Materials and Methods
SUBJECTS

Present study included healthy individuals, patients with OPC and oral cancer patients. Prior consent was taken from all the subjects to participate in the study.

Health and Habit Questionnaire:

One hundred and thirty two healthy individuals, 94 patients with OPC and 126 oral cancer patients were interviewed using a questionnaire designed for collection of socio-demographic details like age, sex, type of tobacco habits, duration and frequency of tobacco consumption as well as clinical details etc. The questionnaire was as outlined below:

<table>
<thead>
<tr>
<th>Registration No</th>
<th>Date:</th>
<th>Sample: Urine/Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name:</td>
<td>Address</td>
<td></td>
</tr>
<tr>
<td>Occupation:</td>
<td>Income:</td>
<td>Education: Age/sex:</td>
</tr>
<tr>
<td>Food test:</td>
<td>Habit</td>
<td>Frequency Duration Stopped</td>
</tr>
<tr>
<td>Spicy/moderate/non-spicy</td>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Food type:</td>
<td>Chewing</td>
<td></td>
</tr>
<tr>
<td>Vegetarian/Non-vegetarian</td>
<td>Snuff</td>
<td></td>
</tr>
<tr>
<td>Chief complains:</td>
<td>Alcohol</td>
<td></td>
</tr>
<tr>
<td>Major Illness:</td>
<td>Family History:</td>
<td></td>
</tr>
<tr>
<td>Diagnosis:</td>
<td>Stage:</td>
<td></td>
</tr>
<tr>
<td>Histopathological Examination:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reports from GCRI</td>
<td>Outside reports</td>
<td></td>
</tr>
<tr>
<td>Follow-up history</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The socio-demographic details of the subjects obtained from health and habit questionnaire are summarized in table-4. All subjects were divided into tobacco habitués and non-tobacco habitués. Tobacco habitués were further classified into abstinence (patients who stopped tobacco habit before 15 days) and non-abstinence (patients who were consuming tobacco at the time of interview) groups as per their tobacco cessation status.
Table-4: Socio-demographic details of the subjects

<table>
<thead>
<tr>
<th>Age (years):</th>
<th>Healthy Individuals (N=132)</th>
<th>Patients with OPC (N=94)</th>
<th>Oral cancer patients (N=126)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>32</td>
<td>29</td>
<td>45</td>
</tr>
<tr>
<td>Range</td>
<td>19-62</td>
<td>16-65</td>
<td>22-75</td>
</tr>
<tr>
<td>Tobacco History</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habit: Non-habitués</td>
<td>N=53</td>
<td>N=4</td>
<td>N=13</td>
</tr>
<tr>
<td>Habitués</td>
<td>N=79</td>
<td>N=90</td>
<td>N=113</td>
</tr>
<tr>
<td>Present status: Abstinence</td>
<td>N=00</td>
<td>N=32</td>
<td>N=55</td>
</tr>
<tr>
<td>Non-abstinence</td>
<td>N=79</td>
<td>N=44</td>
<td>N=54</td>
</tr>
<tr>
<td>Missing</td>
<td>N=18</td>
<td>N=18</td>
<td>N=17</td>
</tr>
<tr>
<td>Type: Chewers</td>
<td>N=58</td>
<td>N=54</td>
<td>N=62</td>
</tr>
<tr>
<td>Smokers</td>
<td>N=17</td>
<td>N=15</td>
<td>N=27</td>
</tr>
<tr>
<td>Chewers+ Smokers</td>
<td>N=03</td>
<td>N=20</td>
<td>N=18</td>
</tr>
<tr>
<td>Income</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 1000</td>
<td>N=28</td>
<td>N=32</td>
<td>N=80</td>
</tr>
<tr>
<td>&gt; 1000</td>
<td>N=87</td>
<td>N=53</td>
<td>N=35</td>
</tr>
<tr>
<td>Missing</td>
<td>N=17</td>
<td>N=9</td>
<td>N=11</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undergraduate</td>
<td>N=73</td>
<td>N=85</td>
<td>N=87</td>
</tr>
<tr>
<td>Graduate</td>
<td>N=54</td>
<td>N=9</td>
<td>N=24</td>
</tr>
<tr>
<td>Missing</td>
<td>N=05</td>
<td>N=15</td>
<td>N=15</td>
</tr>
</tbody>
</table>

Above mentioned subjects were studied for sociodemographic details and odd ratio analysis. The subjects enrolled for study of biomarkers were as follows.

Subjects for study of biomarkers:

Controls: The study included 90 healthy individuals as controls. They were classified into tobacco habitués (n=60) and non-tobacco habitués (n=30). The healthy controls with tobacco habitués were termed as WHT and non-tobacco habitués were grouped as NHT. Urine samples from 10 healthy children were also collected for nicotine and cotinine included for the analysis as controls without environmental tobacco exposure. The age range of healthy individuals was 19 to 62 years with median age of 32 years. It was confirmed that these individuals were not suffering from any major disease and they had no major illness in the recent past.
Patients with OPC: Fifteen patients with OPC were included in the study as pathological controls. The patients were enrolled from the out patients' department of Government Dental College and Hospital, Ahmedabad. The diagnosis of oral submucous fibrosis (n=11) and oral leukoplakia (n=4) was based on clinical and radiological examinations. The age range of patients with OPC was 18 to 58 year with median age of 25 years. All the patients with OPC were tobacco habitués. They were further classified into non-abstinence (n=13) and abstinence group (n=2).

