Adverse Consequences of Areca Nut Consumption on Oral Hygiene, Advances in Molecular Pathology, and Early Diagnostics

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Abstract

Areca Nut (AN) is the most commonly used psychoactive substance in many parts of Asia and in Asian migrant communities throughout the world. The harmful effects of AN with or without tobacco on oral soft tissues have been studied extensively. The active components responsible for the adverse impact of AN are primarily alkaloids, polyphenols and tannins. A higher incidence of periodontal diseases in AN chewer with and without tobacco is observed. The clinical attachment loss of AN chewers was significantly higher, and the plaque index was considered a strong contributing factor. Several studies indicated a causal association between AN/betel-quid chewing habits and oral mucosal disorders such as leukoplakia, submucous fibrosis and cancer. Besides oral, the incidence of cancers in the esophagus, liver, stomach and pancreas was also seen among AN chewer. This review is intended to discuss the various adverse consequences of AN consumption on oral hygiene comprehensively and highlights recent advances in molecular pathology and biomarker-based disease diagnostics. The host-derived biomarkers particularly, immune response-derived biomarkers, can be routinely analyzed in order to obtain a chair-side early diagnosis of periodontal diseases. Upregulation of Securin and occurrence of premature anaphase separation were observed in human blood lymphocytes in cancer patients and in non-cancerous persons who consume AN/tobacco. Using periodontal and cancer biomarkers, it is possible to intercept oral diseases and cancers at a very early stage, when tissue lesions are not yet clinically detectable.

Keywords: Periodontal diseases; Oral cancer; Biomarkers; Gingival crevicular fluid; Early diagnosis; Single nucleotide polymorphisms; Precocious anaphase

Introduction

It has been estimated that more than 10% of the world population chews Areca Nut (AN) or betel nut in different forms [1]. Epidemiological studies have shown that around 40% of the people in India, Nepal and Pakistan have used Betel Quid (BQ) in which AN is one of the constituents. The AN is consumed mainly with a slaked lime wrapped in a betel leaf known as BQ. In India, BQ is commonly known as 'paan'. In northeastern India, traditionally people consume betel quid, which contains raw rather than dry AN, lime and a small part of betel leaf [2]. Several studies have shown a significant relationship between periodontitis and betel quid chewing habit in many countries including, India [3-5]. Studies over the years revealed that betel quid chewing affects teeth and supporting periodontal tissues due to excessive mastication load [5]. Other studies demonstrated a causal association between AN alone or BQ chewing habits and oral mucosal disorders such as leukoplakia, oral submucous fibrosis and oral cancer. In many parts of northeastern India, the whole BQ is swallowed after chewing and such a habit leads to the development of esophageal and gastric cancers [6,7]. The IARC review suggested that in humans, AN is carcinogenic and linked to cancers not only of the oral cavity but also of the pharynx, esophagus, liver, biliary tracts and the uterus [8]. The effects of AN are diverse and several reports for the last decade clearly established its adverse systemic effects and highlighted its molecular mechanisms [9]. This review is intended to discuss various adverse consequences of AN consumption on oral hygiene comprehensively and highlights recent advances in molecular pathology and biomarker-based disease diagnostics.

Description of areca nut and its demographics

AN is the seed *Areca catechu*, and it grows in much of the tropical Pacific, Asia and parts of East Africa. Chewing BQ and AN is an ancient custom in most parts of Asia, and in many places, such practice is deeply entrenched in the populations' culture [10]. Depending upon the type of curing, different forms of AN are available, which are used by the people of other regions [11]. It could be unripe/ripe, whole/sliced, raw/roasted/sun-dried, boiled/soaked in water, or fermented (under mud). Significant chemical constituents (carcinogens) reductions were observed when the AN was subjected to soaking and boiling. The northeast Indian variety of AN is raw, wet and consumed unprocessed with betel leaf and slaked lime, locally known as 'kwai'.

