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**Abstract.** Tobacco abuse and alcoholism cause cancer, emphysema, and heart disease, which contribute to high death rates, globally. Society pays a significant cost for these habits whose first demonstration in many cases is in the oral cavity. Oral cavity disorders are highly curable if a screening procedure is available to diagnose them in the earliest stages. The aim of the study is to identify the severity of tobacco abuse, in oral cavity, as reflected by the emission from endogenous fluorophores and the chromophore hemoglobin. A group who had no tobacco habits and another with a history of tobacco abuse were included in this study. To compare the results with a pathological condition, a group of leukoplakia patients were also included. Emission from porphyrin and the spectral filtering modulation effect of hemoglobin were collected from different sites. Multivariate analysis strengthened the spectral features with a sensitivity of 60% to 100% and a specificity of 76% to 100% for the discrimination. Total hemoglobin and porphyrin levels of habitués and leukoplakia groups were comparable, indicating the alarming situation about the risk of tobacco abuse. Results prove that fluorescence spectroscopy along with multivariate analysis is an effective noninvasive tool for the early diagnosis of pathological changes due to tobacco abuse. © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: [10.1117/1.JBO.19.5.057013](https://doi.org/10.1117/1.JBO.19.5.057013)]

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## 1 Introduction

Tobacco abuse is the leading cause of premature death worldwide. As per World Health Organization reports, about 4.9 million deaths were estimated due to tobacco-related illness in 2000, and by 2020 it is predicted to rise to 10 million deaths per year. Harmful effects of tobacco can be attributed to a variety of cancers, including those of the lung, oral cavity, nasal cavity, larynx, oropharynx, hypopharynx, oesophagus, stomach, liver, pancreas, bladder, ureter, kidney, and cervix. Tobacco carcinogenicity is more than evident, and about 50% of oral cancer cases are attributable to chewing and smoking of tobacco.<sup>1,2</sup>

Exposure of oral cavity tissues to the carcinogens such as polynuclear aromatic hydrocarbons present in tobacco, during smoking, and tobacco-specific nitrosamines during chewing results in malignant changes. Tobacco abuse, either smoking or chewing with alcoholism or areca nut chewing, further increases the risk of oral cancer. Acetaldehyde production due to ethanol oxidization and reactive oxygen species generation due to auto-oxidation of polyphenols in areca nut also increase the chances of malignancy.<sup>1-4</sup>

In South and Southeast Asia, especially countries such as India, Pakistan, and Sri Lanka where one-fifth of the world population exists, incidence of oral cancer is devastating. Abuse of tobacco in the form of both chewing and smoking is termed as the prime reason for higher incidence in these areas. Lack of awareness and cultural traditions are the main causatives. Moreover, people who are in the lower socio-economic background are the main prey of this disease. Unavailability of extensive diagnosis facilities makes this disease more severe.<sup>5,6</sup>

Histopathology followed by scalpel or punch biopsy is the gold standard for oral cancer screening.<sup>7</sup> But this technique based on the changes in tissue pathology most often fails to provide the exact diagnosis.<sup>8</sup> Moreover, by the time the patient reaches the clinic, the disease would have progressed to a stage that can be visibly identified by the clinician. There is no medical demand in diagnosing such a lesion that is easily visible by the naked eye. In order to avoid this delay in diagnosis and also for an effective early diagnosis, bedside optical diagnosis techniques are in more demand. Moreover, these techniques are capable of detecting early-stage biochemical alterations within the tissue in a minimally invasive or noninvasive way.<sup>9-18</sup>

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**Table 1** Clinical report of leukoplakia patients involved in this study.

Sl no	Sex/age	Tobacco chewing		Smoking		Alcoholism		Biopsy report
		Duration in months	Frequency per day	Duration in months	Frequency per day	Duration in months	Frequency per week	
1	F/48	372	8	N	N	N	N	MD
2	M/39	264	10	288	20	228	O	EH
3	M/66	600	4	540	4	N	N	EH
4	M/42	264	8	N	N	264	O	EH
5	M/63	564	5	564	10	480	O	MD
6	M/45	300	6	N	N	240	7	EH
7	M/53	288	5	396	20	348	O	EH
8	F/39	276	12	N	N	N	N	MD
9	M/32	216	10	N	N	192	O	MD
10	M/44	324	6	288	4	312	4	EH
11	F/59	444	8	N	N	N	N	EH
12	M/61	528	6	540	O	N	N	MD
13	M/62	552	4	N	N	456	5	EH
14	F/41	300	12	N	N	N	N	EH
15	M/48	384	3	396	24	300	O	EH
16	F/45	336	8	N	N	N	N	EH
17	F/70	576	4	576	O	456	O	EH
18	M/42	360	10	264	O	264	O	MD

Note: N, nil; O, occasionally; MD, mild dysplasia; EH, epithelial hyperplasia.

Fluorescence, infrared, Raman, and diffuse reflectance spectroscopy are the emerging *in vivo* optical diagnosis tools in medical oncology.<sup>10-18</sup> Among these, *in vivo* fluorescence spectroscopy is widely used for oral cancer diagnosis because of its simplicity to use, less time consumed, and improved patient comfort level.<sup>13-15</sup> In this study, we present variation in auto-fluorescence spectra from the oral mucosa of habitual tobacco users with that of a group of volunteers without any habits in a clinical setup. We have also considered a group of patients with leukoplakia. This group is included to demonstrate the harmful effects of tobacco in the oral mucosa among the habitual tobacco users. Leukoplakia is a potentially malignant disease with potentiality greater than 30%. This disease is six times more common among habitual tobacco users than nonusers.<sup>19</sup> In the Indian sub-continent, incidence of oral leukoplakia is three times higher than in the Western population. Smoking and chewing of areca nut with tobacco cause this high incidence.<sup>20</sup>

To strengthen the spectral observations in assessing the variation caused by habits associated with tobacco, quantification of hemoglobin concentration and porphyrin levels was performed. Feasibility of using a single system to analyze the changes in fluorophores and chromophores within the tissue was done

using the spectral filtering modulation (SFM) effect on the fluorescence spectra due to hemoglobin absorption.<sup>18,21</sup> Principal component analysis followed by linear discriminant analysis (PCA-LDA) is used for the exact differentiation of spectra among the groups and to obtain the performance level of the clinical trial.

## 2 Materials and Methods

### 2.1 Study Population and Protocols

The study subjects included 30 volunteers without any habits (nonhabitueés), 30 volunteers with habits (habitueés), and 18 clinically diagnosed leukoplakia patients. The patient selection was done by an experienced oral oncologist based on the clinical manifestation of leukoplakia in this group. The other two groups had no history of oral cavity diseases and none of them was taking any medications. The subjects who had any tobacco-based habits were excluded from the nonhabitueés group. The procedure was explained and informed consent was obtained from all patients and volunteers who participated in this study. Age, sex, and details of oral habits of habitueés and leukoplakia patients were recorded.

