Stages of Oral Cancer

- Beta quid ingredients
- Tobacco Alcohols
- Nitrosamines
- Environmental toxins

Initiation → Promotion

Normal Cells → Initiated Cells → Precancer Cells → Transformation

Introduction &
Review of Literature
Global Burden of Cancer

Cancer, the dreaded disease is a major cause of morbidity and mortality worldwide. The term “cancer” is derived from the Latin *cancer* and from the Greek *karkinos*, both meaning “crab”. Its definition has changed over the centuries, as the number and types of lesions that are grouped under this term have expanded. Today’s glossaries define cancer as malignant tumor, which spreads to rest of the body. In 2000, an estimated 10.1 million new cases of cancer were diagnosed and 6.2 million deaths were recorded worldwide. In the year, 2020, the numbers of new cancer cases are expected to soar as high as 20 million with mortality account of 12 million (Notani, 2001). The burden of new cases for the top ten sites for five time periods: 1975, 1980, 1985, 1990 and 2002 are shown in table-1 for men and table-2 for women (Notani, 2001; Parkin et al, 2005).

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>464.3</td>
<td>513.6</td>
<td>676.5</td>
<td>771.8</td>
<td>965.2</td>
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<tr>
<td>Stomach</td>
<td>421.7</td>
<td>408.8</td>
<td>472.5</td>
<td>510.0</td>
<td>603.4</td>
</tr>
<tr>
<td>Colon and rectum</td>
<td>251.2</td>
<td>286.2</td>
<td>331.0</td>
<td>401.9</td>
<td>550.4</td>
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<tr>
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<td>235.8</td>
<td>291.2</td>
<td>396.1</td>
<td>679.0</td>
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<tr>
<td>Liver</td>
<td>182.5</td>
<td>171.7</td>
<td>214.2</td>
<td>316.3</td>
<td>442.1</td>
</tr>
<tr>
<td>Mouth and Pharynx</td>
<td>232.9</td>
<td>257.3</td>
<td>269.6</td>
<td>257.7</td>
<td>337.9</td>
</tr>
<tr>
<td>Esophagus</td>
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<td>202.1</td>
<td>195.9</td>
<td>212.6</td>
<td>315.4</td>
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<tr>
<td>Bladder</td>
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<td>167.7</td>
<td>181.7</td>
<td>202.5</td>
<td>273.8</td>
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<td>139.9</td>
<td>180.8</td>
<td>192.5</td>
<td>175.1</td>
</tr>
<tr>
<td>Leukemia</td>
<td>100.3</td>
<td>106.9</td>
<td>120.5</td>
<td>130.3</td>
<td>171.0</td>
</tr>
</tbody>
</table>

Interpretation of these tables points to a trend of steady increase in cancer cases from year 1975 to 2002. The incidences have gone up by 45% in men and 31% in women. Furthermore, considering the cancer incidences by economic divide, i.e. developed and developing countries, there appears significant difference in the number of new cancer cases (Notani, 2001). Of the 8.1 million cases reported, 4.0 million occurred in the developed and 4.1
million (50.5%) in the developing countries while of the 5.18 million estimated deaths 55% (2.85 million) occurred in the developing countries including India (Notani, 2001). In India, the annual estimates of cancer for the year 2001 was 0.98 million and the annual mortality in 2000 was 0.7 million (ICMR bulletin, 2001).

Table-2 : Estimated number of new cancer cases (thousands) worldwide in women (Notani, 2001; Parkin et al, 2005)

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>541.2</td>
<td>572.1</td>
<td>719.1</td>
<td>795.6</td>
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<tr>
<td>Colon and rectum</td>
<td>255.6</td>
<td>285.9</td>
<td>346.5</td>
<td>381.0</td>
<td>472.7</td>
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<tr>
<td>Cervix</td>
<td>459.4</td>
<td>465.6</td>
<td>437.3</td>
<td>371.2</td>
<td>493.3</td>
</tr>
<tr>
<td>Stomach</td>
<td>260.6</td>
<td>260.6</td>
<td>282.3</td>
<td>287.2</td>
<td>330.5</td>
</tr>
<tr>
<td>Lung</td>
<td>126.7</td>
<td>146.9</td>
<td>219.3</td>
<td>265.1</td>
<td>386.9</td>
</tr>
<tr>
<td>Ovary</td>
<td>NA</td>
<td>137.6</td>
<td>161.5</td>
<td>165.5</td>
<td>204.5</td>
</tr>
<tr>
<td>Corpus uteri</td>
<td>NA</td>
<td>148.8</td>
<td>140.0</td>
<td>142.4</td>
<td>198.8</td>
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<tr>
<td>Liver</td>
<td>76.7</td>
<td>79.4</td>
<td>100.7</td>
<td>121.4</td>
<td>184.0</td>
</tr>
<tr>
<td>Mouth and Pharynx</td>
<td>106.6</td>
<td>121.2</td>
<td>142.8</td>
<td>105.4</td>
<td>146.7</td>
</tr>
<tr>
<td>Esophagus</td>
<td>102.3</td>
<td>108.2</td>
<td>107.6</td>
<td>103.2</td>
<td>146.7</td>
</tr>
</tbody>
</table>

The cancer burden for the top 10 sites for 2002 time periods indicates that oral cancer in men (figure-3) and women (figure-4) is one of the leading sites in both developed and developing countries. The global incidence of oral cancer is about 3,00,000 cases per year. It is also clear from the figures that incidence of oral cancer is higher in developing than developed countries. Over a third of global oral cancer burden is contributed by the countries of South Central Asia, which also include the Indian subcontinent. (Nagler, 2003; Parkin et al, 1999). Figure-5 and figure-6 show the number of new cancer cases of the ten most commonly affected sites in Indian men and women along with the number of deaths. In India, about 80,000 new cases of oral cancer are diagnosed every year (Nair et al, 2004). Both, oral and pharyngeal cancers combined are the leading cancer sites in men and account for 19 % of the estimated total of 319.7 thousand cancer cases (figure-5). While in women, these sites account for about 7% of 350.3 thousand total cases. (figure-6).
Figure-3
Estimated number of new cases and death of cancer (in thousand) in developed and developing countries (males) (Parkin et al, 2005)