Chewing of AN is the most common psychoactive substance used globally more than 700 million people are AN chewer, most of whom are from Asian countries [12,13]. Only AN chewing or combined with scents, condiments or sweeteners is an accepted practice in many parts of the Western Pacific and South East Asian countries [12]. In India, due to easy accessibility of AN and its cheaper rate, it is attractive to youth and even teenagers mostly from less educated families with lower socioeconomic status [14].

Constituents of AN, its metabolism and toxic effects

The major constituents of AN are carbohydrates, fats, proteins, crude fiber, alkaloids, polyphenols (flavonoids and tannins) and water, whose concentration may vary due to geographical locations and climatic conditions. Usually, a higher concentration is observed in raw and wet variety of AN compared to dry and processed types [2]. It has been demonstrated that both the alkaloids arecoline and arecaidine undergo conjugation with glutathione and form mercapturic acid in the murine system [15]. The conversion of arecoline to arecoline 1-oxidase by flavin-containing monooxygenases happens mainly in the kidney and thus kidney plays an important role in the metabolism and toxicology of areca alkaloids [15]. Areca alkaloids in the saliva at alkaline pH (due to lime) lead to the formation of several nitrosamines and most of them are carcinogenic [16]. Stich et al. [17] monitored the release of tannic acid into the saliva of the local people of Meghalaya after chewing one-quarter of RAN+lime and proposed appreciable genotoxic activity.

In fact, the toxicity of AN is usually mild and frequently overlooked and therefore the medical community is not generally considered of its carcinogenic effects or complications of AN chewing. Hepatotoxicity and testicular toxicity can be induced by alkaloids of AN by generating Reactive Oxygen Species (ROS) [18]. Genotoxicity of AN-alkaloids, polyphenol and tannin fractions have been reported and it was suggested that AN-alkaloids are the major factors for its toxicity [19,20]. In Chinese hamster ovary cells, the genotoxic potentiality of the saliva of raw AN-chewers of the tribal population of Meghalaya state of the north-eastern region of India was demonstrated [21]. AN extract can induce DNA strand breaks, sister chromatid exchanges, and micronuclei in various kinds of cells [22]. A stronger genotoxicity of arecoline was observed when administered orally compared to intraperitoneal injection [23]. Higher DNA damage, delay in cell cycle kinetics, p53 over-expression and lower GSH levels in the blood lymphocytes in heavy AN-chewers than in nonchewers were observed [24]. The damage in DNA can be induced by the binding of alkaloids to nucleic acid after losing one of its methyl groups [25]. Apart from this, the production of nitroso-derivatives from AN-alkaloids is evident and these AN-specific nitrosamines

form DNA adducts that show carcinogenic activity. Furthermore, a high pH (>9.5) was found to be a major determinant of reactive ROS induction from AN extract in isolated DNA [26]. Kumpawat et al. [27] also demonstrated that the generation of ROS by arecoline could partially contribute to the induction of DNA damages, since the frequency of such damage was reduced either by post-treatment with superoxide dismutase or treatment performed in anoxic conditions [24]. From the epidemiological survey, it is well evident that AN chewing lead to oral submucosa fibrosis and arecoline is considered to be the main causative factor [28]. In fact, arecoline interferes with the molecular processes of deposition of more collagen causing an imbalance in the normal process [29]. Simultaneously, it was also reported that inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) were significantly increased in AN-chewing OSF patients [30].

Besides the oral, a significant rise in the incidence of cancers of the esophagus, liver, stomach and pancreas was seen among AN chewer [31]. Moreover, a long-term habit of AN use can not only lead to cancers but also various other diseases in the brain, nervous system, heart, lungs, skeletal system, gastrointestinal tract, and reproductive organs [32].

AN-chewing habit, dental problems and oral health consequences

Due to the complexity of several related factors, the clinical impact of AN chewing on periodontal status is inconclusive. Almost all male AN chewers are tobacco users in India [33], whereas 90% of AN chewers in Taiwan were reported to be cigarette smokers [34]. However, in hospital-based studies, detailed information on full-mouth pocket depth, clinical attachment loss and the diagnosis of chronic periodontitis [35] can be obtained from AN chewers where an association of AN chewing and periodontitis can be established.