Oral Cancer Patients: All 126 oral cancer patients were included in the study for the biomarker. The patients were enrolled from out patients' department of the Gujarat Cancer and Research Institute, Ahmedabad. The diagnosis of cancer was established by clinical, radiological and histological examination. Staging was done according to AJCC norms (1997). Clinical details are illustrated in table-5.

Table-5
Clinical details of oral cancer patients for study of biomarkers

<table>
<thead>
<tr>
<th>Oral cancer patients</th>
<th>N=126</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Site</strong></td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>N=58</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>N=48</td>
</tr>
<tr>
<td>Other</td>
<td>N=20</td>
</tr>
<tr>
<td><strong>Histopathological Type</strong></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>N=95</td>
</tr>
<tr>
<td>Epidermiology carcinoma</td>
<td>N=31</td>
</tr>
<tr>
<td><strong>Stage of the disease ( TNM)</strong></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>N=12</td>
</tr>
<tr>
<td>Stage II</td>
<td>N=20</td>
</tr>
<tr>
<td>Early stage (Stage I+II)</td>
<td>N=32</td>
</tr>
<tr>
<td>Stage III</td>
<td>N=23</td>
</tr>
<tr>
<td>Stage IV</td>
<td>N=54</td>
</tr>
<tr>
<td>Advanced stage (Stage III+IV)</td>
<td>N=77</td>
</tr>
<tr>
<td>Not defined</td>
<td>N=17</td>
</tr>
<tr>
<td><strong>Nuclear Grade</strong></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>N=36</td>
</tr>
<tr>
<td>Moderate</td>
<td>N=66</td>
</tr>
<tr>
<td>Poor</td>
<td>N=11</td>
</tr>
<tr>
<td>Not defined</td>
<td>N=13</td>
</tr>
</tbody>
</table>
SAMPLE COLLECTION:

Tobacco Products:
Tobacco products of 26 brands and pan masala of 6 brand were collected for estimation of NO₂+NO₃. The brands of tobacco products and pan masala selected for study were most favorite and were commonly used by tobacco habitués in the population. Pan masala products were not containing tobacco, but they contained areca nut, lime and catechu. Tobacco products were classified into major groups including cigarette, bidi, gutkha, tobacco alone, pan masala and snuffing products (table-6).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Brands (N=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarettes</td>
<td>4</td>
</tr>
<tr>
<td>Bidi</td>
<td>4</td>
</tr>
<tr>
<td>Gutkha</td>
<td>9</td>
</tr>
<tr>
<td>Tobacco alone</td>
<td>6</td>
</tr>
<tr>
<td>Pan masala</td>
<td>6</td>
</tr>
<tr>
<td>Snuffing Products</td>
<td>3</td>
</tr>
</tbody>
</table>

Urine:
Urine samples were collected from the subjects prior to initiation of any anticancer therapy and stored at -20°C until analyzed for estimation of nicotine and cotinine and thioether levels. Urine samples for estimation of NO₂+NO₃ were diluted with borax buffer and stored at -20°C until analyzed.

Blood:
Blood samples were collected from controls (n=90), patients with OPC (n=15) and cancer patients (n=126) by venipuncture in heparin and EDTA vials between 9 a.m. and 11 a.m. on every occasion. Blood samples from the patients were drawn before initiation of anticancer therapy. The oral cancer patients were followed-up at various intervals after initiation of anticancer treatment. Follow-up blood samples (n=70) were also collected from the
Materials and Methods

patients, during their subsequent visits to the hospital. The clinical status of the patients at time of follow-up collection was recorded. The patients with no evidence of disease at the time of follow-up were classified as responders (CR, n=42). Whereas, the patients with locoregional failure of disease, stable/progressive diseases, metastasis or recurrence at the time of follow-up were classified as non-responder (NR, n=28).

Processing of Blood Samples:
Plasma was separated from heparinized blood and stored at -20°C for estimation of antip53 antibodies, prostaglandin E2, NO₂+NO₃, GST, GR, thiol and MDA analysis. Red Blood Cells (erythrocyte) were washed 3 times with normal saline and packed erythrocyte were stored at -20°C for assays of GST, GR, SOD, catalase. During analysis of the biomarkers, erythrocytes were lysed with chilled distilled water and tubes were vortexed vigorously during three freeze thaw cycles of hemolysates. The hemolysates were then sonicated to ensure lysis and centrifuged at 15000 rpm for 10 minutes. The lysates were diluted in appropriate proportion according to the requirement for analysis of GST, GR, SOD and catalase.

REAGENTS, KITS AND INSTRUMENTATION:
The experiments were carried out using fine chemicals and analytical grade reagents obtained from Qualigens, Mercks and Sisco Research Laboratory (India). Molecular biology and electrophoresis grade reagents which were used wherever required, were purchased from Sigma (USA), Amersco (USA), or Bangalore Genei (India). HPLC assay was carried out using Pharmacia HPLC system with oracle2 software. ELISA Kits prostaglandin E2 and antip53 antibodies were obtained from R&D systems, USA and Immunotech. Kit for polymerase chain reaction (PCR) was obtained from Bangalore Genei (India). Gel Documentation System (Bio-Rad) was used for gel scanning. Photometric assays were performed using Beckman DU-640 spectrophotometer (USA) and ELISA reader.
PARAMETERS FOR STUDY OF BIOMARKES

