

Original Research

# Comparative Evaluation of Salivary Parameters in Tobacco Substance Abusers

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## Abstract

**Background:** Tobacco use by youth is ever-demanding, and it is increasingly distributed not only in India but also globally. Saliva is a complex oral bio-fluid, freely available, performing absolute tasks for maintaining oral health and homeostasis. It contains a plethora of significant constituents such as proline-rich proteins (PRPs), immunoglobulins, IgA, enzymes lysozyme, lactoferrin, peroxidases, amylase, etc. The basic ecological balance of the oral cavity is stabilized via salivary clearance by reduced aggregation and adherence of microorganisms by direct microbial activity. This balance of oral activity is also done by indirect mechanisms by immunological as well as non-immunological means and also by effectively regulating salivary pH flow rate. This institutional observational study was planned to assess and compare salivary parameters (pH, salivary flow rate), total proteins,  $\alpha$ -amylase, calcium, phosphate, and IgA, of unstimulated whole saliva of both tobacco abusers and tobacco non-users. **Methods:** The Study consisted of 270 participants (Tobacco habit) group,  $n = 135$  and Control (Healthy) group,  $n = 135$  and were in the age range of 20–50 years. They were assessed for oral health status, followed by the analysis of salivary pH, flow rate, total proteins, amylase, calcium, phosphates, and IgA of unstimulated whole saliva. **Results:** Comparative evaluation of salivary parameters among groups found that varying tobacco abusers had increased salivary amylase, protein levels, and phosphate whereas decreased salivary pH, flow rate, IgA, and in the whole unstimulated saliva samples than those of non-tobacco users. This difference among groups was statistically significant. ( $p < 0.05$ ), and calcium levels were not altered significantly. **Conclusions:** This study concludes that salivary parameters are altered in tobacco abusers when compared to those of non-abusers, and it was more significant in smokeless tobacco abusers than in any other form of tobacco abuse.

**Keywords:** amylase; calcium; flow rate; IgA; pH; saliva parameters; tobacco abuser

## 1. Introduction

Tobacco abuse in either smoking or smokeless form is in wide use globally, especially by youth and adult populations affecting both genders and presenting a challenge to public health. Tobacco in either form leads to changes in oral health, forms various oral lesions to the extent of oral cancer, and causes serious health damage [1,2]. Toxic chemicals of tobacco invade cells, imparting malignant changes, and resulting in physical and psychological disability affecting the quality of life [3]. Tobacco habits are correlated with a high prevalence of dental caries and higher DMFT scores [4].

Tobacco abuse may lead to alteration in the local environment of the oral cavity as well as saliva and its constituents. Saliva is a clear, viscous, watery, heterogeneous, and complex oral bio-fluid, also known as the mirror of the body, and is an essential component of the oral cavity. Saliva collection is not only rapid, simple, noninvasive, accurate, and inexpensive, but saliva is a chair-side screening medium of diagnostic importance for exploring the health of an individual [5]. The role of saliva as a diagnostic aid is described in various clinical situations, including dental caries. Salivary pH varies from 6.0 to 7.4 and constitutes potassium, bicarbonate, sodium, and chloride ions. Sali-



vary antimicrobial constituents comprised lysozymes, thiocyanate, immunoglobulins, transferrin, and lactoferrin [5]. Saliva maintains the integrity of the oral mucosa and has mechanical cleansing action along with antimicrobial activity; it lubricates the oral cavity, controls pH, and hence remineralization of teeth. Saliva protects the tooth being the mainstay of calcium and phosphate ions, influencing the driving force for the dissolution or precipitation of calcium hydroxyapatite (HAP), the main inorganic constituent of teeth [6]. Post-eruptive maturation of enamel as well as remineralization of incipient carious lesions, is facilitated through the saliva [7]. Salivary proteins are related to the immune response and protect oral tissues by possessing bacteria-killing properties of histatins and defensins [5]. Salivary Alpha amylase cleaves  $\alpha$  (1–4) glycoside linkage in starch and glycogen. It also clears food debris containing starch when retained encircling the teeth and/or dissolves it over oral mucosa [8].

Secretory Salivary IgA acts as the first line of host defense against invading mucosal surface pathogens by neutralizing the bacterial toxins and enzymes and preventing adherence of the bacteria by blockage of bacterial adhesion to the tooth surface, leading to reduced hydrophobicity and agglutination of the oral bacteria [9,10].

The greatest virtue of man is perhaps curiosity. Saliva has a very important role in maintaining tooth structure integrity, and it is influenced by external oral environmental factors such as tobacco and alcohol. Alcohol influences saliva via the microbial oxidation of ethanol by forming acetaldehyde [11]. Ethanol stimulates parotid saliva flow rate initially, but frequent acute alcohol doses may reduce salivary secretion [12]. Saliva is the multi-constituent, first oral fluid to come in contact with tobacco and cigarette smoke. Cigarette smoke contains a large amount of oxidative species and increases reactive oxygen species (ROS) production or initiates radical chain reaction. It also induces oxidative stress and reduces its antioxidant compounds. Tobacco abuse can act as an immunomodulator in the oral cavity. Its effect on bacterial diversity and host response has been found to be altered in smokers when compared to healthy participants. Tobacco abuse, over a period of time, may lead to depressed salivary reflex and hence impaired salivary flow rate. On the contrary, tobacco can lead to parasympathetic stimulation of post-ganglionic neurons in response to its nicotine and hence increased salivation. Evidence suggests that smoking leads to reduced saliva release with altered composition with contradictory results. Additionally, both smoking and smokeless forms of tobacco contain proven carcinogens and toxic substances. Because of this, protective multi-constituent saliva can lose or alter its enzymes, proteins, and hence its protective mechanism. Existing studies have reported contradictory results evaluating salivary parameters in tobacco abusers, and there is a scarcity of studies comprehensively evaluating all parameters; there is a need to study these salivary parameters among tobacco abusers to those of non-abusers. Saliva

