3. Material and Methods
3.1 **Introduction**: The present work is an outcome of a perspective and a prospective study based on the observations made on Siddis of Uttara Kannada district of Karnataka State from the year 1992 through 1997. The inhabitants other than Siddis living in the same environment and almost of the same socioeconomic status were also observed and examined. These subjects other than the Siddis were identified by us as “Non-Siddis”, and it is they who served as ‘Controls’ in our study. We took the assistance of Siddi Development Project (S D P) for their health examination as also of the Non-Siddis. The Chief Co-ordinator of S D P at Yellapur helped us in organising and gathering the Siddis at one convenient place from surrounding settlements on allotted days, for their health examination. Thus at the following places, general health examination was conducted: Gullapur, Manchikere, Gunjavati, Kirwatti and Sambrani. These places are located in different directions from Yellapur, as seen in the Map No 3. Later on we also had to go into the interior regions of the forests to some small settlements, to examine the Siddis over there.

A total of 526 Siddis and 346 Non-Siddis registered for health examination. Some people would submit themselves for only physical examination and refused blood examination. Because of their timid nature, some of them even declined systemic examination, while some others of them refused to reveal personal history too. Many of them felt shy perhaps awkward to bring their stools for examination. Hence for reasons mentioned above, it was not possible to examine all the people in all the parameters, as per the Proforma (Appendix I.) The upper age limit for children has been mentioned differently by different authors. Achar S T & Vishwanathan J. (1982) opine that Pediatric care is extended to children
upto 12 years of their age. While in well-developed countries this care is extended upto the age of 18 years (Udani P M, 1998). Hence in our study we have taken an average i.e. till end of 14 years as the upper age limit for children.

After registration, the subjects who volunteered for examination were asked if they had any complaints, any illness in the past or in the family.

3.2 General Physical Examination (G.P.E) : The GPE examination was undertaken wherein the following parameters were noted.

3.2.1 Height and Weight : was recorded to determine the Nutritional status. For height measurement the subject was asked to stand barefoot, as erect as possible, the arms hanging at the side, heels together and the head held in eye-ear-plane. The anthropometer rod was placed behind the individual. Crossbar was adjusted to touch the vertex. The maximum measurement from ground to vertex was recorded in cms.

The weighing machine was kept on a level surface with needle adjusted to zero kilograms. The subject was asked to stand barefoot and erect on the weighing machine and thus the weight was recorded in Kilograms.

3.2.2 Nutritional Status : The Nutritional status of children was evaluated by the method recommended by Indian Academy of Pediatrics and used in Integrated Child Development Scheme (ICDS) which is as given below: Expected body weight means 50th percentile of the National Centre for Health Statistics (NCHS) standards. Weight for Age was compared with 50th percentile value of NCHS table to determine nutritional status (Lavoipierre G J, 1983).
80 - 100% of Expected body weight - Normal
70 - 79% of Expected body weight - Grade I Malnutrition
60 - 69% of Expected body weight - Grade II Malnutrition
50 - 59% of Expected body weight - Grade III Malnutrition
< 50% of Expected body weight - Grade IV Malnutrition

To determine the Nutrition status in adults, the Body Mass Index (BMI) was calculated using the formula:

\[ \text{BMI} = \frac{\text{Wt in Kg}}{\text{Ht in m}^2} \]

The results were interpreted as follows:

- BMI < 14 - Severe Undernutrition
- 14 - 19.9 - Mild Undernutrition
- 20 - 24.9 - Normal
- 25 - 27.9 - Mild Overweight
- 28 - 29.9 - Overweight
- 30 - 39.9 - Obesity
- BMI > 40 - Severe Obesity.

The Nutritional status was evaluated in 138 Siddi Children and 273 Siddi Adults. Among the Non-Siddi population of the evaluated, 97 were children and 224 were adults.