Following parameters were studied from tobacco/Pan masala products as well as erythrocyte and plasma samples of subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₂+NO₃</td>
<td>Tobacco products/ Pan masala</td>
</tr>
<tr>
<td>Nicotine and cotinine</td>
<td>Urine</td>
</tr>
<tr>
<td>Thioehter</td>
<td>Urine</td>
</tr>
<tr>
<td>NO₂+NO₃</td>
<td>Urine and Plasma</td>
</tr>
<tr>
<td>Antip53 antibodies</td>
<td>Plasma</td>
</tr>
<tr>
<td>Prostaglandin E2</td>
<td>Plasma</td>
</tr>
<tr>
<td>Malonaldehyde (MDA)</td>
<td>Plasma</td>
</tr>
<tr>
<td>Glutathione –S-transferase (GST)</td>
<td>Plasma and erythrocyte</td>
</tr>
<tr>
<td>Glutathione Reductase (GR)</td>
<td>Plasma and erythrocyte</td>
</tr>
<tr>
<td>Superoxide Dismutase (SOD)</td>
<td>Erythrocyte</td>
</tr>
<tr>
<td>Catalase</td>
<td>Erythrocyte</td>
</tr>
<tr>
<td>Total Thiol</td>
<td>Plasma</td>
</tr>
<tr>
<td>GSTM1 genotyping</td>
<td>DNA</td>
</tr>
</tbody>
</table>

**Urinary Nicotine and Cotinine**

**Principle:**
Urbanic nicotine and cotinine were extracted in chloroform under alkaline conditions according to Lequang Thuan et al (1989). These compounds were detected at 254 nm using HPLC and UV detector according to Watson et al (1977). The separation of nicotine and cotinine was done by reverse phase chromatography using C₁₈ coloum.

**Reagents:**
- Chloroform
- Sodium hydroxide (5 M)
- Mobile phase mixture: Ethyl acetate : Isopropanol : Ammonia (80:3:0.4).
Materials and Methods

- Nicotine standard (1gm/ml): Working standard (10μgm /10μl) was prepared from the stock standard.
- Cotinine standard (1mg/ml): Working standard (10μgm /10μl) was prepared from the stock standard.
- Methanol (HPLC grade)

Procedure:

**Figure- 20: HPLC analysis of urinary nicotine and cotinine**

Diagrammatic presentation of HPLC analysis of urinary nicotine and cotinine is depicted in figure-20.
> 5.0 ml of urine, 2.5 ml of 5M sodium hydroxide and 5.0 ml of chloroform were mixed in screw-capped vial.
> The tubes were vortexed for 30 minutes and centrifuged at 2500 R.P.M. for 10 minutes.
> Organic layer were separated and evaporated to dryness. The residue were dissolved in the mobile phase mixture.
> 40 µl of the mixture was injected and flow rate was set at 1 ml per minutes. The quantification was carried out using an U.V. detector at 254 nm. The intra and inter assay variation were also studied.
> Nicotine and cotinine peaks were distinctly isolated by the HPLC method with retention times at 5.08 and 7.03 minutes, respectively (figure-21).

**Figure- 21: Chromatographs for nicotine and cotinine standards**
The standard curves (concentration of standards against area under curves) for nicotine and cotinine are demonstrated in figure- 22. The standards curves for nicotine and cotinine were found to be linear.

Figure-22: Standard curves for different concentration of nicotine and cotinine against area under curve

Calculation:

Urinary nicotine and cotinine values were expressed as μg/mg creatinine.

Thioether

Principle:

Urinary thioether was extracted in ethyl acetate under acidic condition. Thiol (–SH) groups were released after hydrolysis of extracted moieties. The concentration of thiol was determined by the Ellman reaction which was based on the reaction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) with thiol compounds at weakly alkaline pH to produce 1 mole 4-nitro thiophenol anions per mole thiol. This compounds were measured at 412 nm. (Bagwe and Bhisey, 1995)
Materials and Methods

Reagents:

- Hydrochloric acid (4N)
- Ethyl acetate
- Sodium hydroxide (4 N)
- Phosphate buffer (0.2 M), pH7.2
- Hydrochloric acid (4 N) in 0.2 M Phosphate buffer
- Glutathion standard
- DTNB (0.01M) in Phosphate buffer

Procedure:

Part: 1 Extraction and hydrolysis procedure of urine samples

- 2ml of urine sample was centrifuged at 1500 rpm for 5 minutes.
- 1 ml of supernatant was taken in glass tube and used for analysis.
- The supernatant of urine samples was acidified by adding 1-2 drop of 4 N HCL and extracted into 2 ml of ethyl acetate by vigorous shaking in a separating funnel.
- The upper organic layer was taken in glass tube, which was then evaporated at 100°C.
- The contents were reconstituted with 1 ml of DW.
- 0.5ml of 4 N NaOH was added in reconstituted tube and nitrogen gas was passed into the tube. The tube was kept in a boiling water bath for 50 minutes for hydrolysis.
- 0.5 ml of 4 N HCL in 0.2 M Phosphate buffer was added to neutralize the alkali.

Part: 2 Estimation of -SH (Thiol)
(For hydrolysis urine sample)

Tubes were labeled as reagent blank, sample blank, sample/standard and assay was performed as follows.

- One ml of phosphate buffer in reagent blank tube, 0.770 ml of phosphate buffer in sample blank tube and 0.750 ml of phosphate buffer in hydrolysed sample or standard solution tube were taken.
Materials and Methods

- 250 μL of hydrolysed urine sample was added in sample and sample blank tube.
- 20 μL of DTNB was added in reagent blank and sample tube.
- All tubes were vortexed and kept at room temperature for 5 minutes.
- Yellow color was measured at 412 nm against reagent blank.
- Standard curve for thioether (Glutathione concentration against OD) is displayed in **Figure -23**.