contains many biochemical substances, antibacterial components and is the first line of defense. The purpose of the present study was to evaluate the influence of tobacco, either in smoking or smokeless forms, on saliva by comprehensively estimating various salivary constituents and comparing the same with non-tobacco users. Understanding the variation of salivary calcium, phosphates and alpha-amylase, IgA, Flow rate, pH, and protein in tobacco abusers may help us to limit the overall disease burden. By keeping this in mind, the research hypothesis was framed as, “There is variation in salivary pH, Flow rate, Protein,  $\alpha$ -amylase, calcium, phosphates, and IgA levels among tobacco abusers compared to those of non-users”.

## 2. Materials and Methods

270 Participants for the study were randomly selected from the patients visiting the outpatient Department of K M Shah Dental College and Hospital. (Study Approval: SVIEC/ON/DENT/PHD/15002 dated 31 August 2015) The sample size was determined as per the number of patients visiting the outpatient department of the hospital. This study was attempted as a case control study.

Study Groups were as follows:

Group A (n = 135): Tobacco and related substance abuse in any form for a minimum of 5 years duration.

Group B (n = 135): Normal healthy participants without any habits as controls. The inclusion criteria for the study group (Group A) were:

(1) Participants have a history of continuation of tobacco habit for a minimum of 5 years to ensure changes due to tobacco addiction, though tobacco addiction develops over a period of 12 months of use of tobacco substances.

(2) The age range of 20–50 years.

(3) Participants with a habit of smokeless tobacco, e.g., Gutka, padiki, flavored tobacco with betel nut, pan masala, or smoking form of tobacco.

Exclusion Criteria for the study group (Group A) were:

(1) Subjects with systemic diseases, diabetes, patients on medications affecting salivary secretions, and special health care needs.

(2) Individuals with tobacco abuse habits of less than 5 years or occasional tobacco abusers.

(3) Participants having oral mucosal lesions, either pre-cancer or cancer or similar visually detected lesions, were excluded from the study.

(4) Patients on steroids and antibiotics for the last six months were excluded from the study.

(5) Pregnant and adolescent females or females having metabolic or hormonal disturbances.

Participants for the control group (Group B) were selected as age and gender-matched healthy participants having no tobacco or any other related substance habit and were selected from those visiting the Institution for routine health checks. Participants were selected after clinical examination and obtaining information about tobacco habits through

a self-reported questionnaire by the participants. Demographic details were entered in case history proforma, including tobacco and related substance habits, if any, along with the frequency of intake, duration, and quantity of tobacco substances. All study information was explained in detail to all participants, and written consent was obtained from each participant who volunteered to become a part of the study.

### 2.1 Saliva Collection and Processing of Samples

The screening was followed by instructions for saliva collection. To avoid diurnal variation, unstimulated saliva was preferably collected between 9 AM and 11 AM. All participants were informed to avoid eating or drinking for at least an hour just before saliva collection. Participants were seated comfortably in the dental chair and were instructed to rinse their mouths and asked to sit upright as per protocol for saliva collection. The unstimulated whole salivary samples were collected by the method suggested by Colin Dawes [13].

Participants were asked to collect saliva on the floor of the mouth by passive drool and were further spit into a graduated container. The saliva flow rate was assessed as a volume of saliva/sample collection with duration expressed as units of volume/time (mL/min). The saliva sample in the Eppendorf tube was kept in an ice pack box and was immediately transferred to the central biochemical laboratory for analysis of salivary parameters. As far as possible, freeze-thaw cycles were avoided. Salivary pH was noted down by using a digital pH meter. First, the pH meter was standardized. As per the protocol for pH determination, for pH calibration, the solutions used were pH 4, pH 7, and pH 10. The pH meter used was HANAA-pHep, (made in Italy) for assessing the total concentration of hydrogen ions. Analysis of unstimulated whole saliva samples was carried out in the Biochemistry section of the Central Laboratory of Dhiraj Hospital, Sumandeep Vidyapeeth University. Salivary protein, amylase, calcium, and phosphate levels were evaluated by using the autoanalyzer EM 360. (Automated Clinical Chemistry Analyzer - Transasia Bio-Medicals Ltd. Made in Mumbai, Maharashtra, India). For auto analysis, a minimum of 3 mL of saliva was collected. Samples were centrifuged for 10 minutes at 3000 rpm. Once supernatant saliva was procured, 200  $\mu$ L of the saliva supernatant was placed in separate vials in auto-analyzer EM 360 for total salivary protein, amylase, calcium, phosphate, and IgA evaluation, respectively. Samples were placed in Erba 360 for analysis, followed by programmed order for a particular analysis.

### 2.2 Quantification of Total Salivary Proteins

Quantification of total salivary proteins was done as per the manufacturer's instructions by the Biuret method end point of ERBA Mannheim. Peptide bonds of protein react with copper II ions in an alkaline solution to form a blue-violet complex (biuret reaction). Each copper ion

complexes with 5–6 peptide bonds. Tartrate was added as a stabilizer, whilst iodide was used to prevent auto reduction of the alkaline copper complex. The color formed was proportional to the protein concentration and was measured at 546 nm (520–560 nm) [14].

### 2.3 $\alpha$ Amylase

$\alpha$  amylase in the saliva was determined by using an autoanalyzer, and the used reagents were 2-Chloro-4-nitrophenol- $\beta$ -1-4 galactopyranosylmaltotrioxide (CNP-G). Collected saliva was diluted to 1:100 and was added to the reagent (ready-to-use kit) and analyzed using an automatic analyzer [14]. Amylase catalyzed the hydrolysis of a 2-chloro-4 nitro phenol salt to chloro nitrophenol (CNP). The rate of its formation was measured at 405 nm and was proportional to  $\alpha$  amylase activity (U/L).