The chewing habits of AN alone or as a component of betel-quid result in exposure among other things to alkaloids and their derivatives (N-nitroso compounds). In addition, if the habit includes tobacco, then tobacco-specific nitrosamines will be an additional factor for detrimental effects. Usually, AN induces deleterious effects on oral soft tissues. However, its impact on dental caries and periodontal diseases are less well-documented.

Periodontal problems: Globally prevalent periodontal diseases are due to virulent bacterial infections, which are considered to be initiating agents in periodontitis. The host response to the pathogenic infection is critical to disease progression [36]. At its early stage, the gums can become swollen and red, called gingivitis, which may bleed (Figure 1). If untreated, it may lead to tooth loss, which negatively impacts on overall quality of life.

The cytotoxic effect of AN and its metabolites on periodontal fibroblasts are evident and may further exacerbate preexisting periodontal disease and impair periodontal reattachment [37]. The poor periodontal status of chewers happens due to the excessive mastication load and hardness of the betel-quid and interactions among the various ingredients of chewing materials with periodontal tissue [38]. Such damage to periodontal tissue is known as the cholinergic effect of betel quid. This, together with calcium salt in the saliva, produced hypersalivation-caused calculus deposition, which leads to the destruction of gingival tissue and periodontal membrane [39].

Excessive tooth abrasion and fractured teeth: The dental abrasion of the occlusal tooth surface is another form of dental

damage caused by the forces applied to the teeth while chewing the hard, fibrous nature of AN. The incisors become shortened and the molars, premolars, and canine teeth frequently lose their cuspal form [40]. Such enamel loss leads to exposure of the underlying softer dentine and increases dental sensitivity [41]. Regular and frequent chewing of hard AN can also lead to dental attrition. In chronic AN-chewers, root fractures have also been demonstrated due to an increased masticatory load [41].

Furcation involvement (FI): If periodontal disease has caused bone resorption into the bifurcation or trifurcation of a multi-rooted tooth, then the condition is known as FI. Usually, it is assessed with a combination of clinical detection and intraoral radiographs. A curved furcation probe (e.g., the Nabers probe) should be used for this purpose. Since maxillary molars usually have 3 roots, therefore 3 furcation's have to be assessed (buccal, mesio-palatal, disto-palatal). Regarding mandibular molars with two roots, two furcation's should be evaluated (buccal and lingual). Such furcation involvement is observed more in AN chewer with cigarette smoking [42].

Betel quid lichenoid lesion: In many BQ users, oral lichenoid lesions have been reported which are chronic inflammatory lesions of the oral mucosa and mostly developed at the site of the placement of the quid. It is characterized by the presence of fine, white, wavy, non-elevated parallel lines that do not overlap and in some instances radiate from a central erythematous area [43].

Oral leukoplakia

Epidemiological data suggest that heavy AN chewer develop a predominantly white patch or plaque on the oral mucosa [44]. In most cases, leukoplakia is considered a pre-cancerous lesion that can turn into cancer if not properly treated [45].

Oral Submucous Fibrosis (OSF): The chewing of AN is one of the many contributing factors that have been implicated in the development of OSF which is a subepithelial inflammatory reaction followed by fibroelastic changes of the lamina propria, accompanied by epithelial atrophy. This process subsequently leads to stiffness of the oral mucosa, which results in trismus and inability to eat [46]. It has been reported that the alkaloids component of AN not only stimulates fibroblastic proliferation and collagen synthesis, but also decreases its breakdown [47]. Such collagen accumulation and subsequent increase of various cytokines, Transforming Growth Factor beta (TGF-beta), Platelet-Derived Growth Factor (PDGF), and basic Fibroblast Growth Factor (bFGF) facilitate the development of OSF [48]. It was further demonstrated that the expression of heme oxygenase-1 was enhanced in OSF patients in AN chewer and arecoline is responsible for this [49].

