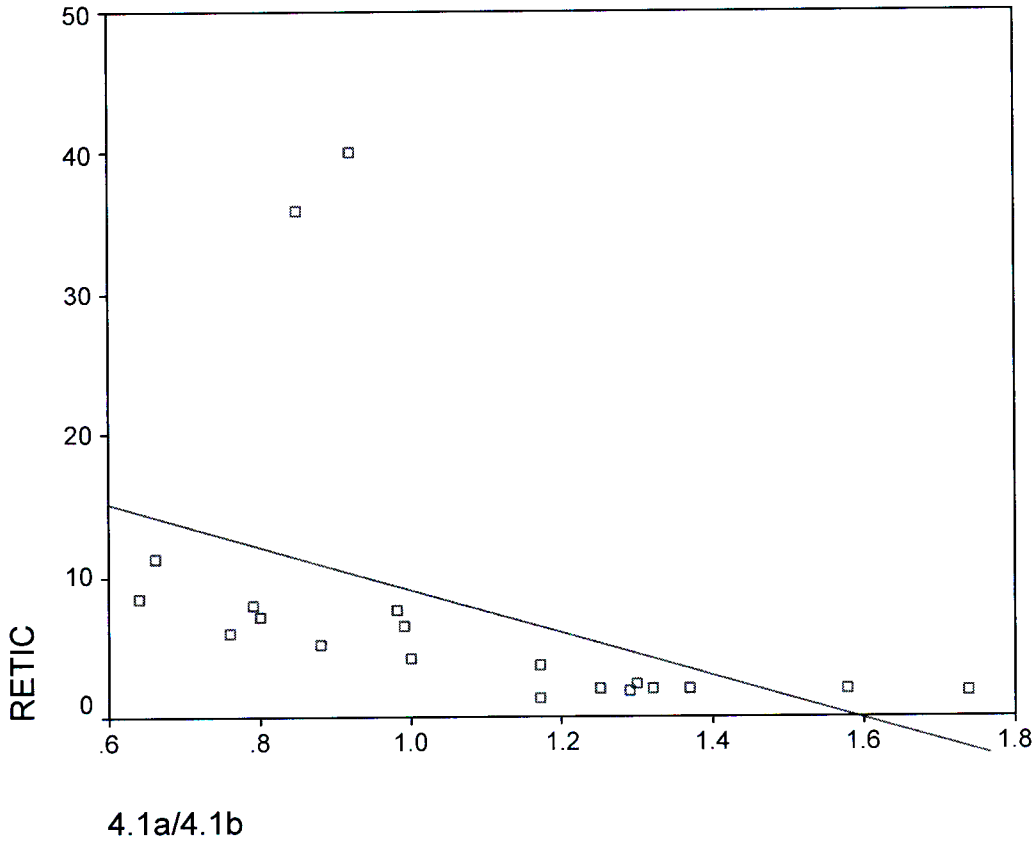


Figure 8 Graph of Reticulocyte count vs Protein 4.1 a/ 4.1b ratio

Fairbanks SDS – PAGE

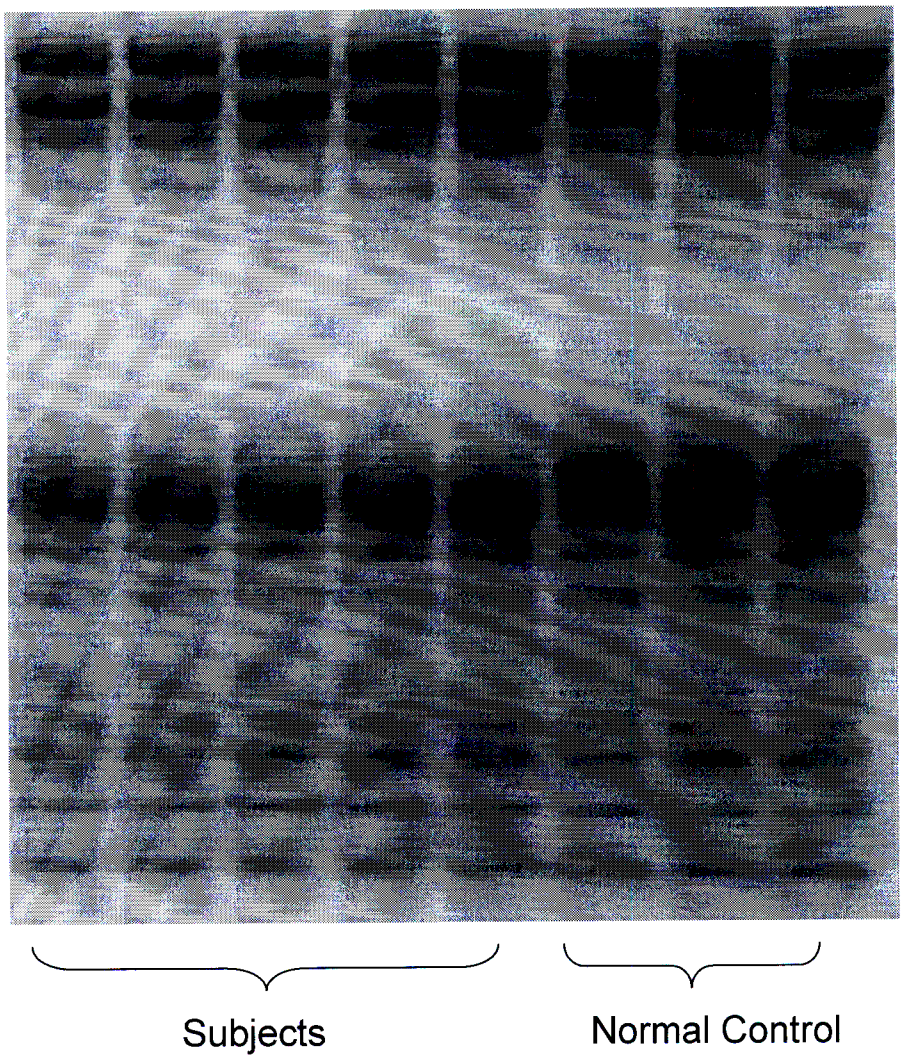


Figure 9 SDS – polyacrylamide gel (Fairbanks) stained with Coomassie blue.

The above (Figure 9) is an example of SDS- polyacrylamide gel (Fairbanks). The Fairbanks electrophoresis shows the subject in the first five lanes with 20ug, 22ug, 24ug, 26ug and 28ug of protein loaded.

The last three lanes are the normal control sample with 24ug, 26ug and 28 ug of protein loaded. The Fairbanks shows a decreased band 3.

The following ratios were calculated from the values obtained from scanning the Fairbanks gel. (Table X)

Table X : Ratios obtained from scanning the Fairbanks gel

	$\frac{\alpha \text{ Spectrin}}{\beta \text{ Spectrin}}$	$\frac{\text{Sp}}{\text{b3}}$	$\frac{2.1}{\text{b3}}$	$\frac{4.1}{\text{b3}}$	$\frac{4.2}{\text{b3}}$	$\frac{4.1}{4.2}$	$\frac{4.1+4.2}{\text{b3}}$
N=	30	26	25	27	30	30	23
X	1.09	1.06	0.19	0.16	0.19	0.88	0.34
x±1SD	1.09±0.04	1.06±0.04	0.19±0.01	0.16±0.01	0.19± 0.02	0.88± 0.07	0.34±0.02
Normal Range	1.03-1.18	0.96-1.09	0.16-0.22	0.13-0.17	0.16-0.22	0.78-0.99	0.3-0.38

Sp = spectrin
b3 = Protein band 3
α = alpha spectrin
β = beta spectrin