### 2.4 Quantification of Calcium Concentration

Quantification of calcium concentration in saliva included the method of Arsenazo III [15]. Estimation of inorganic salivary calcium was done by using the Arsenazo reagent (Erba Mannheim Calcium Arsenazo III Lab Care Diagnostics). The reagent was ready for immediate use and was added to the saliva sample in the ratio of 1:100, incubated for 1 min, and absorbance was measured at a wavelength of 650 nm. Calcium reacts with Arsenazo III in a slightly alkaline medium to form a purple-colored complex that absorbs at 650 nm. Arsenazo has a strong affinity for calcium ions, and it is proportional to the concentration of calcium in the sample. A biochemical assay of saliva samples was carried out by using Erba 360 fully automated auto analyzer (Erba Diagnostic, Mannheim, Germany). Calcium concentration was displayed by the system and was noted in the proforma.

### 2.5 Inorganic Phosphate

Inorganic phosphate concentrations in saliva were determined by using ammonium molybdate reagent under acidic conditions, wherein inorganic phosphorous reacts to form a phosphomolybdate complex [16]. The absorbance of this complex at 340 nm is proportional to the phosphate concentration in saliva. Thus, total phosphorus concentration was displayed in the computerized system, and values were noted down.

### 2.6 Quantification of Salivary IgA

Quantification of Salivary IgA was done by (Santa Cruz Biotech- made in USA) ELISA Method [17]. The supernatant of centrifuged salivary samples was taken into the microtitre plate. Subsequently, a Primary antibody was poured onto the samples, followed by a secondary antibody. The degree of color production based on the quantity of IgA present in the sample was read by an ELISA reader, wavelength 570 nm. All steps were followed as per the manufacturer's instructions, and readings were subsequently noted down. The results thus obtained were entered in a master

chart and subjected to statistical analysis using SPSS Version 20.0 (SPSS Inc, IBM Corp., Chicago, IL, USA).

### 2.7 Statistical Analysis

On testing data, there was a normal distribution of data, and data analysis was performed using SPSS version 22.0 (IBM Corp. Armonk, NY, USA). The data were analyzed using descriptive statistics. Data are presented in tables as percentages. Statistical significance was determined at  $p < 0.05$ . Tests performed were the Chi-Square test, Independent  $t$  test, and One-way ANOVA.

## 3. Results

In the present study, a total of 270 participants satisfied the study selection criteria, and out of them, 63 (60.37%) participants were male, and the remaining 107 (39.63%) participants were female. In our study, we observed that the majority of our study population was young and in the second decade. The difference in the age group was not statistically significant. The mean age of the total 270 participants was 32.53 years, 32.03 years for males, and 33.29 years for female participants, and the difference was not statistically significant. In the habit group, the mean age of male participants was lesser (33.53 years) than those of female participants (35.8 years) (Table 1).

**Table 1. Age and gender distribution.**

Age group	Habit group		No habit group	
	Male	Female	Male	Female
20–29	47 (44.76%)	06 (20%)	35 (60.34%)	36 (46.75%)
30–39	23 (21.90%)	14 (46.67%)	15 (25.86%)	17 (22.07%)
40–49	30 (28.57%)	10 (33.34%)	08 (13.79%)	22 (28.57%)
50	05 (4.7%)	00 (0%)	00 (0%)	02 (2.59%)

Among males and females in both groups, tobacco abuse was a commonly reported habit as compared to other habits. It was found in the present study that abuse of tobacco was significantly higher in the male population as compared to the female population; the  $p$  value is 0.001 (Table 2).

**Table 2. Gender distribution as per type of tobacco habit.**

Type of tobacco	Male frequency %	Female frequency %	$p$ -value
No Habit	58 (35.58%)	77 (71.96%)	0.001
Tobacco (padiki)	35 (21.47%)	17 (15.89%)	0.001
Gutka	43 (26.38%)	06 (5.61%)	0.001
Smoking	25 (15.34%)	01 (0.93%)	0.001
Betel Nut	02 (1.23%)	06 (5.60%)	0.001

Chi-Square test.

Among gender distribution for both the control group and study groups, there were participants in the control

group, and the difference was statistically significant ( $p = 0.001$ ), whereas in the study group, among male participants, gutka abuse was more, followed by tobacco and smoking habit. The lowest reported was betel nuts abuse by male participants in the study group.

Among female participants, tobacco abuse was followed by gutka and betel nuts, whereas smoking was reported by a single female participant among study groups.

The mean value of study parameters like salivary pH and Flow Rate, protein, amylase, calcium, phosphorus, and Immunoglobulin A (IgA) between tobacco abusers and non-users were compared (Table 3).

**Table 3. Distribution of salivary parameters among the total population.**

Investigation	Tobacco user		Tobacco non-user		$p$ -value
	Mean	SD	Mean	SD	
pH	5.47	0.58	6.76	0.60	0.0001
Flow Rate (FR)	0.97	0.17	1.79	0.41	0.0001
Protein	1.59	0.88	0.98	0.48	0.0001
Amylase	801.46	545.64	600.02	388.92	0.0020
Calcium	12.37	2.79	12.55	2.23	0.2430
Phosphorus	38.39	34.35	23.46	6.33	0.0001
IgA (Immunoglobulin A)	38.93	12.60	51.90	12.50	0.0001

Independent  $t$  test.