3.2.3 Blood Pressure: The blood pressure was measured using an instrument known as Sphygmomanometer, by Auscultatory method. The inelastic cuff of the instrument was snugly placed round the arm about 3 cms above the elbow joint. The cuff pressure was raised to a level at which arterial pulse could not be felt. Then the stethoscope diaphragm was placed in the cubital fossa and the cuff pressure was gradually reduced. Appearance of tapping sound indicated systolic pressure. The reading on
mercury column of Sphygmomanometer was noted. The reading at which sound disappeared was taken, as diastolic pressure.

Blood pressure was noted in subjects above the age of 20 years from 269 Siddis and 224 Non-Siddis.

3.2.4 Skin and Hair: Were observed for fungal infections like Tinea or parasitic infestations like lice, in 521 Siddis and 328 Non-Siddis.

3.3 Oral Health Status: The population studied was not at all examined or treated earlier for dental problems. The mouth was examined for oral health. Here we observed for oral Hygiene status, Dental caries (Decayed teeth) and Periodontal status as per the method of Antony W.J. (1994). Examination for Oral Health status was done on 413 Siddis and 328 Non-Siddis.

3.3.1 Oral Hygiene Status (OHS): OHS was examined using a probe and mouth mirror. The presence of food debris, soft deposits and hard deposits on the necks of teeth and stains were observed. The presence or absence of these parameters were used for evaluating the OHS as: Good, Fair or Poor.

Good OHS indicates, no calculus/plaques/stains

Fair OHS indicates, the presence of soft deposits and stains in inaccessible areas.

Poor OHS indicates, presence of soft deposits on teeth and gums; Heavy calculus and stains on all the surfaces of teeth.

3.3.2 Dental Caries: The Dental caries status was evaluated using a curved probe and a mouth mirror. The Presence of decayed as well as missing teeth in each of the subjects mouth was noted.
3.3.3 **Periodontal Status**: The Periodontal status was evaluated by detection of bleeding gums on probing and the presence of periodontal pockets, using a straight probe and mouth mirror. In children the Periodontal condition was evaluated mainly for the permanent teeth.

3.4 **Systemic Examination**: The various body systems (Respiratory system/Gastro Intestinal system/Cardio vascular system/nervous system) were examined by the methods as given by Swash M. (1989). Depending on the clinical symptoms, findings and investigations, diagnosis of inflammatory/neoplastic/metabolic disorder was done and noted.

3.5 **Blood Examination**: The Oral consent of the subjects was taken before drawing the blood for examination. Venous blood was drawn from antecubital vein using a disposable syringe of 2 ml capacity and 22 gauge needle for adults and 23 gauge needle for children. Immediately after this blood smears were made on 2 separate glass slides. Register number of the subject was written on the blood smear and the glass vial. The remaining blood was added to a glass vial containing dipotassium salt of Ethylene diaminetetra-acetic acid (EDTA) at a concentration of 1.5mgs/ml of blood, and mixed by rotation to prevent clotting. EDTA acts by its chelating effect on the calcium molecules in blood and thus prevents clotting. The blood from EDTA bulb was taken up immediately thereafter for the following tests in the field:

- i. Blood group examination
- ii. Haemoglobin estimation
- iii. Sickling test.

The remaining blood (in vial) was preserved in ice box till we reached the laboratory. Then the following tests were done

- i. Blood smear staining and examination
ii. Fetal Haemoglobin estimation and
iii. Polyacrylamide Gel Electrophoresis for abnormal Hb.

For all blood tests except electrophoresis, samples from 506 Siddis and 317 Non-Siddis were collected and examined.

**3.5.1 Blood Group Examination** : The ABO and Rh Blood group was evaluated in the field itself by "slide technique" method suggested by Dacie JV and Lewis S M (1991) and Bharucha C (1970).

A 5% suspension in saline of Red Blood cells (RBC) was prepared by adding 5 drops of blood to 2 ml of saline in a clean small test tube. One drop each of 5% cell suspension was kept on 3 glass slides labelled as A, B and D. Now one drop of monoclonal Anti A, Anti B and Anti D sera were added to the cell suspension on the slides marked A, B and D respectively.

