Salivary biomarkers and its implication in tobacco users-A narrative review

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INTRODUCTION

Tobacco causes many deleterious ill effects in systemic and local health. Tobacco consumption is avoidable and the associated illness/death can be greatly reduced by cessation of the habit. Tobacco product falls into three categories: smokeless products, heat delivery and combustion. The habit of tobacco consumption influences personal as well community health issues. The chemicals found in tobacco products, as well as other chemicals created by combustion, are readily absorbed by the body. These chemicals and their metabolite can be present in biological samples. The major chemicals are tobacco-specific nitrosamines (TSNAs), nicotine and nicotine metabolites (Schick et al., 2017).

The systemic effects of tobacco products include the altered immune response, oxidative damage, delayed wound healing, psychiatric, teratogenic, toxic/carcinogenic and atherogenic effects to various cells and internal organs. The primary systemic effects are associated with the respiratory and cardiovascular system (Ekezie et al., 2020).
impaired gingival bleeding, periodontal diseases, halitosis, failure of the implant, severe stains and calculus (Jayaram and Anitha, 2019). Tobacco intake is also a risk factor for oral cancer. In India, almost 21 people per 100,000 of the population are affected by tobacco-related oral cancer (Mathur et al., 2020).

The chemical present in the tobacco and their metabolites can be measured in the various body fluids and other samples. These can be used as biomarkers to identify and compare the diseases in tobacco users and for monitoring purposes in tobacco cessation.

The influence of the tobacco chemicals to other biomarker levels such as enzymes, inflammatory markers and proteins, etc. with or without associated diseases can also be measured and used for diagnostic/monitoring purposes.

Monitoring the genomic and epigenomic alterations in tobacco users would be of great use in high-risk cancer patients such that intervention can be applied and early prevention is possible in these stages itself. Thus, the measurement of a biomarker in healthy tobacco users and/or with associated diseases in various biological samples would greatly influence the disease outcome. Currently, Saliva has been used as a biomarker identification sample in various disease conditions and reported to have many advantages.

The objective of this research paper is to narratively review the salivary biomarker studies in healthy tobacco users in reference to the inflammatory biomarkers, oxidative stress markers, growth factors, tissue injury markers etc. and other genetic markers and their inference on human health from the available literature. The summary of the different salivary biomarkers associated with tobacco users is presented in Table 1.

The reported biomarker study variables would include age, sex, type of smoker, type of tobacco product, frequency and quantity of the product used, duration of the habit, intervention history and the biomarker of interest as an outcome. Sample collection and method of assessment also important factors to be considered while assessing the biomarkers in tobacco users.

**DISCUSSION**

**Biomarkers of exposure**

Biomarkers of tobacco exposure and nicotine delivery products are critical methods for recognizing and determining the health consequences of tobacco products. The earliest biomarkers to be used for evaluating exposure to cigarette smoke were nicotine. Cotinine is the key metabolite of nicotine, and its extended t1/2 (16–18 h) renders it a superior biomarker for nicotine absorption in tissues and different biological fluids than nicotine since it has a short half-life (t1/2; ~2 h) and the rate of metabolism is unpredictable. The cut-point of plasma or saliva cotinine is 15 ng/ml (Lewis et al., 2003). The great advantage of cotinine is that the ideal cut-points are minimally influenced by the prevalence of smoking in the sample community. Plasma or saliva cotinine achieve greatest, with 96–97% sensitivity and 99–100% specificity, correspondingly. There is a high correlation of cotinine concentrations in blood and saliva with an average salivary concentration of 15–20% elevated than plasma concentrations.

Cotinine levels indicate the past 3 to 4 days of exposure to tobacco. Immunoetric assays, Gas chromatography, liquid chromatography and point-of-care techniques are commonly used techniques for cotinine assays (Ramzanan et al., 2018).

There are several OTC kits available for saliva use and greater sensitivity has been documented. These include the iScreen OFD Test (30 ng/ml cutoff; Alere) and the 1-Step Cotinine Rapid Saliva Test (20 ng/ml cutoff; Alere, Waltham, MA). To differentiate between tobacco consumers and non-users, a more sensitive saliva test is required; the cotinine level is lower when compared to urine. Normally, these kits are assumed to carryout the results perfectly at mentioned cutoff values. An active smoker are indicated by a positive result; perhaps a negative result signifies a likely non-smoker, occasional smoker, or of a low-nicotine e-cigarette users of low nicotine (Schick et al., 2017).

