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Abstract

Background: Oral submucous fibrosis (OSF) is a chronic, insidious disease characterized by progressive submucosal fibrosis of the oral cavity and the oropharynx. People affected by this disease mostly live in south Asia, but migrants from these countries to the USA and Europe may present with OSF.

Purpose: We provide a historical background of the disease and the objective of this review is to update the current knowledge on the aetiology and aetiopathogenesis of OSF.

Introduction

Oral submucous fibrosis (OSF) is a chronic, insidious disease that affects the lamina propria of the oral mucosa and as the disease advances it involves tissues deeper in the submucosa of the oral cavity with resulting loss of fibroelasticity.¹ OSF is within a group of conditions that is classified under oral potentially malignant disorders.² The disease manifests with blanching and stiffening of the oral mucosa leading to limitation in opening of the mouth. The presence of fibrous bands in lips, cheeks and soft palate is a hallmark of the disease. The disease is
histopathologically characterized by fibrosis that affects oral cavity, pharynx and upper third of the oesophagus. The prevalence of the disease is on the rise as a result of increased use of commercially prepared areca nut preparations in India.3

Historical perspective
OSF was first described by Schwartz in 1952 among five Indian females living in Kenya and he coined the term Atrophia idiopathica (trophica) mucosae oris. Several other descriptive terms have been given by subsequent authors, including idiopathic scleroderma of the mouth, idiopathic palatal fibrosis, and sclerosing stomatitis.5-7 Lal highlighted the diffuse nature of OSF and the condition is no longer considered “idiopathic”.

Our understanding of oral submucous fibrosis has evolved as a clinico-pathological entity over many decades. Jens Pindborg - a Danish pathologist - in his various and extensive travels as a WHO consultant to south and far east of globe in search of tropical diseases, comprehensively described various facets of the condition. Pindborg with several Indian colleagues initiated an extensive study on the natural history of oral precancer in a rural population of India. Their journey began at Tata Institute of Fundamental Research (TIFR) in Mumbai with several field stations spread across India. At the time OSF was an enigma to both Jens and to the TIFR team. Field studies planned by Jens and undertaken by the TIFR team led to a breathtaking study that comprised of examining over 200,000 Indian villagers over a span of 30 years. A great clinician and a scientist, his sincerity to work in the villages of India with no basic amenities available, showed the man’s motivation and caliber of commitment. Pindborg’s art of observations and determined follow up of his observations on this condition over 2 decades lead to several logical conclusions including the premalignant nature of OSF. Supported by the statistical parlance of Prakash Gupta the team led by Fali S. Mehta and Jens Pindborg published their findings in over 30 scientific papers, describing the epidemiology and clinicopathologic aspects of OSF, as we know of today8-15.

Aetiology
Until about a decade and half ago the aetiology of the disease was thought to be multifactorial and several agents have been reported, including local irritants (chilies), nutritional deficiency,
and auto-immune disease. However, more recent studies have confirmed areca nut as the major (and the only) risk factor of oral submucous fibrosis, among people who probably have a genetic predisposition to the disease. Sufficient evidence on its etiological role based on epidemiological, animal and in vitro studies has been assembled by the International Agency for Research on Cancer and is elegantly presented in several of their monographs 16-18. Areca nut (Fig 1) may be consumed alone or as an ingredient of betel quid. The terminology of various areca consumption habits described by Gupta and Warnakulasuriya 19 and IARC (2004)17 is listed in Table 1. The role of other ingredients in betel quid (leaf, slaked lime or tobacco) as causative of OSF has not been established.17 In several cross sectional studies from Taiwan where betel quid is used without added tobacco, significant association of areca/ betel quid use with OSF were found. 18

In 1995, Murti et al published a review on the aetiology of OSF 20 and the topic was discussed during an expert symposium in London 21. At the time reported ecological observations pointed towards a possible hypothesis of an association of areca nut with OSF, but the data was weak for inferring a causal relationship. Over the past 15 years additional data have emerged crystalising our understating on the aetiological role of areca nut in causing OSF. However, only an estimated 1-2% of the population who have an areca nut chewing habit may develop the disease. This suggests a possible genetic predisposition in the affected people (see later).

In this review we update the current evidence from primary studies that lead to the IARC’s conclusions. Eligible studies were identified from the Monographs published by IARC17,18 and more recent literature. A narrative review is present here.

Epidemiological studies
OSF is predominantly encountered in people of South and South East Asia (Fig 2) or among the diaspora arising from these countries. The evidence on the role of areca nut use increasing the risk for development of OSF is based on case reports, case-series studies, prospective cohort studies, and several case-control studies conducted in India, Pakistan Sri Lanka and Taiwan. The relative risk estimates for OSF reported in case-control studies from these 4 countries ranged from 1.8-172 (Table 2). Large confidence intervals noted in many studies are due to “small cell
sizes” in the 2x2 tables in these studies. A summary outcome of these studies by each country is presented below:

**India:** Five case-control studies from India conducted in Bhavanagar, Nagpur, Kerala, Patna and Chennai provide the relative risk estimates on the use of areca nut \(^{22-26}\). Sinor *et al.* provided the first convincing epidemiological study by comparing chewing habits of mawa and betel quid of 60 OSF cases with 60 controls. The reported relative risks were 109.6 for all forms of areca nut chewing, commonly in the form of mawa, primary ingredient of which was areca nut. Subsequent studies from other parts of India (Dharwad) have confirmed these findings \(^{27}\). Hazarey *et al.* \(^{28}\) reported significant differences in chewing habits between men and women (males chewing Gutkha, mawa and kharra and women chewing exclusive areca nut). Men were reported to develop the disease at a younger age.