These fluids (cells and Antisera) were mixed with the help of a clean thin plastic rod. After 5 to 10 minutes the slides were observed for agglutination and interpreted as follows:

<table>
<thead>
<tr>
<th>Reaction In</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti A</td>
<td>Anti B</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.5.2 Haemoglobin Estimation : The Haemoglobin (Hb) was estimated by 'Sahli's Method' (Acid haematin method) suggested by Bharucha C (1970). N/10 HCl was taken in Haemoglobinometer tube upto the lowest mark. To this, 20 cmm of blood was added with the help of Haemoglobinometer pipette. The blood and acid were mixed by shaking the tube and allowed to settle for 10 to 15 minutes. During this period, maximum colour development takes place by formation of acid haematin.
Now distilled water was added, drop by drop and then the solution mixed with a glass rod, till the colour matched with the glass plates in the colour comparator. The reading on the tube corresponding to the lower meniscus was read and expressed in grams per 100 ml of blood. The normal Hb content of blood varies from person to person. The normal range is:

For Adults: Men 14-16 gms%
   Women 13-16 gms%

For Children the values are lower: 12-14 gms%

Anemia is reduction in the concentration of haemoglobin in the peripheral blood, below the normal for the age and sex of the patient.

3.5.3 **Sickling Phenomenon**: The Sickling Test was done as per the method suggested by Dacie J V and Lewis S M (1991). One drop of blood was mixed with 3 drops of freshly prepared 2% solution of Sodium metabisulfite on a glass slide and covered with a coverslip. It was then sealed with vaseline and cells observed after 30 minutes, under the microscope. This test, done to detect the presence of HbS depends on the decreased solubility of Hb at low oxygen tension. Sickle - cells appear as elongated cells with pointed ends and Sickle shaped. Partially sickled cells may appear as “holy leaf”. In HbS disease the sickling of red cells occurs almost immediately.

3.5.4 **Blood Smear Examination**: The Blood smear examination was done after staining with Wright’s stain (Henry J.B, 1989). The blood smear was fixed for 2 minutes with absolute methanol. Wright’s stain was poured on the smear and kept for 2 minutes. Equal quantity of Buffer solution (KH$_2$PO$_4$ 6.63 gms + Na$_2$HPO$_4$ 2.56 gms + Distilled water 1 litre) was added to the stain mixed by blowing and then kept for 8 minutes. Then the stain was washed with tap water and subsequently the slide was air dried. A drop of cedar wood oil was put on the smear and examined.
under the microscope. First the smear was screened in low power objective (X 100) to confirm that the cells were evenly distributed. Then the Blood smear was examined using oil immersion lens (X 1000) for:

1) Morphology of Blood cells (Erythrocytes, Leukocytes and Platelets)

3.5.4.1 Morphology of Blood Cells: The morphology of different blood cells is as follows:

Normal Erythrocyte [Red Blood cell (RBC)] is 6-8 μm in diameter and biconcave in shape. Central pallor occupies 1/3 of the RBC. Normally the RBC is stained pink by Romanowsky stains. When the central pallor was large enough the cell was labelled as “hypochromic”. Variation in size is called “anisocytosis”. Small RBCs are called “microcytes”. Large RBCs are called as “macrocytes”. Microcytes are formed as such or they result from fragmentation in iron deficiency anemia and thalassaemia. Macrocytes are classically found in Megaloblastic anemia due to deficiency of Vit B12 and Folic acid. They are also observed in chronic liver disease and increased erythropoiesis.

Abnormal variation in shape of RBCs is referred to as “Poikilocytosis”. They could be “Elliptocytes” where RBCs are more oval or elliptical in shape, seen in hereditary elliptocytosis, megaloblastic anemia, iron deficiency anemia, and myelosclerosis. “Spherocytes” are RBCs which are spherical in shape with no central pallor. These cells could be seen in hereditary spherocytosis, Acquired Hemolytic Anemia, Physical or Chemical injury to the red cells. “Target cells” are RBCs showing peripheral rim and a dark central area containing Hb. Such cells are observable in Thalassaemia, obstructive Jaundice, post spleenectomy state and HbC disease. “Schistocytes” are fragmented RBCs of varying
shape. Such cells could be found in Megaloblastic anemia, haemolytic anemia and burns.

