Discussion
Oral cancer incidence varies strikingly around the world. In parts of Southeast Asia and India, it is one of the most common cancers, in contrast to western countries where it accounts for only 1 to 4% of total malignancies. It has been also reported that 90% of oral cancer cases in South Asia among men are attributed to tobacco use. Tobacco use is a burning problem and a major factor for high incidence of oral cancer in India. In India, 47,000 oral cancer related deaths occur every year. The poor survival and high mortality rates are due to late presentation, recurrence, and development of second primary tumor. Hence, scientific documentation of hazardous effects of tobacco products and development of biomarkers of tobacco exposure as well as biomarkers in the area of predictive oncology might be helpful for prevention, early diagnosis and management of oral cancer. Considering the role of tobacco, in the causation of OPC and oral cancer, the present investigation included NHT, WHT, patients with OPC and oral cancer patients as the subjects. The current investigation evaluated: (i) NO\textsubscript{2}+NO\textsubscript{3} contents of tobacco products and Pan masala (ii) OR analysis (iii) urinary biomarkers (nicotine, cotinine, thioether and NO\textsubscript{2}+NO\textsubscript{3}) of tobacco exposure (iv) plasma levels of the biomarkers including: NO\textsubscript{2}+NO\textsubscript{3}, prostaglandin E2, MDA, antip53antibodies, GST, GR and thiol and (v) erythrocyte levels of the biomarkers including GST, GR, SOD and catalase.

**NO\textsubscript{2}+NO\textsubscript{3} levels in different tobacco products**

Extensive research on tobacco has conclusively demonstrated that the nicotine-derived nitrosamines like NNK and NNN as well as nornicotine, anabasine and anatabine-derived nitrosamines significantly contribute in tobacco carcinogenesis. The present study found higher levels of NO\textsubscript{2}+NO\textsubscript{3} in tobacco alone, snuffing products and bidi. NO\textsubscript{2}+NO\textsubscript{3} levels in cigarette and gutka were 0.14 and 0.13 mg/gm, respectively. NO\textsubscript{2}+NO\textsubscript{3} levels in pan masala were 0.03 mg/gm (figure-28). It is reported that amount of TSNA is positively associated with nitrite concentration. Therefore, reduced nitrate contents of tobacco products are reported to be less harmful (Hoffmann and
Previous studies have also reported that elevated nitrate levels in tobacco generate significantly higher yield of volatile N-nitrosamines in smoke and, more importantly, raise TSNA levels in smoke. Inhalation of nitrogen oxides (components of cigarette smoke) increases the endogenous N-nitrosamine formation in smokers, which was correlated with the elevated excretion of N-nitrosoproline in smokers' urine (Adams et al, 1988). Pakhale et al (1997) also reported NO\textsubscript{2}+NO\textsubscript{3} contents of various tobacco products collected from various parts of India. They reported higher NO\textsubscript{2}+NO\textsubscript{3} contents in many tobacco products, which is in accordance with current data. The authors further recommended regular evaluation of the parameters in tobacco and tobacco product in India for selection of varieties with lower levels of tobacco alkaloids. They suggested that formation of TSNA can be reduced by adopting modified storage and processing conditions. The NO\textsubscript{2}+NO\textsubscript{3} contents in the tobacco products analysed in the present study were comparable with that observed by Pakhale et al (1997). Recently, Rodu (2004) documented that levels of TSNA in American moist snuff products were higher than those in their Swedish counter parts, but levels in contemporary products were uniformly low.

**Odds Ratio analysis**

Retrospective studies are based on the assessment of set of sample of subjects with the disease (cases) and a separate sample of subjects without the disease (controls). Retrospective determination of the distribution of the risk among cases and controls is calculated, by computing OR from the data. The OR assumes values between zero and \( \infty \) (infinity). A value of zero indicates no association between the risk factors and disease status. A value less than 1 indicates reduced risk of the disease and the OR greater than 1 indicates increased risk of the disease among subjects in whom the risk factor is present. The risk of oral cancer in betel quid and tobacco chewers was suspected as early as in 1908 (Bentall, 1908). By late 1960's, several studies demonstrated association between betel quid chewing and other forms of tobacco use with oral cancer in India (Sinha et al, 2003; Wahi, 1968). The
first case-control study, showing relation of tobacco consumption and oral cancer was reported by Meeletti et al (1989). Several other epidemiological studies have been reported to confirm the relationship between tobacco consumption and oral diseases (Banoczy, 2001; Hashibe et al, 2000; Hecht, 2003; Johnson, 2001). In the present study, small number of cases and controls were included for OR analysis. This study from Gujarat, also found positive association with smoking as well as chewing habits of tobacco with duration and amount of consumption and oral cancer as well as OPC (table-7). These results are consistent with previous data from other parts of India (Znaor et al, 2003). Previous reports also observed that tobacco habits were significantly associated with an increased risk of oral submucous fibrosis and oral leukoplakia (Lee et al, 2003). A significantly positive association between smoking and leukoplakia was consistently observed. Few studies have also reported positive association between betel nut chewing and OPC as well as oral cancer. Shiu et al (2000) reported a statistically significant relationship between pan (a mixture of tobacco and betel nut with betel quid) chewing and malignant transformation. Moreno-Lopez et al (2000) reported that risk of oral cancer was associated with cigarette smoking, which was increased with the increase in the number of cigarette smoked per day. The OR for the consumption of 6-20 cigarettes per day was 3.1 (95% CI, 1.4-6.7) and for more than 30 cigarettes per day were 7.96 (95% CI, 3.29-19.29). It was also reported that the risk of developing oral cancer was five to nine times greater in smokers than non-smokers, and this risk was also 17 times greater for extremely heavy smokers of 80 or more cigarettes per day than non-smoker (Naville and Day, 2002). The data from present study also revealed positive association between frequency and duration of tobacco (smoking and chewing) and risk of OPC and oral cancer (table-7). Znaor et al (2003) reported tobacco chewing as the strongest risk factor for oral cancer in India. They also observed a significant relationship for dose, duration and amount of tobacco consumed by subjects with chewing and smoking habits with the development of oral cancer.
Urinary biomarkers of tobacco exposure in the subjects