Importantly, these over-the-counter kits must be checked until they can be used to accurately detect cotinine at existing biological cut point values in saliva and urine samples to distinguish smokers and non-smokers. They could be utilized in various clinical setups for screening and monitoring the tobacco users and for easy eligibility of smoking status of a patient for various studies.

**Inflammatory markers**

The importance of Inflammation in different health aspects are widely reported in particular chronic inflammatory diseases and autoimmune diseases. The association of immune function and dysregulation resulted in a widespread range of deleterious outcomes. The sympathetic-adrenal-medullary (SAM) system and the hypothalamic-pituitary-adrenocortical (HPA) axis are the important systems which can influence inflammatory response.
to emotional or psychological states. The increased salivary cytokines such as IL-1β, TNF-α and IL-6 has been observed in response to acute stress (Slavish et al., 2015).

A cross-sectional study conducted to estimate the salivary interleukin-8 among the 30 smokeless tobacco(naswar) users, and the collection of morning unstimulated whole saliva with ELISA kit resulted in 100.73pg/ml to 263.93pg/ml with mean and standard deviation of 173.48±46.52pg/ml. A higher level of IL-8 was found in naswar users. A statistically significant association was found between the levels of salivary biomarker IL-8 and frequency of naswar usage (Sohail et al., 2020).

A different cross-sectional study revealed the inflammatory biomarkers among four groups of participants of non-smokers (NS), cigarette smokers (CS), e-cig users (EC) and dual EC and cigarette smokers (DS). Saliva was collected passively in the participant’s mouth until 5ml of saliva were obtained. ELISA and Luminex assay is used to estimate the biomarker of interest. Compared with EC and DS, prostaglandin E2 level was significantly increased in CS and significant differences between groups of DS and NS for En-RAGE (Ye et al., 2020).

A study explored Proinflammatory and anti-inflammatory cytokine/chemokine concentrations in saliva, and levels were calculated by using a Milliplex Human Cytokine/Chemokine Magnetics Bead Panel (TNF-α, IL-12(p70), MDC, IL-10, IFN-γ, TNF-β, IL-1β, IL-5, IL-2, IL-6, IL-4, IL-1RA, IL-13, IL-17, IL-7, and GM-CSF). An UltraSal-2 saliva collection device was used to collect 1ml of passive saliva. Significantly higher levels of IL-4 and IL-2 was observed in smoker samples. Whereas relative to non-smoker samples, MDC, IL-10, IL-5 and IL-7 were downmodulated (Rodriguez-Rabassa et al., 2018).

Oxidative stress markers

The reactive oxygen species that initiate the salivary markers of lipid peroxidation, protein oxidation and DNA injury can be estimated in various oxidative stress-related pathologies. The possible sources are blood plasma, oral bacteria and immune cells. The underlying mechanism could be due to the imbalance between lack of antioxidants or overproduction of reactive oxygen species in response to inflammation (Naresh et al., 2019).

N.Kurtal et al., in their study, obtained saliva samples from three groups as smokers, maras powder users(MPU), a form of smokeless tobacco, and healthy control subjects. Unstimulated saliva samples were collected. Denny’s colorimetric method was used to estimate salivary total sialic acid (TSA) and lipid metabolism peroxidation was analyzed by fatty acid peroxidation end products and amount of MDA with biochemical analysis.

The results were reported that the number of cigarettes smoked and the duration of smoking were significantly correlated with Malondialdehyde (MDA) values. In MPU, duration of MP use and the amount of daily consumed MP were significantly correlated with both MDA and TSA levels. The study concluded MPU and smokers were associated with increased salivary TSA and MDA levels. The MPU group were shown higher salivary TSA and MDA levels than those in smokers (Kurtul and Gökpınar, 2012).

Growth factor markers

Salivary growth factors plays a role in cell migration, proliferation and maturation of oral and other distant tissues once they are taken up from digestive tract mucosa. These factors work in autocrine and paracrine modes to maintain and stimulate salivary gland tissue and distant organs (Mori et al., 2008).

