3. Review of Literature

Search Strategy

- Most widely used information databases were included in the search to answer the research question (PubMed, Cochrane, CINAHL).
- None of the subject specific database was found relevant to research question so subject specific database not included
- As all relevant bibliographic databases were searched and search was successful, so other resources were not searched.
- Initially Singular form of words were used and searched again with plural forms of words were used where appropriate.
- As per my research question use of other search terms like synonyms, acronyms, old terminologies, generic terms and lay terms are applicable to subject area and found appropriate so included.
- Truncation was not used except when searching ‘web of science’. During Initial searches truncation was used but that resulted in lot many unnecessary results which were not appropriate so truncation was not used in later part of search.
- As so many alternative words were already used, the Explode function was not required to be used in research question.

Introduction To Oral Submucous Fibrosis

Oral submucous fibrosis (OSF) is an insidious, chronic disease characterized by progressive submucosal fibrosis of the oral cavity and the oropharynx\(^1\). It leads to restricted mouth opening resulting in restriction of food consumption, difficulty in maintaining oral health as well as impairs the ability to speak. It is a well recognized oral potentially malignant disorder and is predominantly seen in India, Bangladesh, Sri Lanka, Pakistan, Taiwan, Southern China, Polynesia, Micronesia and also among Asian immigrants to the UK and South and East Africa. Recent epidemiological data indicates that, the number of cases of OSF has rapidly increased in India from an estimated 250,000 cases in 1980 to 2 million cases in
1993\textsuperscript{25} to 5 million people in 2002\textsuperscript{26}. Paymaster first described the malignant potential of OSF in 1956\textsuperscript{27}, and the rate has been estimated to be 7\% to 13\% recently\textsuperscript{28}. Upsurge in the popularity of commercially prepared areca nut preparations (pan masala, gutkha and mawa) and increased uptake of this habit by young people\textsuperscript{29} due to easy access, effective price changes and marketing strategies has lead to rapid increase of the disease. Betel quid chewers oral cancer is one of the most common malignancies in South and southeast Asian countries\textsuperscript{30}. The incidence of oral cancer concomitant with oral submucous fibrosis was found to be 25.77 \% in Central India\textsuperscript{31}.

**Historical perspective**

In 1952, Schwartz described five Indian women from Kenya with a condition of the oral mucosa including the palate and pillars of the fauces, which he called "atrophia idiopathica (tropica) mucosae oris"\textsuperscript{32}. Other names given to the disorder were "diffuse oral submucous fibrosis", "idiopathic scleroderma of the mouth", "idiopathic palatal fibrosis", "sclerosing stomatitis" and "juxta-epithelial fibrosis"\textsuperscript{33,34}. Joshi subsequently coined the termed oral submucous fibrosis (OSF) for the condition in 1953\textsuperscript{35}.

**Sir Jens Pindborg**, a Danish pathologist, in his various and extensive travels as a WHO consultant, to south east Asian countries, along with Tata Institute of Fundamental Research (TIFR) team in Mumbai published their findings in over 30 scientific papers, describing the epidemiology and clinicopathologic aspects of OSF, as we know of today\textsuperscript{36-43}. Sir JJ Pindborg has defined submucous fibrosis as “an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx, occasionally preceded by vesicle formation, always associated with
fibrous bands and a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa, trismus, and inability to eat.” Sir Pindborg's observations and determined follow-up work over 2 decades led to several logical conclusions, including the premalignant nature of OSF. SMF is now recognized as the most prevalent potentially malignant oral disorder in South Asia.

**Etiology**

When the disease was first described in 1952, it was classified as an idiopathic disorder. Lal highlighted the diffuse nature of OSF and the condition was no longer considered “idiopathic”\(^3^3\). Subsequently, researchers started to implicate various environmental agents as likely etiological factors such as areca nut, capsaicin in chillies, micronutrient deficiencies of iron, zinc and essential vitamins. In addition, a possible autoimmune basis to the disease with demonstration of various autoantibodies and an association with specific HLA antigens has been proposed\(^4^5\).

However, more recent studies have recognized areca nut as the major and the only etiologic factor of oral submucous fibrosis, among people who probably have a genetic predisposition to the disease. Areca nut consumed alone or as an ingredient of betel quid has been recognized as one of the most important risk factor for OSF\(^4^6,^4^7\). A clear dose-dependent relationship was observed for both frequency and duration of chewing areca nut, without tobacco in the development of OSF\(^4^8\). Severity of OSF varies according to the preparation of areca nut consumed. Commercially available freeze dried products such as pan masala, Guthka and mawa which have high concentrates of areca nut per chew develops OSF more rapidly than conventional betel quid which contain smaller amounts of areca nut\(^4^7,^4^9\).
Pathogenesis

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\textsuperscript{50}. Fibrosis and hyalinization of sub epithelial tissues accounts for most of the clinical features of OSF\textsuperscript{51}.

Areca nut is the endosperm of the fruit of the Areca catechu tree. Euphoria, increases salivation, satisfies hunger, relieves tooth pain, etc. are the various reasons given for chewing areca nut. Various constituents of areca nut have role in the pathogenesis of OSF. The major areca nut alkaloids are arecoline, arecaidine, arecolidine, guyacoline, and guacine\textsuperscript{52}. The important flavonoid components of areca nut are tannins and catechins. Arecoline is the principal etiological agent while tannin has a synergistic role. These constituents interfere with the molecular processes of deposition and/or degradation of extracellular matrix molecules such as collagen, causing excessive deposition of collagen in submucosa resulting in fibrosis. Arecoline causes fibroblastic proliferation and increased collagen formation in OSF\textsuperscript{53}. Tannins cause reduced collagen degradation by inhibiting collagenases. Copper present in areca nut, casuses upregulation of the enzyme, lysyl oxidase. Lysyl oxidase catalyzes final processing of collagen fibers into a stabilized covalently cross-linked mature fibrillar form that is resistant to proteolysis.