Oral squamous cell carcinoma (OSCC): It is an established fact that the AN chewing habit is an important factor to develop OSCC [50,51]. The International Agency for Cancer Research classifies AN as a Group 1 carcinogen. Earlier studies have demonstrated that arecoline is the main etiological factor for the development of OSCC [52]. In an animal model, tumors have been shown to be induced in esophagus, stomach and liver when raw AN extract was administered either by oral intubation [53], or mixed with the diet [54], or by ad libitum administration of raw AN extract with lime in drinking water [55]. The highest incidence of esophageal and gastric cancers besides oral cancer has been reported in the North-Eastern states of India [2].

Early diagnosis

The aim of the early periodontal diagnosis is to provide useful information to clinicians regarding the type, status, and severity of the disease which may be helpful for treatment planning and disease-monitoring phases of treatment. Traditional diagnostic tools commonly used clinically are clinical observation (exam, photography), periodontal probing, plaque index, and radiography for assessing the alveolar bone level [56]. These traditional tools are noninvasive, easy to use, and cost-effective, even though, these procedures have some limitations since only disease history can be assessed, not the current disease status.

Oral cancer is considered a serious health issue due to its low survival rate, which is largely attributable to delayed diagnosis due to the asymptomatic nature of the condition in the early stages. Since nowadays, awareness of the cancer burden among people has grown and therefore cancer screening has become widely accepted as an important health care service. Therefore, advances in diagnostic research are providing information and strengthening the methodology whereby periodontal risk assessment is an ongoing process that requires clinicians' awareness of the various dynamic factors that influence the disease state.

Diagnosis of periodontal diseases and application of biomarkers

At clinical level: Depending on the signs and symptoms of gingival inflammation and periodontal tissue destruction, the dentist will decide the type of clinical diagnostic analysis. It is important to diagnose gum disease early before it progresses to periodontitis. Symptoms of gingivitis may include: Swollen or bleeding gums, bad breath, or a metallic taste in the mouth. For a more comprehensive diagnosis, several risk factors like smoking, AN-chewing, diabetes, stress and genetic susceptibility are systematically examined since these may influence the condition further.

Gums become weakened due to gingivitis and periodontitis (i.e., pocket ulceration) and therefore, bleeding can occur during the probing of disease sites. Therefore, Bleeding on Probing (BOP) has been considered a sign of periodontal disease and is evaluated as a numerical indicator called a BOP score [57]. The BOP score is assessed as a proportion of bleeding sites within six tested sites (mesiobuccal, midbuccal, distobuccal, distolingual, mid-lingual and mesio-lingual) on all present teeth when stimulated by a standardized probe with a controlled force (0.2-0.25 N) to the bottom of the pocket. It is considered that the presence of BOP is a poor predictor of periodontal disease activity, however, the absence of it is an excellent indicator of periodontal stability [58]. Interestingly, this BOP score was accepted as a basic parameter in 2017 World Workshop, for the diagnosis of gingivitis and assures the state of a healthy (<10%) or gingivitis (localized: 10% ≤ BOP score ≤ 30%, generalized: BOP score >30%).

Over the years, advanced research has developed the use of biomarker-based diagnosis of oral and periodontal diseases from plaque biofilms [59], Gingival Crevicular Fluid (GCF) [60], and saliva [61] (Figure 1). Table 1 shows different tools that can be used for the diagnosis of periodontitis from plaque biofilm, GCF or saliva.