Due to logistical reasons, cost implications and the distance of the Department of Molecular Medicine in Johannesburg, where the red cell membrane analysis was carried out, only 21 subjects were analysed for red cell membrane defects.

These results provided the information to formulate conclusions for objectives 6, 7 and 8 of this study.

Table XI : Results of haemoglobin; osmotic fragility and red cell membrane defects:

Subjects & Race	Hb(g/dl)	Osmotic Fragility	Red cell membrane defect
10 IND	14.3	↑↑↑	↓band 3, slightly ↓ 4.2
8 IND	9.1	↑↑	↓band 3, ↓4.2
7 IND	15.2	↑	↓band 3, ↓4.2
35 B	10.1	↑↑	↓band 3, ↓4.2
25 W	15.1	↑↑	None detected
26 * B	6.1	↑	↓spectrin, ↓ankyrin 2.1
39 B	13.4	↑	None detected
36 W	12.2	↑	None detected
37 B	17.7	↑↑	↓spectrin , ↓ 2.1
38 W	14.5	↑	None detected
40 W	14.1	↑↑	None detected
41 B	13.1	↑↑↑	↓spectrin, ↓ 2.1
42 IND	14.1	↑↑↑	↓spectrin, ↓ 2.1
44 W	17.0	↑↑↑	↓spectrin, ↓ 2.1
43 IND	14.7	↑↑↑	↓band 3
45 IND	16.5	↑↑↑	↓band 3, ↓ 4.2
46 * IND	15.7	↑↑↑	↓band 3, slightly ↓4.2
48 IND	9.3	↑	↓band 3, ↓ 4.2
49 * B	11.7	↑↑↑	↑band 6 **
50 * B	7.4	↑↑	↓ band 3
31 B	9.5	↑↑↑	↓band 3, slightly↓4.2

IND = Indian

B = Black

W = White

* indicates that the red cell membrane analysis was performed by the scientist Mr K Naidoo, when it was not possible for the author to be in Johannesburg.