The mean salivary pH in healthy participants was  $6.76 \pm 0.60$ , whereas in tobacco abusers, it was  $5.47 \pm 0.58$ , and the difference was highly significant ( $p$  value = 0.0001). The mean salivary flow rate was  $1.79 \pm 0.41$  in the control group, whereas it was reduced significantly in tobacco abusers to  $0.97 \pm 0.17$  ( $p = 0.0001$ ).

The total salivary protein level was increased in tobacco abusers to  $1.59 \pm 0.88$  as compared to healthy participants ( $0.98 \pm 0.48$ ) with a highly significant difference ( $p = 0.0001$ ).

Similarly mean salivary amylase in tobacco abusers was significantly higher when compared to healthy participants ( $p$  value = 0.0020) and, mean salivary phosphates were significantly higher in tobacco abusers as compared to those of non-users ( $p$  value = 0.0001). Mean salivary IgA was higher in tobacco non-users as compared to those of tobacco abusers ( $p = 0.0001$ ) and a significant mean difference was found in all salivary parameters except for Calcium ( $p = 0.2430$ ) (Table 3).

Tobacco (padiki) was the most commonly consumed form of smokeless tobacco among the abusers studied, followed by gutka, smoking and betel nut habit and difference was statistically significant ( $p = 0.001$ ) (Table 4).

Salivary flow rate, pH, phosphate, and IgA levels were statistically highly significant in tobacco abusers as compared to the tobacco non-user group ( $p = 0.001$ ). However, for salivary protein, salivary amylase, and calcium, there was no difference observed in participants with the tobacco-padiki user and non-user group (Table 5).

**Table 4. Distribution of varied forms of tobacco abuse.**

Type of habit	Total participants	<i>p</i> value
No Habit	135 (50.00%)	
Tobacco (padiki)	52 (19.25%)	
Gutka	49 (18.18%)	0.001
Smoking	26 (9.61%)	
Betel Nuts	8 (2.96%)	
Total	270	

Chi-square test.

Salivary flow rate (SFR), pH, and IgA levels were statistically highly significant in gutka abusers when compared to the gutka non-user group ( $p = 0.001$ ), whereas salivary protein, amylase, calcium, and phosphate were not significantly different in gutka users when compared to gutka non-user group.

SFR, pH, and IgA levels were statistically highly significant in smokers when compared to the non-smoker group of participants ( $p = 0.001$ ), whereas salivary protein, amylase, calcium, and phosphate levels did not show any significant difference in the smoker and non-smoker group.

SFR and pH were statistically highly significant in betel nut abusers when compared to those of the betel nut non-user group ( $p = 0.001$ ). Other salivary parameters such as salivary protein, amylase, calcium, phosphate, and Ig A were not significantly altered in the Betel nut users group when compared to the betel nut non-user group (Table 5).

All salivary parameters were significantly altered with statistically highly significant differences in tobacco abusers except for calcium when the frequency of habit was compared in tobacco abusers.

SFR and pH were increased in tobacco abusers when the frequency of substance abuse was increased, and the difference was highly significant ( $p = 0.001$ ) (Table 6).

Salivary proteins were increased when the frequency of substance abuse was increased, and the difference was highly significant ( $p = 0.006$ ). Salivary amylase was increased as the frequency of habit was increased, and the difference was highly significant ( $p = 0.002$ ). Salivary Calcium levels were increased as the frequency of habit was increased, but the difference was not statistically significant. Salivary phosphates and salivary IgA levels were decreased as the frequency of habit was increased, and the difference was highly significant ( $p = 0.001$ ).

Various salivary parameters were compared as per varying habit duration of tobacco abuse as 5 years, 6 to 10 years, 11 to 15 years, and 15 to 20 years. The mean salivary flow rate in tobacco abusers of 1 to 5 years duration was  $0.58 \pm 0.08$ , whereas 16 to 20 years was  $0.64 \pm 0.11$ , while in no habit group, it was  $1.40 \pm 0.37$  and the difference was statistically highly significant ( $p = 0.001$ ) (Table 7).

The salivary pH in tobacco abusers of different duration of 5 years intervals was gradually reduced as the duration of habit increased and was significantly lower as compared to the healthy group ( $p = 0.001$ ).

The total salivary proteins were found at higher levels in tobacco abusers as the duration of habit was increased to 16–20 years as compared to 1 to 5 years of tobacco abuse ( $1.62 \pm 0.54$ ) and was statistically significant when compared with the no habit group ( $p = 0.001$ ).

Salivary amylase and calcium were increased as the duration of habit increased, and the difference was statistically significant.

Mean salivary phosphates were higher in the 11–15 years duration group, whereas mean salivary IgA was lower in the 16–20 years duration group, and the difference was statistically significant when compared with the no habit group.

## 4. Discussion

Saliva forms a thin film over oral mucosa and plays a multiplicity of roles in the protection of the oral cavity, assisting digestion through amylase, maintaining pH, and flow rate, influencing redistribution of ions between enamel remineralization and demineralization, leading to localized dissolution and destruction of calcified teeth, supporting tooth surface integrity. Through its constituents such as salivary proteins, electrolytes, and small molecules, it protects against abrasion, attrition, erosion, and dental caries, and further prevents injury to oral mucosa through its clearance properties and protecting against resistance to physical damage, antibacterial and anti-fungal effects. Smoking decreases the commensal population of normal oral normal and increases pathogenic microbes and microbial colonization by biofilm formation on oral epithelial cells [18]. There are few studies evaluating the role of tobacco and related substances exposure and oral health status, especially assessing salivary components.

Whole unstimulated saliva was collected in the present study as the basal salivary flow rate is reflected by unstimulated whole saliva, and the same is favored by most of the population studies [19,20].

