RESEARCH COMMUNICATION

Utility of Urinary Biomarkers in Oral Cancer

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Abstract

Objective: Oral cancer is the leading malignancy in India, with tobacco playing a major role in the etiology. The aim of the present study was to quantify nitrate+nitrite (NO2+NO3) in tobacco products as well as to study tobacco exposure related biomarkers in controls, patients with oral precancers (OPC) and oral cancer patients. Materials & Methods: Healthy individuals (n=90) were grouped into without habit of tobacco (NHT, n=30) and healthy individuals with habit of tobacco (WHT, n=60). Oral cancer patients with a tobacco habit were classified into abstinence (n=62) and non-abstinence (n=64) groups according to status at the study time. Urinary nicotine and cotinine levels were analyzed by modified high-pressure liquid chromatography (HPLC) using a UV detector. Levels of NO2+NO3 in tobacco and urine, and urinary thioether levels were estimated by spectrophotometry. Results: NO2+NO3 levels in different types of tobacco product ranged between 0.13 to 3.39 mg/g. The Odds Ratio (OR) analysis indicated positive associations of both smoking and chewing habits of tobacco with high risk of development of oral cancer. Urinary nicotine, cotinine and NO2+NO3 levels were significantly elevated in WHT, patients with OPC and oral cancer patients as compared with the NHT group. This was also the case for urinary thioether levels. Levels of urinary nicotine and cotinine were also higher in the non-abstinence group with oral cancers. Conclusion: The results confirmed that tobacco chewing and smoking habits are prominent risk factors for development of oral cancer in the western part of India (Gujarat). Urinary nicotine, cotinine, NO2+NO3 and thioether levels can be helpful for screening programs for oral cancer.

Key Words: Tobacco - oral cancer/precancers - nicotine, cotinine, thioether and NO2+NO3 markers

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Introduction

Oral cancer is one of the 10 most common cancers in the world. Tobacco products cause around 30% of all cancer death in developed countries (Peto et al.,1996). In spite of this, there are over one billion smokers in the world and millions of people who use smokeless tobacco products (Hatsukami and Severson, 1999; Pershagen, 1996). Exposure to environmental tobacco smoke is also a recognized cause of cancer (Benowitz, 1999). World health organization (WHO) has reported that about 90% of oral cancer in South-East Asia is attributed to use of tobacco. In India, oral cancer is highly prevalent, comprising 35-40% of all malignancies, due to the habit of tobacco chewing in betel quid commonly observed in the population (Daftary et al., 1991).

Millions of people in India consume tobacco in various forms. Many individuals who do not consume tobacco are also exposed to tobacco smoke or smokeless tobacco products. These products contain thousands of chemical constituents including major alkaloid (nicotine) and minor alkaloids (noricotine, anabasine, anatabine etc.). These alkaloids can react with nitrite to form nitrosamines like 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which are called tobacco specific nitrosamines. The components of nicotine, cotinine, nitrate and nitrite are important progenitors in the formation of tobacco specific nitrosamines (Hoffmann and Hecht, 1985).

Nicotine, cotinine and NO2+NO3, the major constituents of tobacco products that are excreted in urine of tobacco-exposed individuals, can be used as the markers of tobacco exposure. Urinary nicotine and cotinine can be estimated by various methods including thin layer chromatography, high performance liquid chromatography (HPLC), gas chromatography and spectrophotometry. Urinary thioether levels can be also useful as biomarkers of tobacco exposure, because the tobacco exposure to electrophilic moieties increases thioether levels in urine (Bhisey et al., 1992). Urinary thioether levels are the index of total electrophilic burden in body. Such data from tobacco products and biological fluids can be helpful for development of preventive stragies. Therefore, aim of the study was to quantify NO2+NO3 in tobacco product and to assess role of tobacco habits as risk factors for development of oral

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Table 1. Details of Healthy Individuals (N=132) and Oral Cancer Patients (N=126) for the Odds Ratio Analysis

		Healthy Individuals	Oral Cancer Patients
Median Age (years):		32	45
Age Range		19-62	22-75
Tobacco History:	Non-habituate	53	13
	Habituate	79	113
Present status:	Abstinence	00	62
	Non-abstinence	79	64
Type :	Chewers	58	62
	Smokers	17	27
	Chewers+ Smokers	03	18