Urinary nicotine and cotinine can be detected using ultra-violet spectroscopy (Schmidt, 1968), HPLC, thin layer chromatography (Beryy and Grove, 1971) and gas chromatography (Feyerabend et al, 1975) after basic extraction. Among these methods, ultra-violet spectroscopy cannot differentiate between nicotine and cotinine. Other methods including gas chromatography and thin layer chromatography are cumbersome, costly or less sensitive. But, the major advantage of HPLC method used in the current study was the rapidity of elution of nicotine and cotinine and distinct separation leading to clear detection of these compounds. In present study, extraction of urinary nicotine and cotinine was done under alkaline condition using chloroform reagent according to the Lequang et al. (1989). Quantification of nicotine and cotinine residues by HPLC using U.V. detector was carried out according to the method of Watson et al (1977). Thus, HPLC method used in the current study was a modified method using combination of previous methods. Standardization was done considering various variables (Figure-21,22). Ghosheh et al (2000) also reported that HPLC method is suitable for determination of cotinine and nicotine metabolite levels in large numbers of samples. Therefore, this HPLC method may be useful for screening of tobacco exposure in large studies. ROC curves showed good discriminating efficacy between NHT and WHT, between NHT and patients with OPC as well as between NHT and oral cancer patients (Figure-34). Thus, the result reveals that this method is highly sensitive and specific for urinary nicotine and cotinine estimation.

Cotinine, the proximate metabolite of nicotine has been extensively used as the biomarker of nicotine uptake from tobacco products. Cok and Ozturk (2000) suggested that cotinine can be widely used as a biomarker of average daily intake of nicotine because (i) it is a major metabolite of nicotine and has a much longer half-life than nicotine. (ii) it is tobacco specific. (iii) Cotinine is easily measurable in urine, plasma or saliva. (iv) it is presumed to be superior
to thiocyanate and carboxyhaemoglobin levels on the basis of specificity and (v) renal excretion of nicotine is influenced by urinary pH, whereas cotinine is not influenced. Several reports have documented that urinary nicotine and cotinine can also be used as biomarkers of environmental tobacco smoke (ETS) exposure (Benowitz, 1996). It is also reported that urinary nicotine and cotinine are the most specific and sensitive biomarkers for ETS exposure (Benowitz, 1996). The measurement of cotinine concentration in biological fluids has been widely used by various scientists to evaluate ETS exposure, because cotinine reflects exposure to nicotine, which is specific for tobacco exposure (Benowitz, 1999). Present study compared urinary nicotine and cotinine levels between children and NHT. Urinary nicotine and cotinine levels were higher in NHT than children (figure-31). The higher levels of nicotine and cotinine in NHT may be due to ETS exposure. Crawford et al (1994) reported that cotinine levels were significantly higher in children whose mother smoked tobacco than the children whose mother didn’t smoke. Hence, present study included those children whose mother didn’t used tobacco. Bhisey et al (1992) also reported that mean levels of urinary cotinine were higher in passive smokers (bidi roller) than unexposed individuals. Cok and Ozturk (2000) suggested that high concentration of cotinine values in passive smokers could be attributed to factors such as duration of exposure and intensity of smoking. The present study also showed that urinary nicotine and cotinine levels were significantly elevated in WHT as compared to NHT (figure-31). The non-abstinence group of oral cancer patients showed significant elevations of urinary nicotine and cotinine levels as compared to abstinence group (table-10). There were only two patients in the tobacco abstinence group of OPC. Therefore, comparison of abstinence and non-abstinence groups of OPC was not carried out in the present study. But representative pattern showed faint peaks of urinary nicotine and cotinine in abstinence group of OPC (figure-30). Further, the healthy individuals and oral cancer patients having tobacco smoking and chewing habits were also found to have higher levels of urinary nicotine and cotinine as compared to
Discussion

NHT (table-8,9). Earlier studies have reported higher urinary cotinine levels in healthy smokers and chewers as compared to non-habitués. Cotinine levels were also associated with frequency of smoke and smokeless tobacco consumption (Cok and Ozturk, 2000; Surmen-Gur et al, 2003). Ong et al (1994) have analysed urinary cotinine levels by HPLC method with U.V. detector and reported higher levels in the smokers than the non-smokers. They also suggested that (i) the amount of nicotine inhaled by smokers depend not only on the number of cigarettes smoked, but also on the amount of nicotine per cigarette, the smoker’s inhalation pattern and the length of each cigarette smoked. (2) there was a great variation in the metabolism of nicotine and cotinine among individuals. Noteworthy observation of the present study was that urinary nicotine and cotinine were also higher in oral cancer patients with no tobacco habit as compared to NHT. This may possibly be due to false tobacco history given by the subjects due to various reasons. However, number of oral cancer patients without habits of tobacco was also very less. All of these results supported that the HPLC method using U.V. detector was sensitive and economic way for estimation of urinary nicotine and cotinine as biomarkers of tobacco exposure.

Several chemicals are metabolized in the body to reactive electrophiles, which are capable of interacting with nucleophilic cellular macromolecules and cause genotoxicity. Glutathione, a cellular nucleophile prevents such interaction by conjugating with the electrophilies, which are further metabolized and excreted as thioethers in urine (Sorsa et al, 1982). In the present study, urinary thioether levels were significantly higher in WHT as well as WHT with only smoking habits than the NHT. (figure-32, table-9).