In our study, the salivary flow rate was considered for analysis as salivary buffering activity, and the clearance depends on the salivary flow rate. Salivary flow rate is altered in patients with increased caries activity, [21] various medical conditions (autoimmune diseases such as Sjogren's syndrome) [22] and with an intake of medications (anti-hypertensive, antihistamines, and antidepressants) [23], also therapeutic radiation affects salivary flow rate.

In our study, the salivary flow rate in tobacco abusers was found as 0.8 to 1.14 mL/min. Whereas in healthy participants, it ranged from 1.38 to 2.2 mL/min. It was similar to the normal range documented as 5.5 to 7.9 for salivary pH and SFR in the range of 0.33–1.42 mL/min by Wu *et al.* [24]. The salivary flow rate varies as age and environmental factors vary. Evidence suggests SFR is increased in children, whereas in adults, it decreases due to the replacement of glandular components by fat and/or atrophy of salivary glands. Variation in the flow rate can also occur be-

**Table 5. Salivary parameters concerning varying tobacco habits.**

	SFR	pH	Protein	Amylase	Calcium	Phosphate	IgA
Tobacco (Padiki)							
Present	0.63 ± 0.08	5.26 ± 0.57	1.66 ± 1.02	1401 ± 417.92	13.27 ± 2.53	48.47 ± 24.94	31.47 ± 6.40
Absent	1.07 ± 0.50	6.08 ± 0.74	1.36 ± 0.57	1195.88 ± 404.38	13.04 ± 2.39	31.99 ± 34.54	52.59 ± 17.59
<i>p</i> -value	0.001	0.001	0.288	0.097	0.751	0.039	0.001
Gutka							
Present	0.57 ± 0.06	5.38 ± 0.55	1.67 ± 0.88	1322.68 ± 507.58	12.83 ± 3.21	55.02 ± 59.74	32.16 ± 8.98
Absent	1.11 ± 0.49	6.09 ± 0.76	1.34 ± 0.58	1205.28 ± 382.24	13.15 ± 2.16	29.30 ± 19.37	53.59 ± 17.20
<i>p</i> -value	0.001	0.001	0.139	0.357	0.691	0.081	0.001
Smoking							
Present	0.59 ± 0.06	5.44 ± 0.40	1.66 ± 0.64	1610.63 ± 684.25	12.31 ± 2.16	34.68 ± 21.50	34.63 ± 7.13
Absent	1.04 ± 0.49	5.99 ± 0.79	1.39 ± 0.67	1192.94 ± 360.43	13.16 ± 2.42	34.74 ± 34.63	50.48 ± 18.21
<i>p</i> -value	0.001	0.005	0.284	0.131	0.321	0.994	0.001
Betel Nut							
Present	0.69 ± 0.01	5.03 ± 0.01	1.30 ± 0.04	1226 ± 175.36	9.96 ± 2.18	50.50 ± 38.89	36.001 ± 14.14
Absent	1.01 ± 0.49	5.96 ± 0.77	1.41 ± 0.67	1230.16 ± 415.79	13.15 ± 2.37	34.37 ± 33.63	49.36 ± 18.10
<i>p</i> -value	0.001	0.001	0.151	0.979	0.279	0.662	0.403

Independent *t* test, SFR, Salivary Flow Rate.

**Table 6. Salivary parameters concerning the frequency of the habit.**

Frequency of habit times/day	Total number	SFR SALIVARY FLOW RATE	pH	Protein	Amylase	Calcium	Phosphate	IgA
1–4	37	0.59 ± 0.07	5.31 ± 0.54	1.58 ± 0.84	1371.86 ± 561.20	12.31 ± 3.18	50.98 ± 27.24	32.07 ± 7.03
5–8	68	0.59 ± 0.06	5.31 ± 0.52	1.69 ± 0.89	1434.67 ± 480.68	12.93 ± 2.77	50.70 ± 54.02	34.54 ± 7.59
9–12	30	0.64 ± 0.10	5.45 ± 0.46	1.67 ± 0.81	1255 ± 505.94	13.16 ± 1.91	40.06 ± 21.15	27.43 ± 8.56
Abuse/no habit	135	1.40 ± 0.37	6.55 ± 0.45	1.17 ± 0.25	1072.96 ± 198.07	13.39 ± 1.97	20.34 ± 6.03	65.47 ± 7.01
<i>p</i> -value	-	0.001	0.001	0.006	0.002	0.526	0.001	0.001

One-way ANOVA.

**Table 7. Salivary parameters concerning the duration of the habit.**

Duration of habit (years)	Total number	SFR SALIVARY FLOW RATE	pH	Protein	Amylase	Calcium	Phosphate	IgA
1–5	47	0.58 ± 0.08	5.26 ± 0.60	1.62 ± 0.54	1224 ± 328.54	12.40 ± 2.24	49.32 ± 27.12	31.08 ± 6.03
6–10	56	0.61 ± 0.07	5.25 ± 0.43	1.74 ± 0.81	1534.21 ± 675.72	11.78 ± 2.55	44.62 ± 18.99	35.64 ± 9.13
11–15	22	0.58 ± 0.06	5.37 ± 0.52	1.28 ± 0.69	1373.92 ± 474.51	13.18 ± 2.73	58.63 ± 75.31	33.17 ± 8.63
16–20	10	0.64 ± 0.11	5.27 ± 0.54	2.44 ± 1.52	1435.80 ± 433.88	14.70 ± 3.66	46.00 ± 18.77	28.60 ± 3.97
Abuse/no habit	135	1.40 ± 0.37	6.55 ± 0.45	1.17 ± 0.25	1072.96 ± 198.07	13.39 ± 1.97	20.34 ± 6.03	65.47 ± 7.01
<i>p</i> value	-	0.001	0.001	0.001	0.00	0.050	0.001	0.001