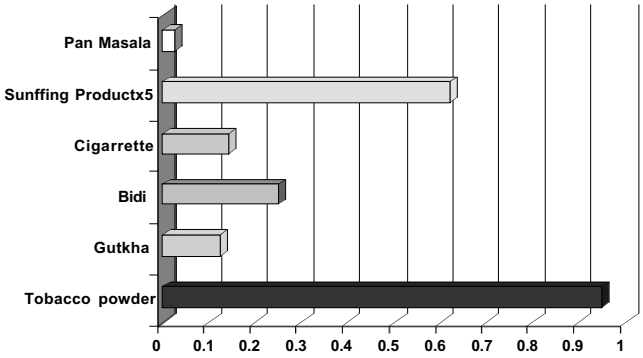


Figure1. NO2+NO3 Levels in Different Tobacco Products

cancer. We also evaluated urinary nicotine, cotinine, thieother and NO2+NO3 levels in healthy individuals without habits of tobacco (NHT), healthy individuals with habits of tobacco (WHT), patients with oral precancers (OPC) and oral cancer patients.

Materials and Methods

Study group sampling for OR analysis

Patient demographics were collected for healthy individuals (n=132) and oral cancer patients (n=126) by interview questionnaires. The information ascertained included details of age, sex, tobacco habits, duration and frequency of tobacco consumption (Table 1).

Study group for urinary biomarkers

The subjects were enrolled from a single centre for analysis of the biomarkers and included 15 patients with OPC, 126 oral cancer patients, 30 NHT and 60 WHT . Urine samples from 10 children without environmental tobacco exposure were also included as controls. All the subjects were divided into tobacco users and non-users. Tobacco users were further classified into abstinence (patients who stopped tobacco habit before 15 days) and non-abstinence (patients who were consuming tobacco at the time of interview) groups as per their tobacco cessation status.

Sample collection

Twenty six brands of tobacco products and six brands of pan masala were collected for estimation of NO2+NO3. The brands of tobacco products and pan masala selected for the study were the most commonly used by tobacco habitués in Gujarat (western part of India). Pan masala products were not containing tobacco, but they contained areca nut, lime and catechu. Tobacco products were classified into major groups including cigarette (n=4), bidi (n=4), gutkha (n=9), tobacco alone (n=6), and snuffing products (n=3). Urine samples were collected from oral cancer patients prior to initiation of any anticancer therapy and stored at -20 ˚C until analyzed.

Methods

NO2+NO3 levels in tobacco products were estimated by Griess reagent method as designed by Pakhale and Maru (1995). Urinary nicotine and cotinine levels were extracted into organic phase under alkaline condition according to Lequang Thuan et al. (1989). These compounds were separated using reversphase chromatography (C18 column) and detected at 254 nm using an U.V. detector (Watson, 1977). Spectrophotometric methods were used for estimation of urinary thioether and NO2+NO3 (Green et al., 1982; Bagwe and Bhisey, 1995; Van Bezooijen et al., 1998). Urinary creatinine levels were measured by alkaline picrate method (Varleg, 1967).

Data were statistically analyzed using the SPSS statistical software (Version 10). Student's unpaired t- test was performed to compare levels between controls and patients with OPC and oral cancer patients. Relative risks of cancer in healthy individuals were estimated by computing OR. 95% confidence interval (CI). Pearson's correlation was studied to assess association between biomarkers. Receivers Operating Characteristic (ROC) curves were constructed to evaluate discretionary efficacy of the biomarker levels between patients and controls. Data was considered statistically significant when p values were 0.05 or less.

Results

NO2+NO3 levels in different tobacco products

Figure 1 shows levels of NO2+NO3 contents in different tobacco products and pan masala. NO2+NO3 contents in the products ranged from 0.13 to 3.39 mg/gm.

Risk of oral cancer in tobacco habitués

Table 2 illustrates the risk estimates for oral cancer in tobacco habitués and related variables.

