3

MATERIALS AND METHODS
MATERIALS AND METHODS

3.1 Selection of subjects, clinical details and sampling

3.1.1 Lung cancer

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3.2 Chemicals

3.3 Instruments used

3.4 Assays

3.5 Statistical Methods
3.1 SELECTION OF SUBJECTS, CLINICAL DETAILS AND SAMPLING:
The present study included 766 blood samples from 619 individuals in different groups as detailed in figure 2.

3.1.1 LUNG CANCER:

**Lung cancer patients:**
Serum levels of the biomarkers were examined in 145 lung carcinoma patients before administering any anticancer treatment. The diagnosis of lung carcinoma was based on their histopathological, cytological and radiological examinations. Lung cancer patients with limited disease was defined as inoperable tumour confined to one hemithorax but including mediastinal extension and ipsilateral supraclavicular lymphadenopathy and malignant disease outside this limit was classified as extensive disease (Ganz et al., 1987).

**Pathological controls:**
61 patients with benign lung diseases (BLD) were included as pathological controls. Pathological and other clinical findings including sputum cytology, chest x-rays, examinations of bronchial washings revealed that 25 of them had tuberculosis and 36 of them had other lung diseases such as pneumonia (n=8) and benign epithelial tumours (n=28).

**Controls**

**Non-smokers:** 104 age and sex matched individuals not consuming tobacco in any form were selected as controls to define normal values of the biomarkers. Healthy subjects were
Figure-2
Distribution of subjects

I Non-smokers (104)
II Normal smokers (53)
III Pts. with BLD (61)
IV Untreated patients (145)
V Responders (38)
VI Non-responders (36)

I Non-chewers (104)
II Normal chewers (50)
III Pts. with OPC (105)
IV Untreated patients (101)
V Responders (49)
VI Non-responders (24)
determined to be free from any major illness by medical examinations and by review of their medical histories.

**Smokers:** 53 age and sex matched healthy individuals with habit of smoking were included in the study to assess effect of smoking on the biomarker values. Each individual smoked at least ten cigarettes per day for minimum 5 years.

Clinical details of, histopathological classification as well as age and sex of patients with benign/malignant lung diseases are provided in table-1 and 2.

**Follow-up study:**

74 follow-up blood samples from lung cancer patients were collected during/after anticancer treatment. The lung cancer patients were treated with combination of chemotherapy, radiotherapy or radical surgery. The disease status and response to therapy at the time of each sample collection was assessed using clinical information from and review of objective independent studies. Lung cancer patients responding to therapy were termed as responders. The response to therapy was defined as absence of the disease for more than one month. When there was an increase in size of lesions, the patients were classified as having progressive disease or non-responders (Ganz et al., 1987). Among 74 follow-up samples 38 were responders and 36 were classified as non-responders.
### Table-1

**Age and sex distribution of patients with benign lung diseases and lung cancer**

<table>
<thead>
<tr>
<th>Age</th>
<th>≤20-40</th>
<th>&gt;40-60</th>
<th>&gt;60</th>
<th>Total</th>
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<tbody>
<tr>
<td>Male</td>
<td>9</td>
<td>32</td>
<td>13</td>
<td>54</td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>38</td>
<td>13</td>
<td>61</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Age</th>
<th>≤20-40</th>
<th>&gt;40-60</th>
<th>&gt;60</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>Male</td>
<td>9</td>
<td>95</td>
<td>26</td>
<td>130</td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>12</td>
<td>2</td>
<td>15</td>
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<tr>
<td>Total</td>
<td>10</td>
<td>107</td>
<td>28</td>
<td>145</td>
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</table>

BLD : Benign lung diseases  
Ca  : Carcinoma
<table>
<thead>
<tr>
<th>Group</th>
<th>No. of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign lung diseases</td>
<td>61</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>25</td>
</tr>
<tr>
<td>Penumonia</td>
<td>8</td>
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<tr>
<td>Benign epithelial tumour</td>
<td>28</td>
</tr>
<tr>
<td>Lung Cancer</td>
<td>145</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
</tr>
<tr>
<td>Epidermoid Carcinoma</td>
<td>79</td>
</tr>
<tr>
<td>Adeno Carcinoma</td>
<td>26</td>
</tr>
<tr>
<td>Small cell Carcinoma</td>
<td>18</td>
</tr>
<tr>
<td>Large cell Carcinoma</td>
<td>6</td>
</tr>
<tr>
<td>Adenosquamous Carcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Undifferentiated Carcinoma</td>
<td>14</td>
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<tr>
<td><strong>Limited disease</strong></td>
<td>115</td>
</tr>
<tr>
<td><strong>Extensive disease</strong></td>
<td>30</td>
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ORAL CAVITY CANCER:

Oral cavity cancer patients:

Pretreatment serum levels of the biochemical markers were analyzed from 101 patients with oral cavity cancer (OC patients). The patients were histopathologically confirmed cases of squamous cell carcinoma of oral cavity. Tumour staging of OC patients was determined as per UICC norms (American Joint Committee on Cancer, 1983). OC patients were further grouped as patients with localized cancer and patients with regional or distant metastases in other organs.