Mononuclear phagocyte survival, proliferation and differentiation have been linked to a variety of inflammatory diseases and were stimulated by Colony-stimulating factor (CSF)-1. An enzyme-linked immunosorbent assay was used to measure the CSF-1 with stimulated saliva. Among the various groups, when compared to non-smokers, smokers had substantially lower levels of CSF-1 (Lira-Junior et al., 2017).

Stress-related markers

Salivary alpha-amylase (SAA) is produced by the salivary glands to help in starch digestion, and these glands have a large number of adrenergic receptors which are stimulated by norepinephrine. The sympathetic adrenal medullary system (SAMS) activation increases the production and release of SAA from parotid and submandibular glands acinar cells in response to increased plasma norepinephrine due to psychological stress. Being a stressful agent, the pain increases the production and secretion of alpha-amylase, with SAMS activating capacity. It can be used as a biomarker of pain sensation (Nater et al., 2006).

Cortisol is secreted in response to stress that reaches through complex hormonal and immune system interactions. This activates the hypothalamus-pituitary axis, contributing to the release of the hormone ACTH that acts on the adrenal cortex and increases the levels of salivary cortisol (Kudiela et al., 2009).

The alterations in the sympathetic nervous system
### Table 1: Summary of the salivary biomarkers associated with tobacco users

<table>
<thead>
<tr>
<th>Type of Tobacco</th>
<th>Salivary biomarker</th>
<th>Inference</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarette smoke</td>
<td>Tobacco exposure and nicotine delivery products.</td>
<td>Significantly higher cotinine levels in smokers than in non-smokers.</td>
<td>Schick et al. (2017); Lewis et al. (2003)</td>
</tr>
<tr>
<td>Smokeless tobacco, Smokers</td>
<td>Inflammatory markers</td>
<td>IL-8, prostaglandin E2, EnRAGE, IL-2 and IL-4 are significantly higher in smokers whereas, MDC, IL-10, IL-5 and IL-7 are downmodulated in smokers when compared to non-smokers.</td>
<td>Sohail et al. (2020); Ye et al. (2020); Rodriguez-Rabassa et al. (2018)</td>
</tr>
<tr>
<td>Smokers and smokeless tobacco (maras powder users)</td>
<td>Oxidative stress markers</td>
<td>Increased Malondialdehyde (MDA) and Salivary total sialic acid (TSA) has been observed in smokers and MPU.</td>
<td>Kurtul and Gökpınar (2012)</td>
</tr>
<tr>
<td>Smokers</td>
<td>Growth factor markers</td>
<td>When compared to non-smokers, smokers had substantially lower levels of CSF-1.</td>
<td>Lira-Junior et al. (2017)</td>
</tr>
<tr>
<td>Smokers</td>
<td>Stress-related markers</td>
<td>People who were less addicted to nicotine had greater cortisol responses to the stressor.</td>
<td>Morris et al. (2016)</td>
</tr>
<tr>
<td>Smokers, Tobacco chewers</td>
<td>Genetic markers</td>
<td>Increased salivary levels of microRNA-21 level in smokers than in non-smokers. Polymorphism in the CDH-1 160 promoter region.</td>
<td>Karkera et al. (2017)</td>
</tr>
<tr>
<td>Smokers</td>
<td>Salivary microbiome</td>
<td>The samples of smokers have shown 40% Proteobacteria, 29% Firmicutes, 23% Bacteroidetes, 5% Fusobacteria and 2% of Actinobacteria.</td>
<td>Rodriguez-Rabassa et al. (2018)</td>
</tr>
<tr>
<td>Smokers</td>
<td>Hormone, enzyme markers</td>
<td>ACTC level is significantly higher in smokers and insulin, and leptin were downmodulated.</td>
<td>Rodriguez-Rabassa et al. (2018); Morris et al. (2016)</td>
</tr>
</tbody>
</table>

(SNS) and hypothalamic-pituitary-adrenal (HPA) axis are the major stress reaction that has also been linked to nicotine dependence. A study observed hypothalamic-pituitary-adrenal axis (cortisol, dehydroepiandrosterone) and sympathetic nervous system (alpha-amylase) reactions to the Trier Social Stress Test (TSST) in smokers of young age. Stress is one of the important factors in the susceptibility to nicotine use and dependence, neural circuits associated in the regulation of stress and reward processing are stimulated when using nicotine for a short span, implying that characteristics of the biological stress reaction can amplify stress sensitivity and augment the fortifying consequences of nicotine.