Patterns of nicotine and cotinine

Figure 2 shows representative patterns of urinary nicotine and cotinine in an NHT and a WHT. Urinary nicotine and cotinine peaks were not detected in urine samples of a child (a). The NHT group revealed either absence (b) or faint presence (c) of nicotine and cotinine peaks. Further, WHT showed prominent presence of nicotine and cotinine peaks in urine samples (d). As clear from representative patterns of urinary nicotine and cotinine in patients (Figure 3), urinary nicotine and cotinine peaks were prominent in tobacco non-abstinence group of patients with OPC and oral cancer patients. These peaks were not found in the tobacco abstinence group of the

Table 2. Odds Ratios of Oral Cancer for Tobacco Habits

		Ca/Co	OR (95% CI)	p value
Non habitual*		12/53	1	
Habitual		90/79	6.3 (3.2-12.6)	0.0001
Smokers		27/17	7.0 (2.9-16.9)	0.0001
Duration	<=10 years	6/4	2.3 (0.4-13.8)	0.002
	11-19 years	7/5	9.6 (2.0-47.5)	0.0001
	> 19 years	14/8	12.7 (4.5-35.6)	0.0001
Frequency	1-10/day	6/5	8.2 (2.1-31.7)	0.003
	10-20/day	11/7	7.2 (2.3-22.3)	0.001
	>20/day	10/5	13.0 (3.6-46.2)	0.0001
Chewers		62/58	4.7 (2.3-9.7)	0.0001
Duration	<=10 years	25/36	4.0 (1.7-9.6)	0.001
	11-19 years	17/16	6.1 (2.3-16.4)	0.0001
	> 19 years	18/14	11.6 (4.9-25.4)	0.0001
Frequency	1 to 4/day	27/33	4.8 (2.0-11.5)	0.0001
	>4 to 8/day	21/17	7.3 (2.8-18.9)	0.0001
	>8/day	11/10	6.5 (2.1-19.7)	0.001
Both Smokers+Chewers		18/3	26.5 (6.7-104.6)	0.0001

Ca: Cases, Co: Controls, OR: Odd Ratio, CI: Confidence Interval,* Reference Category

patients. Weak HPLC signals showing faint positivity of nicotine and cotinine were also observed in few samples of tobacco abstinence group in oral cancer patients.

Levels of urinary biomarkers

Figure 4 displays urinary nicotine, cotinine, thioether and NO2+ NO3 levels in controls, patients with OPC and oral cancer patients (both non-habitués and habitués). Urinary nicotine and cotinine levels were significantly higher (p=0.001 and p=0.03, respectively) in WHT than NHT. Urinary thioether and NO2+ NO3 levels were also higher in WHT than NHT, but the difference was not statistically significant. Patients with OPC showed significantly elevated urinary nicotine, cotinine and NO2+ NO3 levels (p=0.005, p=0.043 and p=0.01, respectively) as compared to NHT. Oral cancer patients (both tobacco non-habitués and tobacco habitués) also showed significantly elevated urinary nicotine (p=0.001 and p=0.001), cotinine (p=0.009 and p=0.009), thioether (p=0.05 and p=0.001) and NO2+ NO3 (p=0.031 and p=0.035) levels as compared to NHT.

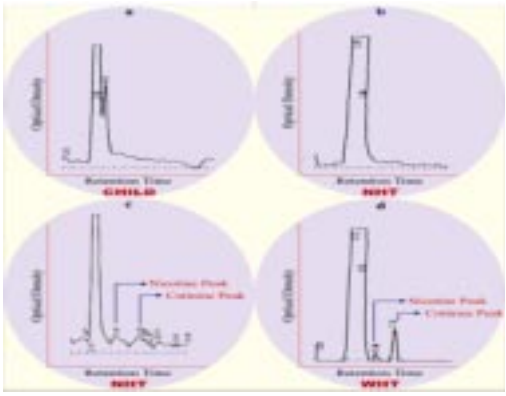


Figure 2. Representative Patterns of Controls a. A child showing absence of nicotine and cotinine peaks. b.A NHT showing absence of nicotine and cotinine peaks. c. A NHT showing faint presence of nicotine and cotinine peaks. d. A WHT showing prominent presence of nicotine and cotinine peaks