The method for determination of total thioethers in urine was applied in a substantial number of studies comparing smokers and non-smokers. Cigarette smokers excrete significantly higher levels of thioether than non-smokers (Hecht, 2002; Scherer et al, 1996). Bhisey et al (1991) and Scherer et al
Discussion

(1996) found higher urinary thioether levels in smokers as compared to the chewers. The authors suggested that smokers receive exposure to large amount of tar, active oxygen generated from smoke and pyrolysates produced in the burning tip, while; chewers were exposed to unburnt tobacco constituents. The differences in the nature of chemicals and mode of exposure may be responsible for the lack of increase in urinary thioether excretion in chewers (Bhisey et al, 1991). Bhisey et al (1991) also reported that urinary thioether excretion was similar in tobacco chewers and controls. Earlier reports also showed that cigarette smoke leads to increased urinary thioether excretion. In the present study, urinary thioether levels were significantly elevated in oral cancer patients than NHT and WHT (figure-32). It has been reported that method for urinary thioether can't provide information about the structure of electrophile, which are detected in urine as a conjugates (Hecht, 2002). Kuralay and Yildiz (2001) suggested that use of non-specific urinary thioether levels and GST activity determination seems to be the reliable indicators for the presence of laryngeal cancer in smokers. ROC curve analysis in this study revealed that urinary thioether levels have good discriminately efficacy between oral cancer patients and NHT as well as between patients with OPC and NHT (figure-34). These results indicate that urinary thioether is a highly sensitive marker for tobacco exposure in the subjects.

Nitrates in biological fluids have been determined by colorimetric assay, either by direct nitration, or by oxidation of organic compounds to produce a colored complex (Nicholas and Nason, 1957; West and Ramachandran, 1966). However, these methods lack specificity due to interference from biological materials. Enzymatic and ion-chromatographic methods (Cortas and wakid, 1990; Lee et al, 1986) were more sensitive and specific but they required expensive reagents and equipments. The method based on reduction of nitrate to nitrite using cadmium metal, followed by estimation of nitrite by Greais reagent (Cortas and Wakid, 1990) is more commonly used because it is sensitive, specific and inexpensive than others (Follett and Ratcliff, 1963;
Hegesh and Shiloah, 1982; Usher and Telling, 1975). Present study also employed the above method for estimation of NO₂+NO₃. ROC curves showed that NO₂+NO₃ could discriminate between NHT and oral cancer (figure-34). These results indicated that this method is highly sensitive which is in accordance with earlier reports (Cortas and Wakid, 1990).

The endogenous formation of N-nitrosamines in humans following ingestion of a source of nitrate has been demonstrated (Malaveille et al, 1989). Increased excretion of N-nitrosoproline and other N-nitrosamino acids by cigarette smoking in the subjects was significantly correlated with a higher exposure to nitrosating agents or nitrosamine precursors present in cigarette smoke (Malaveille et al, 1989). In the current investigation, urinary NO₂+NO₃ levels were higher in WHT, patients with OPC and oral cancer patients than NHT (figure-33). Urinary NO₂+NO₃ levels were also elevated in WHT, patients with OPC and oral cancer patients having habits of chewing or smoking as compared to NHT (table-8,9). The results suggested that urinary NO₂+NO₃ levels were associated with tobacco consumption. A positive relationship between the extent of tobacco exposure and urinary nitrate levels has been reported earlier (Malaveille et al, 1989). In the present study, the values of urinary NO₂+NO₃ were comparable between non-abstinence and abstinence groups of oral cancer patients. The urinary NO₂+NO₃ levels were also significantly elevated in patients with OPC and oral cancer patients (habitues and non-habitues) than WHT. Therefore, the results indicated that urinary NO₂+NO₃ levels might be associated with the etiology of cancer. Wu et al (1993) also suggested that N-nitroso compound or nitrate-derived carcinogens were implicated in the etiology of esophageal cancer in china.

Current data also showed a higher risk of oral cancer is associated with higher quartile groups of urinary cotinine and NO₂+NO₃ levels (table-12). Yi et al (1993) reported that urinary nitrate levels were twice as high in the subjects from the high-risk area as compared to low risk area for nasopahryngeal
cancer. Badawi et al (1998) reported that the OR for oral cancer showed higher risk associated with salivary nitrate > 7.5 \( \mu g/ml \) and salivary nitrite > 40 \( \mu g/ml \). In contrast, it is also reported that salivary nitrate, nitrite and N-nitroso compounds might not be suitable markers for the assessment of the risk of cancer of the upper aero digestive tract (Airoldi et al, 1997).

Thus, the present study observed positive correlation between tobacco exposure and urinary biomarkers including urinary nicotine, cotinine, thioether and \( \text{NO}_2+\text{NO}_3 \). Pearson's correlation test also revealed that the changes in the values of nicotine were positively associated with the alterations in urinary thioether. The alterations in urinary cotinine levels were positively associated with urinary \( \text{NO}_2+\text{NO}_3 \) levels (table-13). A report by Malaveille et al (1989) also showed correlation of urinary nicotine and cotinine with urinary thioether and nitrate levels which also supports the current observations.

**Plasma and erythrocyte biomarkers**

Reactive oxygen metabolites such as superoxide anion, hydrogen peroxide, hydroxyl radical, MDA and nitric oxide are involved in initiation, promotion and progression stages of carcinogenesis. Free radicals produced during auto oxidation of polyphenols in saliva of the tobacco users play a crucial role in the development of oral cancer (Jeng et al, 2001). It is reported that the higher production of free radicals could cause damage at cellular and molecular levels, leading to lipid peroxidation as well as mutations in tumour suppressor gene (p53) or gene of antioxidant enzymes. Nair et al (2004) reported that the calcium hydroxide contents of lime in presence of areca nut are primarily responsible for the formation of ROS that could cause oxidative damage in the DNA of buccal mucosa cells of betel quid chewers. The antioxidants and detoxification enzymes have a vital role in defense mechanisms against detrimental effects of free radicals. The altered levels of antioxidants and detoxification enzymes are reported in cancer (Ray and
Husain, 2002). The damage and protection against free radicals are reflected through circulating biomarkers in blood. It has become an interesting research area for studies on tissue damage, tissue hypertrophy and neoplastic transformation (Prochaska and Fernandes, 1993).

