The present study entitled "A study on tobacco smoking and its relation to cancer laryngopharynx in Assam" was carried out on 2676 number of patients admitted and treated at Gauhati Medical College and Hospital, Guwahati; Assam Medical College and Hospital, Dibrugarh; Silchar Medical College and Hospital, Silchar; and Dr. Bhubaneswar Barua Cancer Institute, Guwahati during the period 1988 to 1992 i.e., for 5 (five) years. Since all the above mentioned medical college and hospitals of Assam and Dr. Bhubaneswar Barua cancer Institute are provided with latest cancer management facilities, almost all the cancer cases from all over the state come to these institutions for investigation and treatment. The detailed history, clinical and laboratory findings were recorded in a proforma specially designed for this study. The proforma has been prepared as follows:
Name of the patient  ---  ---  Age  ---  Sex  ---
Address  -------------------------------------------
Hospital  ----------------------------------------
No  ------------------------------- Date  ---------------
Site involved by cancer  ----------------------
Diagnosed by  --------------------------------
HPE report  ------------------------------------
Types tobacco smoked: - With filter/ Without filter/ Non specific
Daily consumption of tobacco: - Irregular/ Low
   (less then 10)/ Medium (10 to 20)
   / Heavy (more than 20 )
Duration: - Less then 10 years/ 10 to 20 years/
          More then 20 years.
Blood samples: - Collected / Not collected.
Blood nicotine level  ------------------------
Blood Glucose level  -------------------------
Blood lipid profiles:
   Blood cholesterol level  ------------------
   Blood triglycerides level  -----------------
Blood levels of Important enzymes:
   Serum alkaline phosphatase level  --------
Materials for certain cases had been collected from the discharge notes, summaries or bed ticket or from inpatient departments or from the medical records departments of the respective hospitals. Special care was taken to prevent duplication or double entry of the cases. In the medical history sheet name and hospital number were recorded for identification of the cases with date of first entry into the hospital, Age, sex, religion of the cases were also recorded to find out the incidence in different groups of patients. Permanent addresses of the patients were also recorded for future communication as and when necessary. Site of involvement of the disease and histopathological examination reports (HPE) were also recorded along with types of treatment given to the patients. A full history of tobacco smoking with duration since
when the patient was habituated along with quantity of tobacco smoked were also recorded. In this study after few sample surveys we have fixed the intervals as follows:

1. Irregular (Occasional smoker).
2. Less then 10 (Low grade smoker).
3. 10 to 20 (Medium grade smoker).
4. More then 20 (Heavy smoker).

This class interval seems to be the best in the present series of study in this region. Blood samples were collected for laboratory analysis of nicotine level, glucose level, cholesterol level, triglycerides level, serum alkaline phosphatase level, SGOT and SGPT level from some of the cases selecting randomly. A group of patients, other than suffering from cancer but of same age and sex group with similar habit of tobacco smoking were taken as control group and their blood samples were also collected and subjected for similar analysis of blood nicotine level.
Method for estimation of blood biochemical parameters: 6 ml of venous blood were collected from the patients without anticoagulant and preferably in the morning time. Estimation was done on the same day of collection. The serum was separated by centrifugation method. The following blood biochemical parameters were estimated during the study; these are: fasting blood sugar level, serum cholesterol level, serum triglyceride level, serum SGOT level, serum SGPT level and serum alkaline phosphatase level.

1. Fasting blood sugar level: Autopak reagent kit manufactured by Bayers Diagnostics Limited was used for estimation of blood sugar level. The reagent kit contains:

1. Tablets: Buffer/Enzymes (GOD,POD) / Chromogen (4 Aminophenazone, phenolic compound).

2. Glucose 100 mg/dl, ready for use.

1 tablet is gently dissolved in 20 ml of distilled water/deionised water, in a clean beaker, with continuous stirring. Solution was
transfer to a dark bottle and labelled as "working solution", which can be preserved for 2 months at 2-8 °C and for 2 weeks at room temperature i.e., 20 to 30 °C. The samples and the working solution should be brought to room temperature prior to use. The following general system parameters were used with this kit:

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>End point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weave length</td>
<td>505 nm (490-530nm)</td>
</tr>
<tr>
<td>Flow cell temp</td>
<td>30 °C</td>
</tr>
<tr>
<td>Incubation</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Sample volume</td>
<td>10 ul</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>1.0 ml.</td>
</tr>
<tr>
<td>Standard concentration</td>
<td>100mg/dl</td>
</tr>
</tbody>
</table>

Zero setting with reagent blank

To ensure adequate quality control commercial reference control serum was used. The normal serum sugar level is 70-100 mg/dl (Fasting).
2) Serum cholesterol level: Autopak reagent kit manufactured by Bayer Diagnostics Limited was used for estimation of serum cholesterol level. The reagent kit contains:

1. Buffer/Enzymes/chromogen
2. Phenol.

Standard: Cholesterol 200 mg/dl

The reagent is stored in 2-8 oC temperature. Before preparation of the working solution the reagents are allowed to attain the room temperature. Then 64 ml of distilled water was added to one bottle of reagent 1, and mix gently till contents were dissolved completely, and the solution were marked as "solution 1". The reagent 2 is ready for use. Then equal volume of solution 1 and reagent 2 were mixed and this working solution can be stored in dark bottle for 2 weeks at 2-8 oC temperature.

The general system parameters use in the system are:

- Reaction type: End point
- Wavelength: 505 nm (505-530 nm)
- Flow cell temperature: 30 oC
Incubation 30 minutes
Sample volume 10 ul
Reagent volume 1.0 ml.
Standard concentration 200 mg/dl.
Zero setting with Reagent blank.

To ensure adequate quality control, commercial reference control serum was used. The normal serum cholesterol level is 130-220 mg/dl.

3) Serum triglycerides level: Autopak reagent kit manufactured by Bayer Diagnostics limited was used for estimation of serum triglyceride level. The reagent kit contains:

1. Enzymes/Chromogen.
2. Buffer/Chromogen

Standard: 200 mg/dl.

The reagent kit was stored at 2-8 oC temperature. Before preparation of the working solution, the reagent are allowed to attain the room temperature. Then 5.5 ml of reagent 2 and
5.5 ml of reagent 3 were added to one bottle of reagent 1 and mix properly to dissolve completely. The standard supplied with the kit is ready for use. The working solution is stable for one month if stored at 2-8 oC. The samples and the working solution should be brought to room temperature prior to use. The following general system parameters were used:

- Reaction type: End point.
- Wave length: 505 nm (500-530 nm)
- Flow cell temperature: 30 oC
- Incubation: 15 minutes.
- Sample volume: 10 ul
- Reagent volume: 1.0 ul
- Standard Concentration: 200 mg/dl
- Zero setting with: Reagent blank.

To ensure adequate quality control, commercial reference control serum was used. The normal triglyceride level is up to 170 mg/dl.

4) SGOT level: Autopack reagent kit manufactured by Bayer Diagnostics limited was used for estimation
of SGOT level. The reagent kit contain:

1. Asparlate / Buffer 2 bottles

1A. NADH/MDH/LDH 2 vials.

2. Alpha-Ketoglutarate 2 vials.

The reagent kit was preserved at 2-8 oC temperature. Before preparation of working solution the reagents are allowed to attain room temperature. Then one vial of 1A was transfer to one bottle of 1 and mix it thoroughly and it was marked as solution 1. The solution 2 was prepared by dissolving one vial of 2 with 7 ml of distilled water. Then 3 ml of solution 1 was mixed with 0.3 ml of solution 2 and mix it thoroughly and this prepared working solution and was stored at 2-8 oC temperature and use within 8 hours of preparation. The general system parameters used in this system were:

- Reaction type: Kinetic
- Wave length: 340 nm
- Flow cell temperature: 37 oC
- Delay time: 60 seconds
To ensure adequate quality control, commercial reference control serum was used. The normal SGOT level is up to 40 IU/L (37°C).

5) SGPT level: - Autopack reagent kit manufactured by Bayer diagnostics limited was used for estimation of SGPT level. The reagent kit contains

1. Alanine / Buffer 2 bottles
2. NADH/LDH 2 bottles
3. Alpha-deto-glutarate 2 vials.

The reagent kit was stored in 2-8°C temperature. Before preparation of the working solution the reagent kit was allowed to attain room temperature. Then one vial 1A was transferred to one bottle of 1 and mix thoroughly to mix properly, and it was marked as solution 1. The solution 2 was
prepared by dissolving the contents of one vial of 2 with 7 ml of distilled water. Then 3 ml of solution 1 was mixed with 0.3 ml of solution 2 and mix thoroughly and the prepared solution was used within 8(eight) hours of preparation.