** An increase in band 6 (glyceraldehyde-3- phosphate-dehydrogenase), is probably a non-specific finding, reflecting increased adherence of the enzyme to the membrane.

5.11 Clinical Features

Table XII : Clinical features of 43 subjects

Clinical findings	Number of subjects
Pallor	12
Jaundice	13
Splenectomy	13
Splenomegaly	23
Gall stones	2
Leg Ulcers	1

The clinical evaluation for all 43 subjects was performed by Professor V. B. Jogessar.

13 subjects (splenectomised & non- splenectomised) of the 50 subjects had mild to moderate jaundice without anaemia. Twenty subjects had splenomegaly ranging from minimal to moderately enlarged; up to 6 cm below the left costal margin. As a patient's

spleen must be at least twice its normal size before it is clinically palpable, minimal enlargement may be missed if the abdomen is not palpable.

Table XIII shows the Hb, MCHC, RDW and the parameters describing the degree of haemolysis post splenectomy for the 13 subjects

Table XIII : Hb, MCHC, RDW and parameters describing the degree of haemolysis

Subject	Sex	Hb	MCHC	RDW	Retic %	MCF	LDH	Biliru bin	Splenectomy
1	M	14.1	35.7	12.0	3.2	↑	850	22	YES
2	M	17.8	34.6	13.2	3.0	↑↑↑	533	41	YES
3	F	13.2	36.4	22.4	6.0	↑↑↑	650	36	YES
4	F	8.8	36.5	24.0	10.8	↑↑↑	719	37	YES
5	M	16.6	34.3	15.0	3.1	↑↑↑	520	10	YES
9	M	18.8	36.6	12.8	3.7	↑↑↑	571	23	YES
10	M	14.3	35.8	13.0	1.4	↑↑↑	466	16	YES
18	F	7.9	34.9	24.4	4.0	↑↑↑	637	227	YES
32	M	11.1	31.1	19.8	1.7	↑	1626	17	YES
33	F	15.3	33.6	12.2	1.7	↑	615	31	YES
42	F	14.1	35.9	11.6	2.4	↑↑↑	359	14	YES
44	M	17.0	35.8	13.1	2.3	↑↑↑	497	12	YES
47	F	11.7	35.8	16.0	1.9	↑↑↑	518	8	YES

Reticulocytes : 0.2%- 2.0 %
LDH : 266–500 mmol/l
Bilirubin : 0 – 17 mmol/l

Regarding Hb < 11.5 g/dl as anaemia, three subjects (2 females and a male subject) were still anaemic post splenectomy. One subject was iron deficient and the other two subjects had normal ferritin and vitamin B12 and folate levels.

Of the 13 post splenectomy subjects 10 subjects had markedly increased median cell fragility. Subjects 4 and 18 had continued to haemolyse despite the splenectomy as evidenced by her low haemoglobin and increased levels of LDH, bilirubin and reticulocyte count.

In this study, of the 13 subjects who had splenectomy, pre-splenectomy results were available in only five patients. These patients showed an increase in the haemoglobin level and a decrease in reticulocyte counts post - splenectomy. However, the number is too small to make a meaningful comparison and to arrive at conclusions regarding the effect of splenectomy on erythrocyte parameters.

CHAPTER 6

DISCUSSION

6.1 Red cell parameters

Due to the extensive use of automated RBC indices that are easily available worldwide today, it seems reasonable to assess the value of these parameters either singly or combined as a tool for the screening of patients suspected of having HS.

The data of all red cell parameters for all 50 subjects of the study were recorded. Control values were obtained from 50 age and sex matched normal volunteers.

Using the t - distribution of the student t – test to determine the significance for the means of small samples it was found that the HS group vs the normal control for RBC; Hb; MCV and MCH the p value was > 0.05 and hence there was no significance with these parameters for the screening of HS.

Of the parameters evaluated, it was found that the two indices most useful in identifying cases of HS were the Mean Cell Haemoglobin Concentration (MCHC) and the Red Cell Distribution Width (RDW).

