CHAPTER 2

MATERIALS USED

2.0 MATERIALS

This project was designed with the major aim to screen the Saudi population, in different regions of Saudi Arabia, to determine the frequency of the blood genetic disorders, to study the natural history of the diseases identified and to conduct studies at the molecular level to identify the molecular basis of these genetic defects in Saudis. In this section the subjects screened, equipments, materials, chemicals and kits used are presented.

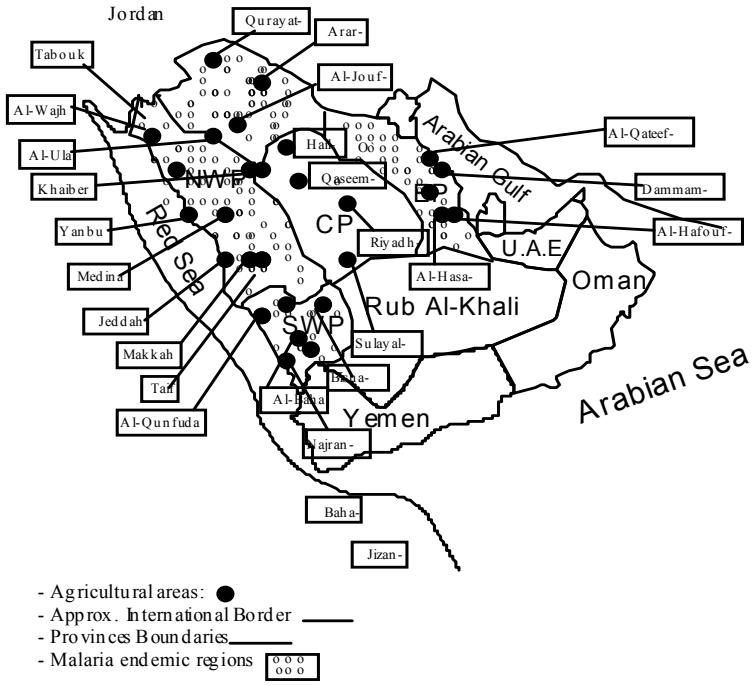
2.1. Subjects and sample collection

The subjects screened included Saudi males, females and children living in different parts of Saudi Arabia. In addition, studies were also conducted on known patients attending the hospitals for routine follow-up.

2.1.1 Screening

The subjects screened in the different areas and in Riyadh were randomly selected. Saudi individuals, who were either attending the outpatient clinics of the Ministry of Health hospitals or health centres for minor illnesses. Other volunteers were included in the study. Blood was also collected from school and university students and blood donors. Known patients were not included in the screening studies. The different areas of Saudi Arabia screened during the course of this study are presented in Figure 2.1. To each area, one-two week trips were made by the research teams. The team was composed of the principal investigator, one co-investigator and a team of 4-5 technical staff well trained to conduct the investigations. The fully equipment teams were based at the central laboratory at a selected Ministry of Health hospitals and the team members made trips in the

Figure 2.1: Sketch map of Saudi Arabia showing the regions screened during this study



surrounding areas to collect blood samples. The essential information about each individual was recorded in specially designed forms and blood was drawn in an EDTA or heparinised or ACD tubes and mixed immediately to prevent clotting. An 0.5-1.0 ml sample in ACD tube was separately placed in a small tube for the enzyme studies. The blood collected by each team was placed in cold boxes and brought to the main hospital laboratory where analysis were immediately carried out. In Riyadh, the analysis were conducted at the Department of Medical Biochemistry, College of Medicine. The total number of samples screened during the investigations are presented in Table 2.1

2.1.2. Prospective and retrospective studies

The subjects investigated during this study included patients known to be suffering from haemoglobinopathies, thalassaemias and enzymopathies and attending the out-patient or inpatient clinics at the various Ministry of Health hospitals and King Khalid University Hospital, Riyadh. Several of the patients identified during screening were registered at the hospitals for follow up and were included in the retrospective and prospective studies. Patients were also included in treatment protocols and were subjected to a regular follow-up.

History, physical data and other relevant information was filled on special forms by a clinician. Blood was drawn in ethylene diamine tetraacetate (EDTA), heparin or acid citrate dextrose (ACD) tubes and stored at 4°C until analysed (maximum of 3-4 days). The patients on the treatment protocols were followed regularly at 2-4 weekly intervals.

2.1.3 Severity Index

In an attempt to provide a quantitative assessment of the clinical presentation in the

sickle cell disease patients special forms for estimation of Severity Index (SI) were

Table 2.1: The total number of samples screened during this study

Analysis	No. of Samples
Period	14.12.1402 to 30.11.1413
No. of Samples	35,926
Analysis conducted	
Haematology	35,830
Hb A_2	18.843
Hb F	20,981
Hb F distribution	21,210
Hb Electrophoresis	35,483
Hb IEF	3,454
Biochemistry	6,708
Hb A_1c	15,483
Immunoglobulin	707
G-6-PD level	21,409
G-6-PD phenotyping	27,937
DNA extraction	11,106

* A number of other specific analysis on plasma, red cells and DNA, were conducted on a limited number of samples from patients (SCD and β-Thal) and controls. The details of these analysis are given in the Method and Materials Section.

designed and were filled for each patient to include all signs, symptoms complications, extent of hospitalization and blood transfusion requirements. The Severity Index (SI) is defined as "The sum of signs, symptoms, hospitalizations and blood transfusion requirements, encountered over a period of one year. The Severity Index was further devided into `Acute Severity Index' which was composed of only the acute symptoms of SCD. this ASI was used to access the clinical status of the SCD patients on treatment protocols (hydroxyurea and erythropoietin - see later). The SI and ASI forms are presented as Table 2.2 and 2.3, respectively.

2.2 Transportation and Storage of Samples

All whole blood samples were stored at 4°C in anticoagulant tubes and were never frozen. The transportation from different regions to Riyadh were carried out in ice boxes by air freight. Plasma and buffy coat samples after separation from the red cells by centrifugation were stored at -20°C or -70°C (if available) until required for analysis. The transportation was also at a low temperature below 0, ideally -20°C. Red cells were kept at 4°C until required for analysis.

2.3 Chemicals, materials, kits and disposables

A list of chemicals, materials, kits and disposables used during the course of this study are presented as Appendix I.

2.4 Equipment

Several routine and specialized equipment were used during the course of this study. A list is enclosed as Appendix II.

Table 2.2: Severity Index of Sickle Cell Disease

	Points
- Severity of anaemia - >110 g/L - 90-110 g/L - 70-89 g/L - <70 g?l	 0 1 2 3
- Chronic pains (bone, abdominal, headache, joints)	1
- Bilirubin level (>35 mmol/l)	1
- Painful crises (Hand-foot syndrome, bone, abdominal)	0-12
- Aseptic necrosis (Head of femur or humerus)	1
- Osteomyelitis	1
- Leg ulcers	1
- Gallstone	1
- Hyposplenism	1
- Polyurea/isothinurea	1
- Priapism	1
- Chest infection	1
- Hypoxia (PO_2 <70 in room air)	1
- Retinopathy	1
- Cerebrovascular accidents (CVA)	1
- Deep Vein Thrombosis (DVT)	1
- Blood transfusion	0-12
- Hospitalization	0-12

Table 2.3*:* Arbitrary Severity Index (ASI)

Reversible Complications	Score	Chronic Complications	Score
Hb 600-100 g/l	1	Stroke/DVT	1
< 60 g/l	2	INT. lung dis. (decreased PO_2)	1
Ret. cytopenia	1	Cardiomyopathy	1
Bil. >2 Norm.	1	Gallstones	1
LDH > 2 Norm	1	PAP. necrosis	1
No. of severe painful crises per year	1 (each)	Aseptic necrosis	1
No. of transfusions per year	1 (each)	Short stature	1

CHAPTER 3

METHODS

3.0 METHODS

Blood samples collected during trips to the different regions were analysed for some parameters on site in the hospital laboratory or the health clinic in that region. The analysis conducted included the preparation of blood smears, determination of haematological parameters and reticulocyte count (on anaemic samples) and separation of the red cells from plasma and buffy coat by centrifugation. All other analysis were conducted at the Department of Biochemistry, College of Medicine, Riyadh.

This section outlines briefly the various analysis conduction and methods used during the course of this study.