OSF is regarded as a disease of collagen-metabolic disorder. Increased collagen synthesis or reduced collagen degradation or both are the possible mechanisms involved in its pathogenesis. In OSF, there is gross imbalance in extracellular remodeling. Deposition of collagen in the oral submucosa is the most important
change seen in initiation of this disorder. Decreased degradation of collagen due to increased cross-linking of the fibers results in progression of the disease. Various growth factors and cytokines such as, Transforming growth factor-beta, beta-Fibroblast growth factors, connective tissue growth factor, tumor necrosis factor-alpha are involved in increased collagen accumulation while TGF-beta appears to be the main mediator of the disease.\[54\]

Deposition of collagen in the oral submucosa is the most important change seen in the initiation of this disorder. **Factor XIIIa** (FXIIIa) also known as fibrin stabilising factor, has been proved to have role in various fibrotic conditions. Increased collagen production being one of the mechanisms involved in pathogenesis of OSMF, the role of FXIIIa needs to be evaluated in this collagen metabolic disorder.

**Factor XIIIa**

**Introduction**

FXIII is a coagulation factor that, beyond a role in haemostasis, plays as a multifunctional protein and functions in a wide range of other physiological and pathological processes, including tissue repair and wound healing.

**History**

FXIIIa was discovered in 1940s when Barkan and Gaspar\[55\] observed that fibrin clots formed in the presence of Ca\(^2\) become insoluble in weak bases. 20 years later, Robbins\[56\] performed the experiment with purified fibrinogen and concluded that in addition to Ca\(^2\), a “serum factor” is also needed to render the clot insoluble in weak acids and bases. Laki and Lóránd\[57\] were the first who realized that the serum
factor, which made the fibrin clot insoluble in concentrated urea solution, was thermo-labile and nondialyzable, and they called this protein as *fibrin stabilizing factor*. Loewy and co-workers then purified fibrin stabilizing factor from plasma, and revealed its enzymatic nature\textsuperscript{58-62}. In 1960, severe bleeding of a patient due to a deficiency of fibrin stabilizing factor was reported. Soon after this clinical finding, the fibrin stabilizing factor was termed as *Factor XIII* in 1963 by International Committee on Blood Clotting Factors\textsuperscript{63}.

The milestones of FXIII research were\textsuperscript{63}:

1) FXIII was discovered to play a highly important role in the regulation of fibrinolysis. 2) The discovery of cellular FXIII (cFXIII) in monocytes and different types of macrophages, including tissue macrophages, revealed that this proenzyme is present in most organs and tissues of the body. 3) The primary protein structure and the genomic structure of FXIII subunits were published in the late 1980s. This was followed by the exact identification of molecular genetic defects in an increasing number of FXIII deficient patients. In 1994 the three-dimensional structure of cellular FXIII was published. 4) The availability of recombinant cFXIII (rFXIII) promoted basic biochemical studies. 5) Simple and reliable methods for the determination of FXIII activity and concentration in the plasma became available and made large-scale clinical studies possible. 6) The discovery of FXIII polymorphisms induced biochemical and clinical studies. 7) FXIII is a multifunctional protein that, beside hemostasis, plays an important role in a wide variety of physiological and pathological processes.
**Structure**

FXIII is a protransglutaminase that circulates in plasma in tetrameric form (FXIII-A2B2)\(^{64, 65}\). It consists of two potentially active, catalytic A subunits (FXIII-A) and two carrier/inhibitory B subunits (FXIII-B). The A subunit (FXIII-A) contains the catalytic domain and the B subunit (FXIII-B) serves as carrier and regulatory protein. In plasma, all FXIII-A exists in a complexed form of FXIII-A2B2. In plasma FXIII (pFXIII), the B subunits are wrapped around FXIII-A2. While the free form, FXIII-A dimer (FXIII-A2), but not FXIII-B, is present in the cytoplasm of certain cells, particularly of platelets and monocytes/macrophages. FXIII-A is detected in a number of monocyte-derived macrophages including macrophages of different serous cavities, alveolar macrophages, tumor-associated macrophages, histiocytic and dendritic reticulum cells of lymph nodes, connective tissue histiocytes, perivascular dendritic macrophages, dermal dendrocytes etc.\(^{64, 66}\) FXIII-A is also present in chondrocytes, osteoblasts, and osteocytes. The gene for XIIIIA spans about 180 kb and is localized to chromosome 6 at bands p24–25\(^{67}\). The gene for XIIIIB is about 28 kb in length and is localized to chromosome 1 at bands q32–32.1. It is composed of 12 exons interrupted by 11 introns, and each of the 10 Sushi domains is encoded by a single exon\(^{68}\). Results of the expression of XIIIIA and/or XIIIIB in a mammalian cell system suggest that XIIIIA is not secreted through the conventional secretory pathway, but is released from the cells by cell damage\(^{69}\). In contrast, XIIIIB is readily released from the synthesizing cells by the secretion pathway because it has a typical signal peptide\(^{68}\). XIIIIA and XIIIIB appear to be a globular particle and filamentous strand, respectively. The A subunit of FXIII (FXIII-A), as the protransglutaminase, is made up of 732 amino acids with a molecular mass of 83,000. It is comprised five domains: The N-terminal
activation peptide (AP-FXIII) (amino acids 1–37), β-sandwich (38–184), catalytic core (185–515), β-barrel 1 (516–628), and β-barrel 2 (629–731). FXIII-B is a glycoprotein consisting of 641 amino acids and its molecular mass is 80,000 \(^{70}\). FXIII-B serves as a carrier protein and is essential for stabilizing FXIII-A. It accelerates cross-linking of fibrin by promoting the formation of a ternary complex between proenzyme FXIII, prosubstrate fibrinogen, and activator thrombin\(^{71}\).