Increased cortisol responses to the stressor were seen by people with a lower level of nicotine dependence, whereas those with a higher level of nicotine dependence showed no difference in the cortisol response to the stressor. Recent nicotine use is linked to increased stress response system activation and lower dependency intensity. More extreme levels of dependency, on the other hand, can reduce stress response systems (Morris et al., 2016).

**Genetic markers**

Different genetic and epigenetic markers have been studied in saliva, and they play a role as diagnostic/prognostic marker in various pathologies. Since smoking is associated with varieties of oral and systemic diseases, the evaluation of these markers could help in the early prediction of disease, espe-
cially in patients with a genetic association of particular diseases. It has been suggested that even lower exposure to tobacco in these patients might influence the disease outcome. Thus, early intervention is possible if the epigenetic/genetic markers are found out in high-risk groups.

Ali and Amer (2017) demonstrated increased salivary levels of microRNA-21 in smokers than in non-smokers and concluded that salivary miR-21 could be a promising new diagnostic marker for cancer susceptibility in cigarette smokers. MicroRNA-21 (miR-21) is one of the most known miRNAs to be over expressed in oral cancer, as well as being capable to distinguish between progressing and non-progressing leukoplasias. miR-21 is considered an oncogene; it down regulates tumor suppressor genes, thus suppresses apoptosis and promotes cell proliferation (Ali and Amer, 2017).

Polymorphism in the CDH-1 160 promoter region and its association was assessed in various oral potentially malignant disorders and in tobacco chewers. The study resulted polymorphism in 43% of the tobacco chewers. This study also revealed that the patients with habits for the lesser duration (0-5 yrs) show C/A polymorphism as well. It has been suggested that genetically predisposed betel-quid chewers are much more susceptible to life style or environment risk factors (Karkera et al., 2017).

Salivary microbiome

A research examined the saliva samples obtained by passive drool from eighteen smokers and sixteen non-smoking individuals. The 16S rRNA gene was used with the Illumina MiSeq platform to classify the salivary microbiome. Proteobacteria (40%), Firmicutes (29%), Bacteroidetes (23%), Fusobacteria (5%), and Actinobacteria (2%) resulted in smoker samples and accounted for 99% of all sequences. Streptococcus (15%), Haemophilus (14%), Prevotella (13%), Neisseria (13%), Porphyromonas (9%), Veillonella (6%), Fusobacterium (5%), Aggregatibacter (2%), Staphylococcus (2%), and Actinobacillus (1%) varied from the nonsmoker community in the bacterial compositions in the smoker group samples (Rodriguez-Rabassa et al., 2018).

Protein/glycoprotein/enzyme/hormone markers

Adrenocorticotropic hormone (ACTC) level is significantly higher in smokers sample. Insulin and leptin were downmodulated compared to non-smokers. The alpha-amylase responses of the TSST were linked to salivary cotinine levels but not to carbon monoxide (CO) (Rodriguez-Rabassa et al., 2018; Morris et al., 2016).

CONCLUSIONS

It is well known that smoking has various deleterious effects on oral health and its physiology and considered as an important risk factor in head and neck carcinogenesis. The above-discussed biomarkers are studied in the saliva of tobacco users. Since saliva is a readily available non-invasive and convenient sample, they can be used as a diagnostic/screening marker for tobacco use and other health related conditions in tobacco users. These markers can be further explored and verified for their accuracy in diagnosing and/or predicting markers in high-risk tobacco users aimed at the applicability in various systemic and oral health conditions. There are various confounding factors for the existence of these salivary markers such as age, gender, lifestyle, history and duration of the habits and other diseases and dietary consumption of anti-oxidant rich food and drinks. These factors might affect the salivary of oxidants and anti-oxidants status. Further, the outliers of the discussed biomarkers can be identified, and the follow-up, patient education of these groups would greatly improve the prognosis of associated diseases as primary prevention. These biomarkers should be further discovered for their validity and their benefit in tobacco users for preventing the progression of tobacco-associated disease conditions.

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Conflicts of Interest

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