3.1. Analysis on whole blood

Fresh whole blood was used for the following investigations. Only on some occasions when analysis could not be conducted immediately, the blood was stored at 4°C for upto 3-4 days.

3.1.1. Red cell morphological studies and differential counts.

Each fresh blood sample was used to prepare a thin blood smear on glass slides. The smears were air dried and stored until required for staining for investigation of the red cell morphology. The staining was carried out using Giemsa stain [Dacie & Lewis, 1975]. Separate blood smears were stained for differential count and a third blood smear was used to stain for Hb F distribution in the cells (Section 3.1.2). The slides were examined by a haematologist under a Zeiss Brightfield Microscope fitted with camera facilities and where ever required slides and prints were prepared. The red cell morphology, differential count and percentage F cells were recorded on the special forms and the data was fed on the

computer.

3.1.2. Estimation of Hb F cells and Hb F distribution

The Hb F cells were estimated by staining a carefully prepared blood smear using commercially available kits (Boehringer Mannheim GmbH, Test combination for fetal haemoglobin Cat. No. 1242). The Hb F distribution was viewed under a Zeiss Brightfield Microscope fitted with camera facilities and the percentage of Hb F cells were obtained in all samples with high Hb F level.

3.1.3. Estimation of the values of haematological parameters and red cell indices

The haematological analytes including total haemoglobin (Hb), red blood cell count (RBC), packed cell volume (PCV), white blood cell count (WBC) and red cell indices (i.e. mean cell volume,MCV, mean cell haemoglobin, MCH and mean cell haemoglobin concentration, MCHC) were estimated in every sample. All analysis were conducted using automated analyzer i.e. Coulter Counter with a haemoglobinometer attachment. The red cell indices, total Hb and PCV values were used to calculate the discriminant factors in each sample i.e. England & Fraser (1973), Shine & Lal (1979), Mentzler (1973). Anaemic samples were differentiated from the non-anaemic ones and classified as normocytic, microcytic, macrocytic, hypochromic or normochromic on the basis of the red cell morphological studies. The discriminant factors were a useful indicator of a possible thalassaemic state and differentiated between hypochromic- microcytic cells due to thalassaemia from those due to iron deficiency.

3.1.4. Estimation of Reticulocyte Counts

Reticulocyte counts were estimated using New methylene blue stain as

recommended by Dacie & Lewis (1975) in all samples showing anaemic parameters. The New Methylene blue stains the reticulo filamantous material in reticulocytes very deeply. Two to three drops of New methylene blue solution (1% in citrate - saline solution) was added to two to four volumes of the patients EDTA blood in a glass tube. After mixing and heating at 37°C for 15-20 min., slides were prepared after gentle mixing to resuspend the cells. The smears were air dried and directly examined to obtain the reticulocyte count.

3.1.5. Cooximeter Studies

Cooximeter (IL Instrument Laboratory, USA) was used for the estimation of methaemoglobin and carboxy-haemoglobin in abnormal samples. Other parameters which were also analysed on the cooximeter included total haemoglobin and oxyhaemoglobin. Normal samples were analysed to establish the normal reference range for these parameters in normal individuals.

3.1.6. Determination of P_{50} - Studies on the Hemoscan

Haemoglobin functional studies were conducted using a Hemoscan (Aminco American Instrument Co. Maryland, USA), to determine the oxygen affinity of the abnormal haemoglobins. The P_{50} for haemoglobin was estimated for the abnormal samples. Normal samples were used as controls and to establish the normal reference range in Saudis.

3.1.7 Red Cell deformability studies

An important determinant of the red cell survival in the circulation is its deformability. As the red cells flow through the microcirculation they undergo rapid changes in their shapes. This allows significant packing of the red cells and provides them

the ability to transverse orifices smaller than their own diameter. Thus the demands of the circulation require that the red cells be deformable. The limits of deformability depends on the surface area/volume ratio of the red cell. Deformability is affected by presence of abnormal haemoglobins and as a result of red cell membrane defects. Decreased deformability produces increased haemolysis and increased selective sequestration in the spleen, since decreased deformability decreases the ability of the red cells to transverse the spleen filters. Red cell deformability may be estimated using either a rheoscope or by an ektacytometer.

We built a home-made instrument for estimation of red cell deformability in sickle cell anaemia patients with severe and benign disease and for patients on treatment protocols [Reid et al, 1977].

Red cell deformability was estimated on fresh whole blood from sickle cell disease patients. The method was standardized using blood from normal individuals and Hb S heterozygotes. The procedure requires the estimation of the rate of blood flow through a microporous membrane in a fixed period of time. Since normal blood cells are highly deformable the rate of flow is fast. Slowest flow is obtained for red blood cells from sickle cell anaemia patients.

3.1.8. Estimation of α /β-globin chain ratio.

To confirm the presence and the type of thalassaemia, the α/β globin chain ratio was estimated in patients provisionally diagnosed as suffering from the thalassaemia syndrome. Fresh whole blood, atleast 10ml, was required and the reticulocyte rich layer obtained following centrifugation of the whole blood was carefully removed, washed with

cold saline (2-3 times) and dialysed against the fresh plasma. Ferrous ammonium sulfate, H^3-leucine and glutamine were added and incubated with the leucocytes for at least 2 hours at 37°C. The cells were removed by centrifugation washed with cold physiological saline and haemolysed. The globin chains were separated and purified by CM-cellulose chromatography. Radioactivity of each peak was measured using the γCounter and the α /β chain ratio was calculated from the total activity of each peak [El-Hazmi and Lehmann, 1978].

3.2. Separation of buffy coat, red cells and blood plasma and preparation of red cell haemolysate.

The fresh whole blood was subjected to centrifugation at 2500 RPM for 15-20 minutes. The layers of plasma and buffy coat were carefully removed from the red cell layer. The plasma and buffy coat were stored frozen at -20°C until required for analysis. The red cells were washed with cold physiological saline two or three times and stored at 4°C. Fresh red cell haemolysate was prepared for the various analysis given below and immediately analysed. The haemoly sate was prepared by addition of cold distilled water or 0.02% digitonin solution to the washed red cells in the ratio of 1:5. Storage of the prepared haemolysate for 5 or more days at 4°C resulted in considerable loss of enzyme activities (If storage was necessary than the haemolysate was stored at -20°C). The cell debris was removed from the haemolysate by centrifugation and the clear supernatant was used for the analysis.

The analysis conducted on the red cell haemolysate, the plasma and the buffy coat are outlined in the following sections.

3.3. Analysis on red cell haemolysate

Freshly prepared red cell haemolysate was used for the following analysis.

3.3.1. Estimation of haemoglobin types

Every new sample, whether obtained during the trips, or locally in Riyadh was subjected to the separation of haemoglobin types by electrophoresis at alkaline pH using cellulose acetate plates (Marengo-Rowe 1965] and the confirmation was achieved by carrying out electrophoresis at acid pH (pH 6.5) using agarose plates (Robinson et al 1957]. The plates were scanned in a densitometer (Corning Medical & Scientific, Corning Glass Work, USA) and the relative concentration of each type of haemoglobin band was obtained.

3.3.2 Isoelectric focussing of haemoglobin and G-6-PD phenotypes

To investigate isoelectric point (PI) differences in the haemoglobin and G-6-PD variants, phenotyping was carried out of G-6-PD using isoelectric focussing techniques.

The isoelectric focussing was carried out using LKB Ampholine PAG plates, pH range 5.5-8.5 (Product No. 1804-103). The anode and cathode solutions were 0.1M Hepes and 0.1M NaOH, respectively. The PAG plates were placed on LKB Multiphor isoelectric focussing unit cooled to 10°C and after placing the electrode strips dipped in the respective electrode solution, samples were applied about 1cm from the cathode. The running conditions were 1600V, 50mA, 25W for 2 1/2 hours. The specific staining of G-6-PD was carried out without fixing at 37°C for 30 min. and the plates were preserved in 10% glycerol solution in ethanol:acetic acid: water (50:16:134) solution. Haemoglobin

phenotypes were stained using the protein stains.

3.3.3 Estimation of Hb A_2 and Hb F

Haemoglobin types i.e. Hb A_2 and F were estimated using an elution procedure following electrophoresis on cellulose acetate plates [Marengo-Rowe,1965] and alkali denaturation [Betke et al, 1959], respectively.