Majority of oral cancer are transformed through OPC. Further, a large number of patients with OPC and oral cancer patients have tobacco habits. Therefore, present study included patients with OPC and oral cancer patients with specific details on their tobacco having habits. In addition, NHT and WHT were also included as controls.

**NO₂⁺NO₃, Prostaglandin E2 and antip53antibodies levels**

The role of NO is multidimensional because it functions as an intracellular messenger and is also implicated as deleterious agent in various pathological conditions including cancer. Chronic inflammation can lead to the production of NO, which in turn has the potentials to mediate DNA damage directly, or indirectly through the generation of more persistent reactive nitrogen species (Li, 1995). Plasma NO₂ + NO₃ levels were used to estimate the levels of NO formation, since NO is highly unstable and has a very short half-life. Evidence of the role of NO in carcinogenesis was provided by the fact that iNOS expression were detected in various human cancers (Prazma et al, 1995; Tanaka et al, 1999; Thomson et al, 1995; Vakkala et al, 2000; Yagihashi et al, 2000). However, it has also been reported that high expression of mutant p53 gene and COX-2 expression in oral cancer patients was associated with iNOS expression (Matsumoto et al, 2003; Park et al, 2003). Jenkin et al (1995) also suggested that biopsy samples of human cancer show the presence of greater expression of iNOS in a high-grade tumor, which tends to be more invasive. Previous reports also observed that end products of nitric oxide were significantly elevated in oral, gastric, colorectal, hepatocellular, breast, leukemia, laryngeal and upper aero digestive tract cancer (Airoldi et al, 1997;
Elevations in plasma prostaglandin E2 levels are observed in various malignancies (Hidalgo et al., 2002; Rishikesh and Sadhana, 2003; Wallace, 2002). It is reported that both COX-1 and COX-2 isoenzymes metabolize arachidonic acid to prostaglandin 15-hydroxy analogue, which is then converted by specific enzyme, to a variety of eicosanoids including prostaglandin E2 (Smith and Marnett, 1991). Rioux and Castonguay (1998) found elevated plasma prostaglandin E2 levels in NNK treated mice. Klapan et al. (1992) also found that serum prostaglandin E2 levels were significantly elevated in head and neck cancer as compared to healthy individuals. In the present study, plasma prostaglandin E2 levels were found to be comparable between NHT and WHT. The levels of plasma prostaglandin E2 were significantly elevated in patients with OPC and oral cancer patients as compared to NHT and WHT (figure-36). Malachi et al. (1981) observed that plasma prostaglandin E2 concentration did not reflect significant difference between the benign and malignant breast tissues, but the levels were higher than healthy controls. It is also reported that induction of prostaglandin by betel quid ingredients might be important for the pathogenesis of oral leukoplakia and oral cancer. It is observed that prostaglandins participate in
the oral carcinogenesis indirectly via inducing inflammatory cell infiltration, cytokine production, production of ROS or immune suppression (Rishikesh and Sudhan, 2003; Wallace, 2002). Therefore, more studies are needed to elucidate clear role of prostaglandin in oral carcinogenesis.

It is well accepted that mutations of the p53 tumour suppressor gene play a critical role in the multistep carcinogenesis. Recent studies on colorectal carcinoma, lung adenocarcinoma and head & neck cancer have revealed significant correlations between iNOS activity and p53 mutations (Ambs et al, 1999; Fujimoto et al, 1998; Matsumoto et al, 2003). Matsumoto et al (2003) demonstrated that iNOS immunoreactivity showed significantly positive association with p53 gene mutation frequency and p53 protein over expression in head and neck cancer. Also, P53 mutations are often reflected as circulating antip53 antibodies (Soussi, 2000). Presence of circulating antip53 antibody levels have been observed in various malignancies like lung, gastric, breast cancer, head and neck cancers etc (Gottschlich et al, 2003; Nakajima et al, 1999; Soussi, 2000; Wollenberg et al, 1997). In head and neck cancer patients, the prevalence of p53 antibodies reported by previous workers ranged from 17 to 44%. Previous study observed 30% of patients with OPC showing antip53antibodies positivity in Indian population (Raihan et al, 1998). In the present study, none of healthy individuals including NHT and WHT, 13% of patients with OPC and 25.8 % of oral cancer showed antip53 antibodies positivity in plasma. It was also observed that circulating levels of antip53antibody in patients with OPC and oral cancer patients were significantly higher than that of the normal subjects. Oral cancer patients showed significantly higher antip53antibody levels as compared to patients with OPC (table-14). Various studies have emphasized on the role of antip53antibodies in oral cancer patients (table-27). It is reported that exposure to the carcinogenetic constituents of betel quid contributes significantly to genetic insults caused in the oral mucosa, resulting in accumulation of p53 protein in early stages of oral cancer, thereby accounting
for the presence of circulating p53 antibodies in patients with premalignant lesions. Thus, the present investigation suggested in-depth study of antip53 antibodies in tobacco and betel abused population for identification of high-risk group.