Figure-4
Estimated number of new cases and death of cancer (in thousand) in developed and developing countries (female) (Parkin et al, 2005)
**Figure-5: Estimated number of new cases and deaths due to cancer in India (males) (Notani, 2001)**

![Graph showing cancer cases by site for males](image1.png)

- Total number of cancer cases: $319.7 \times 10^3$
- Total number of deaths: $231.3 \times 10^3$

**Figure-6: Estimated number of new cases and deaths due to cancer in India, (females) (Notani, 2001)**

![Graph showing cancer cases by site for females](image2.png)

- Total number of cancer cases: $350.3 \times 10^3$
- Total number of deaths: $215.6 \times 10^3$

**Figure-7: Incidence of tobacco related cancer at the Gujarat Cancer and Research Institute, Ahmedabad**

![Graph showing tobacco related cancer incidence](image3.png)
Introduction and Review of Literature

It has also been reported that world highest incidence of oral cancer is in India (Nair et al, 2004). Furthermore, the incidence of oral cancer is reported to be highest in the area of Ahmedabad (Shah and Patel, 2001). Hospital based cancer registry of the Gujarat Cancer and Research Institute, Ahmedabad showed incidence of 11,352 new cancer cases at the institute in the year 2001. Overall, 40.41% of all cancers were tobacco related cancers. Further Oral cancer was the leading site in the tobacco related cancers (figure-7).

Multistage Oral Carcinogenesis:

The development of oral cancer is a multi step process involving three subsequent, through overlapping stages- initiation, promotion and progression. In initiation step, normal oral mucosal epithelial cells are continuously subjected to attack of genotoxic agents present in betel quid, tobacco, nitrosamine and reactive oxygen species (ROS) (Jeng et al, 2001) (figure-8). Process of carcinogenesis occurs due to interaction between genotoxic agents and cellular macromolecules such as DNA, proteins and lipids. Majority of genotoxic agents form adducts with DNA. Therefore, DNA is the potential target for initiators of carcinogenesis. The promotion is a lengthy reversible process related to inactivation of tumor suppressor genes (p53 etc). Promotion is followed by progression, which is generally an irreversible phase resulting in a clonal expansion of neoplastic epithelial cells, which first may be precancerous and later may develop to cancer. Silverman et al (1984) have demonstrated that the risk of oral leukoplakia turning into carcinoma was around 5 % per year. It is also reported that 74% of incidence of oral cancer cases are the result of unattended oral premalignant lesions in India (Gupta et al, 1989). Therefore, most common types of oral premalignant lesions, oral leukoplokia and oral submucous fibrosis are important diseases to study for prevention of oral cancer. Presently, incidence of oral cancer in India for both sexes are among highest reported worldwide, mainly attributed to high prevalence of tobacco chewing and smoking habits.
Tobacco
The word tobacco was originally used to denote a "Y" shaped piece of cone or pipe called *tobago* or *tobaca* that used by Maxican Indians to inhale powdered leaves of a plant. Later, the plant came to be known by the name of device, as "*tobacco". Tobacco was introduced in Europe during late 15th century. Some time in the late 16th or early 17th century, Portuguese traders introduced tobacco in India. Since then, its use has spread swiftly to all sections of society. Initially tobacco was smoked, but later it was used for chewing and for application over the teeth and the gingiva (Bonsle et al, 1992). The World Health Organization (WHO) reported that almost 1000 million men and 250 million women are daily smokers. Tobacco consumption by smoking habits is still growing, with approximately 5.5 trillion cigarettes consumed in 2000. The consumption of smokeless tobacco products also continues to be substantial and widespread. It is estimated that, of everyone alive today, 500 million people will eventually be killed by tobacco use, with cancer being one of the main causes of deaths (Hecht, 2003). Oral cancer, in India, is caused mainly by extensive use of tobacco. Millions of people are exposed to tobacco smoke or smokeless products by smoking or chewing habits of tobacco. The differences in the incidence of oral cancer between
western countries and India reported to be due to difference in the form of tobacco usage. Tobacco smoking (cigarette) is more prevalent in western countries while tobacco chewing, smoking (bidi) and snuffing along with other ingredients like betel nuts, gutkha, lime, catechu etc. are most prevalent forms of tobacco usage in India (Jeng et al, 2001) (figure-9)

**Figure-9 : Tobacco habits in India and Western countries**

In South Asia, especially in India, bidi is the most popular form of tobacco use. It is made by tembuni leaf. It produces smaller volume of smoke as compared to cigarette because they contain small amount of coarsely ground tobacco as compared to cigarette. About 12% to 62% of men living in rural areas smoke bidi. In some areas locally made cigars are smoked often with the burning end inside the mouth. It is known as reverse smoking. Several other forms of smoking such as hookah, chillum and clay pipe are also found to be used in various regions of India. Cheroots small cigarettes made by heavy tobacco are also used. Reverse chutka smoking is more common in Andhra Pradesh and Orissa. It is more smoked by women than men. Dhumti smoking is common in Goa. It is a kind of conical cigar made by rolling leaf tobacco in a leaf of jackfruit tree or in a dry leaf of banana plant. About 4% population of Goa is smoking Dhumti. Hookli smoking is more prevalent in various parts of Gujarat and about 11% of men are habituated of hookli smoking. Chillum is a straight 10 – 14 cm long conical pipe made of clay. It is
practiced in northern and eastern states of India. Hookah smoking is also done more commonly in villages.

Tobacco is also chewed as a scented tobacco (Zarda), crude tobacco leaf, and powder. In recent years, the production and use of the commercially manufactured smokeless tobacco products called pan masala or "Gutkha" has increased many folds. Gutkha is a generic name for products that contains tobacco; areca nut and several other substances in powdered or granulated form and are sold in small aluminum foil sachets. As a commercial product, gutkha was introduced less than three decades ago, but its popularity has spread faster than the other forms of tobacco use. Yet another form of tobacco is keeping crushed tobacco and lime in the gingivo-buccal sulcus for longer period. Un-burnt tobacco is used as toothpowder and as a paste. Tobacco powder is also used for snuffing.