3.3.4 Estimation of red cell enzyme levels

For the estimation of red cell glucose-6-phosphate dehydrogenase (G-6-PD), pyruvate kinase, hexokinase and glutathione reductase levels, freshly prepared haemolysate was used. For G-6-PD and PK estimation commercially available kits from Boehringer- Mannheim GmbH were used and for each sample the unit of enzyme activity in $mU/10^9$ erythrocytes/ml were calculated. Using the normal reference ranges established for these enzymes in the Saudi popu lation [El-Hazmi & Warsy, 1987a, b] the samples with reduced activity were considered as severely or partially enzyme deficient. Hexokinase activity was determined using the procedure published by Beutler (1975).

Glutathione reductase (GR), an enzyme essential for the conversion of oxidized glutathione to reduced glutathione and hence for the stability of the red cell, was estimated spectrophotometrically using the procedure described by Beutler (1975). This method utilizes oxidized glutathione as substrate and estimates the GR activity in presence and absence of flavin adenine dinucleotide (FAD). The GR activity coefficient (AC) was calculated as the ratio of the GR activity in presence of FAD to the GR activity in absence of FAD. This was used to differentiate the genetically determined GR deficiency from cases of GR deficiency due to reduced riboflavin level in diet.

$$\text{GRAC} = \frac{\text{OD 340/min with FAD}}{\text{OD 340/min without FAD}}$$

A GRAC value of 1.3 or above was indicative of riboflavin deficiency while a value below 1.3 and low GR level was indicative of GR deficiency due to presence of genetic variants.

3.3.5. Phenotyping of glucose-6-phosphate dehydrogenase.

All freshly prepared red cell haemolysates samples were subjected to phenotyping of G-6-PD. Fresh clear haemolysate was applied on cellulose acetate plates (at pH 8.6) and electrophoresis was carried out, applying 350V for 20 min, using electrophoresis equipment from Helena (Helena Laboratories, Texas). The G-6-PD moves faster than the haemoglobin and the bands of the enzyme were visualised by specific staining for the enzyme activity using the substrates glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate ($NADP^+$) . The G-6-PD bands appear as blue bands. The plates were fixed, air dried and stored in a dark place. Samples with complete G-6-PD deficiency did not have any major enzyme band while those with partial enzyme activity had a faint band. The location of the band was used to identify the phenotypes.

3.3.6. Estimation of Gγ/Aγ ratio.

The estimation of Gγ/Aγ ratio was carried out on samples from sickle cell anaemia patients and on samples with high Hb F level. The procedure of Shelton et al (1984), slightly modified and standardized using High Performance Liquid Chromatography (HPLC) on a Vydac C4 column was used since a better separation was obtained between the AγI and its variant AγT. To obtain the Gγ/Aγ ratio the AγI and AγT values were added and the Gγ value was divided with the total Aγ.

3.3.7. Separation of the globin chains.

Globin chains of haemoglobin (i.e.α, β, δ) were separated by ion exchange chromatography on CM-cellulose, using 0.005M phosphate buffer, pH 6.7 containing 8M urea and β-mercapto-ethanol to separate the globin chains. Only the abnormal samples were subjected to globin chain separation [El-Hazmi & Lehman, 1978].

The freshly prepared haemolysate was used for the separation of globin chains. To the haemolysate [5% (W/V)] was added a solution of concentrated HCl in acetone cooled to 0°C. This causes the precipitation of the globin chains and removal of the haem group from the globin. The precipitated globins were separated from the haem containing supernatant by centrifugation and the precipitated globin chains were washed several times with pure acetone cooled to 0°C. This globin chain mixture was applied to the CM cellulose column and eluted by applying salt gradient in the same buffer. The position of the peaks was located by determining the absorbance at 280nm using a U.V. detector. The sepa rated peaks were individually pooled, desalted using a Sephadex G-25 column, freeze dried and stored for further analysis and structural studies.

3.3.8. Finger Printing of globin chain.

Globin chains separated by CM-cellulose chromatography were stored in a lyophilized form. Abnormal globin chains were subjected to finger printing (peptide mapping) by treatment with trypsin at pH 8.5, 37°C for 1-2 hours. Trypsin cleaves those peptide bonds in which the -C- of the peptide is contributed by either arginine or lysine. The globin chains were cleaved into several peptides each ending at either a lysine or an arginine. The peptides were separated by high voltage electrophoresis using Whatman No.

3 filter paper, pyridine acetate buffer, pH 6.4, 2.5 KV for 35 min. The chromatograms were air dried and the peptides were located by staining with cadmium-ninhydrin. The peptides generated using normal globin chains were used as controls along with the abnormal samples. The purified amino acids arginine, aspartate and glycine were used as markers. The position of the new peptides was noted and the peptide was eluted from the chromatogram, freeze-dried and stored until required for amino acid analysis [El-Hazmi, 1978].

3.3.9. Amino acid analysis of peptides

The abnormal peptides and some normal peptides were subjected to complete digestion using 6N HCl after freezing and sealing under vacuum. The hydrolysis was achieved at 120°C for 22 hours. The hydrochloric acid was removed by evaporation in a vacuum dessicator followed by several washings with distilled water and drying to remove all traces of HCl. The amino acid mixture was dissolved in the buffer and subjected to amino acid analysis on the LKB autoanalyser and the amino acid composition of the peptide was obtained. The molar ratio of the amino acid was calculated in the peptide and the number of each amino acid in the peptide was obtained [El-Hazmi, 1978].

3.4. Analysis on Plasma

The plasma separated from the cellular components of blood, by centrifugation was stored frozen at -20°C or -70°C and was used for the following analysis.

3.4.1. Estimation of biochemical parameters

Biochemical parameters were estimated in the plasma samples as organ profiles and other miscellaneous tests. The organ profiles investigated during this study included:

(i) liver function test profiles i.e. total and direct bilirubin, total protein, albumin, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase. Wherever possible (particularly in sample from abnormal cases) the level of gamma glutamyl transferase and lactate dehydrogenase were also estimated; (ii) renal function test profiles i.e. urea, creatinine, (iii) bone function profiles i.e. calcium, phosphate and alkaline phosphatase; (iv) electrolytes i.e. sodium, chloride, potassium and bicarbonate; and (v) certain miscellaneous tests i.e. total iron, iron-binding capacity, uric acid, glucose and others. Wherever possible isoenzymes of LDH and alkaline phosphatase were separated by electrophoresis on cellulose acetate plates from Helena and the level of each isoenzyme was obtained using a densitomer.

3.4.2. Estimation of immunoglobulins, complements and properdin

The immunoglobulin (i.e. IgG, IgA, IgM) levels were estimated in the samples of the sickle cell disease and the thalassaemia patients. Complements C3 & C4 were also estimated only in the abnormal samples. Radial immuno diffusion (RID) plates from Behring (Behring Diagnostic, Postfach 1140, D-35501, Maborg) were used for these estimations.

The level of properdin factor B, a component of the alternate pathway of complement fixation, was determined by rate nephlometry using a Beckman immunochemistry system (Beckman Instruments Inc., Brea, CA). Specific kits for properdin factor B were purchased from Beckman.

3.4.3 Estimation of protein C & S

Proteins C & S were estimated in the plasma using electro immunodiffusion

(Laurell Rocket Technique). Commercially available kits (Assera (R) - Plate, Protein S and Protein C kits), were purchased from Diagnostic Stage (France). The levels of these proteins were estimated in sickle cell disease patients, haemoglobin S heterozygotes and normal (HbAA) controls. Quality control was achieved by using protein S control plasma (Cat. No. 0808). A standard calibration curve was plotted using a pool of normal human plasma diluted to make 4-5 different dilutions. Patients samples and controls were tested undiluted. The results obtained in the latter were used for the establishment of normal reference range of protein C & S in healthy Saudis.

3.4.4 Estimation of Zinc

Zinc is an essential micronutrient and is necessary for normal growth and sexual development and proper would healing. We estimated zinc level in the sickle cell disease patients by using atomic absorption spectrophotometer.

3.4.5 Estimation of Hormones

The hormones investigated in sickle cell disease and β-thalassaemia patients and normal controls included luteinizing hormone (LH), follicle stimulting hormone (FSH), testosterone, free thyroxine (fT4), free triiodothyronine (fT3), growth hormone cortisol, insulin and C-peptide.