MCHC

The mean MCHC in the HS group was 34.92g /dl, significantly higher (p value <0.001) than in the normal control subjects who had a mean of 33.75g/dl. Spherocytes have a loss of membrane with a decreased surface area to volume ratio. In HS the absolute amount of haemoglobin in a cell is normal, yet the total cell volume is reduced. This results in a higher haemoglobin concentration in the cell (MCHC). The data in this study demonstrate that the automated MCHC, as measured by aperture impedance, remains a useful and an inexpensive test for the screening of patients suspected of having HS. The MCHC proved to be a screening test with a high specificity but was limited by poor sensitivity. This confirms previous studies that have suggested that the MCHC was a useful index for providing a clue towards the diagnosis of HS.[25] A MCHC value of >35.0 g/dl showed a specificity of 100%, noting that all the subjects are known cases of HS.

There are a few causes of an increased MCHC; the common ones being HS and Cold Agglutinin Disease. In Cold Agglutinin Disease the red cell agglutination is obvious on a stained peripheral blood smear and disappears on incubation at 37°C. In addition the

MCHC usually corrects when the specimen is incubated at 37°C and processed immediately on the auto analyser.

Five of the seven iron deficient patients in this study had normal MCHC. Even though it is rare to find a low MCHC value in HS with Iron deficiency, an elevated MCHC value remains useful in the diagnosis of HS.[25,26] In other words, in individuals with concomitant Iron Deficiency Anaemia and HS the presence of Iron Deficiency Anaemia may be masked by normal or raised MCHC levels.

RDW

The RDW is a measure of the degree of anisocytosis of the red cell population. The mean RDW was increased in the HS group. It has been previously reported that the RDW, when measured by aperture impedance is normal in cases of HS.[27,28] However, the RDW in this study was increased in 56% of the subjects of the HS group and these patients also had an increased reticulocyte count thus explaining the increased RDW.

As RDW is a measure of anisocytosis i.e. the variation of red cell size, the presence of microspherocyes, polychromasia and normocytes adds to the heterogeneity of the red cell population thereby increasing the RDW.

The mean red cell distribution width of 16.2% was significantly higher in subjects with HS compared to normal subjects with a mean RDW of 13.5%. (P value < 0.001)

Further statistical analysis was performed to ascertain if the MCHC and RDW values in combination would enhance the probability of diagnosing HS when compared to each index on its' own. While each index individually had specificity of 100%; in combination the sensitivity fell to 26%. Individually MCHC had a sensitivity of 48% and RDW a sensitivity of 52%.

Sensitivity and specificity are estimates in a sample population (percentages). 95 % confidence interval is a range of values of specificity in the population. We can therefore be confident that the real specificity in the population ranges from 91.1 % to 100% even though our estimate from the sample was 100%.

Notwithstanding the above, the MCHC and RDW are useful indices for screening for patients with HS. Therefore elevated MCHC and RDW levels should alert one to the probable diagnosis of hereditary or acquired spherocytosis. Peripheral blood smear; Reticulocyte counts and osmotic fragility tests are essential. Family Members must be screened for the disease. The negative Anti Globulin Test excludes acquired immune haemolytic anaemia, which has similar peripheral blood findings. In a patient with

splenomegaly, jaundice and anaemia, the presence of an elevated MCHC and RDW make the likelihood of HS high.

6.2 Pre- and post- incubated Osmotic fragility

Red cell osmotic lysis is increased in the presence of spherocytes. In this study all 50 subjects exhibited increased red cell osmotic lysis. The increased lysis is accentuated in the post – incubation osmotic fragility test.

Hence the post- incubated osmotic fragility test is useful in instances where the initial pre- incubation tests are equivocal. The osmotic fragility test is simple and cost effective and can be performed in a routine haematology laboratory.

Traditionally, the degree of increased osmotic lysis is estimated by visual inspection of the osmotic fragility graphs and empirically designated as +, ++ and +++ lysis (subjective). Objective assessment of the degree of lysis is obtained by reading the MCF on the osmotic fragility graph. A comparison between the two methods indicated that there is a high association between the subjective and the objective method of assessment. (Table V).

The subjective method tends to over estimate the degree of red cell osmotic lysis. (Table V)

It is recommended that the subjective assessment of the degree of lysis be discontinued. Laboratory comment on the osmotic fragility graph should indicate only that lysis is normal or increased, further it is recommended that the MCF be utilised routinely to assess the degree of osmotic lysis.

Interpretation of Osmotic Fragility tests

The osmotic fragility test is an index of the resistance of the red cells to osmotic lysis. The normal curve is a 'S'- shaped curve. Increased red cell osmotic lysis is not diagnostic of HS. It indicates the presence of spherocytes, which may occur in other conditions such as auto immune haemolytic anaemia and haemolytic disease of the newborn.