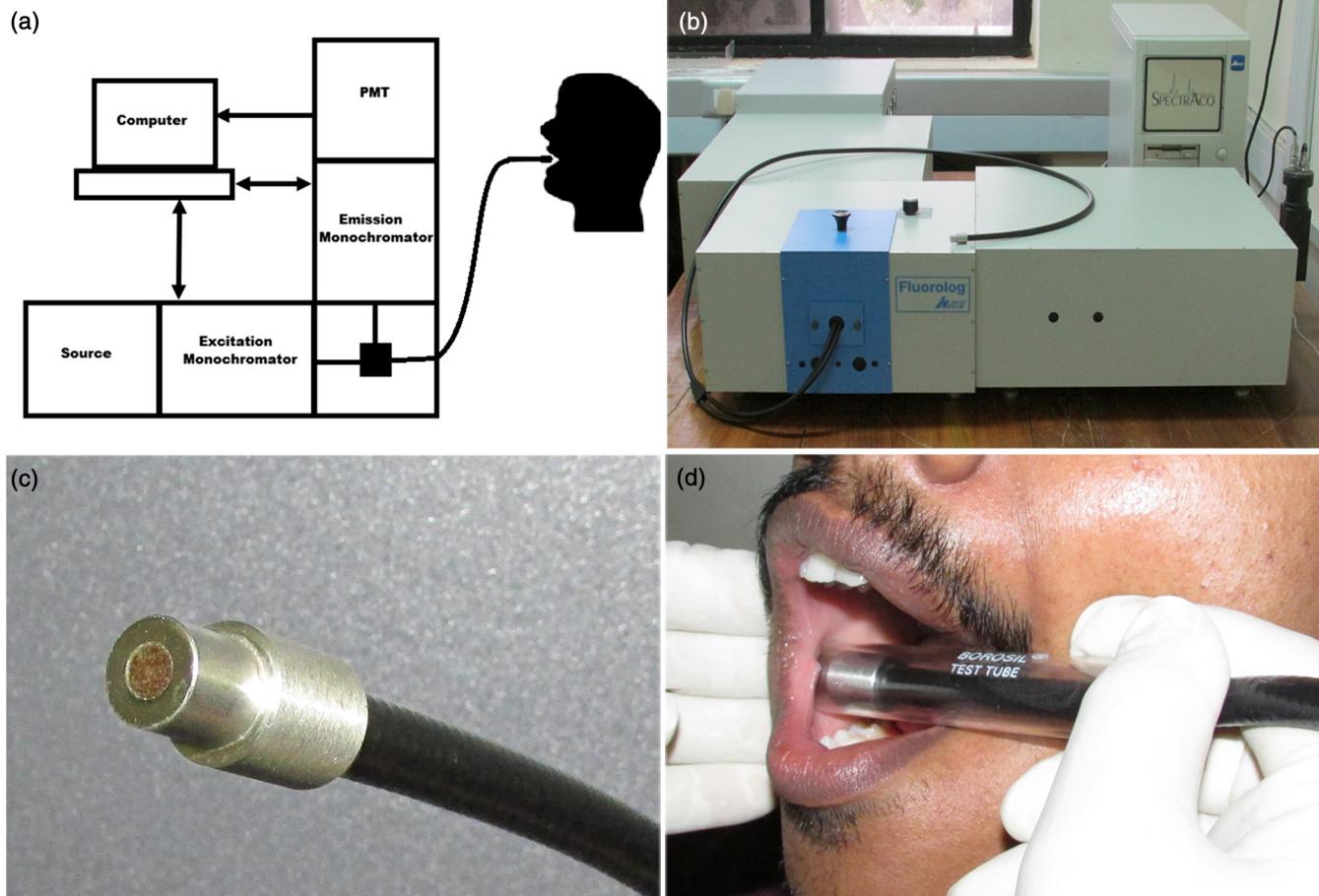
The details of leukoplakia patients such as age, gender, duration, and frequency of tobacco chewing, smoking and alcoholism, and pathological grading are given in Table 1. Age of nonhabitueés ranged from 20 to 52 years (16 men and 14 women) and that of habitueés ranged from 23 to 67 years (18 men and 12 women). Habitueés and leukoplakia patients involved in this study had prolonged tobacco chewing habits with smoking or alcoholism or both together. They were using either betel quid with tobacco or khaini (powdered form of tobacco) available in packets for chewing. Healthy volunteers involved in this study were free from such habits and maintained good oral health and hygiene. Before spectral acquisition, all volunteers and patients were asked to clean their mouth using 1.5% saline solution for 2 to 3 min in order to reduce the effects of recently consumed food.

In the case of nonhabitueés and habitueés, spectral measurements were obtained from different anatomical sites of the oral cavity such as right and left buccal mucosa, palate, and floor of the mouth. Spectra from a total of 22 sites with suspicious lesions were acquired from 18 leukoplakia patients. An experienced clinical oral oncologist identified suspicious lesions in each leukoplakia patient for spectral studies. Since there are reports highlighting the distortion of autofluorescence emission of porphyrin due to bacterial colonization in the tongue, we have excluded spectra of the tongue from this study.

## 2.2 Fluorescence Spectroscopic Study

The optical spectroscopic measurements were carried out using the spectrofluorometer (Fluorolog-III; Jobin Yvon Inc., Edison, New Jersey). The instrument consists of a 450-W Xenon arc lamp, a double excitation monochromator, a double emission monochromator, and a photomultiplier tube. A bifurcated Y type fiber-optic probe is coupled to the sample compartment to enable *in vivo* measurements. This multimode fiber-optic probe consists of illumination fibers and collection fibers. All fibers have a numerical aperture of 0.22.

One arm of the Y type fiber-optic probe was connected to the source. The desired excitation wavelength was selected and the light transmitted to the tissue site through this arm. The received fluorescence signal was directed back to the spectrometer through the other arm. A transparent test tube (Borosil) was used to cover the distal end of the Y type fiber-optic probe in order to avoid contamination. To compensate for the changes made by the test tube, a correction factor was uniformly applied to all spectra. The test tube was cleaned and disinfected with 2% glutaraldehyde solution after each spectral acquisition. The experimental setup is illustrated in Fig. 1. The excitation wavelength of 410 nm is selected using Datamax™ software (Datamax, Round Rock, Texas). The emission spectrum was recorded in the range of 455 to 750 nm in 1-nm increments.



**Fig. 1** (a) Schematic representation of *in vivo* fluorescence measurement setup. (b) Spectrofluorometer with the fiber-optic probe. (c) Fiber-optic probe bundle for *in vivo* analysis. (d) Spectral acquisition from oral cavity using a fiber-optic probe.

During spectral acquisition, care has been taken to maintain a uniform pressure applied at the tip of the probe for all the cases.

### 2.3 Data Processing and Analysis

#### 2.3.1 Processing of spectra

All spectra were baseline corrected and the data values extracted using Datamax™. Spectra were normalized with respect to the maximum intensity of the peak at  $500 \pm 10$  nm. From the normalized data, the peak intensity at the wavelengths of 500, 560, 570, and 635 nm was extracted.

