MATERIALS AND METHODS
1. EPIDEMIOLOGY

All the HD patients on their first attendance to the clinics of Regional Cancer Centre, Thiruvananthapuram during the period 1993-1996 were interviewed on the basis of prestructured questionnaire (Questionnaire 1). Personal details such as (a) Age (b) Religion (c) Socio-economic status (d) History of viral infections (e) History of Tuberculosis (f) Tobacco-alcohol habits (g) Family history and clinical findings etc. were collected. The data was entered into the computer for analysis.

2. CLINICOPATHOLOGICAL DETAILS OF PATIENTS

(a) Selection of Patients and Controls

Only histopathologically confirmed cases of HD were selected in the study. A total of 82 Hodgkin’s disease patients consisting of 70 adult cases and 12 paediatric cases were included. For comparison, 85 age and sex matched healthy normal controls (selected from patient’s relatives, visitors, RCC staff etc) were also included. The age and sex distribution of the subjects included are given in Table 2. Table 3 shows the distribution of HD patients according to age groups. The relative frequency distribution of HD patients by age and sex is shown in Fig. 1.

Classification

Based on clinical and histopathological features, Hodgkin’s disease was classified (Rye classifications) as lymphocyte predominant (LP), Nodular Sclerosis (NS), Mixed
Cellularity (MC), and lymphocyte depletion (LD). Patients were staged according to the Ann Arbor Staging System. Distribution of study subjects according to the various stages and histological subtypes are explained in the table-4.

(b) Sample selection and Processing

Blood

Twenty millilitres of blood was collected from each patient and control, by venipuncture under sterile conditions, for immunological, cytogenetic and virological studies. Of this, 12ml blood was collected in sterile heparinized tubes for the enumeration of total and high affinity rosette forming cells (TRFC and HARFC) and cytogenetic analysis. The rest 8ml was collected in siliconized tubes without any anticoagulant. Serum was separated and stored at -70°C for serological (quantitation of circulating immune complexes (CIC) and Immunoglobulin G (IgG) antibody against Epstein-Barr viral capsid antigen (VCA)) studies.

Tissue

Biopsies from the affected lymphnodes were collected and fixed in 10% buffered formalin for paraffin embedding. 5µm thick sections were cut from paraffin embedded tissues and collected on poly L-lysine coated glass slides and used for histopathological and immunohistochemical studies.

PEDIGREE ANALYSIS

Pedigree tree of Hodgkin’s disease patients with family history was constructed based on family history of cancer and other personal information collected, like age at cancer
diagnosis, present age of the relatives, age of deceased, details of other affected family members, their cancer sites and the pattern of inheritance. The representative pedigrees of 3 HD patients (total 18 patients) included in the study are represented in figures 2 to 4.

**CYTOGENETIC ANALYSIS**

Venous blood was collected from all the subjects. The blood lymphocytes were cultured using whole blood. Peripheral blood lymphocyte microcultures of patients were done as described by Moorehead et al (1960) with slight modification. The reagents used for peripheral blood lymphocyte microcultures are given below:

1. Growth medium RPMI1640 (Sigma Chemicals) 75 ml
   - Fetal bovine serum 25 ml
   - Penicillin & Streptomycin 1.3 ml
     (100 U/ml and 100 μg/ml, respectively)
   - L-glutamine (200 mM or 29.2 mg/ml) 1.3 ml

2. Phytohemagglutinin (lyophilized M-form, DIFCO, USA), as supplied, was dissolved in the appropriate amount of sterile distilled water suggested by the supplier. It was stored frozen.