One-way ANOVA.

cause of many cellular-origin proteins present in saliva. Decrease in SFR when compared to healthy participants may be due to tobacco abusers with a history of tobacco for a minimum of 5 years, and over a period of time, the sensitivity of receptors may be affected because many chemicals leach out in saliva during intake of tobacco, gutka, and related substances. Additionally, ingestion of tobacco substance in either form can lead to depressed salivary gland reflex or degeneration of glandular components and hence impaired salivary flow rate [25]. Another possibility is that alteration in the autonomic nervous system, by increasing plasma levels of epinephrine and norepinephrine, leads to a

decrease in salivary flow rate [26]. Additionally, the heat generated by tobacco smoking affects the blood flow of the mouth over a period decreasing the blood supply and, in due course, reducing the SFR. SFR decreases with an increase in the frequency of smoking. Duration and frequency of smoking have an inverse effect on the resting salivary flow rate [27].

In our study, salivary pH in tobacco users was in the range of 4.89 to 6.05, whereas in healthy participants, it varied between 6.16 and 7.36. In the present study, we found lower levels of salivary pH and flow rate in tobacco abusers when compared to the control group. The mean

salivary flow rate of healthy participants in our study was 1.79 mL/min, which was in contrast to the SFR reported by Rooban *et al.* [28] (3.5 mL/10min) and 3.66 mL/10min by Lalfamkima *et al.* [29] and Dawes *et al.* [19] reported with 0.3 to 0.4 mL/min.

Lalfamkima *et al.* [29] reported increased SFR among gutka chewers without oral submucous fibrosis when compared with oral submucous fibrosis, and this increased SFR in habit chewers may be due to the parasympathomimetic activity of arecoline. Tobacco can result in parasympathetic stimulation of post-ganglionic neurons in response to nicotine, similar to acetylcholine. The membrane of these neurons contains nicotine type of acetylcholine receptors and hence increased salivation during tobacco chewing, but in its absence, again, SFR can decrease.

Long-term effects of smokeless tobacco had decreased SFR, as reported by Kanwar *et al.* [30], whereas Rad *et al.* [31] reported that long-term smoking reduced SFR. Reduced pH and flow rate of saliva may lead to reduced functions of salivary protection in terms of clearing action and xerostomia, leading to caries susceptibility and halitosis; thus, tobacco abuse may lead to compromised oral health. Altered constituents of saliva in tobacco abusers indirectly lead to adverse effects on teeth and oral mucosa by altering the properties of saliva in terms of reduction of flow and pH and constituents alteration leading to aggregation of the microorganism of the oral cavity.

Hypo salivation criteria for whole stimulated saliva is  $<0.7$  mL/min [32], whereas for whole unstimulated saliva is 0.12–0.16 mL/min [19], and unstimulated flow rates  $<0.1$  mL/min or 0.30 mL/min [20]. We collected saliva by using the passive drool method and which was similar to Dawes method 11. Kanwar *et al.* [30] and Rooban *et al.* [28] reported with spitting method. Rudney *et al.* [33] found a negative correlation between unstimulated whole saliva, IgA, and total protein with SFR.

The pH of saliva altered in our study may be because of the high sugar present in the form of sweeteners in tobacco and related substances intake habits of participants and was following to study of Klein *et al.* [34], Schwartz *et al.* [35]. Salivary buffering capacity works in conjunction with phosphates and protein buffer systems [36]. The normal pH of saliva ranges from 6.2 to 7.6; this total hydrogen ion concentration of saliva is related to the constant salivary flow and buffering capacity of saliva [37]. The present study found lower salivary pH, salivary flow rate, calcium, and IgA in tobacco abusers as compared to those of non-users, whereas total salivary proteins, amylase, and phosphates were increased in tobacco abusers as compared to non-users. Reduction in salivary pH in tobacco abusers was in favor of the study reported by Kanwar *et al.* [30] and Khan *et al.* [38]. On the contrary, no difference in salivary pH was observed by Reddy *et al.* [39] between the tobacco chewers and non-chewer, Grover N *et al.* [40] observed lower pH in tobacco chewers  $6.5 \pm 0.29$ , than smokers  $6.75 \pm 0.11$  and control group  $7.00 \pm 0.28$  which was consistent

with the findings of our study. It was in favor of a study by Rooban *et al.* [28], who found that the mean pH turns acidic for those who chew raw areca nut, whereas it was 6.77 in non-chewers.

Lower pH in tobacco chewers observed in our study was in favor of the study by Venkatesan *et al.* [41], Omeroglu *et al.* [42], and Kumar *et al.* [43], and lower values of salivary pH were reported in traditional cigarette smokers and among e-cigarette users when compared to non-smokers by Cichonska *et al.* [37]. However, it was in contrast to a study by Nakonieczna *et al.* [44], who did not find any change in salivary pH in traditional cigarette smokers.

Saliva acts as a diluent for acid. Dawes [19], any alteration in ions and electrolytes can alter the pH due to their interaction with the buffering systems of saliva. This pH difference in tobacco abusers can be because of the various components of tobacco, lime, and ingredients of Gutka and other tobacco substances. Lime in betel quid can cause high alkaline content of saliva and alters the pH. Various chemicals leached out through chewing tobacco, as well as particulate smoke substances, can also affect salivary pH in tobacco abusers when compared to those of non-users. Moreover, the reduced flow rate observed in the tobacco abusers influences the pH of saliva, pH becomes highly acidic. Additionally, the pH of saliva is altered in tobacco abusers, depending upon the pH of smokeless tobacco and the proportion of free base form of nicotine available for absorption [45].