In patients with HS as a result of the decreased surface area to volume ratio, this osmotic lysis is accentuated.

This difference is accentuated by the post- incubation osmotic fragility test. The post- incubation osmotic fragility test is done as a small proportion of HS patients do not display increased osmotic lysis in the pre- incubated test.

The increased osmotic fragility of normal red cells which occurs after incubation is mainly caused by the swelling associated with an accumulation of sodium which exceeds the loss of potassium. Such cation exchange is determined by the membrane properties of the red cell which controls the passive flux of ions and the metabolic competence of the cell which determines the active pumping of cations against concentration gradients.

During incubation for 24 hours the membrane of the red cell becomes stressed and the sodium / potassium pump mechanism tends to fail, one contributing factor being a relative lack of glucose in the medium.

The osmotic fragility of red cells with abnormal membrane such as those patients with HS increase abnormally after incubation. During the 24-hour incubation, HS cells have greater loss of membrane surface because of the relative membrane instability. The measurement of the red cell osmotic fragility provides a useful indication as to whether a patient's red cell membrane is normal. Other tests to detect spherocytes are cryohaemolysis test and the acidified glycerol lysis test. As the Osmotic Fragility test is more widely used in the routine diagnostic laboratory, it was the preferred test method in this study.

6.3 Serum Vitamin B12 and Folate

The serum vitamin B12 and folic acid levels were normal in the study group. Folic acid deficiency is common in patients with chronic active haemolysis. The absence of folic acid deficiency in this group probably indicates a good nutritional status. In addition, patients who had been previously deficient were taking folate supplements.

Further, on the peripheral smear examination, hypersegmented neutrophils and other features to suggest folate deficiency were absent.

6.4 Serum Ferritin

Seven patients (14%) of the subjects were iron deficient. (Serum ferritin level of <15ng/ml). The reason for iron deficiency anaemia in these subjects was not obvious.

Five subjects (10%) had raised ferritin levels ranging from 377- 940ng/ml. Four of these subjects had received multiple red cell transfusions. In conditions of chronic haemolysis iron absorption is generally increased. However, 38 HS subjects (76%) had normal ferritin levels. Hence, iron overload on the basis of increased absorption is not a feature of HS.

6.5 Haemoglobin Electrophoresis

Co- existence of sickle cell trait and HS is unusual and only 16 cases have been reported in literature. [29]

One patient, a 31 year old diabetic female presented with splenomegaly. Her full blood count showed a haemoglobin of 9.3 g/dl with microcytic hypochromic indices. The peripheral blood smear showed polychromasia and spherocytes. In view of the microcytic hypochromic indices, haemoglobin electrophoresis to exclude Thalassaemia or Haemoglobinopathy was performed. This revealed the presence of Hb S of 41, 7% which was confirmed on Paragon Acid haemoglobin electrophoresis to be heterozygous for the sickle haemoglobin. The sodium metabisulphite test for sickling was positive, hence confirming the presence of sickle cells. Her Hb A2 level was 2.3 % and Hb F was 0.8%.

Rarely, sickling episodes may occur in individuals with sickle cell trait resulting from stress such as infections and hypoxia. This case study highlights the fact that even when a diagnosis of Hb S trait or haemoglobinopathy is satisfactorily established, the chance association with an unrelated haematological disorder is always a possibility.

6.6 Serum LDH, Bilirubin, Haemoglobin and Reticulocyte count

The degree of haemolysis in the study population varied from mild to moderate as determined by the reticulocyte count, serum bilirubin and the lactate dehydrogenase levels (Table VII).

Sixty eight percent of the subjects had normal haemoglobin levels and were typically asymptomatic. Seventy six percent of the patients had haemoglobin levels of >10.g/dl.

4 subjects (8 %) with normal haemoglobin had slightly raised reticulocyte counts (2.1%; 2.7%; 2.2% and 2.4%) with normal bilirubin and LDH levels. (Table VII)

The serum LDH, serum bilirubin and reticulocyte count were increased in 26 (52%) subjects. It could be suggested that in patients with normal or slightly raised reticulocyte counts with normal bilirubin and LDH levels, HS could be missed.

The parameters serum bilirubin, LDH and reticulocyte count when considered together with the full blood count would greatly enhance the laboratory diagnosis of HS.

The mild reticulocytosis was the only clue for marrow response to haemolysis. Thus it is important to have a high index of suspicion when the full blood count indicates isolated mild reticulocytosis.