   Colcemid solution (GIBCO, USA)
   Colcemid Solution (as supplied) 10 μg/ml
   or
   Colcemid, lyophilized 10 μg/ml
   Diluted with suggested amount of distilled water
4. Hypotonic solution (0.56% or 0.075 M KCl)
   Potassium chloride 5.6 g
   Distilled water 1 L

5. Fixative
   Methanol-absolute (three parts) 75 ml
   Acetic acid - glacial (one part) 25 ml
   Prepared fresh before use

6. Giemsa stain
   Stock soln. Giemsa Powder 380 mg
   Methanol 25 ml
   Glycerol 25 ml
   Leave overnight at 37°C. Filter the stain and store.
   Working soln. Stock soln. 2.5 ml
      Methanol 1.5 ml
      Giemsa 50.0 ml
   Giemsa water 0.2 M Na₂HPO₄ 80 ml
      Distilled water 800 ml
   pH was adjusted to 6.8 with 0.1 M citric acid and then made up the volume to 1 litre (Verma and Babu, 1989).

**Peripheral Blood Lymphocyte Microculture Procedure (for chromosome preparations)**

1. 2 ml of venous blood was drawn under complete aseptical conditions. Transferred the blood into a tube containing 100 units of heparin and mixed gently to avoid clotting.

2. 0.3 ml of whole blood was added into a sterile 15 ml screw capped culture bottle containing 5 ml of RPMI growth media which contained 25% of fetal calf serum/
human serum, 0.1 ml of PHA-M and Penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively).

3. Two parallel cultures were set up for each test sample (1) Culture A (for detecting constitutional chromosome abnormalities and (2) Culture B (for detecting the mutagen induced chromosome sensitivity).

4. Culture bottles were incubated at 37°C for 72 hours with the stoppers tightly closed. The bottles were shaken each morning. The caps were loosened in order to release excess of carbon dioxide.

HARVESTING AND SLIDE PREPARATION

5. 10 μg/ml colcemid was added to each culture bottle 2-3 hours before harvesting to arrest mitosis and incubated at 37°C.

6. After colcemid treatment, the culture tubes were centrifuged at 800 rpm for 8 minutes.

7. Discarded the supernatant by pipetting off media, leaving as little medium as possible over the cell button.

8. Resuspended the cell button in 5 ml of hypotonic solution (0.075 M KCl) prewarmed at 37°C and incubated 10 to 15 minutes in a waterbath at 37°C.

9. 5 drops of freshly made fixative were added to each tube and mixed gently by inverting the tubes once or twice. Centrifuged the tubes at 800 rpm for 8 minutes.
10. Discarded the supernatant. Disturbed the pellet thoroughly by tapping at the bottom of the tube. Resuspended the pellet in 5 ml of fixative and allowed to stand at room temperature for about 10 minutes.

11. Again centrifuged the tubes, discarded the supernatant, and suspended the cells in fresh fixative. Repeated this step for three times.

12. After the final centrifugation, suspended the cells in a small volume of fixative (approximately 0.5 to 1 ml, depending on the size of the cell button) to give a slightly opaque suspension.

13. Slide preparation is one of the most important and critical step in obtaining quality chromosome spreads. 3 to 4 drops were dropped evenly on a cold wet slide and allowed to dry. After the slide was completely dry, examined under a microscope using low magnification (10 X) phase objective to check the cell density and spread of metaphase chromosomes.

Slides were coded and scored blindly. The coded slides were scanned under low magnification (10 X) and selected for scoring on the basis of good staining and chromosome number. Only cells with 46 chromosomes were scored for chromosomal aberrations under oil immersion (100 X).
G-BANDS BY TRYPSIN USING GIEMSA (GTG)

The Giemsa bands (G-bands) obtained by digesting the chromosomes with proteolytic enzyme trypsin are most widely used in clinical laboratories for routine chromosome analysis. This technique is described as GTG (G-bands by trypsin using Giemsa) (ISCN, 1978).

SOLUTIONS

1. Dulbecco’s phosphate - buffered saline - (calcium and magnesium free (Dulbecco’s PBS-CMF)

2. Trypsin solution (0.05%)
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin (1:250)</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Dulbecco’s PBS-CMF</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

   Freshly prepared solution was used at room temperature for 3 to 4 hours. Discarded the solution as soon as turbidity developed or the slides showed an indication of contamination.

