3. RESEARCH DESIGN AND METHODOLOGY

3.1 Study groups

The material for the present study comprised a total of 200 subjects reported to various hospitals in and around Mangalore. All these individual were age-matched in range of 3rd and 5th decade of life. These subjects were placed into two groups:

Group 1 (Control Group)/E 100 healthy subjects in control group without any habit and oral lesions.
Group 2 (Case Group)/E 100 patients who were chronic smokers without any other habit and oral lesions.

3.2 Inclusion criteria:
1. Smokers with frequency of more than 15 cigarettes per day and duration of 10 or more years
2. Patients are males in age group in range of 3rd and 5th decade of life.
3. Patients with absolutely no other habits other than smoking.
4. Patients who are otherwise healthy with no evidence of oral or systemic diseases.

3.3 Exclusion criteria:
1. Patients who have any other habits other than smoking.
2. Patients who have any evidence of oral or systemic diseases.
3. Patients with metabolic disorders either congenital or acquired.

3.4 Method of collection of samples & Analysis

5ml of venous blood was drawn and was subjected to centrifugation, serum were then separated and transferred into vacutainers for transport to laboratory for analysis.

- Estimation of serum ceruloplasmin was done using Diamine Oxidase Method. [Ravin; 1961]
- Serum glutathione level estimation was done using DTNB Method (5,5-Dithio Bis-2-Nitro Benzoide). [Sharma SM; 2009]
- Estimation of serum b-carotene was done using Bradley & Hornbeck method. [Bradley & Hornbeck; 1973]
- Estimation of serum copper and zinc was done using Atomic Absorption Spectrophotometry. [Mert and Hinkin; 1971]
- Estimation of serum iron was done using Bathophenanthroline Method and photometrically determined. [Perry and San; 1977]

3.5 ESTIMATION OF SERUM CERULOPLASMIN

**Diamine Oxidase Method**

Ceruloplasmin, also called as ferrooxidase or iron II: oxygen oxidoreductase is a blue copper containing protein laden with six atoms of copper, which has a role in iron metabolism.

**PRINCIPLE:**

Ceruloplasmin in serum is measured by its property to catalyze the oxidation of colourless p-phenylenediamine to violet coloured compound known as Bandrowski base. The intensity of the blue colour formed is proportional to the amount of ceruloplasmin. The non-enzymatic oxidation acts as the serum control which is set up by the addition of sodium azide before incubation for blanking the non-enzymatic oxidation.

SAMPLE: Serum
SAMPLE VOLUME: Serum- 60µL.

**CHEMICALS REQUIRED:**
- Sodium acetate (CH3COONa)
- Glacial acetic acid (CH3COOH)
- Sodium azide (NaN3)
- p -phenylenediamine hydrochloride (C6H8N2).
PREPARATIONS:

¾ Acetate Buffer 0.2M, pH 5.6
¾ Sodium Azide (3%)
¾ Substrate
  o Always prepared freshly by dissolving 36mg of p-phenylenediamine in 25mL of 0.2M acetate buffer pH 5.6

PROCEDURE:

Ö Three clean test tubes are taken and 34abeled as “TEST”, ”BLANK” and “CONTROL”.
Ö 1.5 mL of the freshly prepared substrate is added to all the three tubes and incubated at 37°C in a water bath.
Ö To the test tube 34abeled as “TEST” 30µL of serum was added, to the test tube 34abeled as “CONTROL” 30µL of serum was added, immediately followed by the addition of 0.3mL of sodium azide. To the test tube 34abeled as “BLANK” 30µL of water and 0.3mL of sodium azide is added.
Ö All the tubes are then incubated in a water bath at 37°C for 15 minutes.
Ö Following incubation, 0.3mL of sodium azide is added to the test tube 34abeled as “TEST” to stop the oxidation reaction.
Ö The absorbance of the “TEST” and the “CONTROL” was read at 546nm against “BLANK”.
Ö The concentration of ceruloplasmin is then calculated using the formula and expressed as mg/dL.

CALCULATION:

\[
\text{Ceruloplasmin in mg/dL} = \frac{0.142 \times 100}{0.03} = 473.3 \times (\text{O.D of “TEST” - O.D of “CONTROL”})
\]

Wherein 0.142mg is the standard ceruloplasmin to give extinction coefficient of value 10 under assay conditions.
0.03mL is the amount of sample taken for assay and 100 converts to one deciliter of serum.
3.6 ESTIMATION OF SERUM GLUTATHIONE

DTNB Method (5,5-Dithio Bis-2-Nitro Benzoide)

Glutathione is a tripeptide that is biologically significant, as it acts as a useful antioxidant. It exists in two forms, the reduced form (GSH) and the oxidized form (GSSG). The sulphhydryl (thiol) group in reduced glutathione (GSH) acts as a proton donor and is responsible for its biological function.