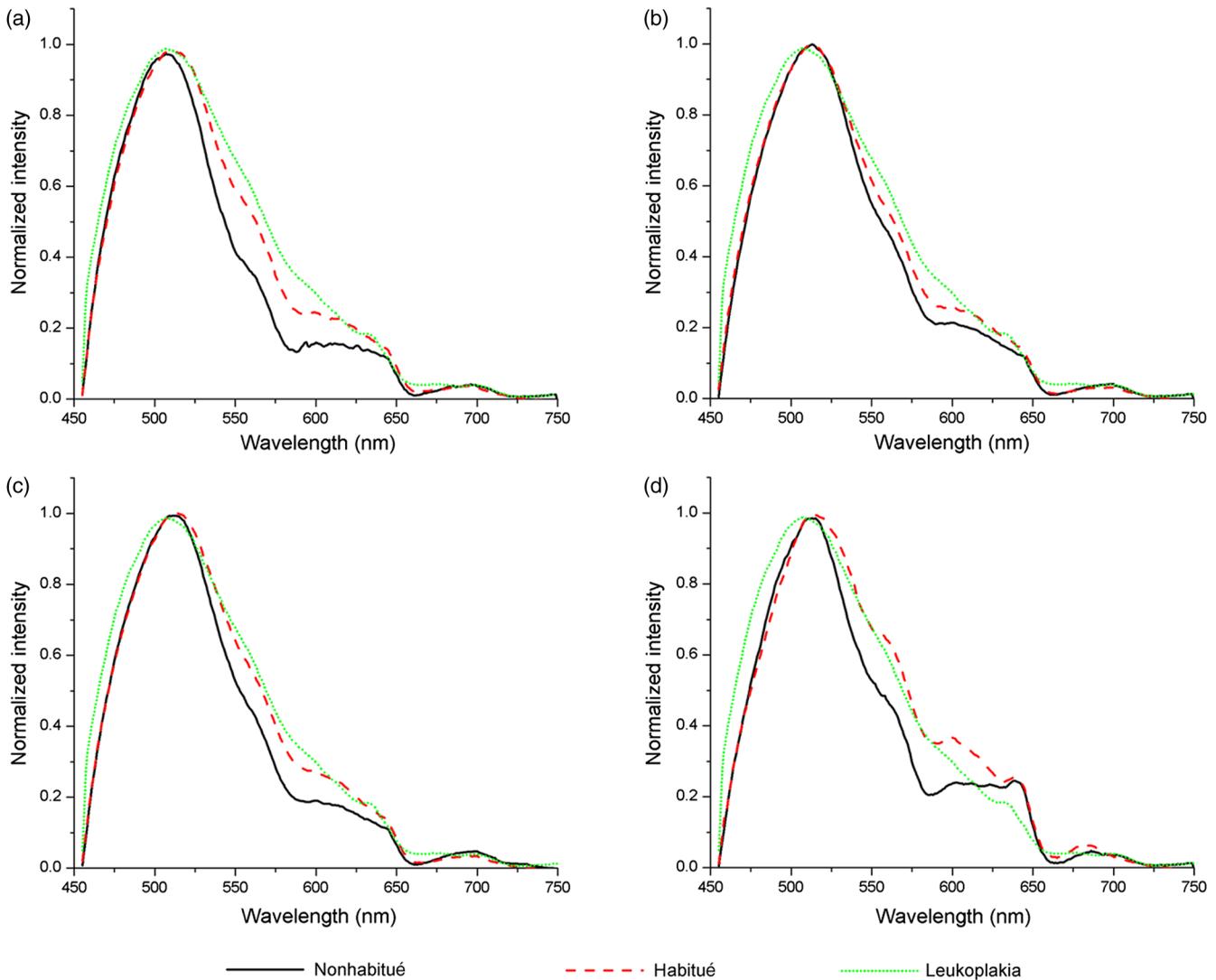
#### 2.3.2 Estimation of total hemoglobin concentration

The ratio of fluorescence intensity at 500 and 570 nm was used to estimate the concentration of total hemoglobin as per the method of Liu and Vo-Dinh.<sup>21</sup> These specific emission intensities were chosen because the extinction coefficients of oxygenated and deoxygenated hemoglobin at these two wavelengths are equal. Hence, it is theoretically and experimentally proved

that the ratio between fluorescence intensity at these two wavelengths will give the total hemoglobin concentration within the tissue. Total hemoglobin concentration of brain tissues has been reported earlier by the same method.<sup>18</sup>

#### 2.3.3 Multivariate statistical analysis

PCA-LDA is a dimension reduction way of classification. In this study, we have used PCA to reduce the high dimension of fluorescence spectral data (455 to 750 nm with a set of 295 intensities) which may cause computational complexity in the optimization and implementation of LDA.<sup>16</sup> PCA on the normalized spectra of each category was performed using SPSS-17 (SPSS Inc., Chicago, Illinois). The objective of dimension reduction in PCA was to achieve more compact representation of the original data that capture the information essential for higher-level decision making. In PCA, dimension reduction is performed by processing the number of competitiveness indicators into a small number of independent indicators through transforming the internal structure of correlation matrix into a specified number in the original indicator variables.<sup>22</sup>



**Fig. 2** Average fluorescence emission spectra from different anatomical sites of nonhabituated, habituated, and suspected sites of leukoplakia patients: (a) floor of the mouth, (b) left buccal mucosa, (c) right buccal mucosa, and (d) palate.

In order to reduce the dimension of the spectral data, we have used PCA to extract a set of orthogonal PCs comprising loadings and scores that account for the maximum variance in spectral datasets.<sup>23</sup> These loadings and scores of the PCA model provide a reduced dataset which is a compact replica of the spectroscopic data. These significant PC scores ( $p < 0.05$ ) were selected as input for the LDA model for oral cavity tissue classification. LDA determines the discriminant function that maximizes the variances in the dataset between groups while minimizing the variances between members of the same group. The performance of the diagnostic algorithms based on LDA models to predict the tissue groups was estimated by leave-one-tissue-site-out, cross-validation method. This method of cross-validation produces a confusion matrix that compares predicted versus actual group membership.<sup>16</sup> Diagnostic sensitivity and specificity of autofluorescence spectroscopy technique for oral tissue differentiation were assessed using these classification results based on PCA-LDA models.

### 3 Results

#### 3.1 Fluorescence Spectral Features

Averaged fluorescence spectra from different sites of nonhabitué, habitué, and suspected sites of leukoplakia patients are given in Fig. 2. The spectra show the major peaks around 500, 560, 635, and 690 nm. One-way analysis of variance (ANOVA) on peak intensity at 560 and 635 nm obtained for different sites of nonhabitué, habitué, and suspected sites of leukoplakia patients are given in Table 2.

When the spectra of nonhabitué to habitué to leukoplakia are considered, a clear and gradual increase in intensity but decrease in spectral resolution of 560-nm peak is observed. The peak intensity varied significantly ( $p < 0.05$ ) for

**Table 2** One-way analysis of variance values on peak intensities and total hemoglobin concentration ( $p < 0.05$  is given in bold letters).