Pakistan: Only case-control study from Pakistan to evaluate the etiology of OSF was conducted in Karachi by Maher *et al.* \(^{29}\). They reported various chewing habits of 157 subjects diagnosed with OSF, and estimated RR by comparison with 157 hospital controls. This study estimated a high relative risk of 154 (95% CI 34-693) for areca nut alone users in this Karachi population.

Sri Lanka: One study conducted in a hospital setting (74 cases and 74 controls) confirmed a strong association of OSF with Betel Quid (BQ) chewing (OR = 171.83, 95% CI: 36.35–812.25). In Sri Lanka, areca nut is the primary ingredient of BQ. \(^{30}\) The numbers chewing areca nut alone were meager, and the study did not find any significant risk.

Taiwan: The significance of Taiwanese studies that add weight to areca nut as causative relates to the use of betel quid without any added tobacco by this population. Three forms of betel quid are used in Taiwan, all with areca nut as the main ingredient: betel quid, lao-hwa quid and stem quid. \(^{31}\) All reported studies have indicated a significant association of OSF with areca nut or betel quid use. \(^{31-36}\)
The percentage of subjects with an areca nut habit was close to 100% among OSF cases in these observational studies. Case reports that describe fibrosis in non chewers, probably had falsified habit histories or fibrosis arising from other inflammatory disorders.

There are also case-series reports among Indian migrants living in other countries particularly South Africa and the UK which indicates continuation of their areca nut chewing habit following migration. Few cases are however, reported among citizens of non-Indian origin.

**Dose response**

In toxicological studies it is necessary to demonstrate a dose response to confirm a causal effect of an agent under study. In the case of areca nut and betel quid several studies have demonstrated a dose response by examining the frequency and the duration of use. Most studies conducted so far show an increased relative risk with longer duration of use and higher daily consumption (Table 3). There is also clear evidence indicating that with an upsurge of manufactured products containing areca nut (pan masala and Gutka – see Table 1) arriving in markets in India the disease prevalence has increased significantly and OSF is being diagnosed at younger ages. This could be due to a rapid development of the disease or starting the chewing habit at a very early age.

**Experimental animal studies**

Few studies have reported in-vivo data on the ability of the areca nut extracts to produce OSF in animal models. Huang, Ling and Wu described a rat model of OSF in Hunan Medical University, China and earlier in-vivo experiments of Khrime et al showed histopathological findings akin to OSF induced by pan masala on the rat mucosa. The characterizations of these models were not complete and the experimental evidence was neither convincing nor reproducible. The relevance of a particular animal model to a human disease rests on its ability to parallel the biological changes that characterize the disease in humans. Perera et al described an OSF animal model in female albino mice of BALB/c strain. They applied an aqueous areca nut extract prepared from fresh, mature endosperms of Areca catechu
by dissolving nuts in 0.9% normal saline (50 mM NaCl) on the buccal mucosae of mice (n=40) for 600 days. Their study showed fibrosis of treated buccal mucosa as a continuous process occurring in the subepithelial buccal mucosal tissues of treated mice. (Fig 3) The amorphous areas confirmed by van Gieson and Masson’s trichrome stains, was an indication of early hyalinization, reflected the presence of young collagen or altered ground substance or both. The findings confirming the excessive deposition of collagen in the treated animals reported by them did bear a close similarity to human OSF.

In this in-vivo mouse model the effects of areca nut extract on epithelial thickness leading to atrophy, connective tissue fibrosis, progressive reduction of fibroblasts, and inflammatory changes, were closely similar to that found in human OSF. The experimental data presented by Perera et al, further supports areca nut contributing to the causation of OSF.

**In vitro studies**

Several investigators have studied the effects of constituents of areca nut, such as arecolene and arecaidine on oral fibroblasts in vitro in order to provide corroboratory evidence of its cause and effect. The addition of arecoline and arecaidine has shown stimulatory effects on fibroblasts in culture. In a later study, fibroblasts when subjected to different concentrations of aqueous concentrations of raw or boiled areca nut showed morphological alterations. In other in vitro studies fibroblasts from OSF specimens showed a more than a 1.5 fold increase in production of collagen compared with fibroblasts from age and sex matched and passage-matched normal controls.

**Summary on aetiology**

A comprehensive evaluation of data reported from epidemiological, in vivo and in vitro studies led the IARC (2004) to confirm the etiological role of areca nut as the causative agent of OSF. It is extremely uncommon to find signs of OSF in a non areca nut chewer. Few case reports that describe OSF in never users appear to be misclassification of the disorder due to finding of sclerotic fibrous bands rarely encountered in other chronic inflammatory disorders (eg ulcerative lichen planus or o-GVHD).

**Aetiopathogenesis**
Although the disease was described in 1950s, its pathogenesis has not been clear until recently. Three previous reviews \(^{51-53}\) had undertaken to critically examine the scientific data available on the pathogenesis of OSF published till 2013. This review updates these research findings.

Several mechanisms and biological pathways have been proposed for the pathogenesis of the disorder, all based on the constituents of areca nut and genetic susceptibility to the disease. The flow chart shown below illustrates the possible biochemical and molecular events known in the pathogenesis of OSF (Figure).