PRINCIPLE:

This method is based on development of a relatively stable yellow color, when 5, 5’-dithiobis (2-nitro benzoic acid) is added to sulphhydryl compounds including glutathione.

SAMPLE: Whole blood, saliva or tissue homogenate.
SAMPLE VOLUME: Whole blood-500µL, Saliva-250µL, Tissue homogenate-500µL.

REAGENTS:

- M-phosphoric acid (HPO₃and NaPO₃).
- Sodium chloride (NaCl).
- Ethylene diamine tetra acetate (EDTA)-(C₁₀H₁₆N₂O₈).
- Di sodium hydrogen phosphate (Na₂HPO₄).
- 5,5’-Dithiobis (2 nitro benzoic acid) (DTNB)- (C₁₄H₈N₂O₈S₂).

PREPARATIONS:

¾ PRECIPITATING SOLUTION

- 1.67g glacial m-phosphoric acid (A mixture of HPO₃ and NaPO₃), 0.2g disodium or dipotassium ethylene diamine tetra acetic acid (EDTA) and 30g of NaCl per 100mL of distilled water. The mixture may contain suspended fine precipitate of EDTA, which may not interfere, but added just to avoid the errors caused by metal ions in the preparations. This solution is stable for approximately 3 weeks at 4°C.

¾ PHOSPHATE SOLUTION (0.3M Na₃HPO₄)
¾ DTNB REAGENT
   o 40mg DTNB per 100mL of phosphate solution. The reagent is stable for at least 13 weeks in the refrigerator.

¾ REDUCED GLUTATHIONE STANDARD (100µg/mL)

PROCEDURE:

¾ STANDARDISATION
   The assay is carried out referring to the table 1.1. The solutions mentioned are added in their appropriate measures. The optical densities obtained are plotted against their respective concentrations on a graph.

ESTIMATION OF GLUTATHIONE IN THE SAMPLE
   • SAMPLE PREPARATIONS:
      o WHOLE BLOOD: 500µL of whole blood anticaogulated with fluoride is centrifuged and 100µL of the erythrocytes are taken and diluted to 1mL with distilled water. (Dilution is 1:10)
- **SALIVA:** The collected saliva is centrifuged and the supernatant is used for the assay. 100µL of the saliva is diluted to 500µL with distilled water (Dilution is 1:5). If glutathione is not detected with this dilution then the dilution is lowered to (1:2).

- **TISSUE HOMOGENATE:** 1g of the tissue is weighed and homogenized with 10mL of phosphate buffer pH (7.0). The homogenate is then centrifuged and 1mL of the supernatant is used for the assay (Dilution is 1:10).

- **ASSAY**
  - The diluted samples are treated with 1.5mL of precipitating solution and kept for 10 minutes for the precipitation to complete.
  - The solutions are then filtered through a whatmann Number 1 filter paper.
  - 500µL of the filtrate is taken and to this 2mL of phosphate solution and 250µL of DTNB solution is added.
  - Simultaneously a blank is maintained containing 200µL of distilled water, 300µL of precipitating solution, 2mL of phosphate solution and 250µL of DTNB.
  - The intensity of the yellow color formed is spectrophotometrically read immediately (within ten minutes) at 412nm against the blank.
  - The optical densities obtained are plotted against the standard graph.

**CALCULATION:**

Concentration of glutathione is calculated graphically and multiplied with the respective dilution factors and the total glutathione in the sample is expressed as µg/mL.
3.7 ESTIMATION OF SERUM BETA-CAROTENE

**Bradley & Hornbeck method**

β-carotene is a precursor of retinol in human body. The beneficial effects of β-carotene are mainly due to its high pro-vitamin activity and its antioxidant nature (Hilbert & Mohsenin, 1996). They can inactivate the free radicals and singlet oxygen by a process termed as quenching (Masci, 1991). They also inhibit the lipid peroxidation in membrane but only at low O2 concentration.

**PREPARATION OF SERUM:**

Blood was transferred to centrifuge tube and centrifuged at 2500 rpm for 7-10 min. The supernatants (serum) were separated with the help of 3 mL venoject tube, stored at 4°C and analyzed for β-carotene and retinol within 72 h.

**DETERMINATION OF B-CAROTENE IN SERUM:**

Serum β-carotene and retinol were determined using TFA method.