The plasma was used for the estimation of hormones by radioimmunoassay (RIA) or by enzyme immunoassay. The kits were purchased from Amersham Kodak (Amerlite), C-peptide kits were obtained from Incester.

3.4.6 Estimation of transferrin and ferritin

The analysis of the ferritin level was carried out by Radioimmunoassay (RIA)

using kits from Amersham Kodak (Amerlite). The ferritin level was used as a measure of serum iron status. Transferrin was estimated using RID plates from Behring (Behring Diagnostica). Mostly these analysis were conducted on abnormal samples, however, wherever available, normal samples were used to establish the `normal reference values' for these parameters in Saudis.

3.4.7 Estimation of haptoglobin

Haptoglobin level was estimated in patients with sickle cell disease and in normal controls by radioimmuno diffusion (RID) using kits from Behring.

3.4.8 Estimation of vitamin E

The level of vitamin E was estimated in plasma from sickle cell disease patients and normal healthy age and sex matched control. The estimation was carried out by high performance liquid chromatography (HPLC). The vit E was analysed as three fractions i.e. α, β, and γ -tocopherol. The major fraction was α-tocopherol in all samples.

3.4.9 Estimation of hepatitis antigens and antibodies

The hepatitis B virus (HBV) and hepatitis A virus (HAV) markers were estimated by radioimmunoassay using kits from Abbotts Diagnostics. The HBV markers included hepatitis B surface antigen (HBsAg), hepatitis Be antigen (HBeAg), antibody to hepatitis B surface antigen (anti HBs) and antibody to hepatitis B core antigen (anti-HBc). The HAV, an antibody to hepatitis A virus (anti HAV) was estimated. In addition anti Delta antigen was estimated in samples positive to HBsAg. Antibody to HIV was estimated in a multitransfused patients & controls.

3.4.10 Estimation of hydroxyurea

The level of hydroxyurea was estimated in patients who were subjected to hydroxyurea clearance studies and in those patients who were on hydroxyurea treatment protocol. In the former group of the patients the level of hydroxyurea was estimated every hour for 6 hours following an oral dose of hydroxyurea (25 mg/kg body weight). In the latter group of patients the level of hydroxyurea was estimated with the aim to follow the compliance of the patient to hydroxyurea oral therapy.

The estimation of hydroxyurea [Fabricius & Rejewsky, 1971] was carried out using the following method:

> "One millilitre of whole blood was diluted with 4 ml distilled water and 5 ml perchloric acid (IM). After standing for 10 min, the supernatant was separated by centrifugation at 30000g for 20 min and filtered using 0.2 nm Millex filter paper. Two millilitres of the filtrate were added to 1.0 ml of phosphate buffer, 1 ml of sulphanilic acid and 0.1 µl of NaOH. The solution was mixed thoroughly and 0.1N I_2 in 2.5% KI (100 µl) was added. After further mixing 100 µl of 0.1M $Na_2S_2O_3$ are added and after mixing 1 µl of N - (1-napthyl-) ethylene diamine dihydrochloride (NED) solution was added. The absorbance of the solutions was read after 20 min at wave length of 540 nm using 0.5 M perchloric acid solution as blank. Standard curve was regularly plotted using standard solutions containing known amount of hydroxyurea and the concentration of the unknown was obtained from the standard curve. The original concentration is obtained after multiplication with the dilution factors."

Hydroxyurea estimation in the urine was also carried out on the patients on hydroxyurea therapy and in patients on hydroxyurea clearance studies.

3.4.10.1. Hydroxyurea clearance curve

All patients included in hydroxyurea protocol were included in this study to determine the dose of hydroxyurea. Each patient was admitted to the King Khalid University hospital under supervision of one of the coinvestigator or collaborators. Approved informed consent was obtained from each patient or his/her guardians. Hydroxyurea clearance studies were conducted at least twice to calculate the individual dose for each patient. The baseline haematological and biochemical parameter values (at least on three separate occasions) were determined prior to initiation of the hydroxyurea therapy.

The test dose of hydroxyurea according to the approved protocol was 25mg/kg body weight. This was given orally in a single dose with water. Blood samples were collected prior to hydroxyurea intake and every one hour following the intake for six hours. The level of hydroxyurea was estimated in each sample and in a urine sample.

The level of hydroxyurea (mg/l) was plotted against the time and the area under the curve at six hours (AUC_6) was calculated [Nienhius - personal communication].

The treatment dose depends on the AUC_6 as follows:

AUC_6	HU Dose (mg/kg body weight)
< 1000	20 mg/kg body wt.
1000-1500	15mg/kg body wt.
> 1500	10mg/kg body wt.

The hydroxyurea clearance studies were repeated on each patient and the final dose depen-

ded on the average values obtained in the two studies. Only adult patients with a good compliance and patients without any renal or hepatic complications were included in this protocol.

3.5. Analysis on Buffy Coat

The buffy coat was stored frozen preferably at -70°C until required. It was used for the extraction of DNA.

3.5.1. Extraction of DNA

The extraction of DNA from the buffy coat (lymphocyte) was carried out by the method of Kunkel and coworkers [1977]. This involves the following steps:

i) Mix the buffy coat obtained from 10 ml blood with 5 ml normal saline and 0.125 ml of 0.5M EDTA (pH 8.0). Freeze at -20°C.

ii) The cells are lysed by mixing with 40 ml lysing solution.

iii) Centrifuge to collect nuclei.

iv) Suspend pellet in 30 ml of resuspending solution.

v) Add 0.3 ml of 10% SDS.

vi) Mix with 10 μl of proteinase K solution (20 g/ml) and incubate overnight at 30°C.

vii) Add 3.0 ml phenol and mix for 10 minutes.

viii) Remove upper aqueous layer, add 3.0 ml equilibrated phenol and mix for 10 minutes. Centrifuge.

ix) Separate the aqueous phase and add 3.0ml chloroform/iso-amyl alcohol (25:1). Mix for 5 minutes and centrifuge for 5 minutes at 1700 RPM.

x) Remove upper phase and re-extract.

xi) Remove upper phase and add 1.5ml of ammonium acetate.

xii) Add 9.0 ml ethanol and mix.

xiii) Hook out DNA and dissolve in 1.0 ml Tris/EDTA.

xiv) Read optical density of the diluted DNA solution at 280nm and 260nm and calculate the concentration of the DNA (μg of DNA/ml).

Initially, DNA was extracted from several thousand samples by the manual method listed above. Recently an automated DNA extractor (i.e. Applied Biosystem Model 340A) was purchased and used for DNA extraction. The blood samples were directly loaded on to 8 vessels in the instrument where they undergo digestion, phenol/chloroform extraction and precipitation with alcohol. After dissolving in the buffer the concentration of the DNA is estimated by measuring optical density at 260nm and 280nm.

3.5.2 Studies using restriction endonucleases

Restriction endonucleases were used throughout the course of this study for:

i) Direct detection of mutation in the gene by identifying the appearance of a new restriction site or disappearance of an already existing one.

ii) Detection of polymorphism (RFLPs) in the flanking regions or in introns.

iii) Detection of HVR in the flanking regions.

iv) Detection of gene deletions.

v) Determination of linkage of specific genetic markers.

vi) Determination of haplotypes using several restriction endonucleases.

For different genes different restriction endonucleases and specific probes were used. A brief list of restriction endonucleases used for the disorders under study is presented as Table 3.1.

A brief outline of the procedure for restriction endonuclease digestion is as follows:

i) The amount of restriction endonuclease required for digestion was calculated.

ii) The components i.e. water, restriction endonuclease, buffer and DNA solution were mixed and digestion was initiated by addition of the enzyme (The optimal condition of digestion by each enzyme vary and are provided by the manufacturers i.e. New England Biolab or Bethesda Research Laboratory, Rockville MD).

Table 3.1*:* Restriction endonucleases used during this study

Bam HI	Rsa I
Ava II	Bgl II
Hpa I	EcoRI
Hind III	Mst II
Hinc II	Xmn I
Dde I	

iii) The DNA was incubated with the restriction endonuclease for 2 hours to overnight.

iv) The extent of digestion was determined by using monitoring mini gels with the same amount of undigested DNA as control.