Wavelength (nm)/calculated parameter	Locations in oral cavity	Nonhabitué versus habitué	Nonhabitué versus leukoplakia	Habitué versus leukoplakia
560	Floor	<b>0.000</b>	<b>0.000</b>	0.187
	Left	<b>0.041</b>	<b>0.000</b>	0.136
	Right	<b>0.000</b>	<b>0.000</b>	0.223
	Palate	<b>0.001</b>	<b>0.000</b>	0.439
635	Floor	0.123	0.117	0.616
	Left	<b>0.016</b>	<b>0.046</b>	0.552
	Right	<b>0.000</b>	<b>0.034</b>	0.549
	Palate	0.716	0.121	0.108
Total hemoglobin concentration	Floor	<b>0.002</b>	<b>0.000</b>	0.192
	Left	<b>0.029</b>	<b>0.009</b>	0.408
	Right	<b>0.002</b>	<b>0.000</b>	0.577
	Palate	<b>0.003</b>	<b>0.022</b>	0.470

nonhabitué versus habitué and nonhabitué versus leukoplakia for all the sites. But for habitué versus leukoplakia, there is no significant difference in the peak intensity for any of the sites.

The 635-nm peak is more prominent in habitué and leukoplakia than nonhabitué. Significant difference ( $p < 0.05$ ) in peak intensity is observed for left and right buccal mucosa when nonhabitué versus habitué and nonhabitué versus leukoplakia are considered. For habitué and leukoplakia, the peak intensity is found to be nearly the same with no significant change. The peak around 690 nm has nearly the same intensity for all groups considered irrespective of the site.

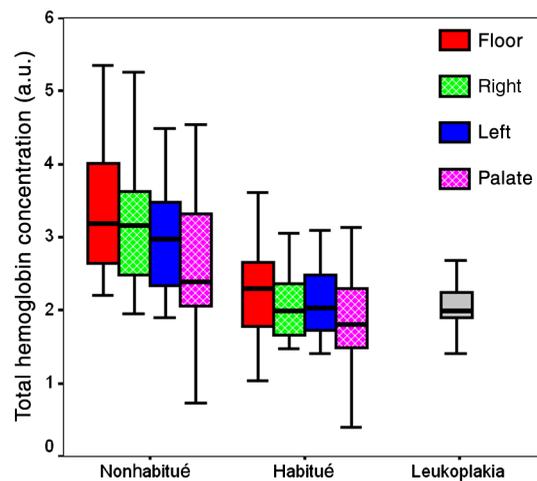
#### 3.2 Estimation of Total Hemoglobin Concentration

Total hemoglobin concentration observed for nonhabitué, habitué, and leukoplakia patients are given in a box plot (Fig. 3). Considerable decrease in the total hemoglobin concentration is observed for habitué and leukoplakia patients from that of nonhabitué. Total hemoglobin concentrations observed for habitué and leukoplakia patients are found to be nearly equal. One-way ANOVA on total hemoglobin concentration obtained for different sites of nonhabitué, habitué, and suspected sites of leukoplakia patients are given in Table 2. A statistically significant difference ( $p < 0.05$ ) in the total hemoglobin concentration was observed between nonhabitué and habitué and nonhabitué and leukoplakia irrespective of the sites. But the difference between habitué and leukoplakia was found to be insignificant.

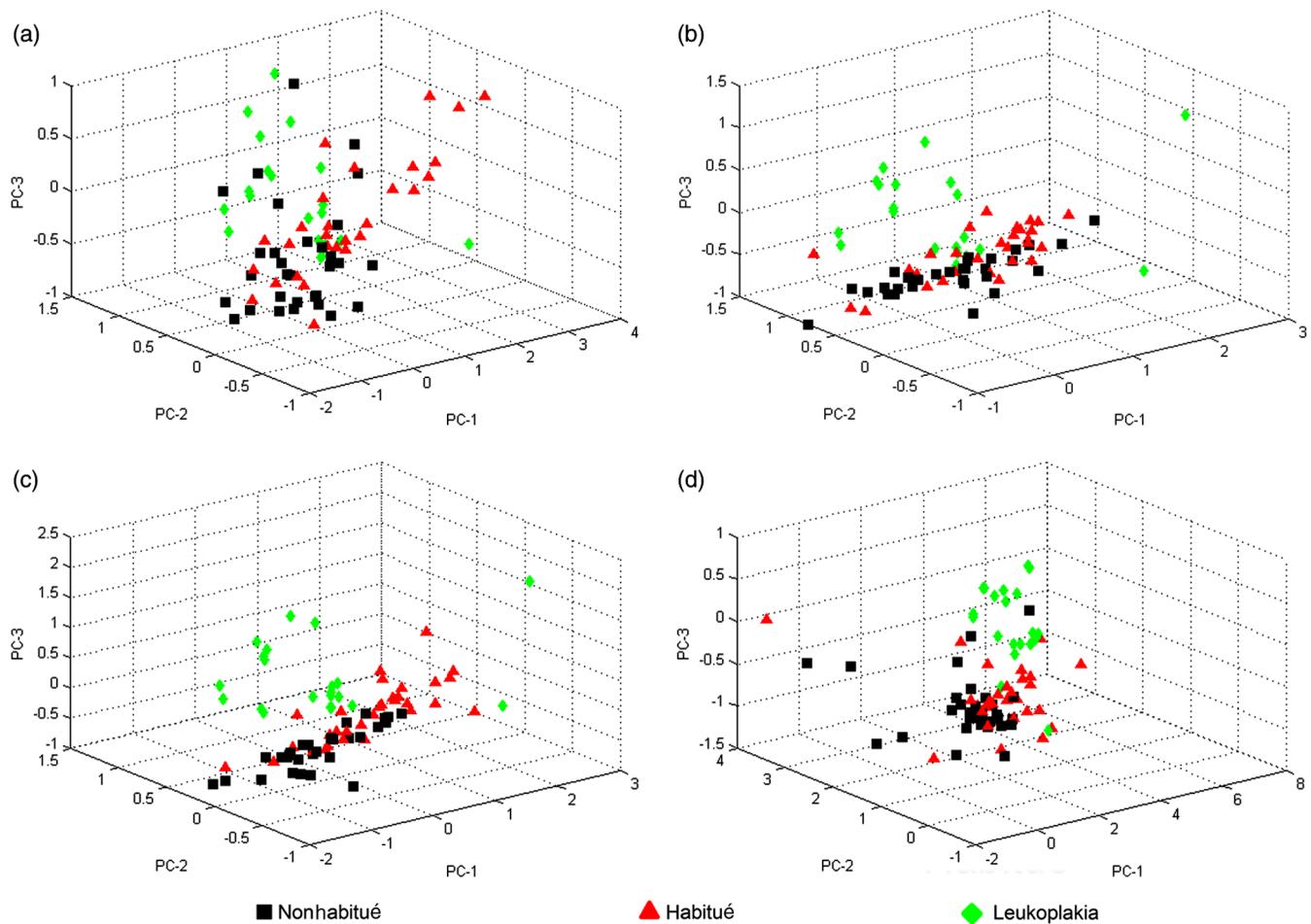
#### 3.3 Multivariate Analysis

To strengthen the accuracy of spectral measurement-based tissue classification, multivariate analysis, PCA-LDA was employed. PCA was first performed on spectra acquired from each site of all groups for understanding the critical spectral features of autofluorescence spectra as well as for looking at the group behavior of the oral cavity tissue.