Pathological controls:

105 patients with oral precancerous conditions (OPC) were included in the study as pathological controls to assess the specificity of serum markers for diagnosis of oral cavity malignancy. Departments of oral diagnosis, oral medicine and dental radiology (government Dental College and Hospital, Ahmedabad) have diagnosed oral precancerous conditions with clinical/pathological findings and classified as (i) Oral Submucous Fibrosis (OSMF). It is characterized by an insidious, chronic, fibrotic change affecting any part of oral mucosa (Pindborg and Sirat, 1966) (ii) Leukoplakia: identified as persistent white patch on the mucous membrane of mouth.

Age, sex and other clinical details of OC patients and pathological controls are described in tables 3 and 4.
<table>
<thead>
<tr>
<th>Age</th>
<th>≤15-30</th>
<th>&gt;30-50</th>
<th>&gt;50</th>
<th>Total</th>
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<tbody>
<tr>
<td>Male with OPC</td>
<td>45</td>
<td>34</td>
<td>11</td>
<td>90</td>
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<tr>
<td>Female</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>15</td>
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<tr>
<td>Total</td>
<td>52</td>
<td>39</td>
<td>14</td>
<td>105</td>
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<tr>
<td>Male with OC</td>
<td>13</td>
<td>37</td>
<td>18</td>
<td>68</td>
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<tr>
<td>Female</td>
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<td>Total</td>
<td>14</td>
<td>58</td>
<td>29</td>
<td>101</td>
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</tbody>
</table>

OPC: Oral precancerous conditions
OC: Oral cavity cancer.
Table-4
Clinical details of the patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No. of individuals</th>
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<tr>
<td>Oral precancerous conditions</td>
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<tr>
<td>OSMF</td>
<td>82</td>
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<tr>
<td>OL</td>
<td>23</td>
</tr>
<tr>
<td>Oral cavity Cancer</td>
<td>101</td>
</tr>
<tr>
<td>Different Anatomic sites</td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>24</td>
</tr>
<tr>
<td>Lower alveolus</td>
<td>17</td>
</tr>
<tr>
<td>Buccal/cheek mucosa</td>
<td>32</td>
</tr>
<tr>
<td>Hard palate</td>
<td>8</td>
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<tr>
<td>Floor of mouth</td>
<td>5</td>
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<td>Lip</td>
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<td>Retromolar region</td>
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</tr>
<tr>
<td>Histology</td>
<td>101</td>
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<tr>
<td>Squamous cell carcinoma</td>
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</tr>
<tr>
<td>TNM classification</td>
<td></td>
</tr>
<tr>
<td>Stage-I</td>
<td>3</td>
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<tr>
<td>Stage-III</td>
<td>42</td>
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<tr>
<td>Stage-IV</td>
<td>33</td>
</tr>
</tbody>
</table>

OSMF : Oral submucous fibrosis
OL : Oral leukoplakia
Controls: Non-chewers:

As detailed in control non-smokers for lung cancer the same individuals not consuming tobacco in any form were considered as non-chewer controls also.

Chewers:

To evaluate effect of tobacco chewing on the levels of the biomarkers, 50 age and sex matched healthy individuals having habit of tobacco chewing were included in the study. The healthy chewers consumed at least 5 gm of tobacco per day for minimum five years.

Follow-up study:

73 samples from the patients who had undergone treatment by surgery, radiotherapy, chemotherapy or a combination of these modalities were collected for follow-up study and were further grouped as responders and non-responders according to the response to anticancer treatment. The responders showed complete disappearance of the disease as indicated by clinical examinations. Non-responders were the group of patients having recurrence or progressive disease as an increase in tumour size or the appearance of new lesions (Xing et al., 1991).

SAMPLE COLLECTION:

Blood samples were collected from controls, normal smokers, normal chewers, pathological controls and lung as well as OC
patients by venipuncture. To avoid possible diurnal variations the sample were drawn between 9 a.m. and 11 a.m. on every occasion. The blood samples were allowed to coagulate at room temperature and were centrifuged at 2500 rpm for 15 min. and resulting sera were separated. Measurements of LDH activity as well as total protein content were carried out on the same day or on the following day. Sera were stored frozen (-20°C) in small aliquotes till assayed for the remaining parameters. Serum once thawed was not reused. Each sample was analyzed in duplicate.