In our study, we found increased levels of salivary proteins in tobacco abusers when compared to those of the non-habit group. It was in favor of the study by Avsar *et al.* [46] on passive smokers. Poor oral health in the habit group may lead to microbial aggregation and hence increased salivary proteins. The role of salivary proteins and peptides is already predicted in monitoring diseases not only in the oral cavity but also in the whole body [47]. Several salivary proteins perform a defensive role and include mucins, proline-rich proteins, immunoglobulins, mucins, etc. [48]. Salivary proteins such as lactoferrin inhibits bacterial growth and biofilm formation by binding and chelating ions of iron [47,48].

Few studies have reported both diminished [49–51] and increased total proteins [52] in caries active participants, whereas few other studies concluded with no consistent relationship between total salivary proteins and dental caries [53]. Salivary Proteins and peptides with effects on calcium phosphate chemistry have a role in regulating dental caries- and in maintaining the integrity of teeth [54–56].

Salivary alpha-amylase is of salivary origin, and it not only initiates the breakdown of carbohydrates present around the teeth but also has a digestive function [57]. It also binds with bacteria and hence affects tooth decay. Increased salivary amylase was in favor of Aysun *et al.* [46], whereas it was in contrast to Granger *et al.* [58], who reported lower salivary amylase activity when exposed to to-

bacco smoke and Goi *et al.* [59], Callegari and Lami [60] found decreased amylase levels in smokers when compared with non-smoker group. Reduced amylase secretions may lead to changes in salivary amylase levels. Salivary amylase not only has a role in metabolism and for colonization of streptococci, but it also acts as a receptor for aggregation and tooth surface adhesion of microorganisms [7]. Lindermeier reported that nicotine promotes the growth of cariogenic *Streptococcus mutans*. Smoking leads to vitamin C deficiency and further affects salivary glands [61]. Increased amylase in response to tobacco smoke may occur as nicotine activates SNS [62,63]. Whereas, Zappacosta *et al.* [64] reported decreased salivary alpha-amylase activity in healthy smokers when smoking a single cigar. Similarly, decreased amylase levels were seen by Nagler *et al.* [65] *in vitro* studies when whole human saliva was exposed to cigarette smoke, and he reported with 34% decrease in amylase activity after 3 hours of incubation with intermittent smoke exposure. Greabu *et al.* [66] documented an 85% decrease in amylase after 1 hour of incubation with cigarette smoke.

Thus, the noxious effects of tobacco smoke affect salivary amylase, specifically by aldehydes present in tobacco smoke react and modify sulphhydryl groups of salivary enzymes [64,67]. It was also in contrast to a study by Nagaya and Okuno [68] and Zuabi *et al.* [69] did not find a significant difference in salivary amylase and protein levels in the smoking and drinking habit of healthy male and female participants. Nater *et al.* [70] reported the diurnal activity of salivary alpha-amylase. The salivary alpha-amylase level is predominantly influenced by SNS activity in the cervical sympathetic pathway, and salivary alpha-amylase levels rise in response to stress [70,71].

Salivary calcium in our study was not significantly different in tobacco users and non-users though it was slightly lower in tobacco abusers when compared to those non-users. Reduction in salivary calcium has been reported in smokers when compared to non-smokers by Tjahajawati [72], and it was found to be further decreased when the duration of smoking was longer. Lower calcium levels are also reported by Fattahi Bafghi *et al.* [73] and Zuabi *et al.* [69], who were in favor of our study whereas it was in contrast to Abed *et al.* [74], who reported an increase in salivary calcium of male smokers when compared to non-smokers. Khan *et al.* [75] and Arimilli *et al.* [76], and Varghese *et al.* [77] also reported higher levels of calcium in the saliva of long-term tobacco abusers and smokers when compared to non-users. They also found that an increased flow rate of saliva decreases salivary calcium levels.

Smoking leads to decreased calcium absorption and hence detrimental effects on many aspects of the body [78]. Additionally, nicotine reduces estrogen and parathyroid hormone levels and hence affects salivary calcium levels. Furthermore, in smokers, the Parathyroid does not work optimally, and hence lower calcium levels in saliva are seen [79,80]. Tobacco smoke exposure results in inflammation

of humoral immunological consequences of sensitization and altered local immunity in response to various toxic and metallic elements released from chewing tobacco as well as particulate smoke [81].

Increased serum phosphate levels were reported by Haglin *et al.* [82] in smokers when compared to non-smokers. Omar [83] reported that cigarette smoking leads to increased levels of calcium in smokers, whereas reduced phosphate levels were seen in cigarette smokers. Haglin *et al.* [84] found high serum calcium and low levels of phosphates in smokers when compared to the non-smokers' group. They predicted high BMI and smoking to be associated with all causes of mortality in both males and females of the cardiovascular risk cohort group. IgA levels in tobacco abusers were decreased in our study when compared to those of non-users. It was in favor of Avşar *et al.* [46], who reported decreased salivary IgA levels in passive smokers when compared to the control group. A decrease in IgA levels can be indicative of a decrease in local immunity. A highly significant decrease in salivary IgA levels in smokers when compared to the control group was reported by Shilpashree *et al.* [85], Kadri *et al.* [86], Andersen *et al.* [87], Al-Ghamdi *et al.* [88], Barton *et al.* [89], Golpasand [90], Giuca, *et al.* [91], Bennet *et al.* [92], and Doni *et al.* [93].