**CONCENTRATION OF SERUM B-CAROTENE**

It was calculated by following formula:

\[
\text{Serum } \beta\text{-carotene (mg/L)} = \frac{A_{450\text{ of Unknown}}}{A_{450\text{ of Standard}}} \times \text{Concentration of Standard} \times 3
\]

*Absorbance values of carotene working standards of different concentrations and calculation of factor } A_1^*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Conc. of carotene working standards (mg/L)</th>
<th>(A_{450}) of carotene working standards</th>
<th>Calculations Conc./Obs X 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.17</td>
<td>0.5/0.17 x 3 = 8.8</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.3</td>
<td>1/0.3 x 3 = 10</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>0.59</td>
<td>2/0.59 x 3 = 10</td>
</tr>
<tr>
<td>4</td>
<td>3.0</td>
<td>0.85</td>
<td>3/0.85 x 3 = 10</td>
</tr>
<tr>
<td>5</td>
<td>4.0</td>
<td>1.1</td>
<td>4.0/1.1 x 3 = 10</td>
</tr>
</tbody>
</table>

\[
\text{Average} = 10.04 = A_1
\]
Absorbance of each working standard (carotene) at 450 nm having different concentration is given in the Table 2. By putting this value to the formula finally the serum carotene value was calculated as:

$$\text{Serum Carotene} = A_{450} \text{of Unknown} \times A_1$$

### 3.8 ESTIMATION OF SERUM COPPER

**Atomic Absorption Spectrophotometry**

Micronutrient status is estimated by Atomic Absorption Spectroscopy (GBC Avanta, ver; 2.02)

Digestion of sample;
250µL of sample + 500 µL of nitric acid
Above sample is digested over heating sand bath for an hour. Make up the sample to 5mL in nitric acid. This solution is used to analyze in AAS

### 3.9 ESTIMATION OF SERUM ZINC

**Atomic Absorption Spectrophotometry**

Micronutrient status is estimated by Atomic Absorption Spectroscopy (GBC Avanta, ver; 2.02)

Digestion of sample;
250µL of sample + 500 µL of nitric acid
Above sample is digested over heating sand bath for an hour. Make up the sample to 5mL in nitric acid. This solution is used to analyze in AAS
3.10 ESTIMATION OF SERUM IRON

Bathophenanthroline Method

Iron present in the body is mainly found to be bound to molecules of hemoglobin, myoglobin and stored as ferritin. At a lower rate, it occurs as circulating iron in the serum where it is bound to transferrin molecules. Transferrin molecules are capable of binding two molecules of iron (III). Transferrin molecules are essential when the stored iron has to be moved and used.

**PRINCIPLE:**

Iron in serum is present as Fe$^{+++}$ bound to transferring. In the assay, the proteins are precipitated and consequently the iron (Fe$^{3+}$) bound to ferritin is released by mild acid treatment. The iron thus released is reduced to (Fe$^{2+}$) by reducing agents. (Fe$^{2+}$) in turn reacts with Bathophenanthroline to form pink color complex whose intensity can be measured at 535 nm.

SAMPLE: Serum or Saliva
SAMPLE VOLUME: 100µL.

CHEMICALS REQUIRED:

- Trichloro acetic acid (CCl$_3$COOH)
- Thioglycolic acid/ Mercaptoacetic acid (C$_2$H$_4$O$_2$S)
- Hydrochloric acid (HCl)
- Bathophenanthroline Di salfonic acid (C$_{24}$H$_{14}$N$_2$Na$_2$O$_6$S$_2$.3H$_2$O)
- Sodium Acetate (CH$_3$COONa)
- Ferrous ammonium sulphate [FeS0$_4$ (NH$_4$)$_2$So$_4$.6H$_2$O]

PREPARATIONS:

¾ PROTEIN PRECIPITATING SOLUTION

- Aqueous solution made to contain 10% TCA 30ml of thioglycolic acid and 2ml HCl per 1 L of deionised water. This should be stored in a dark brown bottle and is stable for at least 2 months.
3/4 SODIUM ACETATE (2M)

3/4 CHROMOGEN SOLUTION
   o 25 mg bathophenanthroline sulfonate is dissolved in 100mL of sodium acetate (2M)

3/4 IRON STANDARD (STOCK SOLN)
   o The iron concentration of the solution is 10µg /ml.

3/4 IRON STANDARD (WORKING)
   o 40ml of stock solution was diluted to 100ml with deionised water to attain a concentration of 4µg /ml.

PROCEDURE:
3/4 ESTIMATION OF IRON IN SAMPLE
   100µL of the sample (serum/saliva) is taken in a clean microfuge tube and made up to 250µL with deionised water.
   Then to this diluted sample 500µL of protein precipitating solution is added.
   The mixture is then centrifuged at 2000rpm for 10 minutes.
   500 µL of the supernatant is taken and added to 500µL of the chromogen solution.
   The optical density of pink color formed is read immediately (within 10 mins) at 535nm against a blank treated in a similar method as the test wherein the sample is replaced with the deionised water.

CALCULATION:
The concentration of iron in the sample is deduced by plotting the optical densities of the test against the standard graph.
The obtained concentration is then multiplied by 2.5 (dilution factor).
Note: The glassware’s, tips and other materials used for the test are washed thoroughly with deionised water to increase the accuracy of the assay.