3. Giemsa staining solution (5%).

<table>
<thead>
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<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
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</tr>
<tr>
<td>Buffer solution (Gurr’s, pH 6.8)</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Giemsa stain</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

   The staining solution was made freshly and used for 2 to 3 hours. The buffer solution (Gurr’s, pH 6.8) was prepared by dissolving a Gurr’s buffer tablet in 1 litre of distilled water. RPMI media, and antibiotics Penicillin and Streptomycin were purchased from Sigma Chemical Company USA, Phytohemagglutinin and Trypsin from DIFCO laboratories USA, and Colcemid from GIBCO laboratories USA.
PROCEDURE

1. Aged, air-dried preparations were kept overnight at 55°C to 60°C in an oven.
2. The slides were treated with trypsin solution (0.05%) for 5 to 10 seconds.
3. Rinsed the slides briefly in cold Dulbecco’s PBS-CMF (2°C to 5°C, kept in the refrigerator).
4. Stained the treated slides in Giemsa staining solution for 4 to 6 minutes.
5. Rinsed with distilled water and allowed to dry.
6. The slides were mounted using DPX for oil-immersion objectives.

Chromosomes stained by this protocol exhibited light and dark stained regions (light and dark bands) along the length of the chromosomes (Verma and Babu, 1989). For each sample, 20-30 metaphases were counted and 5-10 metaphases were karyotyped to detect the constitutional chromosome abnormalities. Karyotypes were prepared according to the International System for Human Cytogenetic Nomenclature (ISCN, 1991).

MUTAGEN INDUCED CHROMOSOME SENSITIVITY ANALYSIS

Mutagen induced chromosome sensitivity analysis was done for each subject for assessing individual genomic instability by challenging peripheral blood lymphocytes with a radiomimetic antibiotic Bleomycin. Peripheral blood lymphocyte microculture technique was adopted as described above, in which the mutagen bleomycin was added to induce chromosomal breakage according
to the method of Hsu et al., 1985. The mutagen bleomycin (Bleomycin hydrochloride was obtained from M/s. Nippon Kayaku Co. Ltd., Japan marketed in India by M/S. Khandelwal laboratories Pvt. Ltd., Bombay) was added in all the B cultures during the last 5 hours of culture ensuring that the damage induced in the late S and G2 phase of the cell cycle could be evaluated at metaphase. After adding colcemide at the 70th hour, all the B cultures were harvested by standard cytogenetic procedures as described earlier.

**SCORING OF ABERRATIONS**

For mutagen sensitivity, chromosome breaks were scored on 100 metaphases per sample. All slides were coded. During scanning with a low magnification objective, the following mitotic figures were rejected: prophase, prometaphases with sister chromatids not well separated, distorted metaphase plates, obviously broken metaphase plates, and metaphases with crowded chromosomes.

In recording chromatid type aberrations, we followed the recommendation of the Chatham Bars Inn Workshop Conference (1971). An achromatic lesion whose length was smaller than the diameter of the chromatid was classified as a chromatid gap and a lesion whose length was equal to or longer than the diameter of the chromatid was regarded as a chromatid break. In the final computation, each chromatid break was recorded as one break. The frequency of breakage was expressed as breaks per cell (b/c) for comparison. Any individual value >0.8 b/c was considered mutagen sensitive and b/c values above 1.0 was considered mutagen hypersensitive to bleomycin.
induced chromosome damage. However, some metaphases exhibited a large number of countable breaks. In such metaphases, an arbitrary limit of 12 chromatid breaks was set to record the number of chromatid breaks ie, a metaphase with more than 12 breaks was counted as 12 breaks. Breaks were scored and the frequency of breaks were expressed as b/c, for comparison. The number of chromatid breaks of each sample was finally converted into the number of chromatid breaks per cell (b/c) to facilitate comparison. The mean number of breaks per cell (b/c), based on evaluation of 100 metaphases, was taken as a measure of mutagen sensitivity. 'Student's 't' test was used to measure statistical significance.