Table-27: Various reports on antip53 antibodies in healthy individuals, patients with OPC and oral cancer patients

<table>
<thead>
<tr>
<th>Healthy individuals</th>
<th>Reference</th>
<th>Positive /total</th>
<th>Reference</th>
<th>Positive /total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mudenda et al, 1994</td>
<td>1/76</td>
<td>Polge et al 1998</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Mayerhofer et al, 1999</td>
<td>0/17</td>
<td>Green et al, 1995</td>
<td>1/69</td>
<td></td>
</tr>
<tr>
<td>Machara et al, 1999</td>
<td>0/15</td>
<td>Maass et al, 1996</td>
<td>0/41</td>
<td></td>
</tr>
<tr>
<td>Saffroy et al 1999</td>
<td>0/40</td>
<td>Gansauge et al, 1996</td>
<td>0/60</td>
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</tr>
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<td>Sthoeger et al, 1997</td>
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<td></td>
</tr>
<tr>
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<td>Friedrich et al, 1997</td>
<td>0/9</td>
<td></td>
</tr>
<tr>
<td>Hallak et al 1998</td>
<td>0/195</td>
<td>Werner et al, 1997</td>
<td>0/41</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oral Cancer/ OPC</th>
<th>Reference</th>
<th>Positive /total</th>
<th>Comment</th>
</tr>
</thead>
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<tr>
<td>Ralhan et al 1998</td>
<td>24/70</td>
<td>Leukoplakia</td>
<td></td>
</tr>
<tr>
<td>Ralhan et al 1998</td>
<td>15/50</td>
<td>Association with poor differentiation and p53 accumulation in the tumor</td>
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</tr>
<tr>
<td>Lavieille et al, 1998</td>
<td>32/74</td>
<td>No clinical correlation detected</td>
<td></td>
</tr>
<tr>
<td>Maass et al, 1997</td>
<td>18/82</td>
<td></td>
<td></td>
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<tr>
<td>Maass et al, 1996a</td>
<td>23/117</td>
<td>Association with p53 accumulation in the tumor</td>
<td></td>
</tr>
<tr>
<td>Bourhis et al 1996</td>
<td>15/80</td>
<td>Association with p53 accumulation in the tumor and short survival</td>
<td></td>
</tr>
<tr>
<td>Friedrich et al, 1997</td>
<td>9/39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Werner et al, 1997</td>
<td>39/143</td>
<td>Associated with therapy failure</td>
<td></td>
</tr>
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<td>37/97</td>
<td></td>
<td></td>
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<tr>
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<td>11/30</td>
<td>Patients with recurrence</td>
<td></td>
</tr>
<tr>
<td>Mass et al 1996b</td>
<td>47/177</td>
<td>Associated with tumor size</td>
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<td>32/73</td>
<td>Association with p53 accumulation in the tumor</td>
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<td>Warnakulasuriya et al, 2000</td>
<td>7/26</td>
<td></td>
<td></td>
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<tr>
<td>Gottschlich et al, 2003</td>
<td>9/32</td>
<td>Patients a correlation with the clinical course of the disease</td>
<td></td>
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</tbody>
</table>

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**MDA, SOD, Catalase, GST, GR and Thiol levels**

It is documented that high levels of oxidative stress result in peroxidation of membrane lipids with the generation of peroxides that can decompose to multiple mutagenic carbonyl products. MDA is a well-characterized lipid peroxidation end product. Earlier, Gupta et al. (2004) have found significantly higher MDA levels in patients with OPC as compared to healthy individuals. It is also reported that lipid peroxidation is significantly elevated in untreated oral cancer patients as compared to the controls (Subapriya et al., 2002). Taysi et al. (2003) reported that MDA levels were significantly elevated in advanced stage of laryngeal cancer as compared to the controls. Significantly higher plasma MDA levels were also found in patients with gastric carcinoma (Choi et al., 1999) and breast cancer (Ray et al., 2000a). On the contrary, decreased plasma MDA levels have also been reported in cancer patients (Alagol et al., 1999; Gerber et al., 1996). Gerber et al. (1997) demonstrated lower MDA concentrations in breast cancer patients. They demonstrated a negative correlation between MDA levels and tumor size in the patients. There are number of observations that indicate an apparently negative correlation between levels of cellular lipid peroxidation and rate of cell proliferation (Ray and Hasain, 2002). The contradicting reports on MDA levels in cancer patient are documented by various investigations. Nagini et al. (1998) found that MDA levels were lower in oral malignant tissue than in normal tissues. Yigitbasi et al. (2000) reported that MDA levels were lower in tissue with larynx squamous cell carcinoma than the normal tissues. The present study found no significant difference in the MDA levels between healthy individuals and patients with OPC and oral cancer patients (*figure-37*). Present study showed higher plasma MDA levels in non-abstinence group of oral cancer patients than the abstinence group (*table-17*). Mircea et al. (2003) have also reported higher MDA levels in healthy individuals having habits of tobacco than non-habitués.

Antioxidant enzymes such as SOD and catalase can directly counter balance the oxidant attack and may protect cells against DNA damage. SOD inhibits...
Discussion

OH\(^-\) production, therefore it acts as inhibitor at the initiation and promotion stages during carcinogenesis. In the present study, erythrocyte SOD activities were found to be higher in WHT as compared to NHT. Erythrocyte SOD levels were higher in patients with OPC than WHT and NHT (figure-38). Gupta et al (2004) reported that erythrocyte SOD levels were significantly elevated in patients with OPC as compared to healthy individuals. This might be due to tobacco associated changes in response to free radical generation in WHT and patients with OPC. It has been reported that SOD and catalase play an important role as first line of defense in response to ROS mediated changes (Ambrosone, 2000). Moreover, contradictory reports for SOD activity in cancer patients are documented. Several authors observed that erythrocytes SOD activities were significantly elevated in Hodgkin’s disease, leukemia and breast cancer patients (Abdel-Aziz and El-Naggar, 1997; Deliconstantinos et al, 1995; Koksoy et al, 1997). Other studies reported decrease erythrocytes SOD activities in various malignancies (Abiaka et al, 2002; Gonzales et al, 1984; Poongothai et al, 2004; Sabitha and Shyamaladevi, 1999; Zima, 1996). Present study showed that erythrocyte SOD activity was decreased in oral cancer patients than healthy individuals (NHT and WHT) and patients with OPC (figure-38). Similar results indicating reported decreased activities of erythrocyte SOD in oral cancer have also been reported. (Sabitha and Shyamaladevi, 1999). The low activity of erythrocyte SOD in the present study might be due to the depletion of antioxidant defense system, which could occur as a consequence of overwhelming free radicals (figure-54). Syed Sultan et al (2004) also suggested that the low activities of antioxidant enzymes in oral cancer patients might be due to the depletion

Figure-54 Malignant phenotype
Discussion

of the antioxidant defenses system. This could occur as a consequence of overwhelming free radicals by the elevated levels of lipid peroxides in the circulation of oral cancer patients.