Our finding was in contrast to Prajapati *et al.* [94], who found no change in IgA levels of smokers and gutka chewers as compared to controls. It was also in contrast to Tarbiah *et al.* [95] found smoking to be associated with increased IgA concentrations in both saliva and serum when compared to those of non-smokers. McMillan *et al.* [96] reported increased IgA in alcohol consumers as well as with increased age. Gonzalez-Quintela A *et al.* [97] also found increased IgA in males and were positively correlated with heavy alcohol intake and age. Along with Prajapati *et al.* [94] and Tarbiah *et al.* [95], Norhagen Engstrom *et al.* [98] reported an increase in IgA levels in smokers. Nakonieczna-Rudnicka M *et al.* [99] found significantly higher sIgA concentration in non-stimulated saliva when compared to stimulated saliva. No change of IgA in smokers and control group was reported by Calapai *et al.* [100], Mcmillan *et al.* [96], Gonzalez *et al.* [97], Lie *et al.* [101], Olayanju *et al.* [102], Nakonieczna-Rudnicka *et al.* [99], and Koss *et al.* [103]. In smokers, various studies have reported variations in the serum as well as saliva levels of IgA. There is no clear consensus on whether there is an increase or decrease or no effect of smoking on Salivary or serum IgA levels. Additionally, there is no exact reason reported for the same.

Tobacco and Cigarette smoke have numerous toxic constituents which can affect the immune system either by immunosuppression or can lead to an increased risk of infection [104]. Few studies [87,88] suggest that cigarette smoking may be associated with the suppression of B-cell function and immunoglobulin production. Further smoking-associated functional antibody deficiency may



compromise the body's response to infection and result in a predisposition to the development of autoimmunity. Reduced phagocyte activity of neutrophils leading to increased susceptibility of smokers to infections reflects multifunctional alteration of their local and systemic inflammatory and adaptive immune responses [102,105]. Immunoglobulins production and their levels in saliva can vary depending upon the need for its production, as well as the presence of bacteria and streptococci in the oral cavity. Increased IgA concentrations in high caries risk or with active caries when compared to caries-free patients were reported by Al Amoudi *et al.* [106], Bagherian *et al.* [107], and Yang *et al.* [108], whereas Doifode *et al.* [109], Pal *et al.* [110], and Kuriakose *et al.* [111] reported higher total IgA in patients with low caries.

Reduction in IgA found may be because smoking has a prolonged negative impact on both innate and adaptive immunity as well as on local and systemic host immune responses [112]. Mucosal immunity is reflected by salivary immunoglobulin A (IgA) levels and is also influenced by psychological stress. In tobacco users, the production of immunoglobulin may be suppressed may be due to unidentified chronic stress, and hence decreases IgA levels [113]. The meta-analysis by Wu *et al.* [114] on an association of dental caries and salivary IgA concentration found that salivary IgA levels in patients with dental caries were lower than those of the healthy control group and can be considered valuable biomarker to evaluate the clinical status of caries patients.

## 5. Conclusions

Tobacco and related substance addiction showed alteration of salivary parameters significantly when compared with those of tobacco non-user participants, affecting salivary pH, flow rate, and local immunity IgA, as well as calcium, phosphate, and amylase.

Tobacco addiction leads to a reduction of salivary pH, Salivary flow rate, and IgA when compared to the non-user group, whereas there was an increase in salivary amylase, phosphates, and salivary proteins. These salivary parameters were altered as per the increased duration and increased frequency of tobacco intake. So comprehensive evaluation will lead to assessing salivary biomarkers exactly in tobacco addiction as well as in dental caries. Additionally, unstimulated saliva is found to be simple, easily available, and non-invasive bio-fluid acts as a diagnostic marker.

Further studies to find out the exact correlation between dental caries, oral health status, and salivary parameters all together comprehensively can lead to the point of care service to participants regarding saliva as a diagnostic marker. Dentists need to be a part of the educational team for tobacco and related substance cessation program or educational counseling for quitting habits.

## Limitations of the study

Saliva is noninvasive, easy to collect, has diagnostic value for biomarkers, and is not compulsory to have specially trained personnel, requiring minimal sample processing as per protocol.

Contrarily, apart from diagnostic value, saliva is a multi-constituent bio-fluid, and salivary diagnostic markers are present at very minute levels. Additionally, variation in diagnostic and analytical procedures gives a wide range of results for analyses. But we need to remember that overall health relies on a local, oral immune response being inherently varied in saliva than that observed in the blood. Additionally, changes in immune markers and various constituents in saliva may respond to extrinsic factors, such as exposure to environmental pollutants and antigens (e.g., tobacco smoke and pollen), and intrinsic factors related to oral health (e.g., dental caries and bacterial load). Hence further studies and standardization is required for the correct interpretation of salivary biomarkers for dental caries or tobacco addiction interpretation between and within-person differences of various salivary parameters.

## Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

## Author Contributions

Conceptualization, RahB, FMA, ATR and RasB; methodology, NAA, PT and RasB; software, RahB, NAA, FMA and VS; validation, PT, RasB, KJA and SB; formal analysis, VS, RasB, KFA and PT; investigation, RasB, VS, IFH, ATR and NAA; resources, RahB, PT and NAA; data curation, NAA, RahB, RR, SB and VS; writing—original draft preparation, SB, ATR, RahB, KJA, FMA and RasB; writing—review and editing, VS, NAA, KFA, IFH, RR, SB and PT; visualization, RahB, RasB, KJA, LT and SB; supervision, SB, LT and RasB; project administration, VS, KFA, IFH and PT; funding acquisition, SB, KJA and VS. All authors read and approved the final manuscript.

## Ethics Approval and Consent to Participate

The study was approved by the Sumandeep Vidyapeeth Institutional Ethics Committee (protocol code 115002 and 31st Aug, 2015).

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## Conflict of Interest

Given Rodolfo Reda's role as Guest Editor and Luca Testarelli's role as Guest Editor and Editorial Board member of the journal, they had no involvement in the peer-

review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Ru Chen. The authors declare no conflict of interest.

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