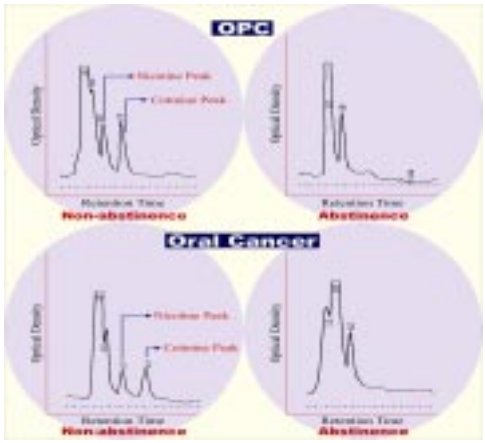


Figure 3. Representative Patterns of OPC and Oral Cancer Patients. 1.A patient with OPC (tobacco non-abstinence) showing presence of nicotine and cotinine peaks. 2. A patient with OPC (tobacco abstinence) showing absence of nicotine and cotinine peaks. 3.A oral cancer patient (tobacco non-abstinence) showing presence of nicotine and cotinine peaks. 4. A oral cancer patient (tobacco abstinence) showing absence of nicotine and cotinine peaks.

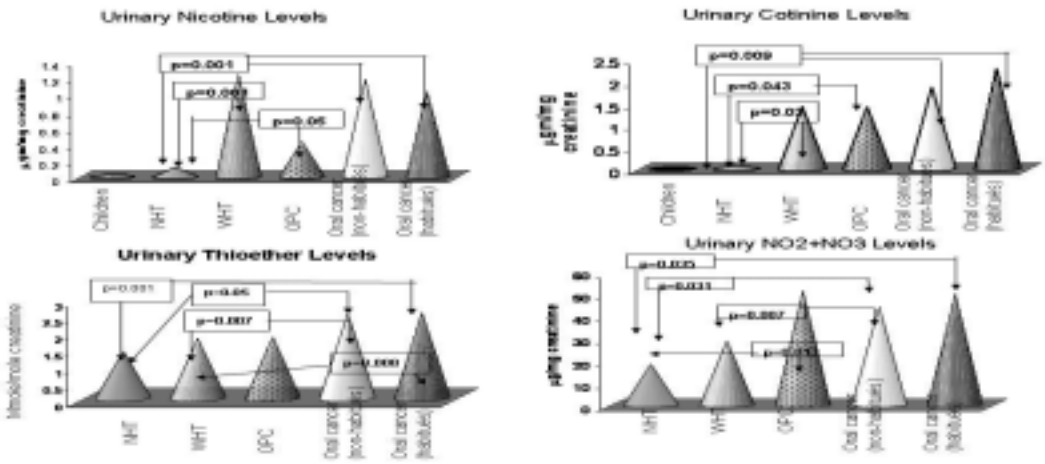


Figure 4. Urinary Nicotine, Cotinine, Thioether and NO2+NO3 Levels in the Subjects

Table 3.Urinary Parameters in NHT, WNT and Patients

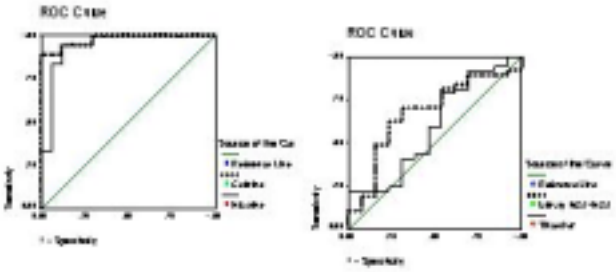
	NHT	WHT		Patients	
		Chewers	Smokers	Chewers	Smokers
Nicotine	0.12 ± 0.09	1.18 ± 0.25[1]	1.60 ± 0.26[4]	0.70 ± 0.27[4]	0.83± 0.2[4]
Cotinine	0.06 ± 0.03	1.25 ± 0.32[1]	1.40 ± 0.22[4]	0.23 ± 0.99[3]	0.85± 0.3[4]
Thioether	1.39 ± 0.19	1.59 ± 0.17	2.37 ± 0.31[7]	2.64 ± 0.37[5]	2.30± 0.3[8]
NO2+NO3	18.5 ± 2.83	27.2 ± 3.43[2]	28.3 ± 5.3	52.10 ± 8.1[6,10]	49.90± 8.0[9,11]

Values are Mean±SE 1: p=0.0001, 2:p=0.005, 3:p=0.05, 4:p=0.001,5:p=0.04, 6:p=0.032, 7:p=0.018, 8:p=0.006, 9:p=0.002 compared to NHT10:p=0.001 and 11:p=0.002 compared to WHT