Figure 4 shows the PC score plot of autofluorescence spectral analysis of the four different sites of oral cavity of nonhabitué, habitué, and leukoplakia. The first three PCs are plotted along



**Fig. 3** Total hemoglobin concentration within different anatomical sites of nonhabitué, habitué, and suspected sites of leukoplakia patients.



**Fig. 4** PC score plot of the first three principal components for the spectral dataset acquired from different anatomical sites of nonhabituated, habituated, and suspected sites of leukoplakia patients: (a) floor of the mouth, (b) left buccal mucosa, (c) right buccal mucosa, and (d) palate.

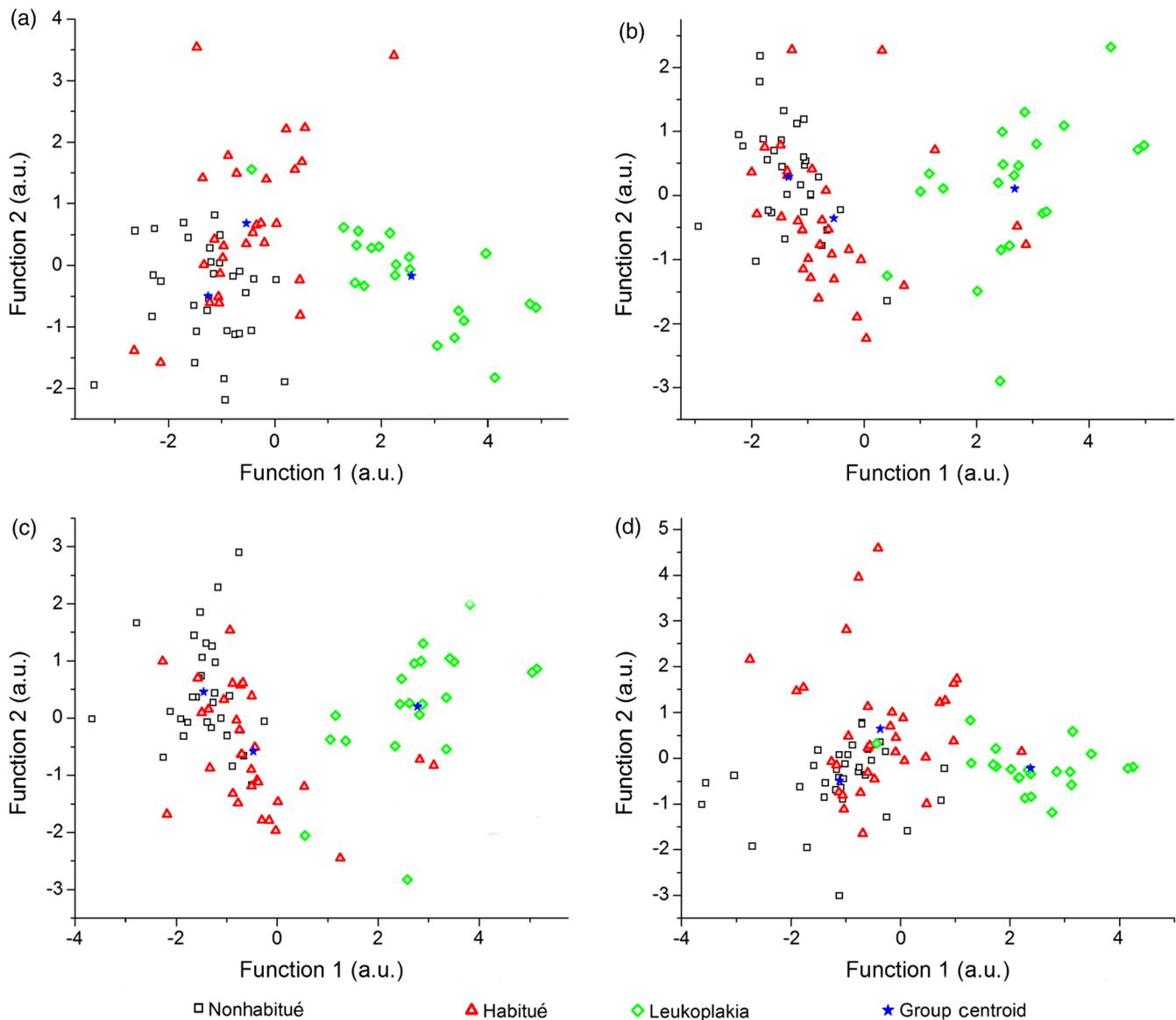
$x$ -,  $y$ -, and  $z$ -axes. Apart from that of palate, the PC scores of other sites tend to cluster and at the same time remain fairly separated from other classes. Among these, leukoplakia appears to be the easiest to distinguish based on the distance from the nonhabituated and habituated. This provides a new outlook to the tissue-tissue intraclass variations. Even though PCA is a valuable tool for characterizing similarities between tissue types, due to its unsupervised nature, it does not automatically provide class memberships. To assign class membership to these site-specific spectra, we have adopted the method of LDA.

A discriminant function scatter plot for the sites floor of the mouth, left and right buccal mucosa, and palate is given in Fig. 5. The first two discriminant functions are plotted along  $x$ - and  $y$ -axes. Group averages are represented by centroids. The distances between these centroids are found to be nearly equal for all the sites. Classification based on the variation in this Mahalanobis distance is a multivariate measure of separation between groups. The sample is classified into the group from which it has shorter Mahalanobis distance. Figure 6 shows the pairwise discriminant score for all the sites considered in this study. Discrimination line drawn at 0 gives a good separation. Positive and negative predictive values were obtained using this position of pairwise discriminant score. These values are used for binary calculations in order to obtain the performance level of PCA-LDA, and the results are given in Table 3.

## 4 Discussion

The extent and nature of structural and biochemical changes that take place during the transformation from normal to precancerous state due to lifestyle oral habits are poorly understood. These changes can lead to cancer in the oral cavity. Although there are many reports on the use of fluorescence spectroscopy in differentiating normal from cancerous oral mucosa, early tissue transformation stages due to tobacco-associated habits has not been investigated in detail. In this study, we have attempted the *in vivo* analysis of biochemical changes caused by lifestyle oral habits, which can lead to oral cancer, using fluorescence spectroscopic technique. In order to compare the harmful effects of habits, a group of habituated who had potentially malignant lesions of clinically confirmed leukoplakia cases were also included in the study. Variation in the concentration of endogenous fluorophores such as flavin adenine dinucleotide (FAD), porphyrin, and chromophore hemoglobin was specifically evaluated in achieving this goal.

To obtain better efficiency in classification, preprocessing methods such as baseline correction and normalization with respect to a specific autofluorescence peak were also done.<sup>14,18</sup> Normalized datasets can provide a comparative quantification of a specific fluorophores and better classification efficiency. Therefore, we have used normalized spectra throughout this study.