3.2 CHEMICALS:

N-acetyl neuraminic acid (NANA), Bovine serum albumin (BSA), Tris, L (-) fucose, Resorcinol, Glucosamine hydrochloride, Galactose, Mannose, Nicotinamide adenine dinucleotide (NAD), Nicotinamide adenine dinucleotide reduced form (NADH) were purchased from Sigma Chemical Company, St. Louis, U.S.A. Phosphotungstic acid (PTA), Cysteine, Trichloroacetic acid (TCA), Sodium arsenite and Thiobarbituric acid (TBA) were obtained from Loba Chemie, Australia. Lithium sulfate was procured from BDH (India) Pvt. Ltd. Glucose-6-phosphate barium salt, fructose-6-phosphate barium salt, p-nitro phenol and p-nitro phenyl phosphate were purchased from Sisco Research Laboratories. All other chemicals and reagents were of Analytical or Guaranteed Reagent grade obtained either from Glindia Ltd., Sarabhai Chemicals, Merck, Alembic Chemical Co.
3.3 **INSTRUMENTS USED:**

In the present work following instruments were used for estimation of various parameters.

i) Beckman DU-2 spectrophotometer,

ii) Digital pH meter (Systronics make),

iii) Analytical balance (sartorius make digital model with 0.0001 gm weighing sensitivity) and

iv) Other routine clinical laboratory instruments like waterbath, centrifuge, hot air oven, magnetic stirrer, vortex mixer etc.

3.4 **ASSAYS:**

**ESTIMATION OF TOTAL SIALIC ACID (TSA):**

Serum TSA levels were determined as suggested by Aminoff (1961), duly modified by Skoza and Mohos (1976).

**Principle:** After oxidation with periodic acid followed by heating with TBA, sialic acid develops stable chromophore with dimethyl sulfoxide. The colour intensity of the chromophore is directly proportion to sialic acid content.

**Reagents:**

1) \(1\text{N }\text{H}_2\text{SO}_4\)

2) \(10\% \text{ TCA}\)

3) \(0.025\text{M periodic acid in }0.125\text{N }\text{H}_2\text{SO}_4\)

4) \(2\% \text{ sodium arsenite in }0.5\text{N HCl}\).
5) 6% TBA: 0.6 g TBA was weighed and transferred in a graduated test tube and 5.0 ml distilled water (DW) was added. pH of the solution was adjusted to 10.0 with 10N sodium hydroxide and volume was made to 10.0 ml with DW.

6) 0.85% sodium chloride (Normal saline)

7) Standard solution: 20mg NANA in 1.0 ml of DW.

**Procedure:**

Acid hydrolysis for the liberation of sialic acid:

0.1 ml serum, 0.1 ml 1N H₂SO₄ and 0.8 ml normal saline were taken in a test tube. The mixture was hydrolyzed at 80°C for 60 min. After hydrolysis, 2.0 ml 10% TCA was added to each sample and centrifuged at 2500 rpm for 10 min.

TBA assay for sialic acid:

0.1 ml of the hydrolysate was added to 0.4 ml DW and 0.25 ml periodic acid. The mixture was oxidized at 37°C for 30 min. The oxidation was terminated by the addition of 2 ml sodium arsenite. This was followed by the addition of 0.5 ml TBA. The chromophore was developed by heating the reaction mixture in boiling water bath for 7.5 min. The colour was intensified by the addition of 1.5 ml dimethyl sulfoxide. Optical density (OD) was measured at 549 nm. TSA concentration was calculated using a standard curve developed from the standard NANA solution.

**Calculation:**

To calculate the concentrations of the biomarkers assayed
by spectrophotometric methods, following formula was used:

\[
\text{mg of substance/dl} = \frac{\text{O.D. of test} \times \text{Concentration of standard in mg}}{\text{O.D. of standard} \times \text{Volume of Test}} \times 100
\]

The formula was abbreviated as under:

\[
\frac{\text{ODT}}{\text{ODS}} \times \frac{\text{Conc S}}{\text{Vol T}} \times 100 = \text{mg substance/dl}
\]

For TSA

\[
\text{Serum TSA (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times \frac{2}{0.1} \times \frac{2}{0.1} \times 100 \quad (\text{Conc S} = 2 \mu g)
\]

Therefore,

\[
\text{Serum TSA (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times 40
\]

**ESTIMATION OF LIPID BOUND SIALIC ACID (LSA):**

LSA concentration was measured by Resorcinol reagent as suggested by Katopodis et al. (1982).

**Principle:** Serum gangliosides are treated with chloroform:methanol mixture and extracted in aqueous phase followed by precipitation with PTA. The precipitates, when boiled with resorcinol reagent, gives blue colour which is directly proportional to the amount of LSA present.