Epstein Barr Virus (EBV) Serology

Many antigens have been identified in EBV-infected and EBV-transformed cells. The viral capsid antigens (VCA) are detected in cells producing EBV particles (Henle and Henle, 1966). These are latent antigens because their expression occurs after the onset of vegetative viral DNA synthesis in the life cycle of the virus (Hummel and Kieff, 1982).

The measurement of immunoglobulin G (IgG) antibody to the Epstein-Barr viral capsid antigen by indirect immunofluorescence is the principal technique used to define the serologic epidemiology of EBV infections. IgG antibody against VCA was detected using the standard indirect immunofluorescence method of Henle and Henle (1966), using P3HRI human cell line which was obtained from National Centre for Cell Science, Pune.
Methodology

P3HRI cells were grown for 4-5 days in RPMI 1640 medium supplemented with 20% fetal bovine serum. To prepare slides, cells were centrifuged and resuspended at $1-2 \times 10^6$ cells/ml in phosphate buffered saline (PBS). Aliquotes (25 ml) were spotted on a slide. The cells were air-dried and fixed in acetone for 10 minutes at room temperature. The slides were kept at $-20^\circ$C until staining was performed. Dilutions of test sera (kept at $-70^\circ$C) starting at 1:10 were made in PBS, and 25 ml was spotted on to P3HRI cells. Slides were incubated at 37°C for 2 hours, washed twice with PBS. The slides were incubated with 25 ml of fluorescein labelled Goat anti human IgG (IgG – FTC obtained from Banglore Genei Pvt Ltd) at a dilution of 1:50 for 1 hour at 37°C. Following this, the slides were washed twice in PBS and counterstained with 1:400 Evan’s blue solution for 5 minutes covered with a coverslip using a drop of PBS-Glycerine (1:1) mountant, examined under fluorescence microscope. Those which were scored as positive were diluted two-fold until the titre value reached 640. EBV positive and negative sera served as controls.

An elevated titer was defined as the one which equalled or exceeded at least 85% of the controls. The minimal value of elevated titre for VCA-IgG was 160. Statistical significance was carried out using Student’s ‘t’ test and chi-squared analysis.

IMMUNOHISTOCHEMISTRY

Immunohistochemical evaluation was done using the EBV specific latent membrane protein (LMP-1) monoclonal antibody
(CS1-4). The standard Avidin Biotin Complex (ABC) immunoperoxidase method was employed for the localization of antigens in tissue sections (Hsu et al., 1981). For immunoperoxidase staining of tissue sections, the paraffin embedded tissue sections were dewaxed and incubated with unlabelled primary antibody. Specifically bound antibody was then visualized by incubation with a biotinylated secondary antibody followed by avidin-biotinylated horse radish peroxidase macromolecular complex and substrate (ABC technique).

The avidin biotinylated horse radish peroxidase complex, consists of many biotinylated horse radish peroxidase molecules crosslinked by avidin to form a three dimensional array. The complex apparently has few exposed biotin residues but retains at least one biotin binding site, formation of the complex was achieved by mixing defined amounts of avidin and biotinylated horse radish peroxidase in dilute solution prior to use. This complex remains stable for several hours after formation.

SOLUTIONS AND REAGENTS

1. The primary antibody used was a pool of four monoclonal antibodies CS1-4 specific for Latent Membrane Protein (LMP-1) obtained from Dako, Glostrup, Denmark.

2. Biotinylated Rabbit anti-mouse immunoglobulin was used as second step antibody.

4. ABC reagent (made just before use)

5. Phosphate buffered saline (PBS) - 8.0 gm sodium chloride; 1.92gm dibasic sodium phosphate and 0.92 gm monobasic sodium phosphate dissolved in 1000 ml distilled water and adjusted to pH 7.6.