At the microbiological factor level: By analysis of 16S ribosomal RNA bacterial genes, the existence of several hundred bacterial species at subgingival plaque is known [62]. The presence of members of the red complex bacteria such as Tanerella for synthesis, *Porphyromonas gingivalis*, and *Treponema denticola* at the gingival crevice triggers the release of inflammatory mediators such as Interleukin (IL)-1, Tumor
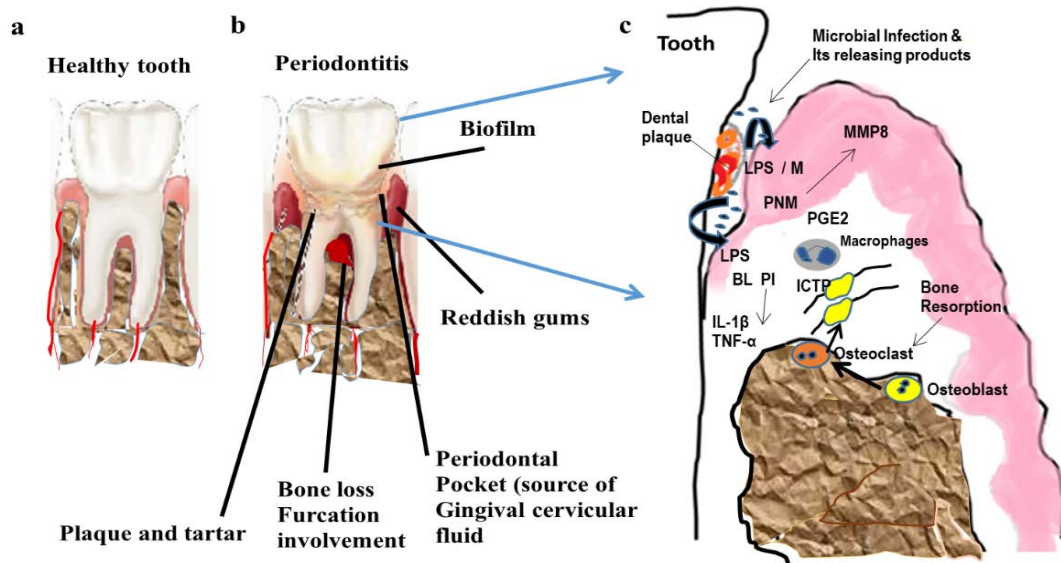
Figure 1: Schematic overview of the involvement of several factors at the cellular and molecular level in the pathogenesis of periodontal diseases. a) Healthy tooth, b) a tooth with periodontitis and c) an enlarged view showing biomarkers involved in the pathogenesis of periodontal diseases. Lipopolysaccharides (LPS) from Gram-negative bacteria are an important agent that causes inflammatory changes and bone resorption in periodontitis. Increased serum LPS is observed due to the activation of macrophages that act as regulatory agents on the immune system of the body. Thus, macrophage-target therapy for inflammatory conditions in periodontitis may prove to be beneficial. The most important cells involved in the pathogenesis of periodontal diseases are Polymorphonuclear Leukocytes (PMN), Macrophages (Mø), and Osteoclasts. PMN leukocytes, representing the first line of defense of periodontal tissues and Mo represents the second line of defense and controlling bacterial diffusion in the connective tissues and represent an important source of enzymes, cytokines, different inflammatory mediators such as interlukin -1β, prostaglandin E2 and tumor necrosis factor-ά. Many substances (PGE2, IL-1, IL-6, TNF-α) secreted by Mø, fibroblasts, plasma cells (PI), B lymphocytes (Bl) and T lymphocytes are involved in osteoclastic activation. An enzyme Metalloproteinase-8 (MMP8) is released by PMN during the immune reaction and is considered to be a valuable biomarker. Subsequent to osteoclastic bone resorption and collagen matrix degradation, pyridinoline, deoxypyridinoline and amino-, and carboxy-terminal cross-linked Telopeptides of type I Collagen (ICTP) are released into the circulation.

Necrosis Factor (TNF)-α, and prostaglandin E2 from monocytes, neutrophils and macrophages of the host cells [63,64]. The recent results show the possibility of diagnosing periodontitis even at early stages by having the oral microbiome profiles from the buccal mucosa and supragingival space [65].

At cellular and molecular level: In the pathogenesis of periodontal diseases, the Polymorphonuclear Leukocytes (PMN), macrophages, and osteoclasts are involved [66]. PMN leukocytes are considered the first line of defense of periodontal tissues and can cause tissue damage as a result of their accumulation in gingival epithelial tissues. The Matrix Metalloproteinase-8 (MMP-8) of PMN is one of the most representative enzymes involved in the breakdown of the extracellular matrix in Periodontitis [66]. The initial lesion of periodontitis is marked by a plaque that results in vascular changes and increased intercellular gap formation that leads to increased amounts of GCF. It has been demonstrated that the severity of periodontitis is associated with an increase in IL-1β, TNFα, prostaglandin E2 and MMPs in tissues or GCF, whereas inhibition of these substances significantly reduces periodontal diseases [67]. It has also been reported that the presence of a large proportion of B lymphocytes and plasma cells and enhancement of the level of immunoglobulins in GCF indicate the advanced stages of periodontal lesions [67]. Therefore, saliva and GCF from the patients, which are easily accessible, are beneficial for the analysis of the current periodontal status and a means of monitoring response to treatment [68]. In addition, other various oral fluid biomarkers like host origin, bacteria and bacterial products, ions and volatile compounds have also been used for periodontal diagnosis [69]. Another valuable diagnostic aid in periodontics is the pyridinoline cross-linked with the telopeptide of