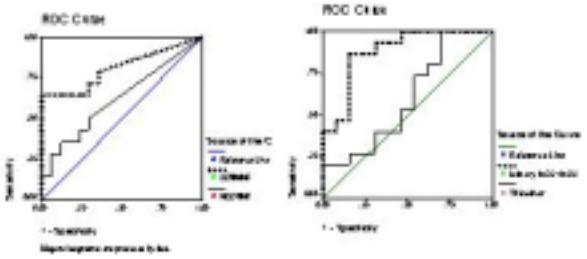
Table 4. Urinary Parameters in Non-abstinence and Abstinence Groups of Oral Cancer Patients

Parameters	Abstinence	Non- abstinence	p value
Nicotine	0.18 ± 0.11	0.44 ± 0.16	0.05
Cotinine	0.12 ± 0.06	0.92 ± 0.25	0.05
Thioether	2.37 ± 0.45	2.84 ± 0.59	NS
NO2+ NO3	55.5 ± 11.6	44.2 ± 11.28	NS

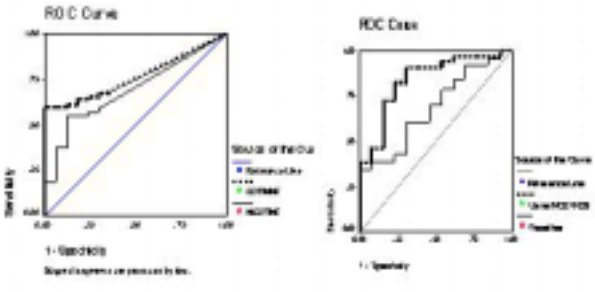
Values are Mean±SE. NS: Not Significant



(a) Between NHT and WHT



b) Between NHT and patients with OPC



(c) Between NHT and oral cancer patients

Figure 5. ROC curves for Comparison of Urinary Nicotine, Cotinine, Thioether and NO2+NO3 Levels

Comparison of urinary biomarkers with reference to tobacco habits

Table 3 provides mean urinary nicotine, cotinine, thioether and NO2+ NO3 levels in NHT and only tobacco chewers and smokers in controls and oral cancer groups. Mean urinary nicotine, cotinine and NO2+ NO3 levels were significantly higher in WHT (both smokers and chewers) and oral cancer patients (both smokers and chewers) than NHT. Mean urinary thioether levels were significantly higher in WHT (only smokers) and oral cancer patients (both smokers and chewers) than NHT. But the levels were comparable between NHT and WHT (only chewers). Oral cancer patients (both smokers and chewers) showed significantly elevated NO2+ NO3 levels as compared to WHT (both smokers and chewers).

Comparison of tobacco abstinence and non-abstinence groups with oral cancer

Comparison of urinary nicotine, cotinine, thioether and NO2 + NO3 levels in tobacco non-abstinence and abstinence groups of oral cancer patients are shown in Table 4. Mean values of urinary nicotine and cotinine levels were significantly higher in tobacco non-abstinence groups than the abstinence group (p=0.05). However, urinary thioether and NO2+ NO3 levels were comparable between tobacco abstinence and non-abstinence groups.

Receiver's operating characteristic (ROC) curves

ROC curves (Figure 5a) revealed that urinary nicotine, cotinine, and NO2+NO3 levels had significant efficacy to discriminate between NHT and WHT (p=0.0001, p=0.0001 and p=0.045, respectively). Urinary nicotine, cotinine and NO2+NO3 levels also showed high accuracy in discriminating between NHT and patients with OPC (Figure 5b). The ROC curve for urinary thioether could not exhibit high sensitivity and specificity for discriminating between NHT and patients with OPC. The ROC curves in Figure 5c depicts that urinary nicotine, cotinine, thioether and NO2+NO3 had high accuracy to discriminate between NHT and oral cancer patients.

Correlation between biomarkers by Pearson's test

Nicotine and cotinine are well known biomarkers for tobacco exposure. However, there are few reports on thioether and NO2+NO3 as biomarkers of tobacco exposure. Hence, the role of urinary thioether and NO2+NO3 as biomarkers of tobacco exposure needs to be validated. Therefore, Pearson's correlation was performed to evaluate correlation of urinary nicotine and cotinine with urinary thioether and urinary NO2+NO3 levels . The alterations in urinary thioether levels were positively associated with urinary nicotine (r=0.152). The

changes in urinary NO2+NO3 levels were positively associated with urinary cotinine levels (r=0.113).