**Mechanisms of pathogenesis of oral submucous fibrosis**

**Changes in the extracellular matrix**

Areca nut alkaloid, arecoline is now identified as the principal causative factor for OSF. Arecoline appears to be involved in the pathogenesis of OSF causing fibroblastic proliferation and increased collagen formation.

Involvement of connective tissue growth factor (CTGF) in fibrosis in many human tissues is well established. CTGF is only produced by hepatic stellate and kidney mesangial cells in adults under normal conditions. Deng *et al* \(^{54}\) have shown expression of CTGF in OSF fibroblasts and endothelial cells in all the OSF cases included in their study. Their in vitro data have shown that arecoline stimulated CTGF production in buccal mucosal fibroblasts through generation of reactive oxygen species (ROS), and by the activation of NF – kappa B, JNK and p38 MAPK pathways. It is also known that NF- kappa B, JNK and p38 are strongly activated by ROS. Significance of ROS in the pathogenesis and malignant transformation of OSF. \(^{55}\) has been highlighted.
Arecoline influences deposition of extracellular matrix (ECM) by increasing the production of TIMP-1 and the effect is enhanced when fibroblasts are co-cultured with keratinocytes.\textsuperscript{56} This data suggested that an interaction of oral keratinocytes and fibroblasts may play an important role in the pathogenesis of OSF.\textsuperscript{56} Interaction of arecoline and keratinocytes may induce the differentiation of myofibroblasts from fibroblasts.\textsuperscript{57} Another study proposed that areca alkaloids induce buccal mucosal fibroblast contraction and persistent fibroblast contraction may induce fibrotic process in OSF.\textsuperscript{58} The fact that arecoline influences ECM was further supported by the evidence for Transglutaminase-2 (TGM – 2) over expression in OSF and its regulation by arecoline. TGM – 2 stabilizes ECM protein by cross linking and has been implicated in several fibrotic disorders.\textsuperscript{59}

Reduced vascularity is another hallmark of OSF. In order to highlight this aspect, effects of arecoline on endothelial cells have been investigated. It is reported that at concentrations of 0.4 and 0.8 mM, arecoline induces cytotoxicity and also $G_2/M$ cell cycle arrest and increase sub- $G_0$ / $G_1$ population, a hallmark of apoptosis. Further it is observed that prolonged exposure to arecoline (0.1mM) significantly suppress endothelial cell proliferation. Also the exposure to $>0.2$ mM arecoline decreases the proportion of endothelial cells residing in S phase but increases the cells arresting in G2/M phase. These findings suggest that the anti-proliferative and cytotoxic effects of arecoline are possibly associated with the alteration of specific cell cycle regulatory proteins. Therefore, it is reasonable to suggest that the endothelial damage leading to impairment of vascular function contribute to the pathogenesis of OSF\textsuperscript{60} These findings explain the decreased vascularity that is observed in histological sections, especially with the progression of the disease. Loss of vascularity may lead to atrophy of the epithelium and the resulting hypoxic environment may predispose the tissue to carcinogenesis.
In addition to arecoline, arecanut contains more active components including other alkaloids (arecaidine, guvacine, guvacoline and arecolinidine), polyphenols (catechin, flavanoids, flavan-3:4-diols, leucocyanidins, hexahydroxyflavans and tannins) and trace elements (sodium, magnesium, chlorine calcium, vanadium, manganese, copper and bromine). Polyphenols of arecanut such as flavanoid, catechin and tannins cause collagen fibers to crosslink, and thereby make them less susceptible to collagenase degradation. The resulting decrease in collagen breakdown in turn leads to increased fibrosis which is the mainstay of the pathogenesis of OSF. 61

Matrix metalloproteinases and Tissue inhibitors of matrix metalloproteinases (MMPs and TIMPs)

Accurate and balanced collagen metabolism is essential to maintain the normal integrity of connective tissue. Equilibrium between two enzyme groups, MMPs and TIMPs is mandatory to achieve the above. In OSF the equilibrium between MMPs and TIMP is disturbed in such a manner that it ultimately results in increased deposition of ECM.. We demonstrated an attenuated expression of MMP1 in OSF tissues compared with normal oral mucosa. 62 Since MMP-1, is the main human enzyme that degrades fibrillar collagen, its downregulation may lead to a reduction in collagen degradation. In addition, stronger intensity of TIMP-1 in fibroblasts of OSF than in normal oral mucosa suggested improper regulation of proteolytic equilibrium as one of the main factors responsible for the excessive fibrosis in OSF. 62 The fibroblasts in OSF have a reduced replicative life span as they accumulate senescent cells during the progression of the disease. This is due to the increased amount of ROS and DNA double strand breaks (DDBs)
produced intrinsically by damaged mitochondria. TIMP -1 & 2 are increased in fibroblast cultures of OSF relative to normal and non-diseased pan user controls. Following the introduction of unrepairable DDBs into normal cultured oral fibroblasts TIMP-1 and TIMP-2 secretion increased within 5 days. Furthermore, TIMP-1 production is enhanced when fibroblasts are co cultured with keratinocytes pretreated by arecoline.\textsuperscript{63,64} This highlights an interaction of oral keratinocytes and fibroblasts in the pathogenesis of OSF. Endogenous collagenase activity in OSF tissues is shown to be 3-5 folds less than that in normal oral mucosa, which may also be responsible for collagen accumulation.\textsuperscript{64} Our data have been reproduced in a recent study that has revealed a decrease in intensity of MMP-1 expression in the epithelium and connective tissue of buccal mucosal tissue of OSF compared with normal tissue.\textsuperscript{65}