3.5.3. Analysis of DNA fragments after enzymatic cleavage - Agarose gel electrophoresis

The DNA fragments produced by restriction endonuclease digestion were separated on the basis of their size on an agarose gel electrophoresis. The distance migrated on the gel was inversely proportional to the logarithm of the molecular size. Using known size standards, the gels were calibrated and the size of the DNA fragments produced was determined. The general procedure for 0.8% agarose gel was as follows and could be modified by changing the agarose concentration, buffer and polarization conditions:

i) 0.8% agarose gel was prepared by adding 2.4g of agarose in 300ml of lXTris acetate EDTA (TAE) buffer and boiling in water bath.

ii) Add 10µl of 10 mg/ml of ethidium bromide solution.

iii) Cool gel to 60°C and pour evenly on the gel tray. Allow to set.

iv) Place the gel on electrophoresis tank and pour lXTAE buffer to a depth of 1-2 minute on the gel.

v) Carefully load the DNA digest (5µl in 50µl loading buffer, 0.25% bromophenol blue and 40% W/V sucrose), in the slots.

vi) Run the samples by turning ON power at 35-40V. Run overnight.

vii) Switch OFF power.

viii) Examine gel under UV light.

ix) Photograph ethidium - bromide stained bands in gels using a UV transilluminator and a polaroid camera.

3.5.4. Southern Blotting

To transfer the DNA fragments separated on the agarose gel to a nitrocellulose or Hybond N filters, Dr. E. Southern in 1975 devised an ingenious technique which is known as `Southern blotting (Southern, 1975). The DNA fragments were denatured, neutralized and the gel was placed under the nitrocellulose sheet, covered with piles of filter paper. The transfer of the DNA fragments involved a capillary action using a high salt buffer. The single stranded DNA was permanently bonded to the filter by baking and was hybridized to a `probe' to detect the hybridizing DNA species. The out line of the Southern blot procedure is as follows:

i) The agarose gel is soaked in denaturing solution, and washed with distilled water.

ii) The gel is then neutralized by soaking in neutralizing solution.

iii) Fill the Southern blot tray with SSC (0.15M NaCl and 0.15M sodium citrate pH 7.0) buffer.

iv) Place the gel onto the surface of the Southern blot tray. Place a nitrocellulose or Hybond N sheet over the gel and cover four sides of the buffer tray with wrapping plastic.

v) Lay a pile of filter paper over the nitrocellulose and cover with a plastic

plate. Apply suitable weight.

vi) Leave for 20 hours for transfer to be complete.

vii) Carefully dismantle and bake between two sheets of filter paper at 80°C for two hours (in a vacuum oven for nitrocellulose sheets).

viii) Seal in plastic bag and keep at 4°C until required for hybridization.

Southern blotting is routinely carried out in our laboratory and both nitrocellulose and Hybond N sheets have been successfully used. The later have the advantage that they may be hybridized with different probes following washing.

3.5.5. Hybridization with different probes

Probes are DNA fragments, complementary to the whole or to a portion of the DNA fragment containing the gene of interest. The plasmid-derived gene fragments, cDNA or small synthetic oligonucleotides may be used as probes.

3.5.5.1. Plasmid-derived probes - Nick Translation.

Plasmid-derived probes of α, β, δ and β genes were used during the study. The bacterial cells containing the hybrid plasmids were continuously cultured and purified to keep a fresh stock available.

The gene probes in the plasmid was obtained by restricting with specific restriction endonuclease, separation of fragments on agarose gel and electro-elution of the required fragment. Prior to using these probes, to detect DNA fragment containing the gene of interest in Southern blots, the probe was radiolabelled by introducing radioactive nucleotide phosphate. This was carried out by the process of `Nick translation'. This reaction utilises the ability of the enzyme DNA polymerase I to initiate DNA synthesis at

free 3' OH groups, which are exposed as nick in unlabelled DNA. These nicks were generated by random digestion with DNAase. ^{32}P labelled nucleotide triphosphates were used by the DNA polymerase I to synthesise new DNA fragments in the 5' → 3' direction.The DNA fragment so synthesised was radioactive and of value as a probe.

The following steps are involved in Nick Translation:

i) In a tube mix plasmid DNA (0.2 μg) with Nick translation kit (5μl) (contains dATP, dCTP,dTTP) and ^{32}P dCTP. Add Nick translation kit enzyme mixture (5μl) and make up the total volume to 50 μl with water. (The nick translation kit is obtained from BRL)

ii) Seal tube, incubate at 15°C for one hour.

iii) Stop the reaction by adding 5 μl `stop buffer' (300 mM Na_2 - EDTA, pH 8.0).

iv) Purify on a Sephadex G-50 column using 10mM Tris, 1mM EDTA buffer, pH 8.0.

v) Count radioactivity of each fraction using a β-counter.

vi) Pool first eluted peak. Calculate specific activity.

$$\text{Specific activity (dpm/μg)} = \frac{\text{Total cpm x 3}}{\text{μg DNA}}$$

vii) Denature DNA at 100°C for 2 minutes.

3.5.5.2 Hybridization

i) Prehybridize the filters in Denhardts solution followed by prehybridization solution in a plastic box.

ii) Seal the box and incubate at 68°C in shaking water bath for at least four

hours.

iii) Degas hybridization solution. Hybridize the filter and seal the plastic bag.

iv) Keep bag at 68°C in shaking water bath overnight.

v) Wash filter at least three times with washing solutions at 55°C.

vi) Place between two X-ray films in a casette with Dupont Cronex lightening plus screen.

vii) Expose film at -70°C for 1-4 days.

viii) Remove film from freezer and develop.

More recently `Nick translation' has been replaced by `End Labelling' techniques. This method is simpler and was later used instead of nick translation.

3.5.5.3. Oligonucleotide-specific probes

Probes may be synthesised oligonucleotides (15-20 nucleotide fragments) with base sequence complementary to a portion of DNA of interest. These probes was either synthesised using an automated DNA synthesiser (Applied Biosystem) or were purchased commercially. The probes were radio labelled by introducing a ^{32}P label from (^{32}P)ATP using polynucleotide kinase, following the removal of the unlabelled 5' phosphate with a phosphatase. Kits available from Bethesda Research Laboratory were used for labelling the 5' end.

i) Place the filter in a heat-sealer plastic bag and add 10 μl of hybridization buffer. Seal.

ii) Incubate at room temperature for 2-3 hours.

iii) Open seal. Add radiolabelled probe. Reseal.

iv) Incubate bag overnight at specific incubation temperature, calculated using the formula T = Tm -15°C.

v) Wash filter using 3 changes of SSC buffer.

vi) Autoradiograph.

More recently, biotinylated nucleotides have been used to label probes by enzymatic reaction and have the advantage that radioactive material is avoided [Leary et al, 1983]. Wherever possible biotina ted nucleotides and other non-radioactive probes were used during this study.

3.5.6. Polymerase Chain Reaction (PCR)

Polymerase chain reactions (PCR) is described as one of the most powerful tools of modern biology which involves enzymatic amplification of the DNA sequence of interest, by a factor of 10^5-10^6 in a short period of time. The applications of PCR are many and the most significant ones are in the field of diagnosis, both prenatal and postnatal. It has also been used for the amplification of viral and bacterial DNA and hence in diagnosis of acquired diseases. We carried out PCR using a `Thermocycler' (Perkin-Elmer).

The PCR procedure requires 2 primers of known sequence for each DNA fragment (eg the gene) to be amplified. (These primers were either purchased commercially or synthesised using DNA synthesizer). It also requires a highly specific thermostable DNA polymerase known as Taq DNA polymerase which has been obtained and purified from Thermus aquaticus. The steps in PCR are as follows:

i) Mix the genomic DNA (1μg) with primer 1 and 2, deoxy ribonucleotide triphosphates, amplification buffer and distilled water.

ii) Place in the DNA Thermal Cycler. Program cycle profile and fill in details of temperature and incubation times for each step.

iii) In the first step, the DNA is denatured by heating at 96°C for 10 minutes. Add Taq polymerase.

Mix and overlay with 100µl of light mineral oil to prevent evaporation during the denaturation and amplification stages. The denatured DNA is subjected to different number of cycles each covering the following steps:

i) Denaturation at 96°C for 30 seconds.

ii) Annealing (hybridization) at 56°C for 30 seconds.

iii) Extension (elongation) at 72°C for 45 seconds. In the last cycle the elongation stage is extended to 10 minutes at 72°C.

iv) Remove the aqueous phase which contains the amplified DNA carefully with a pipette and store at -20°C until required for analysis.

v) The PCR products can be used for:

a) Direct sequencing.

b) Restriction endonuclease studies.

c) Dot blot hybridization.

d) Synthetic oligonucleotide probe hybridization.

e) Denaturing gradient gel electrophoresis.