**Reagents:**

1) Chloroform:Methanol mixture (2:1 v/v)
2) 0.5 g/ml PTA in DW

3) Resorcinol reagent: 0.2 g resorcinol was dissolved in 10.0 ml DW. To this 80.0 ml of concentrated HCl and 0.5 ml of 0.05M CuSO₄ were added and was diluted to 100.0 ml with DW.

4) Butyl acetate: N-Butyl alcohol mixture: 85:15 (v/v) mixture of the two organic solvents was prepared.

5) Standard solution: 100 μg/ml NANA in DW.

Procedure:

0.15 ml ice cold DW and 0.05 ml of serum were added to a test tube. The contents were vortexed for 5 seconds. The tube was transferred to crushed ice. 5.0 ml cold (4-5°) chloroform:methanol mixture was added to the tube and the mixture was vortexed for 30 seconds. To this mixture, 0.5 ml of ice cold DW was added and the contents were mixed by repeatedly inverting the tube for 30 seconds. It was centrifuged at 2500 rpm for 5 min, at room temperature. 1.0 ml of the upper layer was taken in another test tube, 0.5 ml of PTA was added, allowed to stand at room temperature for 10 min. The tube was centrifuged at 2500 rpm for 5 min and the supernatent was discarded. 1.0 ml of DW was added and vortexed until precipitates were in suspension without grossly visible particles. 1.0 ml resorcinol reagent was added and the tube was placed in boiling water for 15 min. Immediately after boiling, the tube was transferred to an ice water bath and left for 10 min. To the ice cold tube 2.0 ml of butyl acetate:n-butyl
alcohol mixture was added at room temperature and the tube was vortexed and centrifuged for 5 min at 2500 rpm. The OD of extracted blue colour in the organic phase was read at 580 nm. The amount of LSA was determined using the standard curve developed from the standard solution of NANA.

**Calculation:**

\[
\text{Serum LSA (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times \frac{10}{0.05} \times \frac{100}{1000} \quad (\text{Conc S = 10 \, \mu g})
\]

Therefore,

\[
\text{Serum LSA (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times 20
\]

**ESTIMATION OF FREE SIALIC ACID (FSA):**

**Principle:** As mentioned for TSA estimation.

**Procedure:**

0.1 ml serum sample was directly taken in the assay system described for the TSA estimation. Sialic acid contents were measured with periodate-TBA method as mentioned, and FSA levels were calculated.

**Calculation:**

\[
\text{Serum FSA (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times \frac{2}{0.1} \times \frac{100}{1000} \quad (\text{Conc S = 2 \, \mu g})
\]

Therefore,

\[
\text{Serum FSA (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times 2
\]
ESTIMATION OF FUCOSE:

Serum fucose levels were estimated using cysteine reagent by the method of Dische and Shettles (1948) as modified by Winzler (1955).

Principle:

Fucose content is determined by the reaction for methyl pentoses using the proteins with ethanol and heating with sulphuric acid. In order to correct for colour developed by other sugars, OD is measured at two wavelengths.

Reagents:

1) 95% ethanol
2) 0.2N Sodium hydroxide
3) H_2SO_4: H_2O mixture : 6 volumes of concentrated H_2SO_4 was mixed with 1 volume of DW.
4) Cysteine reagent : 0.3 g cysteine hydrochloride was dissolved in 10.0 ml DW.
5) Standard solution : 50 \mu g L (-) fucose dissolved in 1.0 ml DW.

Procedure:

0.1 ml serum was mixed with 5.0 ml ethanol in a test tube. It was centrifuged for 15 min at 2500 rpm, decanted and precipitates were suspended in 3.0 ml 95% ethanol. The suspension was again centrifuged at 2500 rpm for 10 min. after the centrifugation the supernatant was discarded. The precipitated proteins were dissolved in 2.0 ml 0.2N sodium
hydroxide. 0.5 ml protein solution was taken in each tubes. 4.5 ml H₂SO₄-H₂O mixture was added in both the tubes, boiled for 3 min and cooled. 0.1 ml cysteine reagent was added in one of the tubes. Both the tubes were vortexed. OD of both the tubes were read at 396 nm and 430 nm after 60 min against a reagent blank. Serum fucose concentrations were calculated using the standard curve obtained from standard fucose solution.

**Calculation:**

\[
ODT = [OD_{396(+)} - OD_{430(+)}] - [OD_{396(-)} - OD_{430(-)}]
\]

where, + = with cysteine reagent

- = without cysteine reagent

\[
ODS = OD_{396} - OD_{430}
\]

\[
\text{Serum fucose (mg/dl) = } \frac{ODT}{ODS} \times \frac{10}{0.1} \times \frac{2}{0.5} \times \frac{100}{1000}
\]

(Conc S = 10 mg)

Therefore,

\[
\text{Serum fucose (mg/dl) = } \frac{ODT}{ODS} \times 40
\]

**ESTIMATION OF HEXOSAMINES:**

Serum hexosamine contents were assessed as described by Winzler (1955).