**Figure-23: GSH standard curve for thioether**

![Standard curve for thioether](image)

**Calculation:**
Urinary thioether values were expressed as mmole/mole creatinine

**Urinary Creatinine**
Creatinine in urine samples was determined by alkaline picrate method (Varley, 1967).

**Principle:**
Creatinine was given red color with picric acid under alkaline condition. This color was measured at 500 nm.

**Reagents:**
- Picric acid (40 mmol)
- Sodium hydroxide (750mmol)
Creatinine (Standard).

Procedure:
Urine sample was diluted 1:100.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW</td>
<td>3 ml</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>3 ml</td>
</tr>
<tr>
<td>Picric acid</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Kept at room temperature for 15 minute
Read at 500 nm

Calculation:
Urinary creatinine values were expressed as mg/dl and mol/Lt.

Nitrite plus Nitrate (NO$_2$+NO$_3$)

(1) Estimation of NO$_2$+NO$_3$ from tobacco products
Extraction of NO$_2$+NO$_3$ levels in tobacco products were estimated by Griess reagent method as designed by Pakhale and Maru (1995).

Reagents:

- Glacial acetic acid
- Sodium hydroxide (2%)
- Borax buffer (pH 9.6):
- Zinc sulphate (0.42M)
- Sulphnilamide (1%): 0.1 gm of sulfanilamide was dissolved in 7 ml of distill water and 3 ml of glacial acetic acid.
- N-(1-nepthyl) ethylene diamine dihydrochloride: 0.01 gm of N(-1-nepthyl) ethelene diamine dihydrochloride was dissolved in 10 ml of 60% glacial acetic acid.
- Griess reagent: Equal volume of sulfanilamide solution and N-(1-nepthyl) ethylene diamine dihydrochloride solution were mixed in glass beaker.
- Sodium nitrite
Materials and Methods

Cadmium Concentration (gm)

Procedure:

❖ 1 gm of tobacco products were blended for 5 minutes with 15 ml of distills water and then 1.2 ml of 2% sodium hydroxide solution was added. The pH was adjusted to 7 by sodium hydroxide solution.

❖ Slurry was transferred into 50 ml volumetric flask and heated in water bath at 50° C for 15 minutes.

❖ 1.0 ml of 0.42M ZnSO₄ was added and was kept at room temperature for 15 minutes. Then solution was filtered and diluted to 25 ml with DW and mixed.

❖ 500 μl solution was used for estimation of NO₂+NO₃ levels.

❖ Oxidised cadmium (Cortas and Wakid, 1990):
Oxidize cadmium was prepared as mentioned by which was as follows. 2.5 gm of cadmium granules and 2.0 ml of HCL (2%) were mixed in glass tube and continuously shacked for 30 minutes. Supernatant was discarded. Then 2.0 ml of HCL (2%) was added and shacked for 30 minutes. The cadmium granule was washed with distills water (three times) and the oxidized cadmium was used for assay. Standard curve of cadmium concentration against OD is displayed in figure-24. The standard curve was linear after 2.5 gm cadmium concentration.

**Figure-24: Standard curve for cadmium concentration**

![Standard curve for cadmium concentration](image-url)
Materials and Methods

❖ It was incubated with 2.5 gm of oxidized cadmium and 0.5 ml of borax buffer at room temperature for 90 minutes. It was vortexed every 5 minutes.
❖ Subsequently, 0.2 ml aliquot was taken another tube. 0.8 ml of borax buffer (pH; 9.6), 0.5 ml of 60% acetic acid, 1 ml of Greiss reagent and 2.6 ml of DW were added.
❖ Pink color was measured at 550 nm.
❖ Standard curve (nitrite against optical density) was plotted which was found to be linear.

Calculation:
The values of NO$_2$+NO$_3$ in tobacco were expressed as mg/gm tobacco product.

(2) Estimation of NO$_2$+NO$_3$ from plasma and urine samples

Principle:
After protein precipitation of plasma and urine with zinc sulphate, nitrate was converted into nitrite using acid washed cadmium granules. The nitrite formed pink color by Griess reagent, which was measured at 550 nm. (Green et al., 1982; Van Bezooijen et al, 1998)

Reagents:
➢ Zinc sulphate (ZnSO$_4$) solution (10%)
➢ Sodium hydroxide (0.5N)
➢ Borax buffer pH 9.6:
  ➢ Greiss reagent: 1gm of sulfanilamide (1%), 100mg of naphthylethylen diamine (0.1%) and 2.8ml of 98% orthophosphoric acid (2.5%) were dissolved in 100ml DW.
➢ 2 % Hydrochloric acid (HCL)
➢ Oxidized cadmium

Procedure:
➢ 200µL of plasma/diluted urine sample was taken in tube and 400µL of sodium hydroxide and 400µL of zinc sulphate were added.
Tubes were centrifuged for 15 minutes at 8000 rpm.

500\(\mu\)L of supernatant was taken in glass tube and 500\(\mu\)L of borax buffer and 2.5 gm of oxidized cadmium granules (acid treated) were added in the tube.

The tube was kept at room temperature for 90 minutes and was vortexed every 5 minutes.

Supernatant was taken in another glass tube and 1 ml greiss reagent was added.

Pink color was developed which was read at 550 nm. The wavelength scan revealed absorption maximum at 550 nm (figure-25).

**Figure- 25: Wavelength scan for NO\(_2\)+NO\(_3\) estimation**

![Wavelength Scan](image)

The standard curves for nitrite concentration against optical density are displayed in figure-26.

**Figure-26 : Standard curve for NO\(_2\)+NO\(_3\) concentration**

![Standard Curve](image)
Calculation:
Urinary NO$_2$+NO$_3$ values were expressed μg/mg creatinine. Plasma NO$_2$+NO$_3$ values were expressed as μM.