(For amplifying different genes the experimental conditions were modified slightly).

3.5.7. DNA sequencing

A rapid method for DNA sequencing was first developed by Sanger in 1975. It involves synthesis of a complementary strand on the DNA fragment to be sequenced and inhibition of the synthesis by using a dideoxynucleotide triphosphate (ddNTP) in four separate reactions. Each such reaction contains the 4 dNTP (one of which is ^{32}P labelled) and one specific ddNTP. Depending on the ratio of the concentration of the inhibitor (ddNTP) to that of the substrate (dNTP), different size fragments are produced each ending at the specific dideoxynucleotide (i.e. ddATP or ddCTP or ddGTP or ddTTP). The fragments so generated are separated on high resolution denaturing acrylamide gels in four separate slots (one for each experiment) and the sequence is directly read from the gel. The sequence of the DNA fragments of interest is complementary to this sequence. We used a DNA sequencer (LKB, Macrophore 2010) for the sequencing studies.

3.5.8. Dot Blot Analysis

This method is a rapid method for determining the presence of a specific DNA or RNA fragment in a sample. A direct transfer of the DNA or RNA is made on the nitrocellulose sheets as `spots' or `dots'. The spotted filter is hybridized with a labelled probe. The presence of the DNA fragment of interest is seen as a dark spot following autoradiography. Non radioactive probes, e.g. biotinylated nucleotides are used successfully.

i) Place Whatman 3MM filter paper wetted in SSC buffer in the Dot blot apparatus (Bio-Rad). Place nitrocellulose sheet on top.

ii) Cover with lid. Hook to vacuum source.

iii) Rinse wells with SSC buffer. Place 2µl of dye in several outer wells as

position markers.

iv) Place 1-2µg of DNA in 10µl of TE buffer. Heat to 95°C for 5 minutes. Add 40µl of 20xSSC buffer to the samples. Rinse wells wth SSC under vacuum.

v) Bake nitrocellulose filter in vacuum oven at 80°C for 2 hours.

vi) Hybridize filter with radiolabelled probe.

3.5.9. Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a gel system that separates DNA fragment on the basis of their melting properties. Each double stranded DNA fragment has a specific Tm value i.e. the temperature at which half the DNA melts (i.e. changes to a single strand). The Tm depends on stacking inter action between adjacent bases and is highly sensitive to differences in stacking brought about by changes in its nucleotide sequence. Even a single base substitution may cause the Tm to change significantly. The DGGE system was designed to separate DNA fragment differing by single-base changes depending on their Tm [Myers et al, 1985a; 1987]. It consists of a polyacrylamide gel containing a linearly increasing gradient of DNA denaturants, such as formamide and urea, from the top to bottom. DNA fragments migrate depending on their molecular weights and when the concentration of the denaturant in the gel equals to the specific Tm, each fragment denatures and the migration rate decreases. DNA fragments differing by a single base substitution begin melting in the gel at different positions and are separated. Brief outline of the procedure is as follows:

1. Acrylamide gels (eg 6.5%) are prepared by mixing two solutions of different denaturant concentration in a linear gradient made of formamide

and urea.

2. Electrophoresis is carried out at 60°C using TEA buffer (40mM tris, pH 7.4; 20mM sodium acetate and 1mM EDTA) at 150V for 130 min.
3. The gels are stained by ethidium bromide staining.

This method can be made more sensitive by attaching GC-rich segments called GC-clamps to genomic DNA fragments [Sheffield et al, 1989; Myers et al, 1985b].

3.5.10. Amplification Refraction Mutation System (ARMS)

ARMS is a simple and rapid method for detecting point mutations, and small nucleotide insertions or deletions. The technique is based on allele specific priming of PCR. To detect a specific mutation, two oligonucleotide primers are required. They are of identical sequence except for terminal 3' nucleotide. The normal primer has 3' nucleotide sequence complementary to the normal DNA sequence, while the mutant primer has its 3' terminal nulceotide complementary to the mutant DNA sequence. No amplification occurs if the primer and DNA are unmatched even in a single base.

The technique requires two PCRs involving four primers. Two primers are control primers which amplify segment away from site of mutation, while the other two primers are ARMS primers, one specific for mutant or normal DNA as required, while the other is common primer which matches same sequence in both normal and mutant DNA.

After PCR amplification, the PCR product is subjected to agarose gel electrophoresis (3% agarose gel) at 100V for 45 min. The gels are stained using ethidium bromide staining. Gels are photographed using a UV transilluminator.

3.6 Statistical Analysis

The computers at the Computer Centre, King Saud University were used to enter the entire data obtained during this study. The results from the different regions were fed separately. All analysis were conducted using the Statistical Analysis System (SAS). Mean, median, mode, standard-deviations, percentiles, quantiles, variance, coefficient of variation, frequency distribution histograms, normal probability plots, shewness, kurtosis and degrees of freedom were obtained. Correlation studies were conducted using the General Linear Model (GLM) programme of SAS and correlation coefficient and statistical significance of the correlation were obtained. The significance of the difference in the mean of any two groups was obtained using the 2x2 contingency tables or by chi square analysis. P value < 0.05 was considered statistically significant. For certain analysis paired `t' tests were used to determine the significance of difference in groups of results e.g. between patients and control groups or before and during treatment.

CHAPTER 4

ANAEMIAS IN SAUDI POPULATION

4.0 Anaemias in Saudi Arabia

Anaemias are a group of disorders resulting from a decrease in the total haemoglobin concentration in red cells, reduction in packed cell volume (hematocrit) or decrease in the number of circulating erythrocytes, and are physiologically characterized by an inability to maintain the normal tissue oxygenation (Hardisty and Weatherall, 1982; Edwards et al 1991; Chandrasoma and Taylor, 1993). Generally, as the oxygen-carrying capacity is reduced, other compensatory mechanisms come into play, such as increased cardiac output, increased pulse rate, increased erythropoietin production, altered oxygen affinity of haemoglobin and diversion of blood from less vital to more vital organs e.g. the brain. The signs and symptoms of anaemia are a consequence of the hypoxic state produced and the compensatory changes taking place in the body.

The prevalence of anaemias is significantly different in different populations and depends largely on the age, sex, socio-economic status, genetics and environmental factors. Generally, the prevalence of anaemias is higher in children and in females of child bearing age, particularly in the low socio-economic groups. Genetic anaemias also show considerable differences depending on the gene frequencies. Frequently two or more genes coexist in the same population and produce a severe anaemia.

The symptoms of anaemia depend on the severity of the anaemic state and are listed in Table 4.1.

The diagnosis of anaemias can be easily made and is generally based on the signs and symptoms and laboratory data. However, differential diagnosis requires specific confirmatory tests, which are often complicated. As is the case with some of the genetic

anaemias. In the last 10-15 years the molecular biology techniques (Dacie and Lewis, 1991; Chandrasoma and Taylor, 1993) have been used to reach the diagnosis of genetic

Table 4.1*:* General Symptoms of Anaemia

- Pallor
- Rapid pulse
- Shortness of breath
- Fatigue
- Headache
- Dizziness
- Irritability
- Difficulty in concentrating
- Decreased appetite
- Nausea
- Loss of libido or potency
- Menstrual irregularities
- Angina Pectoris
- Heart murmers
- Heart failure
- Coma

anaemias at molecular level by identifying the molecular pathogenesis i.e. the basis defect in the DNA. Table 4.2 lists the investigations required to reach a confirmatory diagnosis of anaemias.

Anaemias have been known as a common problem in certain regions of Saudi Arabia from early times. However, no studies have reported the exact prevalence of anaemias in this population. Both acquired and genetic anaemias have been described and are known to exist. The latter are particularly common in those areas of Saudi Arabia that have a past or present history of malaria endemicity.

We investigated Saudi male and female adults and children (<14 years) living in the different areas of Saudi Arabia. The diagnosis of the anaemic state was based on the routine clinical and laboratory data using the normal haematological values established during this study, for the adult male and female and children (Table 4.3). The samples were classified as normal and anaemic depending on whether the haematological parameters were in the normal range or were less than the normal. The prevalence of anaemias in the Saudi population is presented in Table 4.4.