Comparison of the pre- and post- splenectomised erythrocyte parameters and osmotic fragility patterns in splenectomised patients

Earlier studies have shown that splenectomy in HS subjects has a significant effect in that the haemoglobin level increases and the degree of haemolysis is reduced[3].

However, the pre-splenectomy results for only 5 subjects is too small to make a meaningful comparison and to arrive at conclusions regarding the effect of splenectomy on erythrocyte parameters.

10 of the 13 subjects post splenectomy had markedly increased MCF (0.81-0.9). This shows that MCF is a good indicator of need for splenectomy.

As a patient's spleen must be at least twice its normal size before it is clinically palpable, minimal enlargement may be missed if the abdomen is not palpable.

A limitation of this study is that ultrasound of the abdomen was not performed for financial reasons. This would have correctly ascertained splenic size and the presence or absence of gallstones.

6.7 Assessment and comparison of clinical severity with the number of spherocytes on the smear

There was no correlation between the number of spherocytes present on the peripheral smear and the clinical severity of the disease.

22 patients who had between 30-88% spherocytes on their peripheral smear had haemoglobin > 14.0g/dl and were in good health. Of the 13 post splenectomy subjects, one subject had 30 % spherocytes present and the remaining subjects had > 62 % spherocytes. There was no correlation between the number of spherocytes present and the level of bilirubin, LDH and the reticulocyte count. The percentage of spherocytes was neither a good discriminator of HS nor an indicator of disease severity.

The percentage of spherocytes present in the peripheral blood did not correlate with the type of membrane protein defect present.

6.8 Polyacrylamide gel electrophoresis – SDS PAGE

Laemlli gel electrophoresis resolves protein 4.1 into two isoforms 4.1a and 4.1b. The latter isoform is predominant in reticulocytes and is converted into the 4.1a isoforms by deamidation of Asn502.[12] The 4.1 gene is subject to alternative 4.1mRNA processing of a cell differentiation and tissue specific nature.

A decreased 4.1a / 4.1b ratio in individuals correlated with an increased reticulocyte counts. The discrepancy in one subject with a 4.1a/ 4.1b ratio of 1.17 and an increased reticulocyte count (3.6%) is probably due to red cell membrane preparation and reticulocyte count processed at different times.

The Fairbanks SDS – PAGE resolves all major membrane proteins. It does not however resolve the isoforms of protein 4.1. Membrane protein analysis was performed on 21 patients. As per Table XI, in 5 subjects no abnormality was detected. This apparent normality may be due to a minor deficiency of one of the proteins that is not detectable by SDS-PAGE or to a structural or functional abnormality which was not investigated. 10 subjects had decreased levels of band 3 deficiency (with or without decreased band 4.2 levels), 5 subjects had decreased spectrin levels with decreased band 2.1 levels) and 1 subject had an increase in band 6.

During the past few years, primary biochemical defects responsible for HS have been identified as alterations of different red cell membrane proteins. Spectrin, ankyrin, band 3 or protein 4.2 deficiency have been described. Spectrin deficiency is a common

underlying cause of HS in population groups originating from northern Europe and is also found in the majority of southern African blacks. The 5 subjects with spectrin deficiency in this study comprised South African Whites and South African Blacks.

Ankryrin deficiency, resulting in a concommitant decrease in spectrin, was first described by Coetzer and co-workers[30], and subsequently shown to be caused by decreased ankyrin mRNA production and synthesis of an unstable protein molecule.[31] A combined ankyrin and spectrin deficiency is the most common abnormality underlying HS in Europe and North America.[32,33] In this study 5 subjects (3 Blacks,1 Indian and 1 White) had combined band 2.1 and spectrin deficiency.

Band 3 deficiency is found in approximately 20% of American and European HS but is more common among Japanese and South African Whites, in whom it comprises nearly half of the cases. The decreased band 3 content often results in a secondary deficiency of protein 4.2 as was the finding in this study, which included 8 cases of combined band 3 and band 4.2 deficiency.(6 Indian and 2 Black subjects) and 2 cases of decreased band 3 levels only. (1 Indian and 1 Black)

There was no correlation between the red cell membrane abnormalities and the clinical aspects of the disease e.g. pallor, jaundice, splenomegaly.

6.9 Value of red cell membrane analysis

One of the objectives of this study was to assess the value of red cell membrane analysis in the management of patients with HS. There was no correlation between the need for splenectomy and the red cell protein deficiency.

The initial laboratory tests together with the clinical features were consistent with cases of HS which was our study population.

The clinical management is determined by the severity of the disorder.