**Anti p53 Antibodies**

**Principal:**
Anti-p53 antibodies were detected by ELISA using microtiter plates coated with recombinant wild-type human p53 protein (to detect non specific interactions). A peroxidase-conjugated goat anti-human IgG binds anti-p53 antibodies. The specific p53/anti-53/conjugate complexes were revealed by addition of a peroxidase substrate (TMB) resulting in a colorimetric reaction (figure-27).

**Figure-27: Principle of Anti-p53 Antibodies ELISA**

**Reagents:**
1. Human p53 protein coated or control protein coated microtiter plate
2. Wash Buffer (20X) (Sodium chloride-Tween)
3. Sample diluent (Sodium phosphate-Casein)
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4. Conjugate (Peroxidase-conjugated anti-human IgG antibodies)
5. Substrate solution [3,3', 5,5' tetramethylbenzidine (TMB)]
6. Stop solution [H₂SO₄ (2N)]
7. Conjugate (Peroxidase-conjugate anti-human IgG antibodies)
8. Standards (anti p53 antibodies) : Standards were diluted 1/100 in diluent

All the reagents were provided with the ELISA kit.

Procedure:
Each sample or standard were tested simultaneously in 2 distinct wells (one well coated with p53 protein and one well coated with control protein)

100 µl of diluted samples or standard were added in coated with p53 protein and coated with control protein wells and kept at 20-25°C with shaking for 60 minutes

Wells were washed 4 times with 350 µL of wash solution

100 µl of conjugate solution was added in each well and plate was covered with microtiter plate and incubated at 20-25°C with shaking for 60 minutes

Wells were washed 4 times with 350 µL of wash solution

100 µl of substrate solution was added in each well and incubated at 20-25°C in dark for 30 minutes

100 µl of stop solution was added in each well and kept at shaker for 30 second

Color was measured at 450 nm by using ELISA micro plate reader.

Calculation:
❖ The average absorbance for each blank, standard and sample for wells coated with p53 protein as well as coated with control protein were calculated.
Materials and Methods

❖ The net absorbance was determined by subtracting the assay blank average absorbance from the sample or standard average absorbance.
❖ Specific signal for anti p53 antibodies levels was calculated as suggested by kit manufacture.
   Specific signal : \[ p53 \text{ net absorbance} - \text{control protein absorbance} \]
❖ Calibration curve (specific signals of standard against standard concentration) was found to be linear. Unknown anti p53 antibodies levels in samples were calculated using standard curve.
❖ Interpretation: Less than 0.85 U/ml was considered as absence of anti p53 antibodies level and more than 0.85 U/ml was considered as presence of antip53 antibodies level.

Prostaglandin E2

Plasma prostaglandin E2 was estimated by using ELISA kit.

Principle:
The prostaglandin E2 assay was based on the competitive binding technique in which prostaglandin E2 present in a sample competes with a fixed amount of alkaline phosphatase labeled prostaglandin E2 for sites on a mouse monoclonal antibody. During the incubation, the mouse monoclonal antibody was bound to the goat anti mouse antibodies coated onto the micro plate following a wash to remove excess conjugate and unbound sample, a substrate solution was added to the wells to determine the bound enzyme activity. Immediately following color development, the absorbance was read at 405nm. The intensity of the color was inversely proportional to the concentration of prostaglandin E2 in the sample.

Reagents:
- Diluted wash buffer
- Stop solution (Tri sodium phosphate)
- pNPP substrate (p-nitrophenol phosphate in buffer solution)
Mouse monoclonal prostaglandin E2 antibody
Prostaglandin E2 standard (50000 pg/ml)
Micro plate wells: 96 well coated with goat antimouse polyclonal antibody

All the reagents were provided with ELISA kit.

Reagent Preparation:
1. Wash Buffer: 30 ml wash buffer make up to 300 ml distilled water
2. Prostaglandin E2 standard (Preparation before 60 minutes)

Reagent Preparation:
1. Wash Buffer: 30 ml wash buffer make up to 300 ml distilled water
2. Prostaglandin E2 standard (Preparation before 60 minutes)

Procedure:

<table>
<thead>
<tr>
<th>TA</th>
<th>Blank substrate</th>
<th>NSB</th>
<th>Std Bo</th>
<th>Std or sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>No</td>
<td>150 µl buffer</td>
<td>100 µl buffer</td>
<td>100 µl Std or sample</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>50 µl conjugate</td>
<td>50 µl conjugate</td>
<td>50 µl conjugate</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>No</td>
<td>50 µl PGE2 antibody</td>
<td>50 µl PGE2 antibody</td>
</tr>
</tbody>
</table>

Plate was covered and incubated for one hour at room temperature
Wells were washed for two times and liquids were removed from well every step.

<table>
<thead>
<tr>
<th>50 µl conjugate PGE2</th>
<th>No</th>
<th>No</th>
<th>No</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 µl pNPP</td>
<td>200 µl pNPP</td>
<td>200 µl pNPP</td>
<td>200 µl pNPP</td>
<td>200 µl pNPP</td>
</tr>
</tbody>
</table>

Incubated for one hour at room temperature

<table>
<thead>
<tr>
<th>50 µl Stop Soln</th>
<th>50 µl Stop Soln</th>
<th>50 µl Stop Soln</th>
<th>50 µl Stop Soln</th>
<th>50 µl Stop Soln</th>
</tr>
</thead>
</table>

OD was read at 405nm with wavelength correlation at 570 nm
Four wells with duplicate were allotted for total activity (TA), non-specific binding (NSB), maximum binding (BO), and substrate blank, which were used for calculation of prostaglandin E2 as well as quality controls.