As a rough guide, normal red cell size appears to be the same as that of the nucleus of a small lymphocyte.

The morphology of various leucocytes White Blood Cell (WBC) could be as follows:

“Neutrophil” (Polymorphonuclear neutrophilic leukocyte): the average size is 12 μm in diameter. The nuclei stain blue to purple. The nucleus is segmented. The lobes of nucleus are connected by thin filaments. The number of lobes range from 2 to 5 with a median of 3. The cytoplasm is packed with tiny pink granules.

“Eosinophil” (Eosinophilic granulocyte): The average size is 14 μm in diameter. The nucleus usually has 2 lobes and the cytoplasm is packed with large round to oval eosinophilic granules.

“Basophil” (Basophilic granulocytes): The average size is 12 μm in diameter. The nucleus is less segmented. The granules are large and blue-black in colour.

“Monocyte”: is the largest cell of the leukocytes. The average size is 15 to 18 μm in diameter. The nucleus is large, partially lobulated or horseshoe shaped with fine nuclear chromatin. The cytoplasm is abundant and blue gray.

“Lymphocyte”: A majority of circulating lymphocytes are small lymphocytes. The small lymphocytes are 6 to 10 μm in diameter, and have a thin rim of pale blue cytoplasm and round sharply defined nucleus with condensed chromatin. About 10% of circulating lymphocytes are large
lymphocytes, which are larger (12-15μm) in diameter, with more abundant cytoplasm and less densely staining nucleus.

"Platelets": are 1 to 4 μm in diameter. They are round to oval or irregular in shape with fine red granules within the cytoplasm. The normal platelet count is 150,000 to 400,000/cmm.

3.5.4.2 Differential Leucocyte Count: On the stained blood smear, one hundred leukocytes are counted to determine the percentage distribution of different leukocyte count. The normal differential leucocyte count (in adults) is as follows:

<table>
<thead>
<tr>
<th>Leukocyte</th>
<th>Absolute Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Neutrophil</td>
<td>2500-7500/cmm</td>
<td>40-75%</td>
</tr>
<tr>
<td>b. Lymphocyte</td>
<td>1500-3500/cmm</td>
<td>20-50%</td>
</tr>
<tr>
<td>c. Monocytes</td>
<td>200-800/cmm</td>
<td>2-10%</td>
</tr>
<tr>
<td>d. Eosinophils</td>
<td>40-440/cmm</td>
<td>1-6%</td>
</tr>
<tr>
<td>e. Basophils</td>
<td>15-100/cmm</td>
<td>0-1%</td>
</tr>
</tbody>
</table>

Neutrophilia refers to an increase of neutrophils in the blood than in the case of the normal upper limit. The causes of neutrophilia could be a) Infections due to bacteria, fungi; (b) Toxic conditions like uremia, gout, eclampsia; (c) Drugs and chemicals like lead, mercury; (d) Hemorrhage; and (e) Tissue destruction like burns, infarction, injuries.

Lymphocytosis refers to an increase in the lymphocyte count than in the case of the normal upper limit. The causes of lymphocytosis could be (a) Infections both bacterial and viral; (b) Infectious hepatitis; (c) Infectious mononucleosis and (d) Chronic lymphocytic leukemia.

Monocytosis is an increase in the monocyte count than in the case of the normal upper limit. Monocytosis may be present in (a) Chronic
inflammatory conditions like tuberculosis, Crohn's disease; (b) Chronic
myeloid leukemia; (c) Hodgkins lymphoma, (d) Carcinomas.