**Activation of Factor XIII in the Plasma and Within Cells**

FXIII is a zymogen (proenzyme) which requires activation for its action. In the plasma FXIII becomes activated only on the surface of newly formed fibrin, which accelerates the activation process 80-100-folds\(^{63, 72}\). The activation of pFXIII occurs in the final phase of the clotting cascade by the concerted action of thrombin and Ca\(^{2+}\). Thrombin removes the activation peptide from the N-terminus of FXIII-A by cleaving the Arg37- Gly38 peptide bond. Then, in the presence of Ca\(^{2+}\), FXIII-B dissociates and the remaining FXIII-A dimer assumes an enzymatically active configuration (FXIIIa). The truncated active dimer of FXIII remains associated with its substrate. Following dissociation, FXIII-B becomes detached from fibrin.

In extracellular conditions, cFXIII could be activated by thrombin and Ca\(^{2+}\) the same way as pFXIII, excluding the dissociation of FXIII-B. However, in the intracellular environment, cFXIII does not need proteolytic cleavage for activation, and during platelet activation, no proteolytic truncation of FXIII-A occurs\(^{73, 74}\). The activation of cFXIII in the cytoplasm occurs through a nonproteolytic mechanism and the rise of intracellular Ca\(^{2+}\) concentration is sufficient to bring about the active
configuration\textsuperscript{74,75,76}. The elevation of intracellular Ca\textsuperscript{2+} concentration, as a consequence of cell activation, is sufficient to bring about the enzymatically active configuration (FXIIIa). The activation of cFXIII by the nonproteolytic mechanism (i.e., by the elevation of intracellular Ca\textsuperscript{2+} concentration) is the physiological way of cFXIII activation in platelets\textsuperscript{74,76} and in monocytes\textsuperscript{77} as well.

Apart from thrombin, several other proteases, including batroxobin marajoensis\textsuperscript{78}, thrombocytin\textsuperscript{79}, trypsin\textsuperscript{80,81}, and activated factor XIIIa\textsuperscript{82} have also been reported to be able to activate FXIII. Up to 140 substrates, including fibrinogen, fibronectin, and vitronectin have been reported as substrates for FXIII\textsuperscript{83}.

**Functions**

For a long time, coagulation factor XIII was regarded just as a neglected coagulation factor at the less interesting end of the clotting cascade beyond the diagnostically more relevant thrombin generation and fibrin polymerization. However, FXIII research underwent a revival when its role in cardio and cerebrovascular diseases was first suggested\textsuperscript{84,85}. Since then more and more research groups with different backgrounds have studied FXIII and thanks to their great work over the last decade, FXIII is now recognized as a multifunctional protein which is involved in many regulatory and construction and repair processes with possible implications in many areas of medicine. It plays an important role(s) in hemostasis, wound healing, and maintenance of pregnancy. FXIII of tissue macrophages play a role in maintaining and modulating the connective tissue matrix in normal conditions.
1. Role in coagulation

Factor XIII is best known as the final enzyme in the coagulation cascade, where it is responsible for cross-linking of fibrin. FXIII is essential to form insoluble clot in coagulation to stop bleeding. Enzymatically active form of FXIII is FXIIIa. Its main function is to crosslink alpha and gamma chains of fibrin and alpha-2 plasma inhibitor to fibrin. Through this, FXIIIa strengthen fibrin and protects it from the action of fibrinolytic system by incorporating antifibrinolytic proteins. The intermolecular cross-linking of the fibrin monomers converts loose fibrin fibers into a stable fiber network that is able to withstand mechanical stress and resist clot rupture. This is the normal or physiologic action of FXIIIa in coagulation cascade.

2. Role in wound healing (Fig 3.1)

The promotion of wound healing by FXIII is a complex process. It involves its effects on fibroblasts, macrophages, extracellular matrix, and angiogenesis. FXIIIa enhances migration of fibroblasts and macrophages, increases rate of fibroblasts proliferation, and decreases their apoptosis. FXIII has an important role in collagen matrix formation in physiologic as well as pathologic conditions. Proliferation of granulation tissue is one of the major processes of wound healing. FXIII plays an important part in this process. During the first stages of wound healing, formation of a fibrin clot is stabilized by thrombin-activated FXIII (FXIIIa). At this stage, the platelet-rich thrombus is anchored to the vessel wall to prevent further bleeding. Activated FXIII (FXIIIa) catalyzes the cross-linking of fibronectin monomers to each other and to interstitial collagen, such as type I and type III collagen. FXIIIa subsequently cross links adjacent fibrin chains and
fibrinolysis inhibitors such as α2-antiplasmin and PAI-2 to fibrin to form a stable platelet-fibrin clot that is resistant to fibrinolysis. Immobilized FXIIIa in the tissue of the wound supports platelet adhesion through integrins on the surface of the platelet$^{91}$ thus stabilizing the provisional matrix at the very early stage of wound healing. Fibronectin, an important component of extracellular matrix, is cross-linked by FXIIIa at cellular matrix assembly sites$^{92}$, which complements disulfide bonded multimer formation in the stabilization of assembling fibronectin molecules. Bacteria are also immobilized via cross-linking to fibrin fibers by the action of FXIII$^{93}$. FXIII is also able to cross-link with many molecules such as fibronectin and vitronectin, which adheres to the integrin of inflammatory cells that would migrate to the wound later. As a result, adhesion of the inflammatory cells is enhanced$^{83}$. FXIIIa mediates the incorporation of fibronectin and other extracellular matrix proteins into the fibrin clot to form a provisional matrix for migration and proliferation of macrophages and fibroblasts into the wound area. FXIIIa further facilitates new vessel formation by direct stimulation of endothelial cell migration, proliferation and survival by upregulation of Egr-1, c-Jun and downregulation of TSP-1, thereby providing nutrients for the newly formed tissue$^{18}$.