Results on erythrocytes catalase activity in cancer are contradictory. Both increase (Hristozov et al, 2001) and decrease (Kumaraguruparan et al, 2002; Manju et al, 2002; Ray et al, 2000) in erythrocytes catalase activity has been reported previously. In the present study, erythrocyte catalase activities were significantly elevated in WHT and patients with OPC than NHT. However, erythrocyte catalase activities were decreased in oral cancer patients than OPC (figure-39). This decrease in catalase may be due to higher OH⁻ production, which may adversely affect the activity of catalase. Syed Sultan et al (2004) reported that low activities of erythrocyte catalase in oral cancer patients might be due to increased endogenous production of the superoxide anion by increased MDA, or increased nitric oxide end products or decreased activities of GPx and SOD or all of these factors. Yang et al (2002) reported that alterations in antioxidant enzymes might be useful in the molecular diagnosis of oral cancer, as well as monitoring the effectiveness of chemo preventive and therapeutic strategies in oral cancer.

Phase II enzymes involved in the detoxification of xenobiotics and drugs are of prime importance in carcinogenesis. (Saydam et al, 1997; Tsuchida et al, 1989). The present investigation found that plasma as well as erythrocyte GST activities were elevated in WHT as compared to NHT. Moreover, plasma and erythrocyte activities of GST were also elevated in patients with OPC than the NHT and WHT (figure-40,42). Similar results have been reported in previous study from our laboratory (Rawal et al, 1999). The results indicate that the changes may reflect an adaptive response to tobacco carcinogen exposure. GST and GR are involved in the detoxification of many reactive species, as second line of defense, in response to ROS mediated changes. It is also reported that plasma GST activities were significantly elevated in
gastric, colorectal, liver and lung as compared to healthy subjects (Gupta et al, 2000; Mohammadzadeh et al, 2003; Patel et al, 2002; Severini, 1993). Ferruzzi et al (2003) reported that GST activities were significantly higher in head and neck cancer patients than healthy individuals. In the present study, plasma as well as erythrocyte GST activities were elevated in patients with oral cancer as compared to healthy individuals (NHT and WHT) (figure-40,42). Previous study reported four fold higher plasma GST levels in lung cancer patients as compared to healthy controls (Gupta et al, 2000). Do ru-Abbassu lu et al (2002) reported that plasma GST activity was significantly elevated in colorectal and gastric cancer patients than age-matched controls. Kuralay and Yildiz (2001) have also reported significantly elevated erythrocyte GST activity in patients with squamous cell carcinoma of larynx as compared to healthy individuals.

It is very well known that GST M1 is subtype of GST enzymes, which rapidly detoxifies carcinogens found in tobacco smoke. Cheng et al (1999) reported that 53% of oral cancer patients were having GST-μ null genotype. In present study, only small number of oral cancer patients were analysed for GST M1 polymorphism. 32 % of oral cancer patients were of GST-μ null genotype. In-depth study for the GST-μ polymorphism will be useful for risk assessment in healthy tobacco users (table:26). Several other reports from India have shown correlation of GST-μ null genotype in oral cancer patients with type of tobacco habits and risk of development of disease in healthy tobacco users (Sreelekha et al,2001; Buch et al, 2002). Buch et al (2002) reported that increased life time exposure to chewing tobacco was associated with 2-fold increased in oral cancer risk. Individuals with tobacco smoking (bidi and cigarette), chewing with GST-μ null genotype were at higher risk of oral cancer development in India (Sreelekha et al, 2001) Buch et al (2002) reported 50.8% of oral cancer patients with GST-μ null genotype while Sreelekah et al (2001) reported 49% of oral cancer patients with GST-μ null genotype from India. Nair et al (1999) reported that GST-μ and GST T1 are
enzymes known to detoxify ROS, lipid peroxidation products and tobacco-derived carcinogens that have been found in the saliva of betal quid/tobacco chewer. Null genotypes for GST-μ and GST T1 increase the risk of developing leukoplakia in chewer.

Present study showed that erythrocyte GR activities were elevated in patients with OPC and oral cancer as compared to healthy individuals (NHT and WHT) (figure-43). It is documented that, GST utilizes GSH and forms GSSG, which is been converted into reduced GSH by GR. Hence; utilization of GSH by GST is associated with increase in GR activity in erythrocytes. The increase in erythrocyte GST was also found to be significantly higher with increase in erythrocyte GR in untreated cancer patients. It is reported that, many toxins and activated tobacco products are metabolized to safer products by conjugation with GSH in a reaction catalyzed by GST (Ambrosone, 2000). In present study, plasma GR activities and thiol levels were significantly lower in untreated oral cancer patients as compared to healthy individuals (NHT and WHT) (figure-41,44) which may be due to oxidative stress. Subapriya et al (2002) found decreased SOD and catalase activities with increase in the levels of GSH and GSH dependent enzymes.