**Principle:**

Hexosamines after hydrolysis of the serum, acetylation with
acetylacetone, and treatment with alkali form a cyclic oxazole, which if coupled with p-diethylaminobenzaldyhyde (Ehrlich's reagent) form a coloured derivative which can be determined photometrically.

**Reagents:**

1) 95% ethanol
2) 3N HCl
3) 3N NaOH
4) Acetyl acetone regent: 1.0 ml acetyl acetone was mixed thoroughly with 50.0 ml 0.5N sodium carbonate. Reagent was prepared fresh for each batch of estimations.
5) Ehrlich's reagent: 0.4 g p-diethylaminobenzaldehyde hydrochloride was dissolved in 15.0 ml of methanol. 15 ml concentrated solution was added to the solution.
6) Glucosamine standard: 0.1 mg/ml Glucosamine hydrochloride in DW was used as the standard solution.

**Procedure:**

To 0.1 ml serum, 5.0 ml 95% ethanol was added. The test tube was centrifuged at 2500 rpm for 10 min, decanted and the precipitates were suspended in 3.0 ml 95% ethanol. It was then centrifuged at 2500 rpm for 10 min and the supernatant was discarded. To the precipitated proteins 2.0 ml 3N HCl was added. The tube was then kept in hot air oven adjusted at 100°C for 5 min. After 5 min the tube was capped. The mixture was hydrolyzed in hot air oven at 100°C for 4 hours. The hydrolysate was neutralized with 3N NaOH until it was alkaline.
and was diluted to 10.0 ml with DW. To 1.0 ml aliquot taken in another tube 1.0 ml acetylacetone reagent was added and mixed. The tube was placed in boiling water for 15 min. Then, it was cooled and 5.0 ml 95% ethanol was added and mixed. 1.0 ml Ehrlich’s reagent was added to the tube, vortexed and kept at room temperature for 30 min. OD of the solution was read against blank at 530 nm. Hexosamine concentration was calculated from standard curve obtained from standard solution of glucosamine hydrochloride.

**Calculation:**

\[
\text{Serum hexosamines (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times \frac{5}{6} \times \frac{10}{1} \times \frac{100}{0.1} \times \frac{1000}{100}
\]

(Conc S = 10 μg)

Therefore,

\[
\text{Serum hexosamines (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times 83.33
\]

**ESTIMATION OF PROTEIN AND HEXOSES CONTENT OF SEROMUCOID FRACTION:**

Separation of seromucoid fraction and measurement of hexoses content were done according to the method described by Winzler (1955). The protein content (mucoid proteins) of the fraction was determined according to Hartee (1972).

**Separation of seromucoid fraction:**

Principle: Serum seromucoid fraction is soluble in 1.8M
perchloric acid and perceptible with 5% PTA. The concentration of the fraction is expressed in terms of its hexoses and protein contents.

**Reagents:**

1) 0.85% sodium chloride (Normal saline)
2) 1.8M Perchloric acid
3) 5% PTA in 2N HCl
4) 0.1N Sodium hydroxide
5) 95% Ethanol

**Procedure:**

0.5 ml serum was added to 4.5 ml normal saline in a test tube. 2.5 ml perchloric acid was added dropwise while shaking the tubes. The sample was then filtered within 10 min through Whatman filter paper no. 44. 5.0 ml filtrate was taken in another tube and 1.0 ml 5% PTA was added. After 10 min the solution was centrifuged at 2500 rpm for 10 min and the supernatent was discarded. Precipitates were washed with 1.0 ml 95% ethanol, centrifuged and supernatent was again discarded. The residue was dissolved in 1.0 ml of 0.1N sodium hydroxide and hexoses as well as protein contents of the solution (seromucoid fraction) were measured.

**HEXOSES ESTIMATIONS:**

Principle: Hexoses levels are measured by the reaction of its carbohydrate contents in sulfuric acid with orcinol.
Reagents:

1) Orcinol - H₂SO₄ reagent:
Reagent A: 60.0 ml of concentration H₂SO₄ was mixed with 40.0 ml of DW.
Reagent B: 1.6 g orcinol was dissolved in 100.0 ml of DW.
7.5 volume of reagent-A and 1 volume of reagent-B were mixed and was used as orcinol-H₂SO₄ reagent. The reagent was prepared fresh every time.

2) Galactose-mannose standard solution (0.2 g/ml): 0.1 g each of galactose and mannose were dissolved per 1.0 ml DW.

Procedure:

0.5 ml of the seromucoid fraction was taken in a test tube and 0.5 ml orcinol-H₂SO₄ reagent was added. The tube was kept in boiling water bath for 15 min, solution was cooled to room temperature and OD of the resulting colour was read at 540 nm, against a reagent blank. Contents of hexoses were calculated using the standard curve obtained from the standard galactose-mannose solution.