type I collagen (ICTP) because biochemical markers specific for bone degradation may be useful in differentiating between the presence of gingival inflammation and active periodontal or peri-implant bone destruction [70]. To date, the assessment of GCF ICTP levels as a diagnostic marker of periodontal disease has shown promising results [71]. Several studies have established the relationship between GCF osteocalcin, a calcium-binding protein of bone, and periodontal disease [72] (Figure 1).

Early diagnosis of oral cancer

Early detection of precancerous lesions decreases morbidity and mortality, psychological burden, and economic costs to the individual and society [73]. Although, dentists have a definitive role in diagnosing oral cancer [74], the critical role of general medical practitioners in the early identification of such neoplasms cannot be underestimated [75]. To date, identification and diagnosis of potentially malignant lesions rely on biopsies and histopathological examinations, which are still considered to be the gold standard methods for cancer diagnosis [76]. However, these methods are invasive, expensive, and time-consuming procedures. In the recent past, non-invasive visual tools such as toluidine blue staining, autofluorescence (VELscope), and chemiluminescence (ViziLite) have been used solely or in combination as adjuvant tests to detect potentially malignant lesions [77,78]. Thus, there is an urgent need to explore noninvasive, highly sensitive, and specific diagnostic techniques. Therefore, the detailed investigation of various pathways involved in the carcinogenesis of oral cancers and the identification of new biomarkers and therapeutic targets are quite beneficial to optimizing the current therapeutic regimen for treating these deadly diseases.

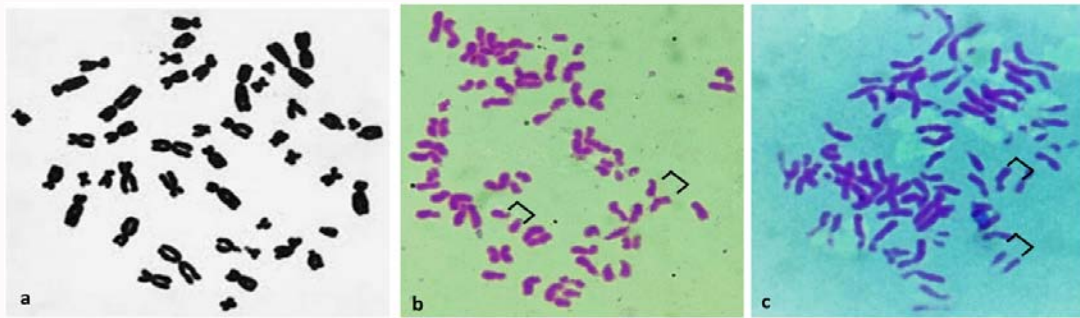**Saliva: A diagnostic fluid:** For early detection of cancer, saliva

Figure 2: Induction of Premature Anaphase Separation (PAS) in blood lymphocytes of AN-chewers with or without tobacco. A) Microphotograph showing a normal human metaphase plate. b and c) Induction of PAS in the lymphocytes of AN chewers with or without tobacco. Brackets show sister chromatids lying separated in mitotic figures that show the phenotype.

Table 1: Different diagnostic tools to measure periodontal disease at various levels identified from plaque biofilm, gingival crevicular fluid, or saliva.