On the basis of the red cell morphology the anaemias were classified as hypochromic-microcytic, normochromic-normocytic and normochromic-macrocytic. The prevalence of each type of anaemia was calculated in the different regions and the results are presented as Table 4.5.

Further data analysis was carried out to determine the frequency of each type of anaemia in the adult males, females and children. The prevalence of the different types of anaemias in the three groups are presented in Tables 4.6 - 4.8 for the adult males, females

and children, respectively.

Table 4.2*:* Diagnosis of Anaemias

A. History
- Symptoms
- Nutritional habits
- Blood loss and bleeding tendency
- Drugs and Toxins
- Family history

B. Physical Examination
- Vital signs
- Skin
- Conjunctiva
- Mouth
- Lymph node
- Cardiorespiratory system
- Fundi
- Neurological examination
- Abdomen
- Pelvic and rectal examination

C. Laboratory Data
- Haematological parameters
- Peripheral blood smear examination
- Red cell indices and discriminant factors
- White cell count
- White cell differential count
- Platelet counts
- Special tests:
  - Hb electrophoresis for abnormal Hb
  - Hb A_2 & F for thalassaemias
  - Schillings tests for Vit B12 absorption*
  - Serum and red cell Vit B12 level*
  - Serum iron and iron binding capacity
  - α/β-Chain ratio for thalassaemia
  - Biosynthetic studies for abnormal Hbs.
  - Molecular biology studies

* Not done during this study

Table 4.3: Haematological Parameters values in normal Saudi population in different provinces

Area	Group	Total Hb g/dl	RBC $x10^{12}$/l	WBC $x10^{9}$/l	PCV l/l	MCV fl	MCH pg	MCHC g/dl
Central								
Riyadh								
	M	14.9±1.2	5.2±0.4	6.5±2.0	0.43±0.033	83.0±5.9	29.0±1.6	34.02±1.3
	F	13.0±1.1	4.7±0.4	6.6±1.94	0.39±3.2	82.5±5.7	28.7±1.4	34.02±1.3
	C	12.9±0.97	4.0±0.34	7.2±1.94	0.38±0.025	78.2±5.7	27.7±1.3	34.2±1.3
Qaseem								
	M	14.4±1.32	5.4±0.57	6.8±2.44	0.43±0.05	82.6±5.0	26.6±1.96	32.1±1.21
	F	12.3±1.49	4.72±0.51	6.8±2.44	0.39±0.05	81.3±5.73	25.9±2.26	31.8±1.42
	C	13.3±1.53	5.1±0.52	7.1±1.99	0.40±0.06	79.75±.55	25.5±2.21	32.0±1.12
Sulayel								
	M	15.1±1.37	5.2±0.58	7.0±5.4	0.45±0.04	85.0±5.40	29.2±1.27	34.0±1.07
	F	13.5±1.65	4.6±0.59	7.2±1.65	0.40±0.046	84.0±3.91	29.2±1.52	33.9±1.44
	C	13.4±1.66	4.65±0.65	7.0±1.75	.40±0.069	82.3±4.93	28.9±1.47	33.7±1.08
Eastern								
Hafouf	M	15.3±1.8	5.6±0.74	7.0±1.8	0.46±0.056	30.7±5.56	27.5±2.92	33.8±1.04
	F	13.2±1.52	4.9±0.66	6.9±2.27	0.40±0.045	79.0±7.75	27.0±3.39	34.0±1.38
	C	13.3±2.0	5.1±0.78	7.3±2.51	0.39±0.05	76.3±9.00	25.6±3.50	33.8±1.37
Qateef	M	12.46±1.31	5.13±0.54	6.10±2.16	0.39±0.04	80.39±8.49	25.6±3.54	32.02±1.75
	F	11.71±1.28	4.86±0.54	6.35±0.54	0.37±0.04	77.55±9.22	24.7±3.4	31.56±1.55
	C	11.43±1.32	4.77±0.61	7.45±2.60	0.35±0.04	75.82±8.76	24.39±3.5	32.05±1.49
Western								
Khaiber	M	15.9±1.83	5.50.8	-	0.45±0.044	80.7±8.55	28.5±2.06	35.6±2.95
	F	14.4±2.13	4.90.78	-	0.41±0.035	83.3±8.17	29.3±1.09	35.3±3.21
	C	14.7±2.1	4.90.78	-	0.40±0.035	83.0±9.16	29.4±1.74	35.7±3.71
Al-Ula	M	14.5±1.8	4.80.56	4.51.97	0.45±0.04	92.9±11.31	29.3±0.87	34.3±2.21
	F	13.1±1.53	4.30.52	3.81.75	0.40±0.07	91.2±12.5	29.8±0.77	34.6±2.5
	C	12.6±1.7	4.20.53	4.011.3	0.39±0.06	91.8±14.0	30.0±0.86	34.7±2.3
Jaizan	M	13.5±2.42	4.9±0.94	6.3±2.67	0.38±0.07	78.0±7.99	27.7±3.48	35.4±1.88
	F	12.6±1.87	4.4±0.74	6.3±2.36	0.33±0.05	75.8±5.03	26.7±3.93	35.4±1.53
	C	12.6±2.2	4.3±0.92	7.8±3.27	0.30±0.06	30.0±6.31	24.9±4.38	35.0±1.36
Najran	M	16.8±1.56	5.1±0.9	7.7±7.06	0.43±0.07	81.1±6.5	33.2±5.37	39.4±5.43
	F	14.5±1.19	4.3±0.78	7.0±3.24	0.36±0.06	81.0±8.1	33.8±5.7	39.6±5.86
	C	14.2±1.29	4.2±0.91	7.8±3.2	0.35±0.07	78.4±7.7	34.7±7.03	41.4±7.34

Table 4.3 …….contd

Area	Group	Total Hb g/dl	RBC $x10^{12}$/l	WBC $x10^{9}$/l	PCV l/l	MCV fl	MCH pg	MCHC g/dl
Makkah	M	-	5.1±0.62	6.8±2.26	0.44±0.05	87.7±6.45	29.4±2.46	33.5±0.84
	F	-	4.5±0.46	7.0±2.4	0.39±0.04	86.4±8.31	29.0±2.42	33.4±0.93
	C	-	4.8±0.64	6.4±2.59	0.41±0.055	83.3±6.77	29.0±3.03	34.2±2.01
Al-Baha	M	15.64±1.85	5.40±0.62	6.82±2.25	0.47±0.050	86.6±6.26	29.23±2.58	33.57±0.87
	F	13.68±1.80	4.85±0.59	703±2.06	0.41±0.05	85.43±7.25	28.39±2.88	33.28±0.91
	C	13.23±2.01	4.85±0.69	7.36±2.42	0.40±0.06	82.3±7.59	27.5±2.94	33.25±0.87
Bisha	M	16.13±2.68	4.79±0.87	3.84±1.51	0.43±0.07	86.44±6.42	34.23±4.87	37.76±3.99
	F	14.46±2.52	4.46±0.78	3.75±1.54	0.39±0.05	86.16±7.16	32.76±5.22	37.02±3.89
	C	14.13±2.76	4.5±0.87	4.16±1.72	0.43±0.07	83.93±7.89	31.94±4.87	37.20±4.12
Yanbu	M	14.51±1.43	4.96±0.59	7.52±1.66	0.43±0.04	85.49±4.35	29.34±1.64	-
	F	12.56±1.51	4.36±0.61	7.79±1.86	0.38±0.05	84.6±5.3	28.93±1.90	-
	C	12.78±1.46	4.41±0.53	7.68±1.98	0.38±0.04	83.2±5.2	28.97±1.99	-

Table 4.4: Prevalence of Anaemias in different Provinces of Saudi Arabia

Province	No. investigated	No. of Anaemic	Prevalence (%)
Central			
- Riyadh	1940	210	10.82
- Qaseem	998	156	15.631
- Sulayel	1304	109	8.359
Eastern			
- Al-Hafouf	906	159	17.550
- Al-Qateef	1040	382	36.730
Western			
- Khaiber	313	46	14.696
- Al-Ula	448	74	16.518
- Abha	1031	271	26.285
- Jaizan	2308	872	37.782
- Najran	1653	175	10.587
- Qunfuda	819	114	13.919
- Al-Baha	1035	119	11.498
- Bisha	1002	153	15.269
- Majarda	451	107	23.725
- Yanbu	783	138	17.624
- Makkah	752	109	14.49
- Safra	199	31	15.578