6. Tris buffered saline (TBS) 6.05 g tris (hydroxy methyl aminomethane) and 8.0 gm sodium chloride dissolved in 1000 ml distilled water and adjusted to pH 7.6

7. DAB (3,3'-diaminobenzidine tetrahydrochloride) substrate solution was prepared by adding 1 mg DAB in 1 ml of tris buffer and 3% H$_2$O$_2$ (Fresh chromogen solution was prepared daily).

8. 0.1% tripsin in PBS adjusted to pH 6.

9. Xylene

10. Absolute Alcohol

11. Harris Hematoxylin

12. DPX mounting medium
Procedure

Deparaffinization and endogenous peroxidase blocking.

1. Sections were deparaffinized in xylene, using 3 changes, 10 minutes each.
2. Xylene : Alcohol mixture (1:1 ratio) 10 minutes.
3. 100% ethanol, using 2 changes, 10 minutes each.
4. Hydrated with 95% ethanol 10 minutes.
5. Incubated for 30 minutes in 3% hydrogen peroxide to quench endogenous peroxidase activity.
6. Rinsed in distilled water.
7. Rinsed in TBS.
8. Enzymatic digestion for antigen unmasking.

Antigenic determinants masked by formalin fixation paraffin-embedding was exposed by enzymatic digestion. The sections were incubated for 12 minutes in 0.1% trypsin in PBS, pH 7.6 at room temperature. The reaction was terminated by repeated washing in distilled H<sub>2</sub>O.

9. Antigen unmasked sections were washed in TBS (pH 7.6) twice for 10 minutes
10. Slides were placed in 0.3% hydrogen peroxide/methanol for 20 minutes.
11. Slides were washed twice in distilled water for 5 minutes. Slides were further washed twice in TBS for 5 minutes.
12. Sections were placed with normal rabbit serum (1:5) in TBS for non-specific blocking.
13. Sections were covered with monoclonal mouse anti-Epstein Barr Virus (EBV), Latent Membrane Protein, CS1-4 primary antibody diluted 1:25 in TBS at room temperature for 2 hours.
14. Sections were washed twice in TBS for 5 minutes.
15. Sections were incubated with secondary antibody diluted 1:200 in TBS for 30 minutes.

16. Sections were then washed twice in TBS for 5 minutes.

17. Slides were incubated with extraavidin peroxidase for 30 minutes.

18. Untreated peroxidase was removed by washing thrice in TBS for 5 minutes.

19. Slides were incubated with DAB substrate and the intensity of staining was checked under the microscope.

20. Slides were rinsed in distilled H₂O

21. Counterstained in Harris hematoxylin solution for approximately 1 minute.

22. Blueing was done in tap water, dehydrated, cleared and were mounted in DPX for permanent slide preparation.

**MICROSCOPIC EVALUATION**

Staining in the cytoplasm and cell membrane of Reed-Sternberg cells, and their variants was observed under a microscope. Staining was graded as EBV -ve (negative) (Reed-Sternberg cells - and their variants -ve) and EBV +ve (positive) (Reed-Sternberg cells and their variants +ve).

**IMMUNOLOGY**

**TOTAL AND HIGH AFFINITY ROSETTE FORMING CELLS (TRFC AND HARFC) BY THE METHOD OF WEESE ET AL (1980).**

A variety of methods for detecting immunological reactivity to surface antigens of human malignant cells have
been described (Morton and Melngreen, 1968; Rosenberg et al., 1977). Rosetting techniques have proved especially useful for this purpose as they have high sensitivity, low background reactivity and do not require special equipments.

The T cells will adhere in vitro to sheep erythrocytes (SRBC) in rosette formation (Brain et al., 1970; Coombs et al., 1970). The rosette formed by the T-cells with SRBC are generally known as the E-rosettes. Two types of rosette forming cells (RFC) were identified by Weese et al., (1980). The total rosette forming cells (TRFC) form rosettes with SRBC at low temperature (4°C) and on prolonged incubation with excess of sheep erythrocytes (SRBC) whereas the high affinity rosette forming cells (HARFC) form rosettes at elevated temperature (29°C) with fewer number of SRBC.