The general system parameters used were:

- Reaction type: Kinetic.
- Wave length: 340 nm.
- Flow cell temperature: 37 oC.
- Delay time: 60 seconds.
- No of readings: 4
- Interval: 30 seconds.
- Sample volume: 100 ul.
- Reagent volume: 1.0 ml.
- Path length: 1 cm.
- Factor: 1749.
- Zero setting with: Distilled water.

To ensure adequate quality control, commercial reference control serum was used. The normal SGPT level is up to 40 IU/L at 37 oC.

6) Serum alkaline phosphatase level: Enzokit
The reagent kit manufactured by Ranbaxy Diagnostic was used for estimation of serum alkaline phosphatase level. The reagent kit contains:

1. DEA Buffer 1 bottle. (Diethanolamine and Magnesium chloride)
2. PNPP Substrate 25 tablet in 5 bottles (Paranitrophenyl phosphate)

The reagent kit was stored in 2-8 °C temperature. Before preparation of the working solution the reagent kit was allowed to attain room temperature. One tablet of reagent 2 was dissolved in 2.2 ml of DEA Buffer 1, i.e., reagent 1 to prepare the working solution. The working solution can be stored at 2-8 °C temperature in a dark bottle for 3 days.

The general system parameter used were:

- Wave length 405 nm.
- Flow cell temperature 25 °C.
- Incubation 1 minutes.
- Reagent volume 1.0 ml.
Sample volume 0.02 ml.
Factor 2713
Zero setting with reagent blank.

To ensure adequate quality control commercial reference control serum was used. The normal serum alkaline phosphatase level is 100-250 IU/L at 37°C.

Method for estimation of blood nicotine level:
Samples of blood were collected from 94 volunteers having malignancy of laryngopharynx. Anticoagulant were not used while collecting blood samples (2.5 ml) from the volunteers. None of these patients were suffering from other chronic disease of serious nature. This could be confirmed by the clinical findings and relevant bio-chemical and other investigations. Samples of blood were also collected from 30 volunteers having no history of malignancy as control group. Both the groups were users of tobacco with high to mild amounts in different forms. Blood samples were collected irrespective of their time of ingestion of tobacco.
Collected blood samples were deprotenised and filtered. The clear filtrates were then acidified with ether and the etherial layer was rejected. The aqueous portions were made alkaline with dilute sodium hydroxide solution, and then extracted with chloroform (at least thrice). The organic layer was collected and evaporated under nitrogen and finally, the volume were made up to 100 ml with chloroform.

Stock solutions of nicotine (Sigma Chemical Company, USA) and tetradecane (Fluka Chemica, Switzerland) in chloroform at a concentration of 0.5 mg/ml of nicotine and 0.004292 mg/ml of tetradecane were prepared. From these stock solutions, calibration standards of 0.50 mg/ml and 0.025 mg/ml nicotine each containing 0.004292 mg/ml of tetradecane were prepared.

100 ml of tetradecane solution (0.004292 mg/ml of tetradecane) were added to each of the chloroform extract of blood samples and the solutions were shaken to mix thoroughly.
Therefore each of the experimental solution contained 0.4292 mg of tetradecane.

Hewlett Packard gas chromatograph (Model 5890 series II) coupled with a computing integrator (Model 3396A) was used for the experiment. Fused silica column of methyl silicone (crossed linked 25 m X 0.32 nm X 0.52 um) was used and the following operating conditions were maintained throughout the experiment.

- Detector: FID
- Carrier gas: Nitrogen @2 ml/min.
- Operating temperature:
  - Oven: 140 °C.
  - Injector and detector: 280 °C.
- Internal standard: Tetradecane.
- Injection mode: Splitless.
- Purge activation time: 0.5 minutes.

Estimation of nicotine was carried out using internal standard method. The instrument was calibrated by injecting about 2 ml of
standard solutions containing known amount of nicotine and the internal standard i.e., tetradecane. After calibration, about 2 ml of the experimental solutions were injected. Here, special care was taken to minimize the procedural error during collection of blood samples and extraction of nicotine in the laboratory.