The above studies highlight the evidence for imbalance between MMPs and TIMPs via different mechanisms leading to accumulation of collagen in OSF. Further, the interaction between keratinocytes and fibroblasts is important in pathogenesis suggesting that OSF is an epithelium driven connective tissue disease.\textsuperscript{63,64}

**Copper and related structural changes of collagen**

The role of copper in the pathogenesis of OSF has been raised as a result of the discovery of a high copper content in arecanut.\textsuperscript{66} Copper dependant enzyme lysyl oxidase is critical for collagen cross linking and organization of ECM.\textsuperscript{67} Salivary copper is found to be higher in arecanut chewers. This finding indicates that soluble copper found in arecanut is released into the oral environment and its buccal absorption may contribute to fibrosis of oral tissues where copper is deposited. This observation suggested a possible local effect of copper in the aetiopathogenesis of OSF.\textsuperscript{68} Salivary copper levels appear to vary from mild OSF to severe cases.\textsuperscript{69} Serum copper levels in OSF is also raised suggesting a systemic effect and increase in serum copper level has
been shown with the advancement of the clinical stage.\textsuperscript{70,71} However, the effect of copper appears to be local in the context of OSF as there is no evidence to suggest that these patients demonstrate any clinical evidence of systemic or organ fibrosis.

**Morphological features of ECM Remodelling in OSF**

Histopathological evidence shows ECM remodeling with the progression of the disease. Stage-specific alterations in ECM have been reported.\textsuperscript{72} In the early stages of OSF over expression of tenascin, perlecan, fibronectin and collagen type III may be found in the lamina propria and submucosa while extensive and irregular deposits of elastin were found around muscle fibres in the intermediate stage, together with the above molecules. In the advanced stage collagen type I appears to dominate the ECM. Their gene expression levels were varied with the progression of fibrosis. This pattern of ECM remodeling steps in OSF is similar to normal granulation tissue formation and maturation process. Difficulty in opening the mouth may be related to loss of various ECM molecules such as elastin and replacement of muscle by collagen type I.\textsuperscript{72}

Heat shock protein (HSP) 47, a known collagen specific molecular chaperone involved in the processing and/or secretion of procollagen is significantly upregulated in OSF. Treating fibroblast with arecoline was found to elevate HSP 47 m-RNA expression in a dose dependent manner through MEK, PI3K and COX-2 signal transduction pathways.\textsuperscript{73} Cystatin C, a non glycosylated basic protein is increased in a variety of fibrotic diseases. Cystatin C was found to be upregulated both at m – RNA and protein levels in OSF and arecoline is responsible for this enhancement in a dose dependent manner.\textsuperscript{74} Malondialdehyde (MDA) is a lipid peroxidation end product with the potential to stimulate fibroblasts and to increase collagen production. MDA is significantly elevated as the grading of OSF progressed.\textsuperscript{75}
Inflammatory cytokines and Growth factors

Upregulation of various cytokines namely, TGF-β1, TGF-β1p, THBS1, SPP1, TIG1, TGM2 and CTGF and down regulation of BMP7 which is a known negative modulator of fibrosis has been reported. TGF β is implicated as one of the main triggers for the increased collagen production and decreased matrix degradation pathways in OSF. It has been shown that the expression of TGF-β1 was significantly upregulated within the connective tissue of OSF compared to normal mucosa. Treatment of cells with arecanut water extract consisting of polyphenols and alkaloids, has shown that 64% of the differentially regulated genes found in test samples matches with the TGF β induced gene expression profile. This suggests that arecoline induces TGF β in keratinocytes and furthermore this is observed only in keratinocytes but not in human gingival fibroblast cells, indicating that keratinocytes could be the source of TGF β in OSF. Further, studies have revealed that keratinocytes secrete TGF β through αvβ6 integrin expression. Same authors have illustrated loss of TGF β expression using the drug tropicamide that blocks αvβ6 integrin confirming that OSF is an epithelial driven connective tissue disease. Phosphorylation of SMAD -2 was observed following treatment of keratinocytes by catechin, tannin and alkaloids, hence the authors state that arecanut mediated activation of p-SMAD - 2 involves upregulation and activation of TGF β. CTGF/CCN2 and COX-2 were found to be over expressed in OSF. The CCN2 synthesis in buccal mucosal fibroblasts is stimulated by TGF β1 and this reaction is mediated via ALK5, JNK and p38 MAPK pathways. Epigallocatechin-3-gallate (EGCG) in turn can completely block TGF β1 induced CCN2 synthesis by suppressing JNK and p38 in buccal mucosal fibroblasts, which may be useful in controlling OSF. Loss of adipose tissue in OSF was found to be due the ability of TGF β to cause lipodystrophy. Level of TGF β secretion is more during the early course of the disease. b-FGF is increased in
fibroblasts and in endothelial cells in early disease, and in advanced fibrosis b-FGF expression was noted more in stroma.\textsuperscript{81} IGF-1 expression is significantly upregulated in OSF both at mRNA and protein levels and arecoline had been responsible for this elevation in a dose dependant manner.\textsuperscript{82} Recently it was reported that the homozygous wild genotype TNF-α2 was significantly associated with an increased risk of OSF and the mutant allele TNF-α2 is about 7 times more efficient in promoter function than the wild allele. Accordingly, TNF-may play a role in pathogenesis of OSF through modulation of collagen metabolism.\textsuperscript{83} With the available literature on growth factors and cytokines, TGF β appears to be the main mediator of the disease and others such as TNF-α, IGF-1, b-FGF and CTGF may contribute to continuous accumulation of collagen with activation of signaling pathways such as ALK5, JNK, SMAD and p38 MAPK. These molecules and pathways can be the target areas for new treatment strategies.