In India, the prevalence of tobacco use in the 10-15 years age group in males is 20% to 25% and in female is about 3%. By the time males reach 25 years of age, close to 60% have acquired the habit, while in women the prevalence is about 15% to 20%. Thus, male dominance of oral cancer has a direct relation to tobacco use (Rani et al, 2003). The main reason for the continued use of tobacco, in spite of its well-known adverse health effects, is dependence on nicotine. This compound is the major alkaloid in tobacco products, typically comprising 1 to 2% of the tobacco. The other tobacco alkaloids are found in significantly lower concentrations than that of nicotine. Important among these are nornicotine, anabasine, and anatabine. They are responsible for formation of nitrosamines compounds.

It was well established that both secondary (nornicotine, anabasine and anatabine) and tertiary amines (nicotine) can react with nitrite yielding nitrosamines (figure-10). The nitrosation of the secondary amines nornicotine, anabasine and anatabine gives the corresponding nitrosamines NNN (N-nitrosonornicotine), NAB (N'-nitrosoanabasine) and NAT (N'-nitrosoanatabine). Nitrosation of the tertiary amine, nicotine, gives NNN by
cleavage of the N-CH₃ bond with loss of formaldehyde or yields NNK or NNA [4-(methylnitrosamino)-4-(3-pyridyl) butanal] by cleavage of either the 2'-N or 5'-N bond, respectively. The formations of NNN, NNK and NNA from nicotine and NNN, NAB and NAT from nornicotine, anabasine, and anatabine have been confirmed in model studies (Hoffmann and Hecht, 1985). These alkaloid-derived nitrosamines are called "tobacco-specific nitrosamines (TSNA)". Therefore, nicotine, cotinine, nitrate and nitrite are important progenitors in the formation of TSNA. Earlier studies have reported varying NO₂+NO₃ levels in different tobacco products used in various parts of India (Pakhale et al, 1997). It is also reported that tobacco products with higher amount of NO₂+NO₃ are more carcinogenic (Malaveille et al, 1989; Hoffman and Hecht, 1985). In India, 250 million kilogram of tobacco is consumed each year. Tobacco is consumed in different forms, among which, smoking accounts for 86%, 13% in smokeless forms other than snuff and 1% as snuff (Sanghvi, 1989). However, NO₂+NO₃ contents of most of the tobacco products are not measured.

**Figure- 10 : Structure of tobacco specific nitrosamines formed by nitrosation of tobacco alkaloids**
Benzo (a) pyrene (BaP), NNK and NNN are important carcinogens found in tobacco products. Previous studies have suggested that; BaP, NNK and NNN are involved in causing oral cancer in tobacco consumers (Hecht, 2003; Hecht and Hoffmann, 1989). BaP, NNK, NNAL, and NNN all require metabolic activation for effective expression of their carcinogenic potential. There are competing detoxification pathways. A major microsomal metabolic activation pathway of BaP is conversion to BaP-7,8-diol, catalyzed by cytochrome p450 and epoxide hydrolase, followed by oxidation to BaP-7,8-diol-9,10-epoxide (Staretz et al, 1997)(figure-11). Other metabolites (such as BaP-4,5-diol,
BaP-9,10-diol, 1-OH-BaP, 3-OH-BaP, and 9-OH-BaP) result from detoxification pathways (Staretz et al, 1997). Primary microsomal metabolites of NNK and NNAL are summarized in figure-12 (Kim and Wells, 1996; Staretz et al, 1997). NNAL-glucuronide and α-Hydroxymethyl NNK-glucuronide are considered to be detoxification products. The metabolic activation of NNK proceeds by hydroxylation of the methylene and methyl carbons adjacent to the N-nitroso group yielding diazohydroxides that alkylate DNA. Thus, these carcinogen compounds have a critical role in the initiation of carcinogenesis. Therefore, the data on tobacco exposure in human can be of a significant value to understand etiology of oral cancer.

Biomarkers of Tobacco Exposure

Earlier studies have reported measurement of tobacco exposure by the means of urinary biomarkers due to certain advantages. Important among these is their quantity; which is generally sufficient for modern analytical methods, which can provide reliable data. Urinary biomarkers potentially can provide vital information on various aspects including: (i) carcinogen dose, which is important for assessing overall carcinogen exposure in tobacco habituates, (ii) mechanisms of carcinogen metabolism in humans and (iii) the distinction between individuals exposed or not exposed to tobacco products (Hecht, 2002). Nicotine and cotinine are addictive agents. Nicotine and cotinine levels in urine can be used as an indicator of tobacco habits and tobacco exposure in human. Epidemiological studies have found correlation between tobacco exposure and various type of cancer (Winn, 2001).

Chemicals with potential alkylating properties (most mutagens and carcinogens) undergo metabolic activation to form electrophilic intermediates that can bind covalently to cellular macromolecules such as DNA, RNA and proteins. One of the protective mechanisms by which the organism combats such electrophilic attack is their conjugation with glutathione. The resultant conjugated product undergo a series of enzymatic reactions to form
mercapturic acid derivative or thioether which, being more polar are excreted via urine. Thus, elevated urinary thioether excretion serves as a signal indicating increased exposure to potentially hazardous alkylating agents (Bagwe and Bhisey, 1995). Therefore, urinary thioether levels are used as the biomarkers of tobacco exposure (Bhisey et al, 1992).

**Tobacco and Free Radicals:**
Various investigators have studied tobacco carcinogenesis. N-nitroso compounds constitute the most abundant carcinogens present in tobacco with TSNA as well as ROS, which are important class of genotoxic carcinogens. The main carcinogens and genotoxic agents in pan masala and gutkha are derived from their ingredients areca nut, lime, catechu and tobacco (Nair et al, 2004). It has been displayed in table-3.