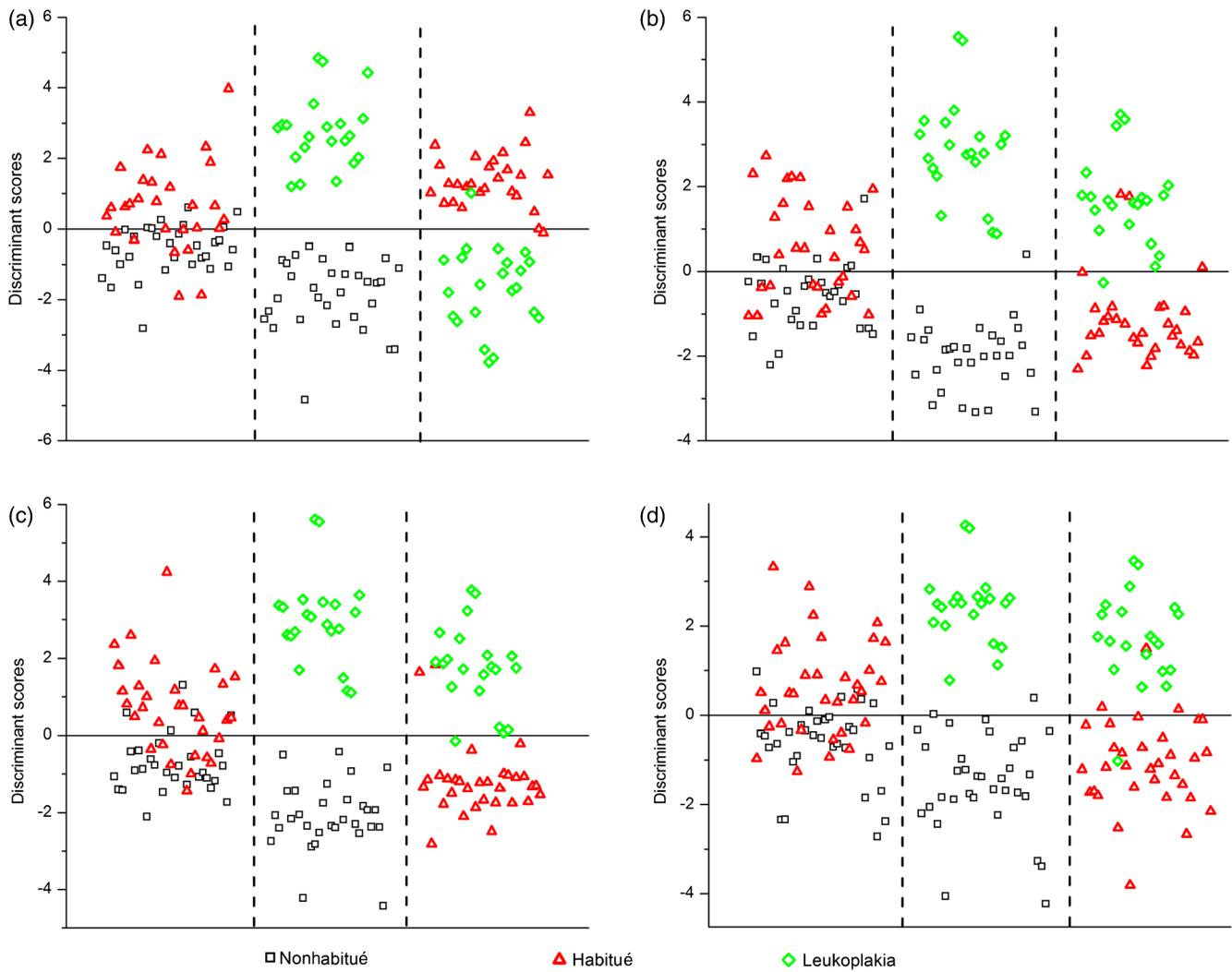
**Fig. 5** Linear discriminant analysis (LDA) score plots of the fluorescence dataset. (a) Floor of the mouth, (b) left buccal mucosa, (c) right buccal mucosa, and (d) palate.

The excitation wavelength around 410 nm is ideal for observing the emissions from endogenous fluorophores FAD and porphyrin. FAD gives emission around 500 nm and porphyrin around 630 and 690 nm.<sup>13,21,24</sup> This excitation also gives peak around 560 nm due to SFM effect of hemoglobin absorption on fluorescence spectra.<sup>18,21</sup>

A considerable increase in the intensity of porphyrin peak is observed for habitués from that of nonhabitués except for the palate site. An elevated level of porphyrin is also observed for leukoplakia patients compared to that of nonhabitués. The porphyrin level is found to be nearly equal for habitué and leukoplakia, indicating the transformation of tissue from its normal pathology due to the effect of oral habits. Variation in porphyrin is an important marker in differentiating oral cavity disorders. Using laser-induced fluorescence spectroscopy on oral cavity cancer, Jayanthi et al. have reported that the level of porphyrin increases with the increase in pathological grading.<sup>13</sup> In this study, habitués who had no clinically observable lesions also showed increased levels of porphyrin. This increase

in porphyrin level can be considered as an indication of the transformation of tissue from normal to diseased conditions in habitués.

Out of the different forms of porphyrin, only protoporphyrin IX is synthesized naturally. protoporphyrin IX is a precursor of heme in its synthesis pathway. It has been reported that excess protoporphyrin IX occurs naturally in cancers and their metastases in organs such as oral cavity and colon.<sup>9,25</sup> The exact mechanism behind this is still unknown. Increases or decreases in enzymes such as ferrochelatase, ALA dehydratase, coproporphyrinogen oxidase, and uroporphyrinogen are termed as the stimulators which cause excess synthesis of protoporphyrin in diseased conditions.<sup>26</sup> Moreover, porphyrin is reported to possess multiple antioxidant properties against benzo[a]pyrene and tobacco-specific N-nitrosamines in cigarette smoke, which induce inflammation and damage in the epithelial tissue. Excess production of porphyrin in the sites that is chronically exposed to tobacco-specific toxicants may be due to either the overexpression of porphyrin in diseased conditions or the



**Fig. 6** Pairwise discriminant score plot based on discriminant function for the pairs nonhabitué versus habitué, nonhabitué versus leukoplakia, and habitué versus leukoplakia for anatomical sites: (a) floor of the mouth, (b) left buccal mucosa, (c) right buccal mucosa, and (d) palate.

autoimmune response by the body to resist harmful effects of tobacco.<sup>27,28</sup>

Consumption of tobacco and alcohol together may also promote excess porphyrin synthesis in oral cavity tissues. Alcohol is reported as a drug that affects porphyrin synthesis in various ways.<sup>26</sup> Excess consumption of alcohol tends to alter activity of

various types of enzymes that influence heme synthesis. Alcohol is reported as a promoter of  $\delta$ -aminolevulinic acid synthase and porphobilinogen deaminase activity and inhibitor of  $\delta$ -aminolevulinic acid dehydratase, uroporphyrinogen decarboxylase, and ferrochelatase activity, which results in transient decreases in intracellular heme that stimulates porphyrin synthesis.<sup>26</sup>

**Table 3** Overall diagnostic accuracies obtained for the discrimination of nonhabitués, habitués, and leukoplakia using PCA-LDA.