**Epithelial–mesenchymal transition (EMT)**

Epithelial–mesenchymal transition (EMT) has gained significant attention due to its implication in cancer and fibrosis. Cell injury caused by areca nut extract produces ROS which in turn triggers both MAPK and NF-κB pathways involved in EMT of OSF. Thereby exposure to areca nut extract causes alterations of normal keratinocyte morphology and induces the cell cycle arrest at G1/S phase and the senescence-associated phenotypes. Keratinocytes secrete a variety of inflammatory mediators such as PGE2, IL-6, TNF–α and most importantly TGF-β, in response to injury. Hif-1 α enhances the EMT in vitro and promotes fibrogenesis by increasing expression of extracellular matrix–modifying factors and lysyl oxidase genes. Further, it is evident that both ROS and HIF-α were necessary for hypoxia-induced TGF-β1 upregulation. In
OSF a possible relationship between Hif-1 α, ROS and EMT has been revealed as we have shown an upregulation of Hif-1 α\textsuperscript{84} in OSF and also production of ROS by arecoline treatment. In summary the above events show possible EMT in OSF leading to fibrosis. Potentially, the transformed epithelial cell may proliferate to expand the fibroblast population, undergo apoptosis, or revert back to epithelial type. The fact that the transformed cell is able to dedifferentiate back to epithelial form directs future research into a novel path as it hints towards a possibility of reversing the disease process.\textsuperscript{85}

**Molecules in cell cycle control and OSF**

There are few studies performed to find out the effects of cell cycle regulation and pathogenesis of OSF. ROS generated by arecoline may cause cell cycle arrest at the G1/G0 phase in human keratinocyte cells without affecting the expression of p21/Cip1. Further, oxidative stress may induce epithelial cell death without eliciting apoptosis at higher arecoline concentration. However, sub-lethal concentrations of arecoline upregulated the expression of several stress responsive genes namely; heme oxygenase-1, ferritin light chain, glucose-6-phosphate dehydrogenase, glutamate-cysteine ligase catalytic subunit and glutathione reductase.\textsuperscript{86} Similar study reported that 0.01-0.04mM arecoline caused late-S and G2/M phase cell cycle arrest in human KB epithelial cells. A decrease in cdc2 and Cyclin B1 protein levels and increasing p21 were identified in gingival keratinocytes by Western blot analysis. It is also revealed that inhibition of KB epithelial cell growth in a dose and time dependant manner and a reduction in
cell number with higher arecoline concentration. Further increase in arecoline concentration has induced both cell necrosis and apoptosis.\(^{87}\)

**Genetic polymorphisms predisposing to OSF**

There are estimated to be over 600 million areca nut chewers worldwide\(^ {19}\). However, only 1-2% of areca nut chewers may develop the disease. This suggests a possible genetic predisposition in the affected people. Rapid development of OSF in young adults or even children reported in clinical case reports\(^ {42}\) further adds weight to this hypothesis. HLA typing has shown certain HLA antigens with a high significantly raised frequency in OSF patients.

Although the exact mechanisms are not clear, various chromosomal, genetic and molecular alterations are associated with the pathogenesis of OSF.\(^ {88}\) A study using Oligonucleotide microarray has shown 716 genes upregulated and 149 genes were downregulated in OSF. It is identified that genes are involved in immune response, inflammatory response and EMT induced by TGF\(\beta\) signaling pathway namely SFRP4, THBS1, MMP2, ZO-1.\(^ {89}\) In another study, differentially expressed genes in OSF had been analyzed using bioinformatic tools and the genes were located on chromosome 1,2,5,6,7,11 and 12. Gene ontology (GO) classification identified these genes to be related to cellular component subgroups associated with extra cellular matrix, cytoskeleton and cell membrane and also biological process subgroups associated with protein binding, signal transducer activity, immune and defense responses.\(^ {90}\)