Discussion

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 (Stepanov et al., 2005). The present study found higher levels of NO2+NO3 in tobacco alone, snuffing products and bidi. NO2+NO3 levels in cigarette and gutka were 0.14 and 0.13 mg/gm, respectively. NO2+NO3 levels in pan masala were 0.03 mg/gm. These results suggested that high amount of NO2+NO3 could be responsible for high amount of tobacco specific nitrosamine in tobacco.

The high incidence of oral cancer in India is linked with smokeless tobacco habits in majority of the population, who chew tobacco in various forms. Smoking habit of tobacco like cigarettes, cigars, pipes etc also cause oral cancer (Stepanov et al., 2005). Several reports have suggested the association of high risk of oral cancer with greater amounts of tobacco used and longer duration of use. But the reduction in risk of oral cancer was also associated with tobacco cessation (Winn, 2001). Population based case-controls studies have reported that cigarette smokers have been found to 2 to 5 times higher risks for oral cancer than that of nonsmokers (Blot et al., 1988 ; Hayes et al., 1999). Former cigar smokers have a lower risk of oral cancer than current smokers (Winn 2001) but even after ten years of abstinence; cigar smokers still have three times the risk for oral cancer than that of non-users (Schlecht et al., 1999). In the present study, small number of cases and controls were included for OR analysis. This study from Gujarat (western part of India) also found positive association between both tobacco smoking and chewing habits of tobacco and development of oral cancer. These results are consistent with previous data from other parts of India (Znaor et al.,2003).

Nicotine and cotinine can be detected using ultra-violet spectroscopy, thin layer chromatography and gas chromatography after basic extraction. Among these methods, ultra-violet spectroscopy will not differentiate between nicotine and cotinine. Other methods are either time consuming, costly or less sensitive. But the major advantage of HPLC method is the rapidity of elution of nicotine and cotinine and distinct separation leading to clear detection of these compounds (Horstmann, 1985). In the 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). Standardization was done considering various variables. 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. Thus, the result reveals that this method is highly sensitive and specific for urinary nicotine and cotinine estimation.

Cotinine, the proximate metabolites of nicotine have been extensively used as biomarkers of nicotine uptake from tobacco products. However, several reports documented that urinary nicotine and cotinine can be used as the biomarkers of exposure to environmental tobacco smoke (Benowitz, 1999). Several authors have reported that nicotine and cotinine in urine appears to be most specific and the most sensitive biomarkers for exposure of environmental tobacco smoke (Benowitz, 1996). Therefore, present study compared urinary nicotine and cotinine levels between children and NHT. Urinary nicotine and cotinine levels were slightly higher in NHT than children. The higher levels of nicotine and cotinine in NHT might be due to environmental tobacco smoke. 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, the present study included children whose mothers didn't use tobacco. Bhisey et al (1992) also reported that mean urinary cotinine levels were higher in passive smokers (bidi rollers) than unexposed individuals. Cok and Ozturk (2000) suggested that high 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. The non-abstinence group of oral cancer patients showed significant elevations of urinary nicotine and cotinine levels as compared to abstinence group. 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. Howver representative patterns showed faint peaks of urinary nicotine and cotinine in abstinence group of OPC. 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 NHT. 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) 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 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, inhalation pattern of smokers and the length of each cigarette smoked. Further, the authors also found that 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 without habit of tobacco 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.

In the present study, urinary thioether levels were significantly higher in WHT (only tobacco smoking habit) than NHT. 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 (1996) found higher urinary thioether levels in smokers as compared to the chewers. They suggested that smokers receive exposure to a 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. It is reported that method for urinary thioether cannot provide information about the structures of electrophiles, which are detected in urine as conjugates (Hecht, 2002). Kuralay and Yildiz (2001) suggested that use of non-specific urinary thioether levels and glutathione S-transferase 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 well discriminating efficacy between oral cancer patients and NHT as well as between patients with OPC and NHT. 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 (Cortas and Wakid, 1990). However, these methods lack specificity due to interference from biological materials. Enzymatic and Ion-chromatographic methods were more sensitive and specific but they required expensive reagents and equipments (Cortas and Wakid, 1990). The method based on reduction of nitrate to nitrite using cadmium metal; followed by estimation of nitrite by Griess reagent is more commonly used because it is sensitive, specific and inexpensive than other methods (Cortas and Wakid, 1990). Present study also employed the above method for estimation of urinary NO2+NO3. ROC curves showed that NO2+NO3 could discriminate between NHT and oral cancer. These results indicated that this method is highly sensitive which is in accordance with earlier reports (Cortas and Wakid, 1990). In the current investigation, urinary NO2+NO3 levels were higher in WHT, patients with OPC and oral cancer patients than NHT. 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. 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 (habitués and non-habitués) 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.