**METHODOLOGY**

Ten millilitres of whole blood containing 100 units/ml of preservative-free heparin was diluted 1:3 with phosphate buffered saline (PBS), pH 7.4, and layered into Lymphoprep (Nyegaard and Co., Norway). After centrifugation at 1500 rpm (400 X g) for 30 minutes, the mononuclear cell interface was carefully collected and washed twice in 50 ml of saline and centrifuged for 10 minutes at 1500 rpm. The lymphocytes were suspended at a concentration of 4 x 10⁶ cells/ml in RPMI 1640 medium containing 10 ml of HEPES buffer (Sigma, USA) and 1% streptomycin. Sheep erythrocytes, drawn once weekly and preserved in Alseiver's solution at 4°C, were washed three times with PBS and resuspended at a concentration of 1 x 10⁸.
cells/ml in RPMI 1640. 0.1 ml of the lymphocyte solution, 0.2 ml of heat-inactivated (56°C, 30 minutes) foetal bovine serum, and 0.2 ml of the sheep erythrocyte solution were added in sequence to 12 x 75 mm plastic tubes. These were gently vortexed and incubated for five minutes at 37°C in centrifuge holders and were then centrifuged at 100 x g for five minutes. The tubes were then incubated overnight in a 29°C water bath. Approximately 18 hours later the cell pellets were resuspended by gentle multi-axle rotation. Lymphocytes binding at least three erythrocytes were visually quantitated. This gave the number of high affinity rosette forming cells.

Quantitation of "total cell levels" was performed in a similar assay. The technique used differed from that in two points. In this assay, sheep erythrocytes were used at a concentration of 3 x 10^8 cells/ml. Additionally, the overnight incubation was performed at 4-6°C instead of at 29°C. Quantitation was similar to the above described method.


Several methods have been used to detect and quantitate circulating immune complexes (Lambert et al., 1978; Rossen et al., 1977). The method employing precipitation with Polyethylene Glycol (PEG) has been found to be easy and reliable and hence this modified PEG technique was employed in this study.
Five ml of venous blood was collected from all subjects and allowed to clot at 37°C for 3 hours which avoided precipitation of cryoglobulins. The serum was separated and then stored at -70°C till use. The circulating immune complexes were then precipitated by poly ethylene glycol (PEG) 6000. A slight modification in the original method of Creighton et al (1973) was made. In brief, 2 ml of 3.3% PEG 6000 was added to 0.2 ml of serum and the mixture was incubated at room temperature for 2 hours and then centrifuged at 2500 x g for 30 minutes at 4°C. The precipitate was washed thrice with 3% ice cold PEG 6000 and the pellet was dissolved in 0.2 ml of distilled water and diluted to 2 ml with 0.1 N sodium hydroxide. The protein content of this solution was estimated by Lowry's method (Lowry et al., 1951).

**Protein Estimation by Folin Ciocalteau**

**Phenol Reagent Method (Lowry et al., 1951)**

**Reagents**

1. 2% sodium carbonate in 0.1 N sodium hydroxide.

2. 0.5% Copper Sulphate in 1% sodium potassium tartrate.

3. Alkaline Copper Solution: Mixed 50 ml of solution 1 with 1 ml of solution 2. Prepared just before use.

4. Stock Folin - Ciocalteau reagent: Dissolved 100 gm sodium tungstate and 25 gm sodium molybdate in about 700 ml water in a 2 litre round bottomed flask. Added 50 ml of syrupy 85% phosphoric acid and 100 ml conc. HCl. Refluxed for 10 hours in all glass apparatus. Added 150
gm lithium sulphate, 50 ml water and a few drops of bromine. Boiled with condenser for 15 minutes to remove excess bromine. Cooled and made upto 1 litre and filtered (Folin-Ciocalteau solution should not have any greenish tint. If greenish tint was present, it was treated with bromine and boiled.