Monocytes/ Macrophages play an important role in wound healing process by removal of cell debris and apoptotic cells. FXIII of the extracellular compartment might act on monocytes/macrophages by influencing their activation, differentiation, and migration, while cFXIII present in these cells might play a role in the mechanism of macrophage migration and phagocytosis. cFXIII is involved in receptor-mediated phagocytosis by monocytes$^{94,95}$. 
3. Role in angiogenesis (Fig 3.2)

FXIII also plays an important role in angiogenesis which is an integral part of wound healing. VEGF receptor-2 (VEGFR-2) and αvβ3 on the surface of vascular endothelial cells are both signal molecules in angiogenesis, which can be activated by fibronectin and vitronectin when cross-linked with FXIII\(^96\). VEGFR-2/Evβ3 complex formation mediated by FXIII further activates the signal pathway of both molecules\(^97\).
Though FXIII can bind to endothelial cells, its proangiogenic effect is exerted only by the active form. FXIIIa enhances endothelial cell migration and proliferation and suppresses their apoptosis. FXIII has shown a direct proangiogenic effect on endothelial cells in vitro and has been shown to promote angiogenesis in several in vivo animal models. Following are the mechanisms by which FXIII is involved in angiogenesis and tissue repair: 1) Covalent avb3/VEGFR 2 (vascular endothelial growth factor receptor complex formation (avb3: integrins are involved in angiogenesis and vasculogenesis); (2) tyrosine phosphorylation and activation of VEGFR-2; (3) upregulation of transcription factors c-Jun and Egr-1; and (4) downregulation of thrombospondin-1 induced indirectly by c-Jun through WT-1 (Wilm’s tumor-1).

Fig 3.2: Schematic presentation of FXIII-induced angiogenesis
Role of FXIIIa in pathology

In fibrosis

FXIIIa can catalyse the crosslinking of fibrin monomers to each other as well as can crosslink fibronectin monomers to each other and to type I and type III collagens. This may be important not only for the stabilization of fibrin clots, but also during the process of fibrosis. It has been shown to play role in various fibrotic lesions including that of oral cavity.

FXIIIa in various studies

M Toida et al\(^8\), 1991, studied the expression of FXIIIa in pulmonary fibrosis induced by bleomycin. The distributions of FXIIIa and collagenous components in fifty five specimens of lung tissue, consisting of 24 normal and 31 pulmonary fibrotic tissues affected by bleomycin were studied immunohistochemically. Authors found that in normal tissues FXIIIa-containing cells were sparse and were detected in few interstitial cells. They were mainly spheroidal or spindle-shaped. Alveolar macrophages also exhibited a weakly positive reaction to FXIIIa but they were numerous in pulmonary fibrotic tissues, especially in the subpleural area and around the blood vessels of alveolar septa, where slight to moderate fibrosis was seen. In these areas, these cells were mainly dendritic in shape. In the collagenous scar-like areas, the FXIII positive cells were fewer in number and their FXIIIa expression was depleted. They were mainly slender, spindle shaped. The findings of the study suggested that cells containing FXIIIa have an important role in the development of pulmonary fibrosis. Their study showed a close relation between the distribution of cells containing FXIIIa and that of collagenous components. Authors suggested that alveolar macrophages have an important role in the
development of pulmonary fibrosis by releasing various fibroblast proliferation factors, such as fibronectin, certain types of platelet derived growth factor and interleukin-1.

Amy C Parsonset et al. 2007, tested the hypothesis that upregulation of transglutaminases contributes to the fibrosis seen in the organs, including skin of renal failure patients exposed to gadolinium contrast. Authors studied immunohistochemical expression of transglutaminase-2, factor XIIIa, transglutaminase isopeptide, and the histiocyte marker CD68 on five archived skin biopsies of nephrogenic systemic fibrosis (NSF) patients. Authors found that the dermal fibroblasts and histiocytes of NSF expressed transglutaminase-2, CD68, factor XIIIa, and transglutaminase isopeptide which indicated increased expression and/or activation of transglutaminases in NSF. Based on these findings, authors recommended further research into the use of transglutaminase inhibitors in the treatment and prevention of NSF.

M Toida et al. 1989, studied the distribution of subunit FXIIIa in various oral and maxillofacial tissues such as normal tongue, gingiva, lip, and submandibular gland, and in Dilantin gingival hyperplasia (one case), pyogenic granuloma (three cases), peripheral fibroma (four cases), squamous cell carcinoma (seven cases), chronic sclerosing submandibular adenitis (two cases), and fibrous dysplasia of the mandibular bone (one case). FXIIIa-containing cells were found to be sparse in the normal tissues but were more abundant in the fibrous connective tissue of inflammatory and neoplastic lesions. In this study authors demonstrated the close relationship between the distribution of FXIIIa-containing cells and that of collagenous components. Authors concluded that FXIIIa-containing cells may play role in the process of fibrosis.
Toida M et al\textsuperscript{11}, 1990, immunohistochemically studied FXIIIa expression in 43 cases of radicular cysts. In each layer of connective tissue wall of radicular cyst, a positive reaction for FXIIIa was observed in certain connective tissue cells. The cells were found to be few in the inner layer where collagenous components were also sparse. In the slightly to moderately fibrous intermediate layer, these cells were found to be more in number and were dendritic or stellate in shape. In the outer densely fibrous connective tissue layer, they decreased in number and were slender and spindle-shaped. Results of the study indicated that there is close relationship between the distribution of FXIIIa-containing cells and of collagenous components which suggested that FXIIIa containing cells play an important role in the process of fibrosis occurring in the radicular cyst wall.