Present study observed positive correlation between tobacco exposure and urinary biomarkers including urinary nicotine, cotinine, thioether and NO2+NO3. Pearson's correlation test also revealed that the values of nicotine were positively associated with the alterations in urinary thioether. The urinary cotinine levels were positively associated with urinary NO2+NO3 levels. A report by Malaveille et al (1989) also showed correlations of urinary nicotine and cotinine with urinary thioether and nitrate levels which also support the current observations.

In the best of our knowledge, there are no reports on simultaneous evaluation of NO2+NO3 levels in tobacco and urinary biomarkers in healthy individuals, patients with OPC and oral cancer patients. This study observed that high exposure to tobacco specific nitrosamines (through production of NO2+NO3) is likely to be the major contributing factor for the epidemic of oral cancer in India. Tobacco smoking and chewing habits are also prominent risk factors for development of oral cancer. The modified HPLC method was sensitive and economic method for estimation of nicotine and cotinine as biomarkers of tobacco exposure. Urinary NO2+NO3 and thioehter levels can be used as additional markers for tobacco exposure. In a nutshell, the results revealed that tobacco chewing and smoking habits were prominent risk factors for development of oral cancer in western part of India (Gujarat). Therefore the present approach can helpful for oral cancer screening programme, which may ultimately decrease the toll of tobacco related cancer in India.

References

Bagwe AN, Bhisey RA (1995). Measurement of exposure to alkylating agents (potential carcinogens)-estimation of urinary thioethers. In Deo MG, Seshadri R, Mulherkar R, Mukhopadhyaya R (eds), Techniques in Cancer Research-a Laboratory Manual, Tata Memorial Centre, Bombay, 322-323.

Benowitz NL (1996). Cotinine as a biomarker of environmental tobacco smoke exposure. *Epidemiol Rev*, **18**, 188-204.

Benowitz NL (1999). Biomarkers of environmental tobacco smoke exposure. *Env Health Perspect*, **107**, 349-55.

Bhisey RA, Govekar RB, Bagwe AN (1992). Toxic effect of exposure to tobacco among bidi rollers. In Gupta PC, Hamner JE, and Murti PR Eds.'Control of Tobacco-related Cancers and Other Disease', International symposium, 1990, Oxford University Press, Bombay, 223-7.

Bhisey RA, Govekar RB, Bagwe AN, et al (1991). Biological risk assessment in tobacco chewers: A population at High risk for oral cancer. In Rao RS and Desai PB (eds), Oral Cancer, Tata Memorial centre, Bombay, 191-9.

Blot WJ, McLaughin JK, Winn DM, et al (1988). Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res*., **48**, 3282-7.

Cok I and Ozturk R (2000). Urinary cotinine levels of smokeless tobacco (Maras powder) users. *Human & Exp Toxicol*, **19**, 650-5.

Cortas NK, Wakid NW (1990). Determination of inorganic nitrate in serum and urine by a kinetic cadmium-reduction method. *Clin Chem*, **36**, 1440-3.

Crawford FG, Mayer J, Santella RM, et al (1994). Biomarkers of environmental tobacco smoke in preschool children and their mothers. *J Natl Cancer Inst*, **86**, 1398-402.

Daftary DK, Murti PR, Bhonsle RR, et al (1991). Risk factors and risk markers for oral cancers in high-risk areas of the world. In Jonson NW. ed. 'Oral Cancer. The Detection of Patients and Lesions at Risk', Cambridge University Press, 2, 29-63.

Ghosheh OA, Brome D, Rogers T, et al (2000). A simple high performance liquid chromatographic method for the quantification of total cotinine, total 3'-hydroxycotinine and caffeine in the plasma of markers. *J Pharm Biomed Anal*, **23**, 543-9.