Polymorphisms of various genes may also contribute to an increased susceptibility to OSF. Polymorphism of Cytochrome P450 3A gene family is considered as a major determinant of
inter-individual variability in chemical pharmacokinetics. Cytochrome P450 had been identified as a genetic biomarker for susceptibility to develop OSF. This may be helpful in identifying high risk individuals according to the genetic polymorphisms in some exclusive regions of the Cytochrome P450 3A, P4501A1 and CYP2E1 genes.\textsuperscript{91,92} Another study revealed the fact that CYP1A1 (m1) genotype and (m2) genotype singly acts as a protective factor but in the absence of GSTM1 and/or GSTT1 gene significantly alters risk towards the disease.\textsuperscript{93} A few genes related to the pathway of CYP metabolism such as CYP2B6, CYP2C18, CYP2F1, CYP3A5, microsomal glutathione S-transferase 2 (MGST2), alcohol dehydrogenase (ADH), UDP glucuronosyl transferase 2B15 (UGT2B15), and ADH1C were found to be down regulated in all stages of OSF, thereby reducing the ability of CYP to metabolize and clear betel nut substances and contributing to the pathogenesis.\textsuperscript{94} Recently, genetic polymorphism of lysyl oxidase, was identified and LOX Arg158GIn appeared to be associated more in elderly OSF patients.\textsuperscript{67} Polymorphisms of collagen-related genes on OSF risk had been investigated. A study focused on the single nucleotide polymorphisms (SNPs) of TGF\textbeta\textsuperscript{-1} gene reported that polymorphism in 5\epsilon UTR C-T in TGF beta 1 gene associated with pro-angiogenic pathway has a significant association with OSF.\textsuperscript{95} Association of SNP (-1171 5A->6A) in the MMP-3 promoter region with the 5A alleles has an increased risk for developing the disease\textsuperscript{96} while SNPs in the MMP-2 and MMP-9 promoter region is not associated with susceptibility to OSF.\textsuperscript{97} Relationship between OSF and TNF-\alpha genetic polymorphism (-308), was not confirmed as the genotype distribution of TNF-\alpha (-308) genetic polymorphism show similar distribution among areca chewers and non-areca-chewers.\textsuperscript{83} N-acetyltransferase 2 locus codes for an enzyme that catalyze acetylation of aromatic amines. Polymorphisms at this specific site can cause improper acetylation of the amines, leading to DNA adduct formation. It is suggested that these polymorphisms can increase
the risk of OSF in men if exposed to arecanut.\textsuperscript{98} It can be summarized that the available evidence support the theory of possible genetic predisposition to the disease.(Table 4)

Conclusion
Oral submucous fibrosis was first described over 50 years ago in Indian migrants to south Africa and in Indian subjects as an idiopathic disorder. Observational studies by Jen Pindborg characterized the potentially malignant nature of this disorder. Oral submucous fibrosis is characterised by the accumulation of excessive ECM in the lamina propria, predominantly type 1 collagen and other collagens. Recruitment and differentiation of fibroblasts from the mesenchymal stem cell compartment is a key event and leads to functioning fibrocytes that lay down collagen. In addition to fibroblasts, myofibroblasts and mast cells may participate in fibrogenesis, tissue repair, and angiogenesis and have been implicated in inducing fibrosis. In this disease areca nut and probably continuous mechanical irritation act as external mediators. Growth factors and cytokines (e.g., TGF\(\beta\)) are key pro-fibrotic factors. We present here a complex sequence of mechanisms that promote fibrosis of the oral mucosa.

Acknowledgements: We thank Dr Dinesh Daftary (Mumbai, India) for helpful comments on life and works of late Jens Pindborg. Fig 1 is reproduced with kind permission from Editor-in-Chief of Journal of Investigative and Clinical Dentistry.

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Legends to figures

Figure 1. Forms of areca nut used in south Asia, pacific islands and China: (a) Ripe areca fruit (b) unripe areca fruit as consumed in Taiwan (c) areca nut (endosperm of Areca fruit shown in (a) commonly consumed in south Asia (d) areca husk used in mainland China (Reproduced with kind permission of J Inv Clin Dent; Reichart PA, Warnakulasuriya S. Oral lichenoid contact lesions induced by areca nut and betel quid chewing: a mini review. J Investig Clin Dent.. 2012 Aug;3(3):163-6

Figure 2. Global map showing areca nut consuming populations

Figure 3. 300-day areca-treated buccal mucosa in albino mice of BALB/c strain. Markedly atrophic epithelium (green arrow), densely deposited oedematous collagen in the lamina propria, inflammatory exudate (yellow arrow), atrophy of the muscles (white arrow) and less vascular connective tissue are prominent (Reproduced with kind permission of John Wiley & Sons, Inc. J Sumeth Perera MW, Gunasinghe D, Perera PA, et al. Development of an in vivo mouse model to study oral submucous fibrosis. J Oral Pathol Med. 2007; 36:273-80).

Figure 4. Schematic diagram showing possible molecules and pathways involved in the pathogenesis of oral submucous fibrosis.
Table 1: GLOSSARY OF ARECA NUT & ITS PRODUCTS

*Areca catechu* L.: see areca nut

*Areca fruit*: fruit of the palm *Areca catechu* L. — see areca nut

*Areca nut*: nut from the fruit of the *Areca catechu* L. (Palmaceae) tree, a palm native to South Asia. The fruit is green when unripe and orange-yellow in colour when ripe and is the size of a small egg. The nut (seed) is separated from the fibrous pericarp and used fresh or dried, or processed by roasting, sun drying, boiling, soaking in water or fermenting. The unripe green areca fruit may also be used. Synonyms include supari (in Hindi and other languages in India), puwak (Sri Lanka), gua (in Sylheti), mak (Thailand), pinang (Sarawak and Malaysia), daka (Papua New Guinea), pugua (Guam) and Kun-ywet (Myanmar). The term ‘areca’ is derived by the Portuguese from Malayalam atrekka and from the Tamil aakkay.

*Betel inflorescence*: flower of the vine *Piper betle* L.

*Betel nut*: the term ‘betel nut’, although commonly used in the scientific literature, has caused considerable confusion and should be avoided. The correct term is areca nut because betel vine and areca palm are different plants.