Expression of FXIIIa, CD 34, S-100 protein, and macrophage antigen (MAC 387) was studied by Regizi JA et al\textsuperscript{90}, 1992, in oral submucosa and oral fibro-vascular lesions with the purpose to determine the presence and distribution of analogous cell types of subsets of monocyte/macrophages lineage in formalin-fixed, paraffin-embedded tissue sections from normal mucosa, peripheral fibroma, peripheral ossifying fibroma, peripheral giant cell granuloma, pyogenic granuloma, lymphangioma, benign fibrous histiocytoma, idiopathic histiocytosis and angiofibroma. FXIIIa positive submucosal dendrocytes (CD34, S-100, MAC 387) were found in abundance in normal tissue in association with collagen, blood vessels, and lymphoid-associated. Authors concluded that FXIIIa containing cells participate in the formation of some oral reactive and fibroblastic lesions.

Regezi JA et al\textsuperscript{101}, 1994, immunohistochemically studied expression of FXIIIa in tissue sections of oral lichen planus to differentiate in dendrocyte populations in lesional and non-lesional areas from the same patient. They found that XIIIa
positive dendrocytes were significantly increased in number and size in lichen planus. Authors concluded that XIII A dendrocytes may have a role in the immunologic mechanisms of lichen planus, and that these cells may act in concert with other immunocompetent cells, such as macrophages and S-100 positive epithelial dendritic cells to activate T lymphocytes in pathogenesis of lichen planus. M Toida et al, 1995, studied the distribution pattern and characteristics of cells containing FXIII A in benign and malignant lesions of human buccal mucosa. Four irritation fibromas and three squamous cell carcinomas were studied by means of double immunofluorescent staining techniques in which the detection of FXIII A was combined with a reaction with CD14 (recognizing a monocyte/macrophage differentiation marker antigen), Mac 387 (reacting with a special subset of macrophages), anti-HLA-DR, Ki-M7 (labelling phagocytosing macrophages) and Ki-67 (visualizing a nuclear antigen associated with cell proliferation) monoclonal antibodies. There was difference in expression of FXIII A expression between benign and malignant tumors. They were spindle-shaped in fibromatous tissues, whereas in the tumor stroma, large stellate cells predominated, and round cells were found around blood vessels. The morphological appearance of the FXIII A+ cells accumulated in carcinoma tissues strongly suggests that they represent tumor-associated macrophages (TAMs). FXIII A + cells were labeled with CD14 and Ki-M7 in both fibromatous and tumoral buccal mucosa; however, they failed to show any reaction with Ki-67 which suggested that they are non-proliferating. FXIII A + cells accumulated in the tumour stroma reacted for HLA-DR as well. These results indicate that in both the benign and malignant buccal lesions FXIII A is contained in a subpopulation of tissue macrophages, which represents a monocyte-derived (CD14 +) and phagocytosing (Ki- M7 +) cell
population. Authors concluded that the FXIII A + cells of the tumour stroma may be actively involved in both antigen presentation and matrix remodelling during tumour progression.

These studies suggest a strong relation between FXIIIa and fibrosis. As found through standard electronic databases (Pubmed, MEDLINE, Google scholar), FXIIIa has not been studied in OSMF till date. Therefore, FXIII need to be studied in SMF where there is fibrosis in submucosa.

**Matrix Metallorptoteinase-9**

**Introduction**

Matrix metallorptoteinases (MMPs) were discovered in 1962, in an effort to establish how the metamorphosing tadpole of a frog lost its tail. Since then, over 66 MMPs (including 28 human MMPs), have been cloned and sequenced. MMPs constitute a set of structurally related matrix degrading proteases. These are endopeptidases that play an important role in tissue remodeling by degrading extracellular matrix. MMPs are produced by various types of cells during various physiological and pathological conditions. They play an important role in wound healing and bone resorption. They are involved in basement membrane disruption, stroma and blood vessel penetration, metastasis, tumor growth and angiogenic events.

**Structure**

Most members of the MMP family have three basic domains namely: an amino terminal propeptide domain, a catalytic domain and a hemopexin-like domain at the carboxy terminal. There is an MMP gene cluster on chromosome 11. MMPs
are products of different genes activation dispersed in the genome. MMPs are structurally similar, but differ in substrate specificity. Each MMP has the ability to degrade a particular subset of matrix proteins. Till date 28 human MMPs have been identified which have been classified according to their substrate specificity and structure. The major subgroups are interstitial collagenases, gelatinase, stromelysin and membrane-bound MMPs. These are:

1. The collagenases (MMP 1, 8 and 13)
2. The gelatinases (MMP 2 and MMP 9)
3. The stromelysins (MMP 3, 10,11 and 19)
4. The membrane bound MMPs. (MT-MMP-1,4)

**Introduction to MMP-9**

MMP-9 belongs to the gelatinase subgroup and is known as gelatinase B due to its ability to degrade gelatin. MMP-9, having molecular weight of 92 kDa, degrade extracellular matrix proteins and acts as a controller of neovascularization. MMP-9 (gelatinase B, 92-kDa type IV collagenase) was first discovered in neutrophils in 1974.

**Structure of MMP-9**

Human MMP-9 consists of an NH_{2}-terminal pro-domain, a catalytic domain, a linker domain, and a COOH-terminal hemopexin-like domain that combine to form a 92-kDa pro- and 88-kDa active enzyme in humans. The catalytic domain of MMP-9 contains two zinc ions, five calcium ions, and three repeats homologous to the type II module of fibronectin. One of the two zinc ions of the catalytic domain
and cysteine switch motif of the pro-domain are structurally coordinated to keep MMP-9 inactive\textsuperscript{110}. The catalytic zinc ion is essential for its proteolytic activity. MMP-9 has collagen binding domain, fibronectin-like domain, within its catalytic domain, which distinguishes it from other MMPs\textsuperscript{107}.