Green LC, Wagner DA, Glogowski J, et al (1982). Analysis of nitrate and (15N) nitrate in biological fluids. *Anal Biochem*, **126**, 131-8.

Hatsukami DK, Severson HH (1999). Oral spit tobacco: addiction prevention and treatment. *Nicotine Tob Res*, **1**, 21-44.

Hayes RB, Bravo-Otero E, Kleinman DV, et al (1999). Tobacco and alcolol use and oral cancer in Puerto Rico. *Cancer Causes Control*, **10**, 27-33.

Hecht SS (2002). Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. *Carcinogenesis*, **23**, 907-22.

Hoffmann D, Hecht SS (1985). Nicotine-derived N-nitrosamines and tobacco-related cancer: current status and future directions. *Cancer Res*, **45**, 935-44.

Horstmann M (1985). Simple high-performance liquid chromatographic method for rapid determination of nicotine and cotinine in urine. *J Chromatog*, **344**, 391-6.

Kuralay F, Yildiz T (2001). Urinary thioether excretion and erythrocyte glutathione S-Transferase activity in smokers and patients with squamous cell laryngeal cancer. *J Toxicol Envl Hlth*, **64**, 447-52.

Lequang, Thuan NT, Migueres ML et al (1989). Elimination of caffeine interference in HPLC determination of urinary nicotine and cotinine. *Clin Chem*, 35, 1456-9.

Malaveille C, Vineis P, Esteve J, et al (1989). Levels of mutagens in the urine of smokers of black and blond tobacco correlate with their risk of bladder cancer. *Carcinogenesis*, **10**, 577-86.

Ong CN, Lee BL, Shi CY, et al. (1994). Elevated levels of benzene related compounds in the urine of cigarette smokers. *Int J Cancer*, **59**, 177-180.

Pakhale SS, Maru GB (1995). Analysis of tobacco and its products for alkaloids, tobacco specific nitrosamines and nitrate and nitrites. In Deo MG, Seshadri R, Mulherkar R, Mukhopadhyaya R (eds), 'Techniques in Cancer Research a Laboratory Manual', Tata Memorial centre, Bombay, 331-336.

Pershagen G (1996). Smokeless tobacco. *Br Med Bull*, **52**, 50-7.

Peto R, Lopez A.D, Boreham J, et al (1996). Mortality from smoking worldwide. *Br Med Bull*, **52**, 12-21.

Scherer G, Doolittle DJ, Ruppert T, et al (1996). Urinary mutagenicity and thioethers in non-smokers: role of environmental tobacco smoke (ETS) and diet. *Mutat Res*, **368**, 195-204.

Schlecht NF, Franco EL, Pintos J, Kowalski LP (1999). Effect of smoking cessation and tobacco type on the risk of cancers of the upper aero-digestive tract in Brazil. *Epidemiology*, **10**, 412-8.

Stepanov I, Hecht SS, Ramakrishnan S, Gupta P (2005). Tobacco-specific nitrosamines in smokeless tobacco products marketed in India, *Int J Cancer*, **116**, 16-9.

Surmen-Gur E, Adnan E, Serdar Z, Gur H (2003). Influence of acute exercise on oxidative stress in chronic smokers. *J Sports Sci Med*, **2**, 98-105.

Van Bezooijen RL, Que I, Ederreen AGH, et al (1998). Plasma nitrate plus nitrite levels are regulated by ovarian steroids but do not correlate with trabecular bone mineral density in rate. *J Endocrinol*, **159**, 27-34.

Varleg H (1967). Practical Clinical Biochemistry, 4th edition, William Heinemann, London and Interscience, New York, 197-198.

Watson ID (1977). Rapid analysis of nicotine and cotinine in the urine of smokers by isocratic high performance liquid chromatography. *J Chromatog*, 143, 203-6.

Winn DM (2001). Tobacco use and oral disease. *J Dent Educ*, **65**, 306-12.

Wu Y, Chen J, Ohshima H, et al (1993). Geographic association between urinary excretion of N-nitroso compounds and esophageal cancer mortality in China. *Int J Cancer*, **54**, 713-719.

Znaor A, Brennan P, Gajalakshmi V, et al (2003). Indendent and combined effects of tobacco smoking, chewing and alcohol drinking on the risk of oral pharyngeal and esophageal cancers in Indian men. *Int J Cancer*, **105**, 681-6.