*Betel quid*: usually prepared by smearing a betel leaf with slaked lime, to which pieces of areca nut are added. Catechu may be added. Crushed leaves of cured tobacco and flavouring agents may also be added. The ingredients are folded in the betel leaf and chewed. Known as paan in Hindi and other languages in India and buyo in the Philippines. Betel quid may be prepared differently in different parts of the world.

*Gutka*: commercial preparation of areca nut and powdered tobacco, slaked lime, catechu and other ingredients. Also spelt gutkha.

*Lao-hwa quid*: specific Taiwanese term for unripe areca nut split in half, with inflorescence of *Piper betle* L. inserted in the middle and slaked lime added

*Mawa*: mixture of predominantly areca nut pieces with some tobacco and slaked lime

*Naswar*: mixture of powdered tobacco, slaked lime and indigo. Popular in Afghanistan and Pakistan. Also spelt nasswar, niswar

*Paan*: see betel quid. Also spelt pan

*Pan masala*: commercial preparation containing areca nut, slaked lime, catechu and other ingredients, but without tobacco.

*Stem quid*: specific Taiwanese name for betel quid consisting of unripe areca nut split in half, with stem of inflorescence inserted in the middle and slaked lime added

*Supari*: see areca nut

*Tamol*: fermented form of areca nut

*Zarda*: tobacco leaf broken into small pieces and boiled in water with slaked lime and spices until evaporation, then dried and coloured with vegetable dyes; usually chewed mixed with areca nut and spices
Reproduced from IARC Monograph Vol 85. Lyon, IARC. 2004
<table>
<thead>
<tr>
<th>Authors, study location, publication year, (ref number)</th>
<th>Characteristics of cases</th>
<th>Characteristics of controls</th>
<th>Exposure categories</th>
<th>Relative risk (95% CI)</th>
<th>Adjustment for potential confounders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinor et al, Gujarat, India 1990 (22)</td>
<td>60 cases</td>
<td>60 controls</td>
<td>Non chewers Areca nut or mawa</td>
<td>1</td>
<td>109.6</td>
</tr>
<tr>
<td>Maher et al, Karachi, 1994 (29)</td>
<td>157 cases</td>
<td>157 controls</td>
<td>Non chewer Pan Pan + TOBACCO Areca nut</td>
<td>1</td>
<td>32 (6-177) 64 (15-274) 154 (34-693)</td>
</tr>
<tr>
<td>Yang et al (2001) (31)</td>
<td>312 participants (119 men, 193 women)</td>
<td>out of a source population of 3623 in Mutan Country (aboriginal Community)</td>
<td>Non chewer Areca/BQ chewer</td>
<td>1.0</td>
<td>1.8 (0.7-4.8) Adjusted for each other, age and gender</td>
</tr>
<tr>
<td>Lee et al (2003) Kaohsiung 1994-95 (33)</td>
<td>125 histologically confirmed cases of OSF (93 men, 1 woman)</td>
<td>876 population controls (844 men, 32 women) matched on age and sex</td>
<td>Never chewed Former chewer Current chewer</td>
<td>1</td>
<td>12.1 (2.8-51.9) 40.7 (16.0-103.7) Adjusted for education and occupation</td>
</tr>
<tr>
<td>Jacob et al (2004) Kerala, India (24)</td>
<td>170 Oral submucous fibrosis</td>
<td>47 773 subjects with no oral mucosal disorders from the same screening study</td>
<td>Non chewer Chewer (betel quid only) Chewer (betel quid with tobacco)</td>
<td>1.00</td>
<td>56.2 (21.8-144.8) 73.0 (32.9-162.2)</td>
</tr>
<tr>
<td>Yang et al (2005) Mutan community, Taiwan (32)</td>
<td>62 subjects Detected by screening</td>
<td>62 controls selected from the same screening programme</td>
<td>Non-chewer Chewer (betel quid only)</td>
<td>1.00</td>
<td>4.51 (1.20-16.94) Non smoker</td>
</tr>
<tr>
<td>Ranganathan et al (2004) Chennai South India (25)</td>
<td>185 cases</td>
<td>185 hospital-based controls</td>
<td>Non chewer Chewer AN Chewer (pan Masala)</td>
<td>1</td>
<td>3.10 (0.83±11.65) 81.50 (4.95-1341.12)</td>
</tr>
<tr>
<td>Chung et al (2005) Taiwan (34)</td>
<td>17 cases</td>
<td>1075 subjects examined</td>
<td>Non-chewer Areca quid</td>
<td>1.00</td>
<td>151.9 (19.1-999) Included smokers</td>
</tr>
<tr>
<td>Ariyawardana et al (2006) Sri Lanka (30)</td>
<td>74 (61 men, 13 women) Hospital-based</td>
<td>74 (61 men, 13 women)</td>
<td>Non-chewer Areca nut Betel quid</td>
<td>1.00</td>
<td>11.79 (0.64-217.2) 16.24 (5.8-44.8) Non Tobacco or alcohol consumption</td>
</tr>
<tr>
<td>Chen et al (2006) Taiwan (26)</td>
<td>23 cases of submucous fibrosis (among 113 pathology archives)</td>
<td>23 control and 27 cases of non-premalignant disorders</td>
<td>Non-chewer Chewer (betel quid only)</td>
<td>1.00</td>
<td>4.2 (0.17-0.54)</td>
</tr>
<tr>
<td>Ahmad et al (2006) Patna, India (26)</td>
<td>157 Oral submucous fibrosis cases hospital based</td>
<td>135 Hospital-based controls with other diseases</td>
<td>Areca nut only Pan masala Pan Gutka</td>
<td>172.8 9 (15.87-723.27) 138.21 (32.97-629.34) 41.5 (12.33-156.59) 234.9 (67.17-900.330)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1: Epidemiological studies confirming the association of areca / betel quid use with oral sub mucous fibrosis in India, Pakistan, Sri Lanka and Taiwan (Adapted from IARC Monographs 2004, 2012). Large confidence intervals noted in many studies are due to “small cell sizes” in the 2x2 tables in these studies.
<table>
<thead>
<tr>
<th>Author, year, reference number</th>
<th>Quids/day</th>
<th>Odds ratio (95% CI)</th>
<th>Duration of chewing (years)</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maher et al (1994) (29)</strong></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1-5</td>
<td>84 (20-360)</td>
<td>1-5</td>
<td>72 (17-316)</td>
</tr>
<tr>
<td></td>
<td>6-10</td>
<td>246 (47-1278)</td>
<td>6-10</td>
<td>137 (29-640)</td>
</tr>
<tr>
<td></td>
<td>10+</td>
<td>100 (19-522)</td>
<td>10+</td>
<td>109 (25-479)</td>
</tr>
<tr>
<td><strong>Yang et al (2001) (31)</strong></td>
<td>1-10</td>
<td>1.0</td>
<td>1-10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>11-20</td>
<td>1.2 (0.7-2.04)</td>
<td>11-20</td>
<td>1.8 (0.7-4.8)</td>
</tr>
<tr>
<td></td>
<td>&gt;21</td>
<td>1.3 (0.7-2.2)</td>
<td>&gt;21</td>
<td>2.4 (1.0-5.0)</td>
</tr>
<tr>
<td><strong>Lee et al (2003) (33)</strong></td>
<td>1-10</td>
<td>31.4 (11.9-82.5)</td>
<td>1-10</td>
<td>30.9 (11.3-84.7)</td>
</tr>
<tr>
<td></td>
<td>11-20</td>
<td>37.4 (12.6-110.4)</td>
<td>11-20</td>
<td>41.9 (14.1-124.9)</td>
</tr>
<tr>
<td></td>
<td>&gt;21</td>
<td>53.5 (16.4-174.8)</td>
<td>&gt;21</td>
<td>39.3 (11.7-131.7)</td>
</tr>
<tr>
<td><strong>Yang et al (2005) (32)</strong></td>
<td>1-9</td>
<td>3.66 (0.71-18.91)</td>
<td>1-10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10-29</td>
<td>4.55 (1.16-17.84)</td>
<td>11-20</td>
<td>3.88 (2.75-5.60)</td>
</tr>
<tr>
<td></td>
<td>30+</td>
<td>10.34 (2.30-44.73)</td>
<td>&gt;21</td>
<td>6.98 (4.96-9.58)</td>
</tr>
<tr>
<td><strong>Yen et al (2008) (36)</strong></td>
<td>Occasional</td>
<td>1</td>
<td>1-10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>11-20</td>
<td>1.26 (0.91-1.74)</td>
<td>11-20</td>
<td>1.26 (0.91-1.74)</td>
</tr>
<tr>
<td></td>
<td>20+</td>
<td>3.88 (2.75-5.60)</td>
<td>20+</td>
<td>3.88 (2.75-5.60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.98 (4.96-9.58)</td>
</tr>
</tbody>
</table>