Calculation:

\[
\text{Hexoses (Galactose+Mannose)} = \frac{\text{ODT}}{\text{ODS}} \times \frac{0.04}{0.5} \times \frac{1}{5} \times \frac{7.5}{5} \times 100
\]

\(\text{(Conc S = 0.04 mg)}\)

Therefore,

\[
\text{Hexoses (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times 2.4
\]

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ESTIMATION OF THE PROTEIN CONTENT (MUCOID PROTEINS):

**Principle**: The reactions of peptide bonds in proteins with copper in alkaline solution and of organic amino acids like tryptophan and tyrosine with Folin ciocalteu (F.C.) reagent forms blue colour complex. The OD of coloured derivative can be read at 660 nm.

**Reagents**:

1) Reagent A: 2.0 g sodium-potassium tartrate and 100.0 g sodium carbonate were dissolved in DW and diluted to 1 litre with DW.

2) Reagent B: 2.0 g sodium potassium tartrate and 1.0 g cupric sulphate were dissolved separately; and were mixed. 10.0 ml 1N sodium hydroxide was added to the solution and was diluted to 100.0 ml with DW.

3) Reagent C: 1 volume of F:C reagent was diluted with 15 volumes of DW. The normality of the solution was between 0.15N and 0.18N when titrated with 1N sodium hydroxide.

**Preparation of F.C. Reagent**:

A mixture containing 100.0 g sodium tungstate, 25.0 g sodium molibdate, 700.0 ml DW, 50.0 ml 85% phosphoric acid and 100.0 ml concentrated HCl was refluxed gently for 10 hours in a round bottom flask. The refluxed solution was allowed to cool. Then 150.0 g Lithium sulphate and 50.0 ml DW were added. 0.5 ml of bromine water was added to the mixture and was boiled for 15 min without condenser to remove excess bromine. The mixture was
then cooled and diluted to 2.0 liters with DW and was filtered. It was confirmed that the reagent had no greenish tint. The acid concentration of the reagent was determined by titration with 1N sodium hydroxide.

4) Standard protein solution: 50 \( \mu \text{g/ml} \) bovine serum albumin (BSA) solution prepared in normal saline was used as standard protein solution.

**Procedure:**

0.1 ml seromucoid fraction and 0.9 ml DW were added in a tube. 0.9 ml of reagent-A was added into the tube and it was incubated at 50°C for 10 min the tube was cooled and 0.1 ml reagent-B was added and mixed. It was allowed to stand at room temperature for 10 min. Then 3.0 ml reagent-C (diluted F:C reagent) was added and kept at 50°C for 10 min. The mixture was cooled and the OD was read against a reagent blank at 660 nm. Protein content of the solution was calculated from the curve obtained from the standard solution readings.

**Calculations:**

\[
\text{Mucoid proteins (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times \frac{10}{0.1} \times \frac{1}{5.0} \times \frac{7.5}{0.5} \times \frac{100}{1000}
\]

(Conc S = 10 \( \mu \text{g} \))

Therefore,

\[
\text{Mucoid proteins (mg/dl)} = \frac{\text{ODT}}{\text{ODS}} \times 30
\]
ESTIMATION OF SERUM TOTAL PROTEINS (TP):

Serum total protein levels were quantitated by Biuret reagent (Wootton, 1964b).

**Principle:** Copper in alkaline condition reacts with the peptide bonds in proteins, producing a violet colour which is proportional to the amount of protein present.

**Reagents:**

1) Biuret reagent: 9.0 g sodium potassium tartrate was dissolved in 500.0 ml 0.2N sodium hydroxide. 3.0 g copper sulphate was added by stirring. Then 5.0 g potassium iodide was added and the volume was made to 1 litre with 0.2N sodium hydroxide.

2) Standard solution: 5 mg/ml Bovine serum albumin solution was prepared in normal saline and kept frozen (-20°C) in small aliquotes.

**Procedure:**

0.1 ml serum and 2.9 ml DW were mixed in a test tube. 5.0 ml Biuret reagent was added and the mixture was kept at 37°C for 10 min. After 10 min OD of the test and standard samples were read at 540 nm against the reagent blank and serum protein concentrations were calculated.

**Calculation:**

\[
\text{Serum total protein (g/dl)} = \frac{\text{ODT}}{\text{ODS}} \times \frac{1}{0.1} \times \frac{100}{1000} \quad (\text{Conc } S = 1 \text{ mg})
\]
Therefore,

\[
\text{Serum total protein (g/dl)} = \frac{\text{ODT}}{\text{ODS}} \times 1
\]

ESTIMATION OF SERUM ALKALINE PHOSPHATASE (ALP) ACTIVITY:

The ALP activities in sera were measured as suggested by Bessey et al. (1946).