The red cell membrane analysis using SDS- PAGE Fairbanks method identifies membrane protein defects. Various deficiencies result in HS. In cases of suspected HS where clinical and laboratory data are incomplete or ambiguous it is important to identify if there is a red cell membrane protein abnormality.

Red cell membrane analysis did not contribute further to an initial clear diagnosis of HS and it did not influence clinical management. The presence of the red cell membrane abnormalities, did not

correlate with the disease severity.

Red cell membrane analysis is useful, however, as a research tool to identify the defective protein and to assess racial or ethnic differences in the red cell membrane abnormalities. Knowledge of the underlying molecular defect allows one to understand the pathogenesis of the disorder. Also, for future treatment, gene therapy may become an option and hence the need to know which gene is defective.

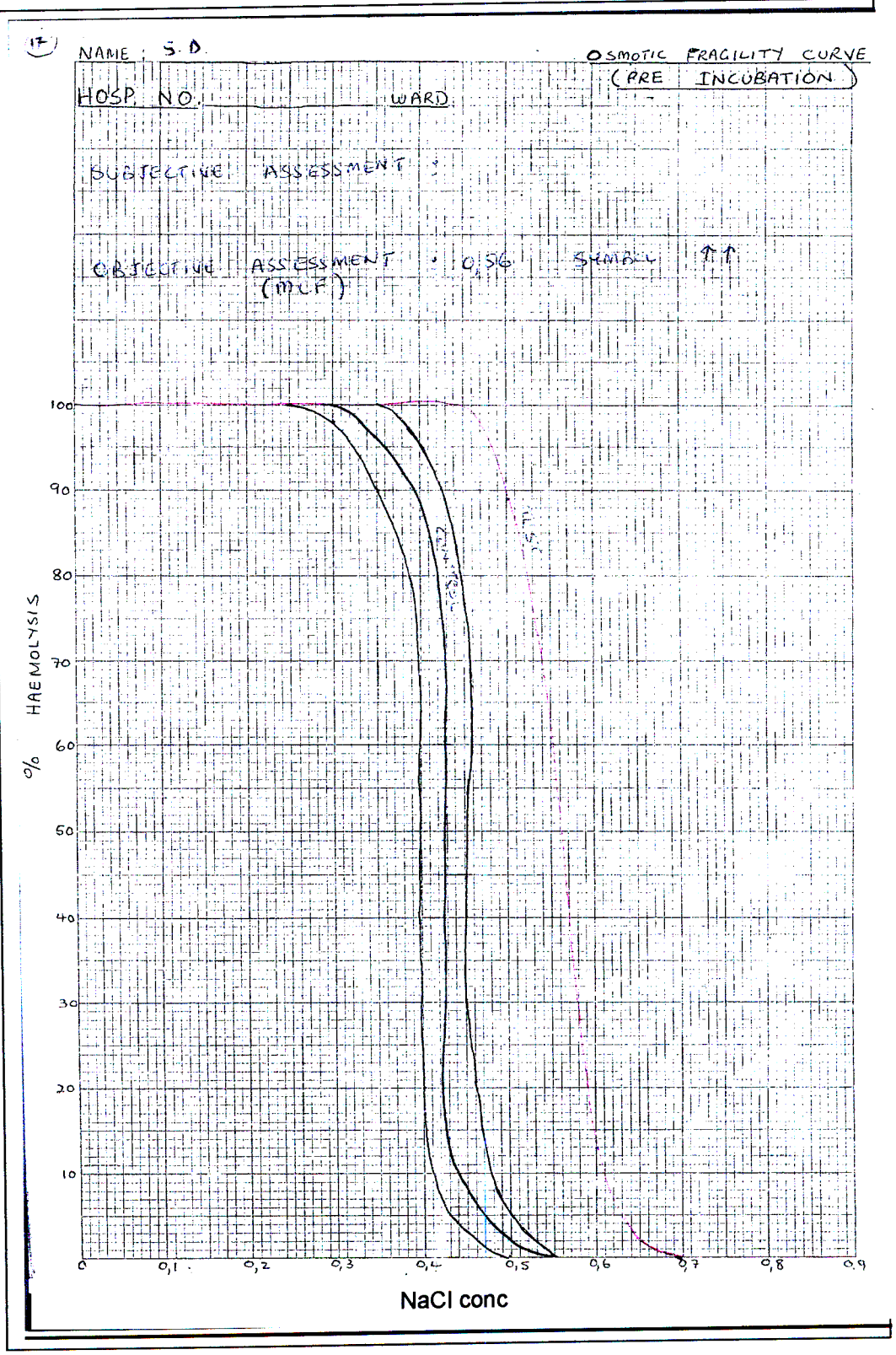
In this study DNA mutation analysis was not performed; since apart from the two hotspot mutations in the band 3 gene, there are greater than a hundred mutations described in HS. These are often family specific. Hence, we cannot speculate as to the mutations of the subjects in this study.