Table 3: Dose response relationship of areca habits and OSF
<table>
<thead>
<tr>
<th>Genetic Polymorphism</th>
<th>Role in pathogenesis of OSF</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450</td>
<td>A genetic biomarker for susceptibility to OSF</td>
<td>91</td>
</tr>
<tr>
<td>Cytochrome P450 3A, P4501A1, CYP2E1</td>
<td>Marks high risk individuals</td>
<td>92</td>
</tr>
<tr>
<td>CYP1A1 (m1) and (m2) genotypes</td>
<td>Confirms protection</td>
<td>93</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOX Arg158Gin</td>
<td>Associated more in elderly OSF patients</td>
<td>67</td>
</tr>
<tr>
<td>TGFβ -1 (single nucleotide polymorphism in 5'UTR C-T)</td>
<td>Increased risk for developing OSF</td>
<td>95</td>
</tr>
<tr>
<td>MMP-3 (single nucleotide polymorphism in -1171 5A-&gt;6A)</td>
<td>Increased risk for developing OSF</td>
<td>96</td>
</tr>
<tr>
<td>TNF-α (-308)</td>
<td>No confirmed relationship with OSF</td>
<td>83</td>
</tr>
<tr>
<td>N-acetyltransferase 2 locus</td>
<td>Increased risk for developing OSF (men only)</td>
<td>98</td>
</tr>
</tbody>
</table>

Table 4: Genetic polymorphisms predisposing to OSF or confirming protection
Figure 4: Possible molecules and pathways involved in the pathogenesis of Oral submucous fibrosis
Statement of clinical relevance

Oral submucous fibrosis remains unfamiliar to clinicians in the Americas and Europe. Knowledge of the aetiology of this disease helps to identify “high risk” individuals and understanding the aetiopathogenesis of OSF may contribute to new research to develop targeted therapies.