**Calculation:**

1. The average net optical (OD) bound for each standard and sample was calculated by subtracting the average NSB OD from the average OD bound;
   
   \[
   \text{Average net OD} = \text{Average bound OD} - \text{Average NSB OD}
   \]

2. Percentage bound was calculated by using following formula.

   \[
   \text{Percentage bound} = \frac{\text{Net OD} \times 100}{\text{Net Bo OD}}
   \]

A graph for percent bound versus concentration of prostaglandin E2 was plotted and prostaglandin E2 in unknown samples were calculated.

**Malondialdehyde (MDA)**

**Principle:**

One molecule of malonaldehyde (MDA) reacts with two molecules of TBA with the elimination of two molecules of water to yield a pink crystalline pigment with absorption maximum at 535 nm (Yagi, 1978).

**Reagents:**

(1) Standard: 1 mmol Tetraethoxypropene (TEP).

   Distillation of TEP standard:
   
   - MDA level was checked TBA reaction at 535 nm \( (ε = 153000) \)

(2) 3N \( H_2SO_4 \)

(3) 10% Phosphotungstic acid (PTA)
(4) 0.67% Thiobarbituric acid (TBA) reagent in acetic acid

**Procedure:**
- 2 ml of 3N H$_2$SO$_4$ was added to 0.1 ml of plasma.
- 0.25 ml of 10% PTA was added and kept at RT for 5 minutes.
- Centrifuged at 3000 rpm for 10 minutes and supernatant was discarded.
- Sediments were mixed with 1 ml of 3N H$_2$SO$_4$ and 0.15 ml of 10% PTA.
- The tubes were vortexed and centrifuged at 3000 rpm for 10 minutes.
- Supernatant was discarded then 2 ml of D.W. and 0.5 ml of TBA reagent were added.
- For Blank and Standards; 2 ml of D.W. and 0.5 ml of TBA reagent were added. (0 to 10 nmol standards were used to prepare standard curve).
- The tubes were vortexed and kept at 95°C for 1 hour.
- 2.5 ml of Butanol was added.
- Mixed vigorously, centrifuged then butanol layers were pipetted out in separate glass tubes.
- Absorbance was measured at 535 nm against blank.

**Calculation:**
Plasma MDA values were expressed as nmole/ml.

**Superoxide Dismutase (SOD)**

**Principle:**
The reduction of nitro-blue by NADH was enhanced by PMS indicating that PMS acts as an electron carrier in the system. Ability of SOD to inhibit the PMS mediated aerobic reduction of NBT by 50% of the maximum inhibition of color development by spectrophotometrically at 560 nm (Kakkar et al, 1984).

**Reaction:**

\[ 2O_2^- + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2 \]

**Reagents:**
Materials and Methods

- 0.052M Sodium phosphate buffer, pH 8.3
- 186 μM Phenazonium methosulphate (PMS)
- 300 μM Nitroblue tetrazolium (NBT)
- 780 μM NADH disodium salt

Procedure:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Control</th>
<th>Sample Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1.5 ml</td>
<td>1.2 ml</td>
<td>1.45 ml</td>
<td>1.15 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>186 μM PMS</td>
<td>-</td>
<td>0.05 ml</td>
<td>-</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>300 μM NBT</td>
<td>-</td>
<td>0.150 ml</td>
<td>-</td>
<td>0.150 ml</td>
</tr>
<tr>
<td>780 μM NADH</td>
<td>-</td>
<td>0.1 ml</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Vortex, Read O.D. at 560 nm after 90 second.

Enzyme Unit: One unit of the enzyme activity was defined as enzyme concentration required to inhibit optical density at 560 nm of chromogen produced by 50% in one minute under the assay conditions.

Calculation:

Erythrocyte SOD activities were expressed as U/gm Hb.

Catalase

Principle:

Catalase activities were measured in terms of decomposition of H₂O₂ into water molecule, which was measured as decrease in absorbance at 240 nm (Aebi, 1984).

Reaction:

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} \text{H}_2\text{O} + \frac{1}{2}\text{O}_2
\]

Reagents:

- 50 mM Sodium phosphate buffer, pH 7.0
Materials and Methods

- 30 mM H$_2$O$_2$, dissolved in phosphate buffer

Procedure:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Reagent Blank</th>
<th>Sample Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1.5 ml</td>
<td>1 ml</td>
<td>1.5 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>2 μl</td>
<td>2 μl</td>
</tr>
<tr>
<td>30 mM H$_2$O$_2$</td>
<td>-</td>
<td>0.5 ml</td>
<td>-</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Read the reagent blank against blank and sample against sample blank. Note the decrease in O.D. at 240 nm at every 15 sec. for 2 minutes.

Calculate Δ O.D./15 seconds

Enzyme Unit: 1 μm of H$_2$O$_2$ converted into H$_2$O in one minute was defined as one unit of enzyme activity.

Calculation:
Erythrocyte catalase activities were expressed as U/gm Hb.

Glutathione-S-Transferase (GST)

Principle:
GST conjugated with GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) gives a complex, (S-2, 4-dinitrophenyl glutathione) which is spectrophotometrically measured as increased in absorbance at 340 nm (Habig and Jacoby, 1981).