5. Working Folin’s reagent : Dilute the stock 1:3 with distilled water.

6. Protein standard : Human serum diluted 100 to 1000 fold usually made about 70 μg/ml.

Procedure

Mixed 0.2 ml of sample with 1 ml of alkaline copper solution and allowed to stand for 10 minutes at room temperature. Then 0.1 ml of working Folin’s reagent was added, mixed immediately and kept at room temperature for 10 minutes. Read at 560 nm in a Spectronic 21 spectrophotometer using the reagent blank for zero setting. The protein standard was treated exactly in the same way and the protein content of the test sample calculated.
QUESTIONNAIRE FOR HODGKIN'S DISEASE PATIENTS (1)

Date:

1. Name :

2. Registration Number (CR No.) :

3. Age :

4. Sex :

5. Religion :

6. Occupation :

7. Economic status (Poor/Middle/High): 

8. Address :

9. History of previous illness :
   (i) Epilepsy (No/Yes - Describe) :
   (ii) Repeated fever with lymphnode enlargement (No/Yes - Describe) :
   (iii) Tonsillectomy (No/Yes - Describe) :
   (iv) Tuberculosis (No/Yes - Describe) :
   (v) Viral infections (No/Yes - Describe) :

10. Diabetes (No/Yes - Describe) :

11. Family history of other cancers (No/Yes - Describe) :
12. Diet (Vegetarian/Non-vegetarian) :
13. History of habits :
14. Smoking (No/Yes - Describe) :
15. Chewing (No/Yes - Describe) :
16. Alcohol intake
   (No/Yes - Describe) :
Table - 2
Age and sex distribution of subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>Sex ratio Male:Female</th>
<th>Age in years Range</th>
<th>Mean ±SD</th>
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<tbody>
<tr>
<td>Controls</td>
<td>85</td>
<td>64:21</td>
<td>3 - 75</td>
<td>34.1 ± 19.4</td>
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<tr>
<td>Patients</td>
<td>82</td>
<td>62:20</td>
<td>5 - 74</td>
<td>34.8 ± 18.8</td>
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Table - 3
Age group distribution of subjects

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<th>HD patients (n = 82)</th>
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<tr>
<td></td>
<td>No.</td>
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<td>35-49 yrs</td>
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<td>17.6</td>
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<tr>
<td>&gt; 50 yrs</td>
<td>22</td>
<td>25.9</td>
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Figure 1

Relative frequency distribution of Hodgkin's disease study subjects by age & sex
Table - 4
Distribution of HD patients according to stage and histological subtypes

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<th>Histology</th>
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<tr>
<td></td>
<td>LP</td>
<td>NS</td>
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</tr>
<tr>
<td>I</td>
<td>1</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
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<td>1</td>
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</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>27</td>
<td>40</td>
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LP- Lymphocyte predominant
NS- Nodular sclerosis
MC- Mixed cellularity
LD- Lymphocyte depletion
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<tr>
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<td>FEMALE</td>
</tr>
<tr>
<td>□→○</td>
<td>MARRIAGE</td>
</tr>
<tr>
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<td>CANCER PATIENT</td>
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NUMBER BELOW SYMBOLS    AGE
P    PROBAND
B    BREST CANCER
L    LEUKEMIA
Li   LIVER CANCER
Or   ORAL CANCER
Hd   HODGKIN'S DISEASE
In this family five members were affected. In the first generation the patient aged 76 and his spouse aged 72 were affected with oral cancer and were deceased. In the second generation there were three patients, two were having oral cancer; one aged 50 was deceased and the other aged 47 is alive. The third patient aged 50 is alive and has Hodgkin’s disease.
Two members were affected in this family. In the second generation one leukemic patient aged 50 was deceased. In the third generation one Hodgkin’s disease patient aged 9 is alive.
There were 5 affected members in this family. In the second generation, two liver cancer patients aged 65 and 50 were deceased. In the third generation one liver cancer patient aged 45 was deceased and one Hodgkin’s disease patient aged 50 and one breast cancer patient aged 33 are alive.