**Table-3**

<table>
<thead>
<tr>
<th>Products</th>
<th>Ingredients</th>
<th>Genotoxic agents/carcinogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gutkha</td>
<td>Tobacco</td>
<td>NNN, NNK</td>
</tr>
<tr>
<td></td>
<td>Areca nut</td>
<td>Arecoline, MNPN</td>
</tr>
<tr>
<td></td>
<td>Areca nut +lime</td>
<td>ROS</td>
</tr>
<tr>
<td></td>
<td>Catechu+lime</td>
<td>ROS</td>
</tr>
<tr>
<td>Pan masala</td>
<td>Areca nut</td>
<td>Arecoline, MNPN</td>
</tr>
<tr>
<td></td>
<td>Areca nut +lime</td>
<td>ROS</td>
</tr>
<tr>
<td></td>
<td>Catechu+lime</td>
<td>ROS</td>
</tr>
</tbody>
</table>

ROS, implicated in multistage carcinogenesis, are generated in substantial amount in the oral cavity during tobacco chewing and smoking (Nair et al, 1992,1995; Syed sultan et al, 2004). Nair et al (1987), first demonstrated that aqueous extracts of areca nut and catechu were capable of generating free radicals at pH>9.5 and further the generation of ROS was aggravated by Fe$^{+2}$, Fe$^{+3}$ and Cu$^{+2}$ present in areca-nut. Thus, these transition metal ions play the catalytic role for ROS generation. But Mn$^{+2}$ is found to inhibit the areca nut-induced production of ROS. Therefore, it is inferred that pH of
Introduction and Review of Literature

Saliva plays a prime role in the generation of ROS and their formation is likely to occur due to autoxidation, redox cycling via quinone/semiquinone radical and iron-catalysed Haber-Weiss and Fenton reactions (Nair et al, 2004). Nair et al (2004) also reported that calcium hydroxide content of lime in the presence of areca nut is a major factor responsible for the formation of ROS, which cause oxidative damage in the DNA of buccal mucosa cells of betel quid chewers. Thus, there is continuous endogenous damage caused to cellular DNA by free radicals and accumulation of such damage has been found to play a significant role in carcinogenesis.

Role of Oxidants and Free Radicals in Cancer

Over the last two decades, a great deal of evidence has linked ROS with many types of cancer. ROS are the chemical species, which are highly reactive owing to the presence of one or more unpaired electrons in their outer orbital. Many free radicals like hydroxyl, superoxide, nitric oxide and lipid peroxyl radicals are unstable and highly reactive. If excess amounts of ROS are generated and when not removed by antioxidant enzymes, can lead to the onset of oxidative stress, which cause severe metabolic malfunction and damage to biological macromolecules (Perwez Hussain et al, 2003) (figure-13). The prime targets of the ROS damage are: (i) Lipids- where the unsaturated bonds in membrane lipids are the major targets. The consequent peroxidation results in a loss in membrane fluidity and receptor alignment and potentially in cellular lysis. Moreover, lipid peroxidation products can cause DNA damage. (ii) Proteins- Free radical damage to sulfur-containing enzymes and other proteins culminates in inactivation, cross-linking, and denaturation. (iii) Nucleic Acids- Attack of ROS on nucleic acids can cause mutations, which may be carcinogenic.
Hence, these free radicals, which are produced during autooxidation of areca nut polyphenols in saliva of tobacco users play a crucial role in initiation and promotion of oral cancer.

**Nitric Oxide (NO)**

The discovery in 1987 that NO accounted for the bioactivity of endothelium-derived relaxing factor (EDRF) (Palmer et al, 1987; Ignarro et al, 1987) rapidly led to an explosion of information on the physiological and pathological roles of this molecules. Half-life of NO is very short (3-30 second). It is an inorganic free radical gas, containing odd number of electrons and can form a covalent link with other molecules by sharing a pair of electrons. NO is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH dependent enzymes called NOS (EC:1.14.13.39) located in various tissues (figure-14), and play an active role in free radical and tumor biology (Felley Bosco, 1998). The three main isoforms of NOS are neuronal (n NOS), inducible (iNOS), and endothelial (eNOS). Generally, nNOS
and eNOS are expressed constitutively in neurons and endothelial cells, respectively, while; iNOS are expressed during pathogenesis of several diseases.

**Figure-14 : Metabolism of L-arginine to citrulline and nitric oxide**

\[
\text{L-arginine} \xrightarrow{\text{O}_2} \text{NO} \xrightarrow{\text{Nitric Oxide Synthase}} \text{Nitrite (NO}_2^{-}) \xrightarrow{\text{Nitrate (NO}_3^{-})}
\]

**Figure-15 : Role of nitric oxide in carcinogenesis**

Chronic Inflammation/carcinogen Exposure

\[
iNOS \xrightarrow{\text{NO}} \text{DNA damage, inactive of repair proteins}
\]

\[
\text{Normal p} \xrightarrow{\text{Mutant p}} \text{Cytostasis Apoptosis}
\]

\[
\text{Transformed Cell} \xrightarrow{} \text{Early Cancer}
\]

NO has mutagenic properties, long-term exposure of cells to high NO concentrations resulting from iNOS induction during chronic inflammation could have an active role in carcinogenesis (Lala, 1998; Murata et al, 1997). Mutagenesis by NO can occur through several mechanisms (figure-15). DNA
damage due to deamination of nucleic acid bases has been shown in cell-free systems, bacteria, and macrophages. Transition and/or transversion of nucleic acid bases (e.g., G:C → A:T, G:C → C:G, G:C → C:G, G:C → A:T) by reactive NO products has been documented in plasmid DNA. Further, inactivation of DNA repair enzymes (e.g., alkyltransferase and DNA ligase) can occur owing to the high affinity of reactive NO species for amino acids containing thiol residues (Lala and Chakraborty, 2001). It has been reported that NO may promote carcinogenesis by inactivating the tumor suppressor oncoprotein p53. Cells containing wild type p53, when exposed to excess NO, accumulated p53 protein (Forrester et al., 1996) with a concomitant loss of its DNA binding activity (Calmels et al., 1997), which has been attributed to nitration of tyrosine residues in the protein (Chazotte-Aubert et al., 2000). Further, mutation of p53 may occur in a high NO environment. A positive correlation between total NOS activity and the prevalent form of p53 mutation (G:C → A:T at CpG dinucleotides) is reported in a study of 118 sporadic human colon tumor (Ambs, 1999) and 27 carcinoma of the head and neck (Gallo et al., 1999).