Reaction:

\[
\text{GSH} + \text{CDNB} \xrightarrow{\text{GST}} (\text{GS-CDNB}) \text{ complex} + \text{HCl}
\]

Reagents:
- 0.1 M Sodium phosphate buffer, containing 1mM EDTA, pH 6.5
- 40 mM 1-chloro-2,4-dinitrobenzene (CDNB), dissolved in 95 % ethanol
- 40 mM reduced glutathione (GSH) in distill water
Materials and Methods

### Procedure:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Reagent Blank</th>
<th>Sample Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1 ml</td>
<td>0.95 ml</td>
<td>0.95 ml</td>
<td>0.90 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

Incubated at 37°C for 30 minutes.

Immediately read O.D. at 340 nm at every 1 minute for 3 minutes.

Calculate \( \Delta \text{O.D/min. of test} \) and subtract from reagent blank.

### Enzyme Unit:

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 \( \mu \text{M} \) of S-2, 4-dinitrophenyl glutathione per minute, using 1 mM GSH and CDNB i.e. the amount of product formed was calculated using the molar extinction coefficient of the product at 340 nm (\( \varepsilon \) for S-DNPG=9.96/M) and the enzyme activity was expressed as nmole product formed /min/ L (for plasma) or gm of Hb (For Whole blood).

### Calculation:

Plasma GST activities were expressed as U/L. Erythrocyte GST activities were expressed as U/gm Hb.

### Glutathione Reductase (GR)

#### Principle:

Glutathione reductase reduces oxidized glutathione (GSSG) into reduced glutathione (GSH) by oxidation of NADPH, which was measured spectrophotometrically by decrease in absorbance at 340 nm (Carlberg and Mannervik, 1985).

#### Reaction:

\[
\text{GSSG} + \text{NADPH} \xrightarrow{\text{GR}} \text{GSH} + \text{NADP}^+ + \frac{1}{2} \text{H}_2
\]

#### Reagents:
Materials and Methods

- 0.2 M Potassium phosphate buffer, containing 2 mM EDTA, pH 6.5
- 40 mM Oxidized glutathione (GSSG), dissolve in distill water
- 4 mM NADPH-Na₄ in 10mM Tris-HCl buffer, pH 7.0

Procedure:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Reagent Blank</th>
<th>Sample Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1 ml</td>
<td>0.95 ml</td>
<td>0.95 ml</td>
<td>0.90 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 30 minutes.

GSSG
- 0.025 ml

NADPH-Na₄
- 0.025 ml

Immediately read O.D. at 340 nm at every 1 minute for 3 minutes
Calculate Δ O.D/min. of test and subtract from reagent blank.

Enzyme Unit:
One unit of enzyme activity was defined as the amount of enzyme that
catalyse one micromole of NADPH in one minute by oxidation of NADPH.
The decrease in absorbance was measured (ε NADPH= 6.3 M at 340 nm).

Calculation:
Plasma GR activities were expressed as U/L. Erythrocyte GR activities were
expressed as U/gm Hb.

Total Thiol

Principle:
5, 5'-dithiobis-2-nitobenzoic acid (DTNB) also known as Ellemen’s reagent
reacts with thiol compounds (-SH groups) to give yellow colored complex,
which was measured at 412 nm (Ellman, 1959)

Reaction:

\[ \text{DTNB} + \text{-SH} \rightarrow \text{Yellow colored complex} \]

Reagents:
- 0.2M Na₂HPO₄ containing 2mM EDTA
Materials and Methods

- GSH standard: Stock standard = 0.003 gm / 10 ml (1 mmole)
- 10 mM 5, 5'-dithiobis-2-nitobenzoic acid (DTNB) in phosphate buffer

Procedure:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Sample Blank</th>
<th>Sample</th>
<th>Sample Blank</th>
<th>Standard 0-10 μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2M Na₂HPO₄</td>
<td>1 ml</td>
<td>0.975 ml</td>
<td>0.995 ml</td>
<td>0.975 ml</td>
</tr>
<tr>
<td>Sample/standard</td>
<td>-</td>
<td>0.025 ml</td>
<td>0.025 ml</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>10 mM DTNB</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>-</td>
<td>0.02 ml</td>
</tr>
</tbody>
</table>

Vortex and wait for 5 minutes. Read at 412 nm against reagent blank.

Calculation:
Plasma thiol values were expressed as mmole/dl.

Glutathione-S-Transferase M1 (GST-μ) genotyping

Reagents Preparation:
In the final phase of the study, GST-μ genotyping was carried out from 25 DNA samples of oral cancer patients. DNA was isolated from buffy coats obtained from the blood samples of the patients.

Reagents Preparation:
(A) DNA Extraction:
1. 1 M Tris, pH 8.0
2. 0.5 M EDTA, pH 8.0
3. 1 M Sodium chloride
4. 10 % SDS
5. 3 mg/ml Proteinase K
6. Lysis buffer: Lysis buffer contained 5 ml of Tris 50 mM, pH 8.0, 2ml of EDTA 10 mM, pH 8.0, 1 ml of NaCl 10mM and 20 ml of 2% make-up with 100ml of DW. Proteinase K was added 150 μg/ ml.
7. 50mM Tris–10mM EDTA (pH 8.0):
8. Tris - saturated Phenol (pH < 8.0):
9. 3 M Sodium Acetate, pH 5.5
10. Erythrocyte lysis Buffer: NH₄Cl(8.29 gm), NH₄(CO₃)₂(0.79 gm), EDTA(0.037 gm) were dissolved in 100 ml of DW.

(B) PCR reaction:
DNA amplification was carried out using PCR kit from Bangalore Genei, India.