Thiol levels were significantly lower in oral cancer patients than the healthy individuals (NHT and WHT) in the present study. Thiol levels were also significantly lower in oral cancer patients as compared to the patients with OPC (figure-44). The possible reasons for this could be: (i) the decrease in blood –SH group content, observed in cancer patients, might be the consequence of peroxidation and oxidation process elicited by free radicals. (ii) The lower level of –SH groups in the blood of oral cancer patients may represent an etiologic element favoring the together and subsequent development of the disease. (iii) the observed decrease of GSH and reduced sulphydrylic groups appears consistent with the decreased vitamin A and E contents of peripheral venous blood (Rovere et al, 2000). It is also reported
that cancer patients show an accelerated shift towards more oxidized condition (Hack et al, 1990). During progression of cancer, the antioxidant property of the cells may progressively decline and this could be related to depletion of thiol (-SH) levels in the redox equation (Bounous and Molson, 2003). Rovere et al (2000) reported lower thiol levels in cancer patients as compared to controls. Mukundan et al (1999) reported lower plasma and erythrocyte glutathione levels in cervical cancer patients as compared to controls. It has been reported that, apart from antioxidant enzymes, low molecular weight nonprotein sulfhydryls, and most importantly glutathione, cysteine and cysteinylglycine play a major role in the cellular defense against active oxygen (Cerutti, 1985).

**Comparison between smokers and chewers**

Present study compared biochemical changes in the two different types (smoking and chewing) of tobacco habits. The current investigation also found that plasma GST levels were higher in WHT (smokers or chewers only) than NHT (table-15,16). Saroja et al (1999) have reported that GSH and GSH dependent enzymes play a crucial role in tobacco carcinogenesis and may be considered as markers of carcinogen exposure. Present study showed higher erythrocyte SOD activity in WHT (smokers or chewers only) than NHT. But erythrocyte catalase activity was higher in WHT (smokers only) than NHT (table-15,16). These results indicate that elevated in erythrocyte SOD and catalase activities were response to tobacco associated ROS which results in oral carcinogenesis. Abouse seif et al (1996) have reported that erythrocyte SOD and catalase activities were significantly higher in smokers than non-smokers. But the enzyme activities were slightly elevated in chewers as compared to non-chewers. In contrast, erythrocyte SOD and catalalase activities were significantly lower in heavy smokers, light smokers and passive smokers than non-smokers (Yildiz et al, 2002). It is also reported that treatment of human lung carcinoma cell with tobacco smoke caused dose dependent single strand break in DNA that could be inhibited by catalase and
SOD (Nakayama et al, 1985). In the present study, no difference was observed in thiol levels between NHT and WHT (smokers or chewers only) (table-15,16). Mircea et al (2003) reported that the free thiol levels were decreased in smokers than non-smokers. It is reported that patients with OPC have lower antioxidant capability (Knak et al, 1998). Previous studies have also demonstrated (Nair et al,1987; Nagler,2003) that saliva inhibits the production of ROS, superoxide free radical and hydrogen peroxide from betel quid tobacco, the most potent inducers of oral cancer. Significant inhibition of all enzymatic antioxidant activities following only cigarettes, probably due to the interaction between smoke aldehyde and –SH groups of the enzyme molecules, was reported by Zappacosta et al (2002). Williams (2000) also showed association of tobacco with p53 mutations in head and neck cancer. In the present study, number of oral cancer patients having habit of tobacco chewing was higher than that of patients with tobacco smoking habits. This might be the reason for higher positivity in patients having tobacco chewing habits. The healthy controls were negative for antip53antibodies levels, therefore, in the present study comparison between subjects with different habits of tobacco could not be performed.

**Risk of oral cancer in relation to circulating biomarkers**

Present study showed that higher levels of plasma NO$_2$+NO$_3$, prostaglandin E2, as well as erythrocyte GST, GR were associated with risk of oral cancer. Further, higher levels of plasma thiol and GR also displayed protective effects (table-19, 20). De Stefani et al (1999) have reported that most of the antioxidants present in diet were associated with a reduction in risk of cancer. The authors further hypothesized that it is biologically plausible that dietary antioxidant could prevent oxidative damage caused to the mucus of organs of upper aero digestive tract that results due to tobacco smoking and alcohol drinking. Previous reports from our laboratory (Raval et al, 2001, 2002) also observed significant decrease in the plasma levels of nonenzymatic antioxidants in tobacco habitués.


**Multivariate analysis**

Multivariate analysis between the markers and clinicopathological parameters as well as tobacco habits showed that GST activities were significantly associated with gender (**table-21**). Tobacco habits were predominant in males than female. This could be the reason for alterations in plasma GST activities in male. It is also reported that GST induction is a part of an adaptive response to any chemical or environmental stress that results into differences in species, strain, age, sex (Hayes and Pulford, 1995). It is also reported that, increase in plasma GST reflects increase in de novo synthesis of GST in liver in response to chemical induction (Bogaards et al, 1994).

**Circulating biomarkers and stage of the malignant disease**

Present study did not find correlation between stage of the malignant disease and plasma NO$_2$+NO$_3$ levels. Gunel et al et al (2002) reported that serum NO$_2$+NO$_3$ levels were significantly higher in breast cancer patients than healthy controls. But there was no significant difference between the patients with metastasis and without metastasis. Taysi et al (2003) also reported that plasma NO$_2$+NO$_3$ levels did not differ significantly between patients with stages III and stage IV laryngeal cancer. In present study, erythrocyte SOD activities were elevated in advanced stage of the disease as compared to early stages of disease (**figure-45**). In contrast to this, Zima et al (1996) did not find any correlation with SOD activities and stage of disease in multiple myeloma patients. No correlation of SOD levels with stages and extent of the malignant disease has been reported by earlier investigations (Gonzales et al, 1984). Further, plasma GST activities were decreased as the stage of the disease increased (**figure-45**). These observations may be due to induction of GST activities in response to carcinogenic activities reflected in terms of detoxification of environmental metabolites in oral cancer. Present study also observed that mean levels of catalase activities were decreased as tumor differentiation increased (**figure-46**). This could be due to oxidative stress in terms of elevated ROS generation resulting into lipid damage and increased
Discussion

H$_2$O$_2$ that may inhibit catalase activities in these patients. In addition, present study also observed more patients showing presence of p53 antibodies in advanced stage of oral cancer (figure-45). Mack et al (2000) have reported that prognosis of the patients with non-squamous cell lung carcinoma was linked to the p53 antibodies status and most of positive results for p53 antibodies were found in advanced stage of non-squamous cell lung carcinoma. It suggests that the positivity of antip53antibodies may be associated with survival of the patients.