**Figure-16 : Nitric oxide exerts direct DNA damage**

![Diagram showing the effects of nitric oxide on DNA damage](image)

Other evidences have also suggested that NO exerts direct damages including DNA base deamination, peroxynitrite (ONOO-) induced adduct formation and
DNA strand breaks (Felley Basco, 1998; Yoshie and Ohshima, 1998) (figure-16). NO can react with O\textsubscript{2} to form nitrogen dioxide (NO\textsubscript{2}\textsuperscript{-}), with O\textsubscript{2}\textsuperscript{-} to form ONOO\textsuperscript{-} compound, which is more reactive, less stable and shorter-lived than NO (Lala and Chakraborty, 2001). ONOO\textsuperscript{-} produced during the reaction of NO and O\textsubscript{2}\textsuperscript{-} likely to be responsible for genetic damage (Wink et al, 1998). The reaction of O\textsubscript{2}\textsuperscript{-} with NO, depending on the relative amount present, can be 5 times higher than the decomposition of O\textsubscript{2}\textsuperscript{-} by SOD. ONOO\textsuperscript{-} is a potent mutagen that can induce transverse mutation (mainly G\textrightarrow{}T) at G-C pairs. Hence, NO through oxidative stress, nitrosative stress and formation of nitrosamine are also associated with direct DNA damage leading to cancer. By causing oxidative stress in human erythrocytes with H\textsubscript{2}O\textsubscript{2} or by increasing the intracellular calcium a gradual increase in both NO and ONOO\textsuperscript{-} is observed. Furthermore, it has been shown that breast cancer erythrocytes are subjected to higher oxidative stress by ONOO\textsuperscript{-} (100 μmoles) with a consequential increase of membrane rigidity as compared to erythrocytes from healthy individuals (Ray and Husain, 2002). Ohue et al (1994) hypothesized that NO may generate the high frequency of p53 mutations that arise at the transition from adenoma to carcinoma in situ. However, exact mechanisms about the association between NO and p53 functions are not clear.

**Role of p53 Tumor Suppressor Gene in Cancer**

The p53 tumor suppressor has been termed as "the Guardian of the Cell". P53 guards two gates: a gate to life and a gate to death. Sensing damage to DNA. p53 can initiate two processes to isolate the damaged cell and prevent its uncontrolled growth. It can halt cell division, freezing the cell at the G1 checkpoint of the cell cycle. If the damage is not repaired, cell is unable to reproduce, where p53 can also initiate the alternative pathway leading to cell death, or apoptosis (Goodsell, 1999).

Alterations in the tumor suppressor p53 gene have been reported in 53-93% of head and neck squamous cell carcinoma (Ahomadegde et al, 1995; Boyle
et al, 1993; Sakai and Tsuchida, 1992). It is also reported that p53 protein is over expressed not only in primary and recurrent oral squamous cell carcinoma but also in premalignant lesions (Kaur et al, 1994). Analysis of p53 gene alterations can be performed either by immunohistochemical, molecular or serological techniques. (Soussi et al, 1994). Serological analysis detected antip53 antibodies in sera of patients with an immune response against an abnormally high levels of p53 protein inside the tumour cells (Soussi, 2000). The serum antip53 antibodies are indirect results of a p53 gene missense point mutation. Abnormal accumulation of the mutant p53 protein in tumour cell nuclei has been found to be significantly associated with the presence of anti p53 antibodies in patients with various malignancies (Soussi, 2000). In head and neck squamous cell carcinoma significant association has been observed between anti p53 antibodies and poor clinical outcome, i.e increase risk of relapse and death (Bourhis et al, 1996). Ralhan et al (1998) have suggested potential usefulness of p53 antibodies in tobacco and betel quid abused populations for identifying high risk individuals. They reported antip53 antibodies as a surrogate marker for early p53 alterations, which can be a potential aid in early detection of oral cancer.

**Lipids and Free Radicals**

It is reported that lipids are the most susceptible targets for the attack by free radicals. Hydroxyl radicals initiate the oxidation of polyunsaturated fatty acids (PUFA) by removal of a hydrogen atom of methylene group of the fatty acid (figure-17). This removal of hydrogen can also be brought about by transition metal ions. This results in the formation of the lipid radicals. The lipid radicals formed, tend to stabilize by molecular rearrangement to form a conjugated diene. The conjugated diene readily combines with oxygen to give peroxyl radical. These peroxyl radicals are capable of removal of hydrogen from another lipid molecule (from an adjacent fatty acid chain) propagating a chain reaction. The peroxyl radical combines with hydrogen atom to give lipid hydroperoxide, generally called as lipid peroxide. The peroxyl radicals are the
Figure-17: Mechanism of lipid peroxidation by ROS

- Fatty acid with three double bonds
- Hydrogen abstraction by Hydroxyl radical
- Unstable carbon radical
- Molecular Rearrangement
- Conjugated diene
- Oxygen uptake
- Peroxyl radical
- Hydrogen abstraction Chain reaction
- Lipid Hydroperoxide