**Activation of MMP-9**

MMP-9 is released from cells as a proenzyme. It becomes activated extracellularly through proteolytic activation where the prodomain is cleaved yielding an active enzyme. Latent MMP-9 can be activated by MMP-3, plasmin, chymotrypsin, MMP-2, MMP-7, MMP-10 and MMP-13\textsuperscript{111-14}. It also gets activated by other mechanisms such as oxidation by reactive oxygen species, s-nitrocylation and allosteric activation\textsuperscript{115,16} which occurs when pro MMP-9 is bound to either gelatin or type IV collagen. Intracellular activation may be an alternative activation mechanism for pro-MMPs in oral cancer.

**Cell Expression of MMP-9**

MMPs are produced by epithelial cells as well as by cells of connective tissue such as fibroblasts, macrophages and neutrophils. MMP-9 is an inducible enzyme, unlike MMP-1 and MMP-2 which are constitutive enzymes\textsuperscript{117}. Hence, its levels are altered during the changes in the tissues. The effectors include growth factors, cytokines, chemical agents such as phorbol esters, actin stress fiber-disrupting drugs, physical stress, and oncogenic cellular transformation\textsuperscript{118}. Macrophages are a potent source of MMP-9. Monocyte entry into the tissue delineates the transition into the macrophage, at which time MMP-9 expression increases\textsuperscript{119}. 


Regulation of MMP

MMP activity is regulated at three levels: gene transcription, posttranslational activation of zymogens, and interactions of secreted MMPs with inhibitors\textsuperscript{121}. They are mostly regulated at transcription level. The mechanism by which gene transcription is mediated is thought to be through prostaglandin E2(PGE) cAMP dependent pathway. Transcriptional activation is stimulated by a variety of inflammatory cytokines, hormones, and growth factors, such as interleukin-1b (IL-b), IL-6, tumor necrosis factor-a (TNF-a), epidermal growth factor, platelet-derived growth factor (PDGF), and basic fibroblast growth factor\textsuperscript{122-26}.

Inhibitors of MMP-9

MMPS are inhibited by specific endogenous inhibitors, Tissue inhibitors of metalloproteinases (TIMPs) which bind MMPs in a 1:1 stoichiometry. All four TIMPs, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 have been identified to inhibit MMP-9 in vitro\textsuperscript{127-130}. After the role of MMPs in cancer invasion and metastasis formation was recognized researchers began developing synthetic inhibitors that inhibit MMP-9. Synthetic inhibitors that inhibited the invasion of ovarian carcinoma, breast carcinoma, fibrosarcoma, Kaposi sarcoma, and melanoma cell lines were developed successfully. It also increased the survival of human tumor xenografts\textsuperscript{131}. A synthetic inhibitor of MMP-9 which inhibited the growth of human tongue cancer cell xenografts, and angiogenesis in nude mice is also developed by researchers\textsuperscript{132}.
Functions of MMP-9

Marxix metaalproteinases are zinc dependent endopeptidases that efficiently degrade the components of the extracellular cellular matrix and basement membrane. Gelatinases are known to cleave native type IV, V, VII, and X collagens along with fibronectin and elastin, as well as the products of collagens types I, II and III after proteolysis by collagenases. They primarily cleave denatured collagen and intact collagen type IV in basal membranes. In addition to gelatin and other forms of denatured collagen, MMP-9 cleaves a number of other physiological substrates particularly substrates containing arginine. MMP-9 plays a major role in the degradation of ECM in a large spectrum of physiology and pathophysiology processes that involve tissue remodeling. MMP-9 is also reported to play a significant role in neovascularization through the proteolytic degradation of the proteins in basal lamina of the blood vessels and a release of the biologically active form of vascular endothelial growth factor. In pathophysiological conditions, MMP-9 is upregulated during development and wound healing, as well as during pathologies that involve inflammatory processes. Its expression is important for embryo implantation, starting from the trophoblastic invasion during the early gestation period. MMP-9 plays an important role in wound healing. MMP-9 could participate in several key areas of wound healing, such as detaching anchored keratinocytes from the basement membrane and remodeling of the extracellular matrix and potentially enabling more efficient cellular migration. It is involved in re-epithelialization after injury. The growth factors epidermal growth factor (EGF) and hepatocyte growth factor (HGF) are found to stimulate keratinocyte migration in wound assays in vitro and this cell migration is dependent on induction of MMP-9 activity and is impaired by the
presence of either function blocking MMP9 antibodies or a general MMP inhibitor. In addition to cell proliferation, MMP-9 could also be involved in epithelial cell differentiation\textsuperscript{141}. Wang et al. showed that cardiac fibroblasts treated with a recombinant protein encoding only the catalytic domain of MMP-9 stimulated cardiac fibroblast migration, increased collagen synthesis, upregulated angiogenic factors, and induced the transition of cardiac fibroblasts to myofibroblasts which play an essential role in post-myocardial infarction healing\textsuperscript{142}. MMP-9 plays important roles in immune cell function. In various pathophysiological conditions, MMP-9 proteolytic properties contribute to stimulate the immune response to initiate pathogenesis and exacerbate disease progression\textsuperscript{119}. Therefore, it can be used as markers of some cancer, neurodegenerative, immune and cardiovascular diseases\textsuperscript{143}.

**Regulation of angiogenesis by MMP-9**

Regulation of angiogenesis is a very delicate balance between pro- and antiangiogenic factors, and MMP-9 plays a dual role in this process. It can act as a proangiogenic factor via VEGF regulation. MMP-9 can have an antiangiogenic role. It causes cleavage of type XVIII collagen, and leads to the release of endostatin, which is a potent inhibitor of angiogenesis and endothelial cell migration\textsuperscript{144, 145}. MMP-9 mobilizes VEGF and initiates angiogenesis. MMP-9 also causes proteolytic degradation of type IV collagen, which produces an angiogenesis inhibitor, tumstatin\textsuperscript{146, 147}. Variations in spatial and temporal MMP-9 expression may switch between two roles: from a proangiogenic to an antiangiogenic molecule.
Interaction of MMP-9 and VEGF

VEGF is a potent mitogen and chemoattractant for endothelial cells and induces the release of MMP-2, MMP-9, and MT1-MMP by endothelial cells. MMPs contribute to angiogenesis by degrading basement membrane and other ECM components, by allowing endothelial cells to detach and migrate into new tissue and by releasing ECM-bound proangiogenic factors such as VEGF, bFGF, and TGF.