**Principle:** At alkaline pH of a buffer system ALP hydrolyzes P-nitro phenyl phosphate into phosphate and p-nitro phenol, which gives yellow colour. The intensity of the colour developed is proportional to ALP activity.

**Reagents:**

1) Carbonate-Bicarbonate buffer (0.1M): 6.30 g Anhydrous sodium carbonate and 3.30 g sodium-bicarbonate were dissolved in DW and the volume was made to 1 litre with DW. pH of the buffer was adjusted to 10.0.

2) 0.05M MgCl₂.

3) Substrate: 0.05M solution of p-nitro phenyl phosphate was used as substrate and was prepared fresh for each batch of estimation.

4) 0.1N NaOH

5) Standard solution: 0.1M solution of p-nitrophenol prepared in DW was further diluted to give 100 n mols/ml solution of p-nitro phenol. The diluted standard solution was used to derive the standard curve.
**Procedure:**

Test: 0.1 ml serum, 0.25 ml buffer, 0.1 ml of MgCl$_2$ and 1.4 ml DW were mixed in a test tube. The mixture was kept at 37°C in a water bath for 3 min. Then 0.15 ml substrate was added and the mixture was further incubated at 37°C for 15 min. The reaction was stopped by the addition of 2.0 ml of 0.1N sodium hydroxide.

Control: Control tube was kept for each test. The mixture containing 0.1 ml serum, 0.25 ml buffer, 0.1 ml MgCl$_2$ and 1.4 ml DW was incubated at 37°C for 15 min. 2.0 ml of 0.1N sodium hydroxide was added and mixed thoroughly. To this mixture 0.15 ml substrate was added.

OD of test as well as control tubes were read at 410 nm against a blank without substrate and enzyme. The enzyme (ALP) activity was calculated using standard curve obtained from the standard solution.

**Calculation:**

Units: The enzyme (ALP) activity was expressed in units/ml/hour. One unit was amount of enzyme liberating 1.0 mmole of p-nitro phenol per min. It was calculated as under:

\[
\text{ALP activity in units/ml/hour} = \frac{\text{ODT} \times \text{Concs in } \mu\text{mols} \times 1.0 \text{ ml serum}}{\text{ODS} \times 0.1 \text{ ml serum} \times 15 \text{ minutes}} \times 60 \text{ min.}
\]

\[
\text{ODT} = \text{OD Test} - \text{OD Control}
\]
Therefore,

Serum ALP activity (units/ml/hour) =
\[
\frac{\text{ODT}}{\text{ODS}} \times \frac{10}{0.1} \times \frac{1}{15} \times \frac{60}{1000} \quad \text{(Conc S = 10 nmoles)}
\]

Therefore,

\[
\text{Serum ALP (units/ml/hour)} = \frac{\text{ODT}}{\text{ODS}} \times 0.4.
\]

**ESTIMATION OF PLACENTAL LIKE ALKALINE PHOSPHATASE (PALP):**

Heat stability of ALP was measured according to Fishman et al. (1968b).

**Principle:** Carcino placental type ALP activity is not destroyed when the serum is heated at 55°C for 15 min.

**Procedure:**

0.4 ml serum was taken in a test tube. The tube was kept at 55°C for 15 min. Immediately after incubation the tube containing inactivated serum was transferred to crushed ice and kept for 15 min. The serum was centrifuged at 2500 rpm for 10 min. ALP activity from the inactivated serum was estimated as described above. The enzyme activity was expressed as units/ml/hour which was calculated as mentioned for total ALP activity.

**ESTIMATION OF LACTATE DEHYDROGENASE (LDH):**

LDH activity in sera was measured by the method of Wroblewski and LaDue (1955) as mentioned by Wootton (1964a).
**Principle:** LDH catalyzes the reaction of pyruvate to lactate with oxidation of NADH to NAD. Since the oxidation of NADH is directly proportional to the reduction of pyruvate in equimolar amounts. The LDH activity can be calculated from the rate of decrease in OD at 340 nm.

**Reagents:**

1) Phosphate buffer (pH 7.4): 7.55 g anhydrous disodium hydrogen phosphate and 1.81 g anhydrous potassium dihydrogen phosphate were dissolved in DW and the volume was made to 1 litre and stored at 4°C.

2) Reduced nicotinamide adenine dinucleotide (NADH): 2.5 mg/ml NADH in phosphate buffer. The solution was prepared fresh for each batch of estimation.

3) Sodium pyruvate (23 nM): 125 mg sodium pyruvate was dissolved in phosphate buffer and volume was made to 50 ml. The solution was stored in small aliquotes at 20°C. Fresh substrate vial was taken for each batch of estimation.