Malondialdehyde
4-hydroxynonenal
ethane/pentane
etc

Carries of the chain reaction. They can oxidize PUFA molecules and initiate new chain producing lipid hydroperoxides that further breaks down (in presence of the transition metal ions) to yield more radical species and to a wide range of compounds, notably aldehydes, many of them being cytotoxic. These aldehydes can diffuse from original site of attack and spread the damage to other parts of cell lipid peroxidation, which has been implicated in a wide range of tissue injuries and diseases. Lipid peroxides are relatively short-lived. They are either reduced by glutathione peroxidases to non-reactive fatty acid alcohols or they react with metals to produce a variety of products, which are reactive (e.g. epoxides, aldehydes, etc.). The major aldehyde product of lipid peroxidation is MDA, which is mutagenic in bacterial and mammalian cells and carcinogenic in rats. MDA reacts with DNA to form adducts to dG, dA and dC. M_{i}G and presumably M_{i}A and M_{i}C also can be made by the reaction of the corresponding bases with propenal, providing an alternate route for their generation by direct oxidation of DNA (Marnett, 2000).
Role of Prostaglandin E2 in Cancer

NNK bioactivation leads to the production of ROS (Kim and Wells, 1996), which are known to activate the nuclear factor Kb (NFkB) which act as positive regulatory element of COX-2 expression (Kosaka et al, 1994; Sen and Packer, 1996) (figure-18). Cyclooxygenases (both COX-1 and COX-2) are key enzymes in the synthesis of prostaglandins (PGs). COX-1 and COX-2 catalyze the same reaction of conversion of arachidonic acid to prostaglandin 5-hydroxy analogue. They have different biological roles. The constitutively expressed COX-1 is known to carry out "house keeping" functions including the production of PGs under normal physiological condition. In contrast, the COX-2 protein is normally absent in most tissues. COX-2 expression can be induced by inflammatory mediators, cytokines, mitogens, growth factors, oncogenes and carcinogens and has been shown to inhibit apoptosis (Wallace, 2002). Over expression of COX-2 is seen in several neoplastic tissues including head & neck and lung cancer (Park et al, 2003; Wallace, 2002). Rioux and Custonguay (1998) have explained that COX-2 metabolized NNK more effectively than COX-1. Cyclooxygenase catalyzes the synthesis of prostaglandin from arachidonic acid. Prostaglandin E2 promotes tumorigenesis by stimulating angiogenesis and inhibiting immune surveillance (Ben-Av et al., 1995; Wallace, 2002).

Figure-18: Pathway of cyclooxygenase induction by NNK in U937 macrophages (Rioux and Castonguay, 2000)
Rioux and Castonguary (1998) have reported plasma prostaglandin E2 levels higher NNK treated mice as compared to untreated mice. Jeng et al (2000) have suggested that areca nut ingredients play a critical role in the pathogenesis of oral submucous fibrosis and oral cancer via their stimulatory effects on the PGs, COX-2 production and associated tissue inflammatory responses.

**Antioxidant and Detoxification Enzymes**

**(1) Antioxidant Enzymes**

Antioxidants are substances that either directly or indirectly protect cell against adverse effects of free radicals. Several biologically important compounds have been reported to have antioxidant functions. SOD, catalase and glutathione peroxidase (GPx) are known enzymatic antioxidants.

The SOD (EC:1.15.1.1) enzyme was discovered in 1968 by McCord and Fridivich (Gutteridge and Halliwell, 1994). This enzyme destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by catalase or GPx reaction. The low levels of superoxide are constantly generated by aerobic respiration. The electron transport chain of mitochondria, which is meant to escort four electrons to molecular oxygen to form water, occasionally leaks a single electron. SOD converts superoxide to hydrogen peroxide and molecular oxygen. Besides, this superoxide reduces Fe(III) to Fe(II), releasing the iron from storage sites so that it can react with hydrogen peroxide and produce hydroxyl radicals, which can damage to DNA.

\[
\text{SOD} \\
2 \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

The metals bound to SOD catalyses the reaction of two \text{O}_2^- molecules with \text{H}^+ ions to form \text{H}_2\text{O}_2 and \text{O}_2. In human, the manganese containing SOD
(Mn SOD) in mitochondria remove $O_2^-$ produced as a result of electron leakage on to $O_2$ from the mitochondrial electron transport chain and by mitochondrial oxidase enzymes. The copper and zinc containing SOD (Cu-Zn SOD) deals with $O_2^-$ from cytosolic oxidases and from the cytochrome p450 enzymes, which are located in the endoplasmic reticulum of the cells. Some Cu-Zn SOD are present in nucleus and some are present in peroxisomes. These are small organelles, bound by a single membrane, that contain a range of oxidase enzymes.

\[
\text{SOD} \quad 2O_2^- + 2H^+ \rightarrow H_2O_2 \quad \text{catalase} \quad H_2O_2 \rightarrow H_2O + 1/2 O_2
\]

Catalase (EC:1.11.1.6) is a tetrameric haeme enzyme consisting of four identical tetrahedral arranged subunits of 60 kDa. Therefore, it contains four ferriprotoporphyrin groups per molecule, and its molecular mass is about 240 kDa. Catalase is one of the most efficient enzymes that react with $H_2O_2$ to form water and molecular oxygen. Therefore, catalase protects cells from hydrogen peroxide within them.

Glutathione peroxidase (EC1.11.1.19) enzyme was discovered by Mills in 1957 (Oberlay,1999). This enzyme catalyzes the reduction of a variety of hydro peroxides (ROOH and $H_2O_2$) using reduced glutathione (GSH), thereby protecting mammalian cells against oxidative damage.

(2) Enzymes of detoxification system

All organisms are exposed continuously to toxic chemicals either exogenously i.e. from surrounding environment including workplace or endogenously due to life style habits e.g. smoking or chewing of tobacco etc. The chemical origin of human malignancies was recognized by observations of unusual cancer incidence in certain occupational groups (Yuspa and Shield, 1997). To ensure survival in the face of a wide spectrum of harmful chemicals, various defense mechanisms have been evolved to protect cells against these toxic
compounds. Metabolism of these foreign compounds in the body usually involves two stages of detoxification commonly referred to as phase-I & II. One of the first steps in the development of head and neck cancer may be the binding of exogenic electrophonic compounds to the DNA of the mucosa cells (Dgawa et al, 1994; Sryfter et al, 1994). Most carcinogens in tobacco require metabolic activation by phase-I enzymes (cytochrome p450, Aryl hydrocarbon hydroxylase) to manifest their carcinogeneic effects (Garcia Closas Met et al, 1997; Goto et al, 1996; Seidergard et al, 1990). These activated metabolites are subjected to further detoxification by phase-II enzymes. Among the phase-II detoxification system, the glutathione-S-transferase (GST) and glutathione reductase (GR) play a significant role in providing protection against the chemical stress.