Role of MMP-9 in fibrosis

In normal tissue, MMPs are expressed at very low levels. Their production and activation is rapidly induced when active tissue remodeling is needed. They have been found to have both inhibitory and stimulatory roles in fibrosis. MMP-9 dependent functions during fibrosis are not limited to effects on extracellular matrix turnover, but rather these proteinases influence cellular proliferation and survival, gene expression, and multiple aspects of inflammation, and can directly and indirectly influence the activation of myofibroblasts. In models of allergic lung inflammation, MMP-9 has been shown to have various functions related to chemokine levels and leukocyte influx. Some studies have demonstrated its ability to cleave and alter the activity of various chemokines. MMP-9 has been found to promote fibrosis by activating latent TGFβ1. Murthy S et al suggested pro-fibrotic role of MMP-9. In kidney fibrosis, MMP-9 has been shown to be a potential activator of latent TGFβ1 and, hence, was considered a pro-fibrotic mediator. Wang et al has suggested suggesting a role for MMP-9 in myofibroblast activation or survival. There is diverse data on role of MMP-9 in fibrosis.
Role of MMP-9 in OSMF

Increased and continuous deposition of extracellular matrix in OSMF may take place as a result of disruption of the equilibrium between matrix metalloproteinases and tissue inhibitors of metalloproteinases (TIMP). The extent to which alterations in the matrix proteolysis play a role in the development and regression of fibrosis is poorly understood. Inhibition of the existing collagenase and decreased generation of active collagenase together results in a marked degradation and a resultant build up of collagen in OSMF. Whether they have role in pathogenesis of OSMF needs to be evaluated as in OSMF decreased degradation of matrix/collagen is thought to be one of the mechanisms responsible for fibrosis.

Studies

Cabrera S et al\textsuperscript{157} studied the effect of MMP-9 overexpression in bleomycin-driven lung fibrosis. Their findings suggested that increased MMP-9 secreted by alveolar macrophages in the lung microenvironment may have an antifibrotic effect and provide a potential mechanism involving insulin-like growth factor binding protein-3 degradation.

Lee CG et al\textsuperscript{158} demonstrate that IL-13 is a potent stimulator and activator of TGF-beta(1) in vivo. They also demonstrate that this activation is mediated by a plasmin-serine protease- and MMP-9-dependent.

Betsuyaku T et al\textsuperscript{159} studied the role of gelatinase B in bleomycin-induced fibrosing alveolitis, for which they instilled bleomycin intratracheally into gelatinase B-deficient mice and gelatinase B+/+ littermates. Authors found that
fibrosing alveolitis develops after intratracheal bleomycin irrespective of gelatinase B.

Kim YJ et al\textsuperscript{160} investigated the changes in the level of MMP-2 and MMP-9 and their various cellular sources in different stages of pulmonary fibrosis by estimating the changes of the activity of those MMPs at different time intervals in bleomycin-induced pulmonary fibrosis in rats. The level of MMPs in BAL fluid of 54 bleomycin-treated rats was assessed by zymography from 1 to 28 days after intratracheal bleomycin instillation. The level of MMPs in lung parenchyma was evaluated by immunohistochemistry. They demonstrated that the activity of MMP-2 and MMP-9 increased rapidly in the early phase. MMP-2 and MMP-9 in this early phase secreted by macrophages and neutrophils may play an important role in inflammatory cell migration.

Rajendran et al\textsuperscript{161} studied the expression of MMP-1, MMP-2, MMP-9 and TIMP-1 and TIMP-2 immunohistochemically. Authors also quantitatively measured enzymatic activity of MMP-2 and 9 and distinguished the active form from the inactive variant of enzymes by gelatin zymography. Positive stromal expression of MMP-9 was observed in all cases of OSMF. While epithelial staining of MMP-9 was seen to be mild in 20\% cases, moderate to intense in 5\% cases while 75\% cases were recorded negative epithelial expression for MMP-9. The results of the gelatinise zymography showed that there was a decreased expression of active forms of MMP-2 and MMP-9 in OSF cases when compared to the controls. According to authors, the excessive collagenisation taking place in the submucosa of OSF could set in to motion a regulatory reflex mechanism causing an upregulation in the matrix enzyme expression. This probably explains the increased immunostaining noticed of the matrix metalloproteinases but due to
functional impairment (steric conformational change?) it fails to degrade the excess tissue collagen. Authors concluded that The defunct tissue proteinases (MMPs) could be envisaged to be refractory to the regulatory influence of TIMPs, which further may lead to excessive fibrosis at tissue sites.

Though at first sight, the MMPs might be expected to be under-expressed in fibrosis or, if present, could function to resolve the excess matrix, there is diverse data on MMP-9 in various fibrosing conditions. Its expression was found to be in less fibrosis in one study \(^{162}\), whereas in another it was pro-fibrotic\(^{158}\), while yet others concluded it had no role\(^{159}\). Thus we do not yet know precisely how MMP-9 functions during fibrosis. In OSMF, although the main pathological change is increased production of extracellular matrix, there is little information on the actual remodeling of connective tissue with the progression of the disease. There are very few studies where role of MMP-9 has been studied in relation to fibrosis in OSMF. Therefore, it is necessary to assess the role of MMP-9 in OSMF.