Eosinophilia indicates an increase in the eosinophil count than in
the case of the normal upper limit. Eosinophilia occurs in: (a) Allergic
conditions like bronchial asthma; (b) Skin disorders like dermatitis and
eczema; (c) Parasitic infestations.

Basophilia indicates an increase in the basophil count than in the
case of the normal upper limit. Basophilia occurs in (a) Myeloproliferative
disorders; (b) Chronic myeloid leukemia; (c) Chronic haemolytic anemia nd
(d) Hypothyroidism.

3.5.4.3 Fetal Haemoglobin (HbF): The Hb F estimation, by Acid Elution
slide method (modified method of Kleihauer and Betke by Shepard, 1962)
is as follows: Ethanol fixed blood-smears are rinsed in water and allowed
to dry. Then the slides are kept in Elution solution for 20 seconds. The
slides are then washed in water and allowed to dry in air.

Elution solution is prepared as follows:
Solution A : 7.5 g/l Hematoxylin in 90% ethanol.
Solution B : FeCl₃ 24 gms + HCl 20 ml + Distilled water to 1 litre
Solution A  5 Vol + Solution B  1 Vol.

The Fetal cells stain red and adult cells appear as ghosts. Normally
1% or less cells contain HbF. Normally HbF ($\alpha_2\gamma_2$) is a major Hb of the
foetus and the newborn. In Adult Hb, there are 2$\alpha$ chains and 2$\beta$ chains.
In Thalassemia a hereditary disorder of haemoglobin synthesis, $\gamma$-chain
production remains high. So HbF level is elevated in blood.
3.5.4.4 Polyacrylamide Gel Electrophoresis (PAGE): PAGE was done to detect any abnormal Haemoglobin, as per the procedure suggested by Anbalagan K (1985). There are 4 steps involved in the PAGE procedure:

The 1st step is preparation of Hemolysate.
The 2nd step is preparation of Gel.
The 3rd step is running of the sample in Gel.
The 4th step is staining.

1st step: Preparation of Hemolysate was done as the method of Sonnenwirth & Jarett (1980). At first the anticoagulated blood was centrifuged at 3000 rpm for 20 minutes in graduated conical tube. Plasma was removed and cells washed 3 times in saline. After the last centrifugation supernatant, was removed, volume of packed cells was measured. To 1 ml of cells, 1.4 ml of Distilled water and 0.4 ml of Toulene was added. The tube was shaken for 5 minutes and centrifuged at 3000 rpm for 20 minutes. Then the superficial layer of the toluene was removed with cotton swab. Lastly the hemolysate was removed with a pasteur pipette.

2nd Step: Preparation of Gel: Discontinuous type of vertical PAGE was used for the study of hemolysates. This procedure included two layers of slab gel one above the other. The lower resolving gel comprised of 10% acrylamide solution and occupied nearly 3/4th of the glass plates. Once this gel was set; then the upper layer of stacking gel (4% acrylamide solution) was prepared and the coomb was placed in position. After the gel was set, the coomb was removed and the gel was used for running the samples.

3rd step: Running of the Sample: Each hemolysate was mixed with sample buffer, containing 40% sucrose to get 1:2 and 1:5 dilutions. In each well a
quantity of 15 µl of the sample was charged and the electrophorosis commenced.

The slab gel was exposed to a current of 2-3 mA/cm till the hemoglobin band moved to about 1 cm from the lower level. Usually this required about 2 to 3 hrs.

After the run was over, the glass plates were dismantled and the gel was fixed in 7.5% Trichlor acetic acid from 2 hrs to nearly 15 hrs.

4th step : Staining : The gels were stained by immersing in a solution of 0.2% Coomasie blue R 250 for a period of 2 hr with continuous shaking. They were de-stained by a solution containing methanol and glacial acetic acid till the background was completely colourless. All the Hb bands appear blue in colour. The thickness and intensity of the stain were found to be directly proportional to their quantity.

Interpretation : With each run a mixture of Hb F and Hb A, hemolysate was included for comparison. By this method, the order of electrophoretic mobility was as follows : HbA₂ was the fastest, followed by Hb A; whereas Hb F lagged behind by a cm. If the HbS were to be present, it would have formed a band above Hb F.