Among the enzymes of detoxification system, the GSTs play a critical role in providing protection against electrophilies and products of oxidative stress. The GSTs are multigene super family of enzymes that catalyze a number of distinct glutathione dependant reactions. The fundamental basis for all the various catalytic activities of GST is the ability of the enzyme to lower the pKa of –SH group of reduced GST from 9.0 in aqueous solution to 6.5 when bound in the active site. Evidence suggest that glutatione exist as the thiolate anion at neutral pH when complexes with GST. It is proposed that once glutathione substrate is formed in the active site of GST, it becomes capable of reacting spontaneously by nucleophilic attack, with electrophilic xenobiotic that are situated in close proximity. The formation of thioether bond between electrophilies and GSH always yields less reactive conjugates than the parent compound and therefore the actions of GST generally result in detoxification. Once formed, the conjugates can be transported from the cell by ATP dependent GST efflux pumps. Majority of GST substrates are either xenobiotic or products of oxidative stress (Hayes and Pulford, 1995).

Human GSTs are encoded by gene superfamily. Five classes of human GSTs including α, μ, π, θ and κ have been described. These classes of human GSTs
are expressed to various degrees in different human tissues. Within each human GST class, there are various isoenzymes, each of which is encoded by a specific gene locus. These isoenzymes are designated as GSTA1-GSTA5 within the α class, GSTM1-GSTM5 within the μ class, GSTT1 and GSTT2 within the θ class, GSTP1 within the π class and GSTK1 within the κ class. But the different classes of human GSTs are expressed in various degrees in different human tissues. For example, GSTs within the alpha class are highly expressed in liver, kidney and testis. The μ class of GSTs is expressed primarily in liver, brain, kidney, testis, oral and lung tissue, with each isoenzyme having a different degree of expression in each type of tissue. The θ class of GSTs are expressed primarily in liver and kidney, with relatively low expression in the lung (Mohr et al, 2003).

Following a 1981 report by Board that first described GST M1 polymorphism (Board, 1981), it has been widely postulated that GST M1 null genotype, which results in a deficiency of the GST M1 isoenzyme, might be associated with increased susceptibility to develop cancer and other diseases related to toxic chemical exposures. This has lead to numerous investigations regarding the effect of the GST M1 null polymorphism on the development of various types of cancer (Mohr et al, 2003).

GR is a ubiquitous enzyme, which catalyzes the reduction of oxidized glutathione (GSSG) to GSH. GR is essential for the glutathione redox cycle that maintains adequate levels of reduced cellular GSH, thus playing a crucial role in detoxification of ROS. The enzyme has got significant role in protein and DNA biosynthesis. This homodimeric enzyme is a member of flavoprotein disulfide oxidoreductase family. Each subunit has four domains; beginning at the N-terminus: an FAD-binding domain, an NADPH-binding domain, a central domain, and an interface domain. The active site of GR is at the dimeric interface. Since the GSSG binding site is composed of residues from both subunits, only the dimeric form is active.
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\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+ + 2 \text{GSH} \]

Most biological reactions, including carcinogenesis, are complex processes involving thousands of compounds, their metabolites and intermediates. Stich and Anders (1989) focused on the pros and cons of ROS being involved in the development of oral cancer among tobacco and areca-nut chewers. They mentioned that bursts of ROS generation occur at different stages of carcinogenesis and are caused by different mechanisms. However, despite considerable efforts, the precise mechanisms by which ROS can directly or indirectly affect neoplastic transformations are still largely unknown. This could be due to the ubiquitous presence of ROS, which makes it difficult to attribute one particular role to them. ROS are formed even during the course of normal metabolism. Whether 'spontaneous' cancers are initiated by endogenously formed ROS is an intriguing but still unresolved issue.

Several antioxidant enzymes have been studied from circulation in cancer. It has been reported that tobacco smoke increases catalase, GPx and GR activities in experimental mice (Koul et al, 2001). Elevated levels of certain antioxidant enzymes in blood of individuals having environmental tobacco exposure at work are also reported (Halliwell and Cross, 1994). Diken et al (2001) have found that long-term smokers have higher antioxidant enzyme activities as compared to short-term smokers or nonsmokers. Ray et al (2000) documented significantly higher rate of superoxide radicals and \( \text{H}_2\text{O}_2 \) production as well as higher activities of SOD and GPx in breast cancer patients as compared to the controls. Serum SOD and erythrocyte catalase activities were significantly lower in patients with tonsillar carcinoma as compared to control group (Sharma et al, 1997). Zima et al (1996) showed that SOD and GPx activities were lower in multiple myeloma patients.
Erythrocyte antioxidant enzymes such as SOD, catalase, GPx, GR and GST were studied in oral cancer and found to be lower as compared to controls (Sabitha and Shyamaladevi, 1999). Immunohistochemical studies in oral and pancreatic carcinoma have demonstrated lower staining intensity of SOD, catalase and GPx in malignant tissues than their corresponding adjacent normal mucosa (Yang et al, 2002; Cullen et al, 2003). Subapriya et al (2002) from India studied antioxidant enzymes from tissue and blood samples from oral cancers. They found tissue levels of SOD and catalase were decreased as well as lipid peroxidation was also decreased in malignant tissues while GSH and GSH dependant enzymes elevated in malignant tissues as compared to normal tissues. In contrast, enhanced lipid peroxidation and decreased antioxidant enzymes were observed from venous blood. Abiaka et al (2002) showed that erythrocyte SOD and GPx activities were lower in gastric, breast and other malignancies as compared to control subjects. SOD, GPx and GST activities estimated from mitochondrial malignant tissues of colorectal carcinoma showed increase in the activity of these enzymes as compared to adjacent normal tissue fraction (Kanbagli et al, 2000).