Table 4.5*:* Prevalence of different types of anaemias in different Regions of Saudi Arabia

Province	No. Inves-tiga-ted	Hypochromic microcytic		Normochromic normocytic		Normochromic Macrocytic	
		No.	Prev.	No.	Prev.	No.	Prev.
Central							
- Riyadh	1940	125	6.44	85	4.38	0	0
- Qaseem	998	63	6.313	93	9.318	0	0
- Sulayel	1304	7	0.537	102	7.822	0	0
Eastern							
- Al-Hafouf	906	94	10.375	64	7.064	1	0.110
- Al-Qateef	1040	230	22.115	146	14.038	1	0.096
Western							
- Khaiber	313	1	0.319	27	8.626	18	5.75
- Al-Ula	448	4	0.893	47	10.491	23	5.133
- Abha	1031	85	8.244	186	18.040	0	0
- Jaizan	2308	422	18.284	447	19.367	0	0
- Najran	1653	97	5.868	78	4.718	0	0
- Qunfuda	819	25	3.05	83	10.134	6	0.733
- Al-Baha	1035	49	4.734	64	6.184	6	0.579
- Bisha	1002	21	2.096	124	12.375	7	0.699
- Majarda	451	41	9.091	66	14.634	0	0
- Yanbo	783	1	0.128	135	17.241	3	0.383
- Makkah	752	30	3.99	79	10.50	0	0
- Safra	199	8	4.020	23	11.558	0	0

Table 4.6*:* Prevalence of anaemias in Saudi Males in different region

Province	No. investi-gated	Prevalence (%)			Total No. (%)
		Hypo-chromic Micro-cytic No. (%)	Normo-chromic Normo-cytic No. (%)	Normo-chromic macro-cytic No. (%)	
Central					
- Riyadh	571	12 (2.10)	22 (3.88)	0	34 (5.95)
- Qassim	355	11 (3.09)	30 (8.45)	0	41 (11.54)
- Sulayel	583	0	33 (5.66)	0	33 (5.66)
Eastern					
- Al-Hafouf	255	12 (4.70)	11 (4.31)	0	23 (9.0)
- Al-Qateef	309	32 (10.35)	37 (11.97)	0	69 (22.33)
Western					
- Khaiber	55	0	0	4 (7.27)	4 (7.27)
- Al-Ula	194	1 (0.5)	16 (8.24)	13 (6.70)	30 (15.46)
- Abha	281	15 (5.3)	34 (12.09)	0	49 (17.43)
- Jaizan	849	93 (10.95)	126 (14.84)	0	219 25.79)
- Najran	421	13 (3.08)	20 (4.75)	0	33 (7.83)
- Qunfuda	385	5 (1.29)	15 (3.89)	2 (0.51)	22 (5.71)
- Al-Baha	443	8 (1.80)	22 (4.96)	0	30 (6.77)
- Bisha	328	9 (2.74)	67 (20.42)	2 (0.60)	78 (23.78)
- Majarda	98	4 (4.08)	8 (8.16)	0	12 (12.24)
- Yanbu	405	0	53 (13.08)	1 (0.24)	54 (13.33)
- Makkah	341	8 (2.35)	39 (11.44)	0	47 (13.78)
- Safra	65	1 (1.53)	4 (6.15)	0	5 (7.69)

Table 4.7*:* Prevalence of anaemias in Saudi Females in different region

Province	No. investi-gated	Prevalence (%)			Total No. (%)
		Hypo-chromic Micro-cytic No. (%)	Normo-chromic Normo-cytic No. (%)	Normo-chromic macro-cytic No. (%)	
Central					
- Riyadh	544	39 (7.17)	24 (4.41)	0	63 (11.58)
- Qaseem	204	22 (10.78)	29 (14.2)	0	51 (25)
- Sulayel	475	2 (0.42)	39 (8.21)	0	41 (8.63)
Eastern					
- Al-Hafouf	353	22 (6.23)	25 (7.08)	0	47 (13.3)
- Al-Qateef	365	84 (23.01)	38 (10.41)	0	122 (33.42)
Western					
- Khaiber	70	0	2 (2.85)	5 (7.14)	7 (0.1)
- Al-Ula	152	0	13 (8.55)	4 (2.63)	17 (11.18)
- Abha	322	36 (11.18)	50 (15.52)	0	86 (26.70)
- Jaizan	873	190 (21.76)	181 (20.73)	0	371 (42.49)
- Najran	484	34 (7.02)	22 (4.54)	0	56 (11.57)
- Qunfuda	218	9 (4.12)	17 (7.79)	3 (1.37)	29 (13.30)
- Al-Baha	301	17 (5.64)	11 (3.65)	2 (0.66)	31 (10.29)
- Bisha	398	2 (0.50)	10 (2.51)	2 (0.50)	14 (3.51)
- Majarda	176	29 (16.47)	11 (6.25)	0	40 (22.72)
- Yanbu	233	1 (0.42)	49 (21.03)	2 (0.85)	52 (22.31)
- Makkah	262	11 (4.2)	20 (7.63)	0	31 (11.83)
- Safra	825	5 (6.09)	10 (12.19)	0	15 (18.29)

Table 4.8*:* Prevalence of anaemias in Saudi children in different region

Province	No. investi-gated	Prevalence (%)			Total No. (%)
		Hypo-chromic Micro-cytic No. (%)	Normo-chromic Normo-cytic No. (%)	Normo-chromic macro-cytic No. (%)	
Central					
- Riyadh	544	74 (13.6)	39 (7.17)	0	113 (20.77)
- Qaseem	439	30 (6.83)	34 (7.74)	0	64 (4.58)
- Sulayel	298	5 (1.68)	30 (10.07)	0	35 (11.74)
Eastern					
- Al-Hafouf	298	60 (20.1)	28 (9.39)	1 (3.35)	89 (29.86)
- Al-Qateef	366	114 (31.1)	71 (19.39)	0	185 (50.54)
Western					
- Khaiber	188	1 (5.32)	25 (13.29)	9 (4.75)	35 (18.62)
- Al-Ula	102	3 (2.94)	18 (17.64)	6 (5.97)	27 (26.47)
- Abha	428	34 (7.94)	102 (23.83)	0	136 (31.77)
- Jaizan	584	141 (24.14)	140 (23.97)	0	281 (48.11)
- Najran	748	50 (6.68)	36 (4.81)	0	86 (11.49)
- Qunfuda	216	11 (5.09)	26 (12.04)	1 (0.46)	38 (17.59)
- Al-Baha	291	24 (8.25)	30 (10.3)	4 (1.37)	58 (19.93)
- Bisha	356	10 (2.80)	47 (13.2)	3 (0.84)	60 (16.84)
- Majarda	177	26 (14.68)	29 (16.38)	0	55 (31.06)
- Yanbu	145	0	32 (22.06)	0	32 (22.06)
- Makkah	149	11 (7.38)	20 (13.42)	0	31 (20.80)
- Safra	52	2 (3.8)	9 (17.30)	0	11 (21.1)

Further attempt was made to identify the causes of anaemias in these populations. The major causes identified included iron deficiency, sickle cell disease, α- or β-thalassaemias and glucose-6-phosphate dehydrogenase deficiency. Macrocytic hypochromic anaemia was identified in some regions, but generally at a low frequency.

It was not possible to separate the genetic anaemias from the acquired, due to a number of causes. Firstly, as these results were obtained during screening studies and follow-up of the individuals was not carried out several confirmatory test could not be conducted. Secondly, om tje hypochromic microcytic group of anaemia, a number of individuals particularly children and females had both thalassaemia trait and iron deficiency occurring together and both contributed to the anaemic state.

The results show that anaemias constitute a significant health problem in the population of Saudi Arabia. Different regions differ in the prevalence of anaemia. The lowest prevalence of anaemia was in Sulayel in the central province, where, on the whole, the population seems to be more healthy. Most of the other areas had prevalence more than 10% and in Al-Qateef and Jaizan almost one-third of the population was anaemic. Majority of the anaemia in some areas was caused by red cell genetic abnormalities as is shown in the following chapters.