**Procedure:**

0.1 ml serum, 2.7 ml phosphate buffer and 0.1 ml NADH solution were mixed in a test tube. The mixture was incubated at 37°C for 30 min to destroy endogenous substrate. After 30 min 0.1 ml pyruvate substrate was added, mixed well and the OD of the solution was measured at 340 nm in spectrophotometer. Readings were taken at an intervals of 30 seconds for 4-5 min. During the period of observation the fall in OD was linear. The
change in OD per min was calculated.

**Calculation:**

Equimolar amounts of substrate and coenzyme were involved in the reaction i.e. each \( \mu \)mole of the substrate reacting is matched by a \( \mu \)mole of coenzyme oxidized. It is known that 1 \( \mu \)mole of NADH in 3.0 ml reaction mixture has an OD of 2.1 thus, the rate (\( \mu \)mole per min) of coenzyme oxidation is equal to:

\[
\frac{\text{OD change per min}}{2.1}
\]

This is the reaction rate caused by the enzyme present in 0.1 ml serum. The activity of the enzyme contained in 1 litre of serum is

\[
\frac{\text{OD change per min}}{2.1} \times \frac{1000}{0.1}
\]

Hence,

Serum LDH (\( \mu \)mole/min/litre i.e. I.U./litre) =

\[
\text{OD change per min} \times 10^3 \times 4.8
\]

**ESTIMATION OF PHOSPHOHEXOSE ISOMERASE (PHI):**

The PHI activity in sera was determined by the method of Bodansky (1954).

**Principle** : PHI catalyses the conversion of glucose-6-phosphate to fructose-6-phosphate. The fructose-6-phosphate reacts when heated with resorcinol in the presence of hydrochloric acid
which develops red colour (Seliwanoff reaction). The intensity of red colour, measured at 490 nm is proportional to PHI activity.

**Reagents:**

1) **Veronal-Acetate Buffer:** 9.71 gm sodium acetate and 14.71 gm sodium-veronal were dissolved in 500 ml of DW to provide 0.143M solution pH of the buffer was adjusted to 7.4.

2) **Buffered Substrate:** 0.030M Na glucose-6-phosphate was mixed with veronal acetate buffer. pH of the buffered substrate was adjusted to 7.4.

3) **10N HCl.**

4) **Resorcinol solution:** 0.1% resorcinol dissolved in 95% ethanol.

5) **5% Trichloroacetic acid (TCA).**

6) **Standard solution:** Fructose-6-phosphate was dissolved in DW to give 100 μg of fructose per ml solution.

**Procedure:**

The reaction mixture containing 0.5 ml diluted serum (0.5 ml serum diluted with 2.00 ml Normal saline) and 2.00 ml buffer substrate was incubated at 37°C for 30 min. The reaction was terminated by adding 2.5 ml of 5% TCA. The tubes were kept in ice for 15 min and centrifuged at 2500 rpm. 2 ml of corresponding supernatant was taken and mixed with 2 ml of 1% resorcinol reagent and 6.00 ml of the HCl and incubated at 80°C for 15 min. The absorbance was measured at 490 nm.
Calculation:

One unit was defined as the amount of enzyme required for liberating one micro mole fructose-6-phosphate under specific assay conditions. The values were expressed as Bodansky units (Bodansky, 1954).

3.5 STATISTICAL METHODS:

The results were analyzed statistically and expressed as Mean ± S.E.M.

A cut off value for each marker was determined as Mean+2S.D. of the controls.

STATISTICAL SIGNIFICANCE:

The level of significance was determined by employing student's 't' test. Only when the 'P' value was less than 0.05 the difference was considered statistically significant.

Paired 't' test was employed to compare the biomarker values in lung and oral cavity cancer patients before and after anticancer treatment. 'P' value less than 0.05 was taken as the minimum level of significance.

SENSITIVITY AND SPECIFICITY:

The individual sensitivity as well as specificity were calculated for each marker in diagnosis of lung and oral cavity cancers.

The criteria to define individual sensitivity and specificity was based on following diagram (Linnet, 1988):
Sensitivity (%) = 1 - $\frac{n(Fn)}{ND} \times 100$

Specificity (%) = 1 - $\frac{n(Fp)}{NR} \times 100$

NR = n(TN) + n(FP), ND = n(FN) + n(TP)

TN = Reference individuals having negative results (True negatives)

FP = Reference individuals having positive values (False positives)

FN = Diseased subjects with negative results (False negatives)

TP = Diseased subjects with positive results (True positives)

Receiver operating characteristic (ROC) curves were plotted by calculating sensitivities (true positive rate) and specificities (false positive rate) of the markers at several cut off points (Feinstein, 1985).

**COMBINED SENSITIVITY AND SPECIFICITY:**

When utility of combination of any two markers was studied, positivity was determined when both or either of the markers were positive.