Circulating biomarkers and tobacco exposure

The present study displayed that the tobacco habits play a significant role in oral cancer development possibly due to increased oxidative stress in these subjects. The habit of tobacco consumption in various forms is studied as a risk factor for oral cancer development. Present study also focuses on comparison between tobacco exposure and the biomarkers. The subjects were grouped into low and high tobacco exposure based on their nicotine and cotinine levels. The results showed that plasma GST as well as erythrocyte GST, GR and catalase levels were elevated in controls with high levels of tobacco exposure than healthy individuals with low levels of tobacco exposure (table-18). Sobczak et al (2004) also observed distribution of antioxidants (vitamin E, alpha and gamma tocopherol) in low and high group of tobacco exposure.

Pearson’s correlation

Correlations between tobacco exposure markers (nicotine and cotinine) and biomarkers by Pearson’s test revealed that alterations in erythrocyte SOD levels were significantly and positively associated with urinary nicotine levels. The changes in erythrocyte catalase levels were also positively associated with urinary nicotine levels (table-24). These results suggested that tobacco consumption is associated with increased risk of oral cancer, which may be mediated through oxidative stress. Morene-Lopez et al. (2000) found that
tobacco and alcohol consumption were significantly associated with increased risk of oral cancer. It was also reported by Winn (2001) that TSNA, aromatic amines, and polycyclic aromatic amines and polycyclic aromatic hydrocarbons present in mainstream tobacco smoke are major carcinogens contributing to the oral cancer risk from smoked tobacco products. For smokeless tobacco, the nitrosamines formed during fermentation and curing occur at relatively higher levels and are thought to be most important. It is also becoming increasingly evident that certain inherited genotype may predispose to tobacco-related oral disease. These represent genes involved in tobacco metabolism such as those coding for N-acetyl transferase, GST and p450 pathway.

**Comparison of circulating biomarkers with treatment response**

Present studies also observed that the circulating biomarkers might be useful to monitor the treatment response in oral cancer patients during/after anticancer treatment (surgery, radiotherapy and chemotherapy). These anticancer therapies mainly radiotherapy and chemotherapy, are based on free radical generation. Hence, it is important to have pretreatment and post treatment levels of circulating biomarkers to monitor the response to anticancer therapy. Therefore, a retrospective study was carried out to evaluate usefulness of these biomarkers in predicting treatment response. Paired 't' test showed that plasma MDA levels remained lower in CR as compared to PT. It has been reported that radiotherapy induced lipid peroxidation resulted into elevated MDA levels in oral cancer patients treated with radiotherapy (Sabitha and Shyamaladevi, 1999). In the present study, mean levels of MDA were increased in NR as compared to PT patients suggesting continued oxidative stress. Also, the increase in MDA levels in NR is in accordance with the clinical appearance of the disease (**figure-51**). No response to treatment indicated presence of tumor, which harbors oxidative stress resulting into higher MDA. Present study also showed that plasma prostaglandin E2 levels were comparable between NR and PT. However,
plasma prostaglandin E2 levels were lower in CR than PT. But, difference was not statistically significant (figure-51). Although the exact mechanisms remain unclear, the inhibition of COX by NSAIDs appears to abort, if not prevent, colorectal carcinogenesis or metastatic tumor progression. In the present study, a representative pattern showed that prostaglandin E2 levels were declined in the CR and elevated in the NR (figure-52). These results suggested that prostaglandin E2 might be associated with disease progression. Shaw et al (1985) reported that elevated levels of prostaglandin E2 may serve as a prognostic marker as well as to identify the high metastatic potentials of tumour. Narisawa et al (1990) suggested that increased plasma prostaglandin E2 might be useful in the detection of metastasis in colorectal cancer. Present study observed that plasma thiol levels were decreased in NRs and increased in CR as compared to PT (figure-51). These results indicate that thiol levels may be associated with the progression of disease. In the present study, plasma GST levels were lower in CR than NR (figure-51). The data from our laboratory (Patel et al, 2002) also suggested that analysis of glutathione and glutathione depleting enzymes could be helpful for treatment monitoring of head and neck cancer patients. Several research groups have demonstrated the presence and clinical utility of serum antibodies in the prognostication of cancer patients (Soussi, 2000). Present study also showed lower antip53 positivity in CR as compared to NR (figure-53).

In the nutshell,

Current investigation observed four aspects: (i) NO$_2$+NO$_3$ levels were higher in various tobacco products, (ii) tobacco habits were the prominent risk factor for development of OPC and oral cancer, (iii) urinary nicotine, cotinine, thioether and NO$_2$+NO$_3$ levels were significantly elevated in WHT, patients with OPC and oral cancer as compared to NHT. (iv) The plasma NO$_2$+NO$_3$, prostaglandin E2 and anti p53 antibody levels in patients with OPC and oral cancer patients were significantly elevated as compared to NHT. Plasma GST
as well as erythrocyte GST, GR and SOD activities were elevated in WHT as compared to NHT. The alterations in GST, SOD and catalase were associated with tobacco exposure as well as smoking and chewing habits of tobacco. Plasma GST and antip53antibodies as well as erythrocyte SOD levels were also significantly associated with stage (early and advanced) of oral cancer. Plasma thiol as well as erythrocyte catalase activities were also significantly associated with tumour differentiation. Plasma prostaglandin E2, GST, thiol and MDA as well as erythrocyte GST levels were also significantly associated with clinical outcome of the patients during post-treatment follow-ups. Hence, the current approach suggests its vital usefulness for prevention, early diagnosis as well as treatment monitoring of oral cancer. If explored to a greater depth, it might turn out to be a very good approach for planning of preventive and management strategies for oral cancer, which is a major health challenge in India.