1. Taq DNA polymerase: 250 units as 3 units/μl and 1.5 to 2 units per reaction are recommended.
2. Deoxy ribonucleotide triphosphate (dNTPs): All four dNTPs are 100 μL each and supplied as 10 mM solutions. For making 2.5 mM stock mixture, add equal volumes of all 4 dNTP’s (10mM stocks) and mix well. This mixture has a final concentration of 2.5 mM of each dNTP and a 10 mM concentration of total mixture.
3. 10 X Taq polymerase buffer: 10 X stock buffer contains 100 mM Tris HCl (pH 9.0), 500 mM KCl, 15 mM MgCl₂ and 0.1% gelatin.
4. φ X 174 DNA/Hae III Digest
5. GSTM1 primers:
   GSTM1 (Forward) 5’ GAA CTC CCT GAA AAG CTA AAG C 3’
   GSTM1 (Reverse) 5’ GTT GGG CTC AAA TAT ACG GTG G 3’
6. β-globin Primers for house keeping genes:
   β-globin (Forward) 5’ CAA CTT CAT CCA CGT TCA CC 3’.
   β-globin (Reverse) 5’ GAA GAG CCA AGG ACA GGT AC 3’.

(C) For Agarose gel electrophoresis:
1. 10 X Tris Borate EDTA (TBE) buffer, pH 8.1 to 8.2
2. Loading dye (5 X) : 0.25% BPB in 40% sucrose and TBE.
3. Ethidium Bromide [1000 X]: 2 mg/ml in water.
4. 1.5 % agarose gel preparation: Agarose powder was dissolved in 85 ml of TBE buffer and boiled in water bath till it dissolved. Cooled to 60°C. Add ethidium bromide 0.5 μg/ml of gel solution. (85 μL from stock ethidium bromide). Pour on submerse gel electrophoresis plate with submerse sample comb.
5. Electrode buffer: 1 X TBE

Procedure:

(A) DNA Extraction:

1. WBC Separation from EDTA whole blood: Whole blood was centrifuged at 500 rpm for 10 minutes at RT. The upper plasma and WBC layers were collected in other vial and centrifuged at high speed to remove plasma layer. The final sediment consisted of WBCs with contaminated erythrocytes. The erythrocytes were removed by 2-3 washes of erythrocyte lysis buffer and finally centrifuged and hemolysate were removed and sedimented. WBCs were stored at -20°C for DNA extraction.

2. WBCs were mixed with 2 ml of lysis buffer and incubated at 50°C for 3 hours and then overnight at 37°C.

3. Equal volume of Tris saturated phenol was added, mixed gently for 10 minutes and centrifuged at 8000 rpm for 20 minutes. The supernatant was mixed again with equal volume of phenol, mixed gently for 10 minutes and centrifuged at 8000 rpm for 20 minutes.

4. The above supernatant was mixed with one half volume of phenol reagent and one half volume of 24:1 Chloroform: Iso-amyl alcohol mixture. The mixture was mixed gently for 10 minutes and centrifuged at 8000 rpm. (This step was repeated when there were high protein impurities).

5. The supernatant was mixed with 0.2 volume of 3M sodium acetate and equal volume of chilled ethanol. The mixer was swirl slowly.

6. The DNA precipitates were washed with 70% ethanol and then with absolute alcohol. The precipitates were air dried and dissolved in TE buffer. Purity of DNA was checked using O.D. ratio at 260/280 nm.

(B) DNA amplification:

50 μl of PCR mixture consisted of 200 ng genomic DNA, 250ng each of upstream and downstream primers, 3 μl of 2.5 mM each of four deoxy
nucleotide phosphate (dNTP), 1.5 U of Taq DNA polymerase and 5 μl of PCR buffer. PCR reactions were performed using thermal cycler (Gene Amp PCR, Parkin Elmer, USA).

PCR conditions were as follows:
An initial denaturing step at 94 °C for 1 minute
- Denaturation - 95°C for 30 second
- Annealing - 64°C for 1 minute
- Extension - 72°C for 1 minute
30 cycles

Final extension step at 72°C for 5 minutes. Cooling at 4°C for 10 minutes. The PCR products were stored at -20°C until electrophoresis.

(C) Detection by Agarose gel electrophoresis

1. 15 μl of the PCR products were mixed with 3 μl of loading dye. The GSTM1 (220 base pairs) and β-globin (265 base pairs) were resolved on ethidium bromide stained on 1.5% agarose gel electrophoresis in submerse condition (Pharmacia LKB Biotechnology, Sweden).
2. 200 ng of φ X 174 DNA/Hae III digest was also run to identify GSTM1 and β-globin products (220 and 265 base pairs respectively).
3. The samples were run electrophoretically (60 V) till the dye front reached near by the anodic end.
4. The presence or absence of the GSTM1 gene was detected under U.V. light using Gel Documentation System (Bio-Rad).

Individuals with absence of 220 base pair GSTM1 band were considered as GSTM1 null genotype.

STATISTICAL METHODS:
Data were statistically analyzed using the SPSS statistical software (Version 10).

- Student’s unpaired ‘t’ test was performed to compare biomarkers levels between controls, patients with OPC and oral cancer. Student’s paired ‘t’ was performed to compare biochemical parameters between before and after anticancer treatment of oral cancer.
Materials and Methods

- Relative risks of OPC and cancer in healthy controls were estimated by computing odds ratio and 95% C.I.
- Receivers Operating Characteristic (ROC) curves were constructed to evaluate discretionary efficacy of biomarkers levels between patients and controls.
- Multivariate analysis was performed to correlate markers with clinico-pathological parameters including gender, tobacco types and duration, stage, grade, differentiation, lymph node involvement etc.
- Person's correlation was studies to assess association between biomarkers.
- 'p' values <0.05 were considered as statistically significant.