Locations in oral cavity	Nonhabitué versus habitué		Nonhabitué versus leukoplakia		Habitué versus leukoplakia	
	Se (%)	Sp (%)	Se (%)	Sp (%)	Se (%)	Sp (%)
Floor	64.29	80.65	100	100	95.24	96.43
Left	60	76.66	100	100	90.48	93.33
Right	70.97	83.33	100	100	80.95	93.55
Palate	69.70	79.41	100	100	96.77	96.97

Note: Sensitivity (Se) = true positive/(true positive + false negative). Specificity (Sp) = true negative/(true negative + false positive).

Inconsistency in the spectra from palate is observed for both nonhabitueés and habitueés. Emission peaks of hemoglobin and porphyrin are found to be distorted. This can be due to reasons including the position of the fiber probe, which is explained more in detail in our earlier report.<sup>14</sup>

Total hemoglobin concentration is an index of angiogenesis.<sup>29</sup> Angiogenesis is the growth of new blood vessels from pre-existing ones. It is a complex phenomenon that is required for the continued growth and survival of solid tumors.<sup>30</sup> Studies based on diffuse reflectance spectroscopy have shown an increase in total hemoglobin concentration in oral cancer compared to normal.<sup>10</sup> But to date, there are no reports on the evaluation of hemoglobin concentration of oral cavity disorders using fluorescence spectroscopy.

Using SFM effect on the fluorescence spectra due to hemoglobin absorption, we have observed a significant decrease in total hemoglobin concentration for nonhabitueé versus habitueé and nonhabitueé versus leukoplakia, irrespective of the site. This finding suggests that due to increased smoking habits, there is decrease in the blood circulation, which is reflected by the reduction in total hemoglobin concentration in oral cavity tissues. Another finding is that habitueés and leukoplakia patients show nearly equal concentration of hemoglobin in all the sites. This may be due to the decrease in interleukin-1 (IL-1) enzyme in the leukoplakic condition.<sup>31</sup> IL-1 is the specific enzyme that promotes tumor angiogenesis.<sup>32</sup> Therefore, the decrease in this enzyme may lead to destruction of blood vessels, which results in a decrease in total hemoglobin concentration.

Results of this study imply that blood vessels within the oral cavity get disrupted initially due to distortion of epithelium and connective tissue as a result of various habits. This result in a decrease in total hemoglobin concentration in habitueés compared to nonhabitueés. Contents in smokeless tobacco, such as tobacco-specific nitrosamines and N-acetyl-L-cysteine, results in increased intracellular ROS levels that cause DNA fragmentation and lipid peroxidation, and decrease in collagen contractability and tissue damage.<sup>33–35</sup> Using smokeless tobacco extracts on human oral fibroblasts, Coppe et al. have shown that tobacco-exposed fibroblasts disrupt epithelial cell–cell interactions and stimulate epithelial migration and proliferation. Tobacco also alters epithelial tissue integrity by reducing the expression and membrane localization of critical cell junction proteins E-cadherin and ZO-1.<sup>36</sup> Continuous exposure of high-temperature smoke due to cigarette smoking can also be one of the reasons for disruption of epithelium and connective tissue.<sup>37</sup>

A combination of tobacco associated habits along with alcoholism and areca nut chewing further increases the chances of disruption of epithelium and connective tissue. Alcoholism also produces lipid peroxidation within the cells, which may also result in oral cavity tissue disruption. This is due to the increase in oxidative stress in cells due to ROS production by the oxidation of ethanol to acetaldehyde.<sup>38,39</sup> Rough surfaces of areca nut and the variation in pH and temperature caused by slaked lime through continuous pan or ghutka chewing further increase damages to the oral epithelium.<sup>14,40</sup>

Sensitivity and specificity of discrimination using pairwise correlation of PCA-LDA score were determined using the cutoff values given in Fig. 6. In this clinical trial, we obtained a sensitivity of 60% to 71%, 100%, and 80% to 96% and a corresponding specificity of 76% to 83%, 100%, and 93% to 96% respectively, for discriminating nonhabitueés from habitueés, nonhabitueés from leukoplakia, and habitueés from leukoplakia

using PCA-LDA analysis. In our earlier study on differentiating nonhabitueés, areca nut chewing habitueés and oral submucous fibrosis patients using LDA, we have obtained overall sensitivity of 69% to 100% and specificity of 76% to 100%.<sup>14</sup>

van Staveren et al. obtained a sensitivity of 64% to 100% and specificity of 82% to 94% in distinguishing leukoplakic lesion from normal oral mucosa using artificial neural network analysis on autofluorescence spectral data.<sup>12</sup> Venugopal et al. have obtained a sensitivity of 96% and a specificity of 100% using spectral intensity ratio analysis, and an overall sensitivity and specificity of 100% using PCA-LDA for the discrimination of leukoplakia and normal oral mucosa.<sup>41</sup> Using LDA on differential path-length spectroscopy data, Amelink et al. showed that nondysplastic and dysplastic leukoplakias can be discriminated with a sensitivity and specificity of 91% and 80%.<sup>11</sup> In this study, we have obtained a similar or better classification efficiency compared to that with the previously reported ones in differentiating nonhabitueés, habitueés, and leukoplakia. Moreover, we have considered the chromophore, hemoglobin, along with the endogenous fluorophores within the tissue in achieving this discrimination.

## 5 Conclusions

This study has proven that autofluorescence spectroscopy along with LDA is an excellent tool for the earliest diagnosis of oral cavity disorders in clinical settings. The highlight of the study is the evaluation of total hemoglobin concentration of oral cavity disorders using fluorescence spectroscopy, which has not been reported so far. Elevated levels of porphyrin and reduced concentrations of total hemoglobin observed in habitueés in comparison with nonhabitueés can be considered as the sign of tissue damage due to habits. These subjects, who had no clinically identified lesions, have a high risk of development of severe oral cavity disorders as evidenced by the hemoglobin concentration and porphyrin levels equal to leukoplakia patients. Considering these findings as an early-stage diagnosis, preventive measures can be taken in the case of these habitueés from further development of oral cancer. The results of this study also suggest that this method can be adopted as an early screening tool among habitueés, especially in rural areas where such habits are alarmingly high, to rule out the tissue transformation, at very early stages. Data from a much bigger population with different duration of habits would give more specific and step-wise biochemical changes that occur in the oral cavity tissues during tissue transformation.