Thiols are extraordinarily efficient antioxidants protecting the cells against consequences of damage induced by free radicals. Antioxidant properties of thiol compounds depend on different mechanisms. These compounds can act as thiol/disulfide component of redox buffer, free radical scavengers and chelators of metal ions.

As a thiol compound, GSH fulfils a very important role of an antioxidant in the cells. GSH is relatively “resistant” to spontaneous oxidation, can enter a non-enzymatic reaction with hydroxyl radical (OH\(^{\cdot}\)) (cytotoxic product of fenton reaction) and react with \(\text{NO}_3^{-}, \text{ONOO}^{-}, \text{O}_2^{-}\) (Wlodek, 2002).
Glutathione also plays a significant role in detoxification of H$_2$O$_2$ and organic peroxides created during lipid peroxidation, in which GPx and GR are engaged (figure-19). In antioxidant reactions, the reduced GSH is oxidized yielding disulfide (GSSG), which can be again reduced to thiol with the participation of GR and NADPH. These reactions constitute an important redox cycle in the cells. When the peroxidative processes remain at physiological level, and at sufficient availability of NADPH and activity of GR, high physiological GSH/GSSG ratio is maintained in normal range. Total thiol and lipid peroxidation were also studied in cancer patients. It is interesting to note that cancer patients show an accelerated shift to more oxidized conditions (Hack et al, 1990). Bounous and Molson (2003) suggested that during the progression of cancers, the antioxidant buffer activity may progressively decline and this could well be related to depletion of the thiol (-SH) in the redox equation. Liu et al (1998) showed that a natural cysteine donor, whey protein concentrate, could also inhibit tumours by directly increasing cellular thiol levels. It has been reported that tumours often use host substrates for growth, and many tumours contain the capacity to use circulating glutathione of the host, which was shown experimentally by decrease in serum GSH
concentration and higher tumour GSH levels in tumour bearing mice (Blumberg et al., 1995). The GSH levels were also observed to be higher in tumours than normal mucosa in head and neck cancer patients (Parise et al, 1994). In contrast, content of GSH in neoplastic tissue was lower than that of corresponding tumour-free tissue in human hepatoma and lung cancer (Corrocher et al, 1986; Saydam et al, 1997). Plasma and erythrocyte glutathione were reported to be lower in cervical cancer patients as compared to controls (Mukundan et al, 1999). Thiol levels were also reported to be lower in cancer patients as compared to controls (Rovere et al, 2000). Study on cigarette smoking and oxidative stress and antioxidant enzymes levels have shown that long-term smokers had significantly lower GSH levels while GSSG increased as compared to controls and short-term smokers (Diken et al, 2001). In contrast, increase in blood GSH in smokers has also been observed in few studies (Costagliola et al, 1990; Sinues et al, 1990).

**Objectives of the Study:**

The points discussed above and the review of literature can be summarized as follows:

(i) India has highest incidence of oral cancer in the world, (ii) tobacco habits (mainly chewing) are highly prevalent in the population (iii) urinary nicotine, cotinine, thioether and NO\textsubscript{2}+NO\textsubscript{3} can be used as indicators of tobacco exposure, (iv) Free radicals (end products of nitric oxide and MDA) and associated biomarkers (prostaglandin E\textsubscript{2} and antip53 antibodies) could be helpful for prevention, early diagnosis and treatment monitoring of oral cancer (v) free radicals including nitric oxide and lipid peroxyl are detoxified by antioxidant (SOD and catalase) and detoxification (GST and GR) enzymes. Therefore, alterations in SOD, catalase, GST and GR levels could be helpful for prevention, early diagnosis and treatment monitoring of oral cancer patients.
Hence, considering of all these facts, the objectives of the study were as follows:

(1) **NO$_2$+NO$_3$ levels in different tobacco products and assessment of tobacco habits as risk factors for oral cancer**

- To study NO$_2$+NO$_3$ levels in various tobacco products.
- To evaluate tobacco habits with duration and frequency of consumption as risk factors for OPC and oral cancer.

(2) **Urinary biomarkers of tobacco exposure in the subjects**

- To estimate urinary nicotine and cotinine by HPLC method as well as urinary thioether and NO$_2$+NO$_3$ in healthy individuals without habit of tobacco (NHT), healthy individuals with habit of tobacco (WHT), patients with OPC and oral cancer patients.
- To compare urinary biomarkers between NHT and the subjects either with tobacco chewing or smoking habits.
- To compare urinary nicotine, cotinine, thioether and NO$_2$+NO$_3$ levels with tobacco abstinence and non-abstinence groups of oral cancer.
- To study association of risk of oral cancer with urinary biomarkers.
- To study comparison of alterations in urinary thioether and NO$_2$+NO$_3$ with urinary nicotine and cotinine levels.

(3) **Study of NO$_2$+NO$_3$, oxidative stress related biomarkers, antioxidants and detoxification enzymes in healthy individuals, patients with OPC and oral cancer**

- To evaluate free radicals and associated biomarkers (plasma NO$_2$+NO$_3$, prostaglandin E2, antip53antibodies and MDA levels in NHT, WHT, patients with OPC and oral cancer patients.
- To estimate antioxidant (erythrocyte SOD and catalase) and detoxification (GST and GR in erythrocyte and plasma) enzymes as well as thiol levels in NHT, WHT, patients with OPC and oral cancer patients.
- To compare circulating biomarker levels in NHT and the subjects with either tobacco chewing or smoking habits.
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- To estimate circulating biomarker levels in tobacco non-abstinence and abstinence groups of oral cancer.
- To study comparison of circulating biomarkers with tobacco exposure.
- To study correlation of circulating biomarkers with clinicopathological parameters.
- To study association of circulating biomarkers with clinical response.
- To study correlation between the circulating biomarkers.