Level of Diagnosis	Targeting subjects	Diagnostic tools
Clinical	Attachment loss	Periodontal probing
	Plaque index	Radiographs
	Bone loss	
Tissue	Downgrowth of junctional epithelium	Histomorphometry
	Bone and connective tissue loss	Immunohistochemistry
	Bone and connective tissue loss	Immunohistochemistry
Cellular antigens	Inflammatory mediators' activation e.g.,	Immunological detection of
	Neutrophils, macrophages, IL-1, IL-6, TNF-α and other cytokines	of pathogenic bacteria ELISA; Immunohistochemistry
	Higher level of IgA and IgG	ELISA
	Level of Osteocalcin	ELISA
	ICTP level of type I collagen	ELISA
Microbial factors putative	Microbial pathogens	DNA probes or culturing of
	(e.g., *Porphyromonas gingivalis*, Tanerella for synthesis, *Treponema denticola*)	periodontal pathogens
Molecular	Activation of receptors for endotoxin; CD-14; Toll-like receptors	Polymerase chain reaction Laser-capture microdissection

is considered a promising approach due to its proximity to cancer cells, accessibility, noninvasive nature and cost-effective sampling. Human saliva is made up of proteins, peptides, electrolytes, organic and inorganic salts secreted by salivary glands as well as GCF and mucosal transudates. Already 100 salivary biomarkers have been identified [79]. Table 2 shows different biomarkers present in the saliva or blood for consideration in the diagnosis of oral cancer. Further research is needed for the reliability and validation of these biomarkers in clinical applications.

Salivary proteomics for oral cancer: About 30% of the proteins that are found in blood are also present in saliva. However, some salivary proteins with specific roles are only produced in the oral cavity [80]. There are six major families of proteins involved in salivary proteomics: Proline-Rich Proteins (PRPs), α-amylases, mucins, Salivary (S-type) cystatins, histatins and statherin [81].

Chen et al. [82] demonstrated that 25 out of 56 salivary proteins were more than 5-fold higher in OSCC patients than in controls. They also suggested a combination of biomarkers, like Complement Factor H (CFAH) and C-Reactive Protein (CRP) with Fibronectin (FINC) for early screening of oral cancer. There are many proteomic biomarkers mentioned in the table (Table 2), and it is true that salivary proteomic research holds great promise. However, there are still some barriers to transferring this technology from the laboratory to clinical practice.

In fact, the post-translational modifications of these proteins are considered to be a big challenge. It is true that standardized protocols for reliable biomarkers are in the process and recent data indicate that the inclusion of "salivary liquid biopsy" has great potential for OSCC diagnosis and management since it improves prognosis, therapy, and follow-up [83].

Cellular and molecular level: Earlier studies demonstrated higher DNA damages, p53 overexpression, a greater delay in cell kinetics, and lower GSH levels in the Peripheral Blood Lymphocytes (PBLs) of heavy AN-chewers than non-chewers [24]. It was reported that 40% of cancer samples from patients in Meghalaya state with only raw AN-chewing habits showed deletion of microsatellite markers located close to exon 1β of the CDKN2A/ARF gene at 9p21 [84]. Almost 27% promoter hypermethylation of the CDKN2A gene was observed in those with the habit of raw AN-chewing alone than in those using both raw AN and tobacco [84]. Increased expression of Securin was observed in human PBLs of both oral and esophageal cancer patients and even in non-cancerous persons with the habit of consuming raw AN [85]. Securin is involved in chromatid separation and its upregulation has been shown to induce aneuploidy, arising from chromatid missegregation in human cells [86,87]. Further, recent studies have demonstrated that an elevated frequency of Premature Anaphase Separation (PAS; Figure 2b, 2c) and sister chromatid

Table 2: Potential sensitive biomarkers for oral cancer detection.