It is also useful as a differential diagnostic tool to distinguish between acquired spherocytosis and an inherited disorder. Limitations of this test are that it is time consuming, costly, laborious and not sensitive enough to detect minor membrane abnormalities. This is not a test for a routine haematology laboratory and is likely to remain a specialised research procedure.

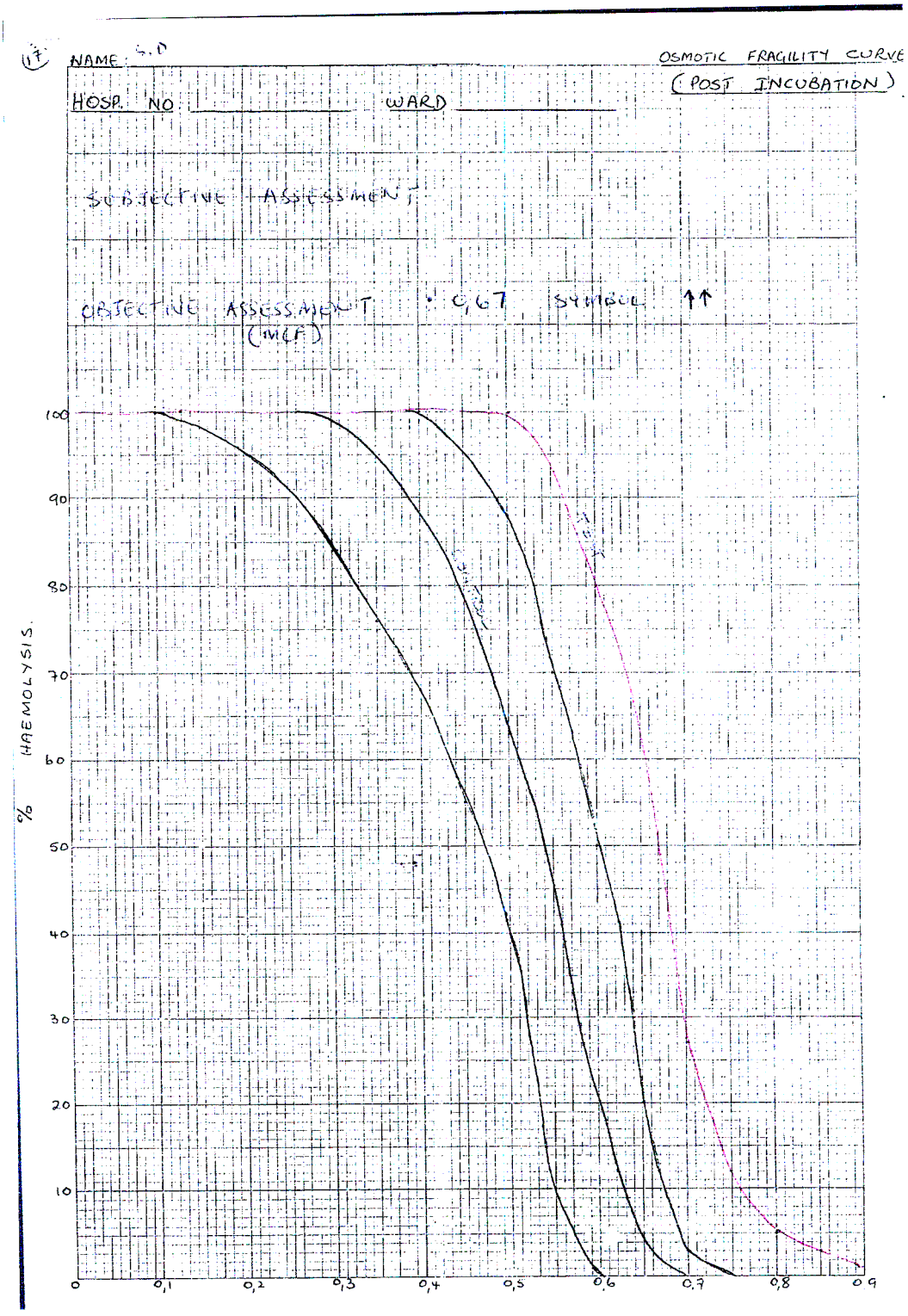
CHAPTER 7

Appendix 1

Pre incubated osmotic fragility curve



Appendix 2
Post incubated osmotic fragility curve



CHAPTER 8

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