3.6 Stool Examination : The subjects were asked to collect their stool samples in a clean bottle or an empty match box and submit them for examination. The slides of stool samples were prepared as follows to detect eggs of intestinal parasites: Two clean slides were taken. On one slide one drop of saline and on the other, one drop of Lugol's iodine solution was kept. A small quantity of stool sample was taken with an applicator stick (or a broom stick) and mixed with saline and iodine solution. A coverslip was put on the saline and iodine preparations and observed under microscope using low power (10x) and high power (40x)
objectives, for the presence of parasitic ova or cyst or trophozoites. (Chatterjee K D. 1967, Sood R, 1993). A total of 105 stool samples was examined in Siddis and 92 samples in Non-Siddis. The morphology of different ova/ cyst/ trophozoites are as follows:

**Entamoeba histolytica** (E. histolytica): Trophozoites of E. histolytica are 15 to 60 μm in diameter. The cytoplasm is granular and contain RBCs. Pseudopodia are also seen. Nucleus is generally visible.

Cyst of E. histolytica measure 5-15 μm in diameter, are round in shape, have refractile wall and 1-4 nuclei.

**Giardia lamblia** (G. lamblia): Trophozoites are pear-shaped measuring 7 x 14 μm. There are 2 nuclei, 2 axonemes and 4 pairs of flagella.

The cysts of G. lamblia are oval-shaped measuring 8-12 μm. The cystwall is thick and have 2-4 nuclei in granular cytoplasm.

**Ascaris lumbricoides** (Round worm): The fertilised egg is oval in shape and measures 60 x 45 μm. It has a thick smooth translucent shell with on outer albuminous coat thrown into rugosities.

The unfertilised egg is larger in size (80 x 55 μm) and is more elliptical. Both are bile stained ova.

**Ankylostoma deodenale** (Hook worm): The eggs are oval in shape measuring 60 x 40 micro, colourless with a transparent hyalin shell membrane. The egg has segmented ovum with 4 blastomeres. There is a clear space between the egg-shell and the ovum.

**Trichuris trichiura** (Whip worm): The eggs are barrel shaped with mucus plugs at each pole, measuring 50 x 25 μm. It has a double shell with a smooth surface. These eggs are bile stained.
3.7 Statistical Techniques: The following statistical formulae were used for analysis of various parameters in the present study:

3.7.1 Mean, Standard Deviation and Standard Error: were calculated with the help of the following formula:

\[
\text{Mean (M)} = \frac{\Sigma fx}{N}
\]

\[
\text{Standard Deviation (SD)} = \sqrt{\frac{\Sigma fd^2}{N}}
\]

\[
\text{Standard Error (SE)} = \frac{\text{SD}}{\sqrt{N}}
\]

Where \( x \) is mid value

\( f \) is the frequency

\( d \) is \((X-M)\) available from frequency table.

\( N \) is sample size.

3.7.2 Body Mass Index (BMI) = \frac{\text{Weight in Kilograms}}{\text{Height in Meters}^2}

3.7.3 Chi Square = \sum \frac{(O - E)^2}{E}

Where \( O \) is observed value; \( E \) is Expected value.
3.7.4. ABO Blood Group Gene Frequencies:

\[ p = 1 - \sqrt{O+B} \]
\[ q = 1 - \sqrt{O+A} \]
\[ r = \sqrt{O} \]

\( p+q+r \) will not in practice be 1. If the deviation from 1 is called \( D \), an improved estimate of the allele frequencies is given by Bernstein which is as follows:

\[ p_c = (1 + 1/2 D)p \]
\[ q_c = (1 + 1/2 D)q \]
\[ r_c = (1 + 1/2 D) (r+1/2D) \]

3.7.5 Rh Blood Group Gene Frequencies:

\[ d = \sqrt{D} \]
\[ D = 1 - d \]