Name of Biomarkers from Saliva [98,99]	Remarks
α-amylase	Quantitative proteomic analysis reveals decreased salivary amylase in oral cancer
IL-1β	in unstimulated whole saliva is a potential biomarker for oral squamous cell carcinoma
IL-8, IL-6, TNF-α	as Potential Diagnostic Biomarkers for Oral Cancer
P53 mutation in exon 4; methylation array (DNA hypermethylation); microRNA expression	Circulating DNA (ctDNA) can easily reach saliva from a local site and bloodstream, carrying information regarding primary tumors and/or metastasis
From Blood [100]	
MMP3 & MMP9	Helpful for diagnosis
Vascular endothelial growth factor	ELISA or RT-PCR
Growth-differentiation factor 15	ELISA
Squamous cell carcinoma antigen	ELISA
TNF αELISA	
Premature anaphase separation Securin overexpression	Culture blood lymphocytes and metaphase preparation by Western Blot/ELISA or by RT-PCR

exchanges in the PBL of both cancerous and non-cancerous people with AN/tobacco consumption habits indicates that these end-points can give relevant information on the genotoxic effect and could become potential intermediate biomarkers for the risk assessment during the early days of such a habit [88].

Most of the oral precancerous and cancerous lesions are primarily caused by life-style risk exposures, and therefore inter-individual and inter-population differences in risk (5) could be partially explained by different distributions of genetic variants (including Single Nucleotide Polymorphisms, SNPs) [87]. Such genetic variants may bring differences in the ability to metabolize carcinogens and/or effectively repair the damage caused by them [89]. Thus, such genetic variations can be considered potential biomarkers for risk assessment of oral cancer development. Meta-analysis of data from multiple studies identified nine SNPs in biologically functional genes that are associated with a higher risk of oral cancer [90].

In unraveling the role of Glutathione S-Transferase P1 (GSTP1) polymorphism (A313G; Ile105Val) as a risk factor in oral oncogenesis, a hospital-based case-control study in North-East India was performed. Individuals with the GSTP1 AA genotype showed a significant association with oral cancer even after adjusting for age, sex and habit. In addition, a separate experiment revealed a higher level of 8-Oxo-2′-deoxyguanosine induction in tumor samples with the AA genotype. Thus, a habit of using RAN/tobacco and GSTP1 AA-genotype together play a significant role in predisposition to oral cancer risk by showing higher DNA-lesions and lower c-Jun phosphorylation that may inhibit apoptosis. Thus, it can be said that the development of cancer is not only due to the type of habit that patients have but also depends on an interaction between the metabolites and the genes that detoxify these metabolites or carcinogens [91].

Nano-detection system

Nano-detection systems are known as new emerging noninvasive strategies that bring the detection sensitivity of biomarkers to the nanoscale. Nanoparticles generate localized surface plasmon resonances at near-infrared wavelengths, providing higher image contrast and resolution [92]. Although these technologies have been studied in tissues and saliva *ex vivo* and in animal models *in vivo*, further efforts should be employed before these strategies can be successfully applied in clinical diagnosis.

Future Direction

There has been a steadily growing trend in the field of oral disease diagnosis to develop tools to monitor periodontitis and other oral disorders. Significant improvements have been made in the understanding of the mechanism at the molecular level in the initiation and progression of periodontitis and other oral disorders, and subsequently developed molecular assays for the detection of biomarkers at the different stages of these diseases. Over the years, developments in genetics and genomics, particularly with DNA sequencing and several genetic factors have become increasingly considered for their potential role in the prediction of predisposition to periodontitis and oral cancer [93]. In order to diagnose severe chronic periodontitis, a genetic susceptibility test is available nowadays where two types of IL-1 genetic alleles are considered: IL-1α +4845 and IL-1β +3954 [94]. The combination of IL-6 and MMP-8 is used to differentiate between periodontitis and healthy gingiva [95]. The American Dental Association (ADA) and others recognize the importance of scientific research on oral fluid diagnostics [96]. It seems that in the near future, the use of a chair-side lab-on-a-chip to detect biomarkers for several dental disorders will be desirable in routine dentistry. Recently, increased hyperphosphorylation of Rb and histone H3 epigenetic modifications both globally and in the promoter region of the Securin gene were demonstrated after AN+lime exposure [97]. Studies looking for biomarkers representing intermediate steps in the pathway from exposure to disease to estimate the risk of cancer in human populations have received increasing attention [64]. Therefore, screening and routine examination in dental and medical clinics for oral disorders, including early cancer lesions can definitely reduce the burden of oral diseases.

Acknowledgement

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