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## References

1. S. Petti, "Lifestyle risk factors for oral cancer," *Oral Oncol.* **45**(4–5), 340–350 (2009).

2. S. Warnakulasuriya, G. Sutherland, and C. Scully, "Tobacco, oral cancer, and treatment of dependence," *Oral Oncol.* **41**(3), 244–260 (2005).
3. U. Nair, H. Bartsch, and J. Nair, "Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms," *Mutagenesis* **19**(4), 251–262 (2004).
4. R. Subapriya et al., "Assessment of risk factors for oral squamous cell carcinoma in Chidambaram, Southern India: a case-control study," *Eur. J. Cancer Prev.* **16**(3), 251–256 (2007).
5. S. Warnakulasuriya, "Global epidemiology of oral and oropharyngeal cancer," *Oral Oncol.* **45**(4–5), 309–316 (2009).
6. D. M. Parkin et al., "Global cancer statistics, 2002," *CA Cancer J. Clin.* **55**(2), 74–108 (2005).
7. M. W. Lingen et al., "Critical evaluation of diagnostic aids for the detection of oral cancer," *Oral Oncol.* **44**(1), 10–22 (2008).
8. D. C. Fernandez et al., "Infrared spectroscopic imaging for histopathologic recognition," *Nat. Biotechnol.* **23**(4), 469–474 (2005).
9. S. S. Nazeer et al., "Fluorescence spectroscopy to discriminate neoplastic human brain lesions: a study using the spectral intensity ratio and multivariate linear discriminant analysis," *Laser Phys.* **24**, 025602 (2014).
10. A. Amelink et al., "Non-invasive measurement of the morphology and physiology of oral mucosa by use of optical spectroscopy," *Oral Oncol.* **44**(1), 65–71 (2008).
11. A. Amelink et al., "Non-invasive measurement of the microvascular properties of non-dysplastic and dysplastic oral leukoplakias by use of optical spectroscopy," *Oral Oncol.* **47**(12), 1165–1170 (2011).
12. H. J. van Staveren et al., "Classification of clinical autofluorescence spectra of oral leukoplakia using an artificial neural network: a pilot study," *Oral Oncol.* **36**(3), 286–293 (2000).
13. J. L. Jayanthi et al., "Comparative evaluation of the diagnostic performance of autofluorescence and diffuse reflectance in oral cancer detection: a clinical study," *J. Biophotonics* **4**(10), 696–706 (2011).
14. S. Nazeer Shaiju et al., "Habits with killer instincts: in vivo analysis on the severity of oral mucosal alterations using autofluorescence spectroscopy," *J. Biomed. Opt.* **16**(8), 087006 (2011).
15. P. S. Haris et al., "Autofluorescence spectroscopy for the in vivo evaluation of oral submucous fibrosis," *Photomed. Laser Surg.* **27**(5), 757–761 (2009).
16. M. S. Bergholt et al., "Combining near-infrared-excited autofluorescence and Raman spectroscopy improves in vivo diagnosis of gastric cancer," *Biosens. Bioelectron.* **26**(10), 4104–4110 (2011).
17. P. Venkatachalam et al., "Diagnosis of breast cancer based on FT-IR spectroscopy," *Aip Conf. Proc.* **1075**, 144–148 (2008).
18. S. S. Nazeer et al., "Fluorescence spectroscopy as a highly potential single-entity tool to identify chromophores and fluorophores: study on neoplastic human brain lesions," *J. Biomed. Opt.* **18**(6), 067002 (2013).
19. J. M. Baric et al., "Influence of cigarette, pipe, and cigar smoking, removable partial dentures, and age on oral leukoplakia," *Oral Surg. Oral Med. Oral Pathol.* **54**(4), 424–429 (1982).
20. V. Vivek et al., "Three-year follow-up of oral leukoplakia after neodymium:yttrium aluminum garnet (Nd:YAG) laser surgery," *Lasers Med. Sci.* **23**(4), 375–379 (2008).
21. Q. Liu and T. Vo-Dinh, "Spectral filtering modulation method for estimation of hemoglobin concentration and oxygenation based on a single fluorescence emission spectrum in tissue phantoms," *Med. Phys.* **36**(10), 4819–4829 (2009).
22. N. Kambhatla and T. K. Leen, "Dimension reduction by local principal component analysis," *Neural Comput.* **9**(7), 1493–1516 (1997).
23. K. Y. Ho et al., "Diagnosis of gastric cancer using near-infrared Raman spectroscopy and classification and regression tree techniques," *J. Biomed. Opt.* **13**(3), 034013 (2008).
24. A. Saraswathy et al., "Optimum wavelength for the differentiation of brain tumor tissue using autofluorescence spectroscopy," *Photomed. Laser Surg.* **27**(3), 425–433 (2009).
25. C. Croce et al., "Naturally-occurring porphyrins in a spontaneous-tumour bearing mouse model," *Photochem. Photobiol. Sci.* **10**(7), 1189–1195 (2011).
26. M. O. Doss, A. Kuhnel, and U. Gross, "Alcohol and porphyrin metabolism," *Alcohol Alcohol.* **35**(2), 109–125 (2000).
27. C. Wang et al., "Addition of porphyrins to cigarette filters to reduce the levels of benzo[a]pyrene (B[a]P) and tobacco-specific N-nitrosamines (TSNAs) in mainstream cigarette smoke," *J. Agric. Food Chem.* **59**(13), 7172–7177 (2011).
28. K. R. Smith et al., "Inhibition of tobacco smoke-induced lung inflammation by a catalytic antioxidant," *Free Radical Biol. Med.* **33**(8), 1106–1114 (2002).
29. Y. Hu et al., "EFEMP1 suppresses malignant glioma growth and exerts its action within the tumor extracellular compartment," *Mol. Cancer* **10**, 123 (2011).
30. R. Hasina and M. W. Lingen, "Angiogenesis in oral cancer," *J. Dent. Educ.* **65**(11), 1282–1290 (2001).
31. M. Wenghoefer et al., "Gene expression of oncogenes, antimicrobial peptides, and cytokines in the development of oral leukoplakia," *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **110**(3), 351–356 (2010).
32. E. Voronov et al., "IL-1 is required for tumor invasiveness and angiogenesis," *Proc. Natl. Acad. Sci. U. S. A.* **100**(5), 2645–2650 (2003).
33. M. Bagchi et al., "Smokeless tobacco, oxidative stress, apoptosis, and antioxidants in human oral keratinocytes," *Free Radical Biol. Med.* **26**(7–8), 992–1000 (1999).
34. S. S. Hecht, "Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines," *Chem. Res. Toxicol.* **11**(6), 559–603 (1998).
35. L. Yin, A. Morita, and T. Tsuji, "Alterations of extracellular matrix induced by tobacco smoke extract," *Arch. Dermatol. Res.* **292**(4), 188–194 (2000).
36. J. P. Coppe et al., "A role for fibroblasts in mediating the effects of tobacco-induced epithelial cell growth and invasion," *Mol. Cancer Res.* **6**(7), 1085–1098 (2008).
37. P. Ermala and L. R. Holsti, "On the burning temperatures of tobacco," *Cancer Res.* **16**(6), 490–495 (1956).
38. S. Warnakulasuriya et al., "Demonstration of ethanol-induced protein adducts in oral leukoplakia (pre-cancer) and cancer," *J. Oral Pathol. Med.* **37**(3), 157–165 (2008).
39. M. J. McCullough and C. S. Farah, "The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes," *Aust. Dent. J.* **53**(4), 302–305 (2008).
40. E. I. Tiktopulo and A. V. Kajava, "Denaturation of type I collagen fibrils is an endothermic process accompanied by a noticeable change in the partial heat capacity," *Biochemistry* **37**(22), 8147–8152 (1998).
41. C. Venugopal et al., "Autofluorescence spectroscopy augmented by multivariate analysis as a potential noninvasive tool for early diagnosis of oral cavity disorders," *Photomed. Laser Surg.* **31**(12), 605–612 (2013).

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