**Vascular Endothelial Growth Factor**

In 1983, Senger and his associates described the partial purification of a protein able to induce vascular leakage in guinea pig skin. This protein was then named vascular permeability factor and was proposed to be a specific regulator of the hyperpermeability of tumor blood vessels\(^{163}\). Ferrara and Henzel\(^{164}\) in 1989, renamed it as vascular endothelial growth factor. VEGF was found to be potent, diffusible and specific for vascular endothelial cells. It is a potent mitogen (ED\(_{50}\) 2-10pM) specifically for micro and macrovascular endothelial cells derived from arteries, veins and lymphatics.
It is a potent angiogenic cytokine involved in angiogenesis. Angiogenesis is the process of formation of new microvessels from the preexisting vasculature. VEGF induces both physiologic and pathologic angiogenesis. It is one of the major positively acting angiogenic molecule.

**Structure or Biological forms of VEGF**

The human VEGF gene has been mapped to chromosome 6p21. Biochemically, VEGF is a heparin binding homodimeric glycoprotein of 45,000 daltons. cDNA sequence analysis of VEGF has indicated that VEGF may exist as one of the four molecular species having 121, 165, 189 and 206 amino acids respectively. The VEGF family currently comprises of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F ad PIGF (placental growth factor). All members have a common VEGF homology domain. They have a common structure of eight characteristically spaced cysteine residues in a VEGF homology domain. They have different physical and biological properties and act through specific tyrosine kinase receptors- VEGFR-1, VEGFR-2 and VEGFR-3.

**Functions/ Biological activity of VEGF**

VEGF is a multifunctional cytokine that regulates various endothelial cell functions such as mitogenesis, permeability, vascular tone, production of vasoactive molecules and the stimulation of monocyte chemotaxis. It is crucial in embryonic development and in physiological conditions such as wound healing. It plays role in pathological conditions including rheumatoid arthritis, ocular neovascularization, tumor progression, endometriosis and cardiovascular diseases.
VEGF family has seven members- VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF). These have a common structure of eight characteristically spaced cysteine residues in a VEGF homology domain and have different physical and biological properties. They act through specific tyrosine kinase receptors- VEGF-1, VEGF-R and VEGFR-3. VEGF-VEGFR receptor system is a key component in the process of angiogenesis. VEGF-A is a key molecule in induction of angiogenesis and vasculogenesis. It causes proliferation, sprouting, migration and tube formation of endothelial cells. VEGF-A also causes vasodilatation and promotes endothelial cell survival. It also has role in vascular smooth muscle cell proliferation and migration and has hematopoietic effects. It promotes monocyte chemotaxis and exerts procoagulant activity via its ability to stimulate the production of the potent indicator of coagulation tissue factor in endothelial cells and monocytes. VEGF-A (mRNA expression) is induced by hypoxia. Certain growth factors, inflammatory cytokines and hormones up-regulate VEGF-A mRNA expression.

VEGF-B is expressed by skeletal tissues, myocardium and brown fat. It has role in inflammatory angiogenesis. VEGF-D is a secreted glycoprotein and is expressed by vascular endothelium, heart, skeletal muscle, lung and bowel. It is involved in tumor and inflammation related lymphangiogenesis. It has been shown to be responsible for proliferation of endothelial cells and it may induce lymphatic vessel growth in adult life in response to pathological conditions. It has been proposed to have a role in tumor angiogenesis and lymphangiogenesis.
Regulation of VEGF

1. Hypoxia

Several mechanisms have been shown to participate in the regulation of VEGF gene expression. Among these, oxygen tension plays a major role. VEGF mRNA expression is rapidly and reversibly induced by exposure to low pO$_2$\textsuperscript{177,178}. In physiology, oxygen is transported by circulating erythrocytes, the production of which is controlled by the glycoprotein hormone erythropoietin (EPO). EPO-producing cells in the liver and kidneys can sense oxygen concentration and respond to systemic hypoxia by increasing EPO gene transcription. There is hypoxia-induced VEGF gene transcription\textsuperscript{17}. Hypoxia can also be restricted to cells within a localized region of a specific organ, usually as a result of insufficient perfusion. VEGF-A plays a central role in angiogenesis and neovascularization, increasing delivery of both oxygen and energy substrates. VEGF-A expression can be induced when cells are subjected to hypoxia or hypoglycemia. This response seems to depend on Hypoxia Regulated/Responsive Element/Enhancer sequences in the 5 and 3 regions of the VEGF-A gene\textsuperscript{179}. The hypoxia-inducible protein complex HIF-1 binds to the enhancer sequences of the VEGF-A gene, EPO gene. According to Jones et al., 2000, both transcription and RNA stability can be enhanced\textsuperscript{180}. In vitro it has been shown that Hypoxia induces an up-regulation of VEGFR-1 mRNA, and an HIF binding site has been described in the promotor region of VEGFR-1 while hypoxia-induced up-regulation of both receptors has been observed in several in vivo models. Both VEGFR-1 and VEGFR-2 are up-regulated in hypoxic conditions\textsuperscript{179,181}. 
2. Reactive oxygen species (ROS)

ROS (superoxide, hydrogen peroxide, and their metabolites) are cytotoxic and mutagenic. Induction of ROS-generating enzymes, such as Nox-1 has been implicated as one of the mechanisms in ROS generation. Nox-1 is shown to be angiogenic. Nox-1 signals angiogenic and tumorigenic effects partly through hydrogen peroxide, resulting in an increase of both the synthesis of VEGF mRNA and the bioactivity of matrix metalloproteinase-9 (MMP-9). It activates the extracellular signal-regulated kinase pathway and NF-kB-dependent transcription, both of which have been implicated in growth and angiogenesis.

3. Growth Factors and Cytokines

Several cytokines or growth factors up-regulate VEGF mRNA expression and/or induce release of VEGF protein. Exposure of quiescent human keratinocytes to serum, epidermal growth factor (EGF), TGF-b, or keratinocyte growth factor has been shown to result in a marked induction of VEGF mRNA expression. Several growth factors, such as tissue growth factor-beta, epidermal growth factor (EGF), and platelet derived growth factor BB (PDGF-BB) induce VEGF-A mRNA expression. It has been shown that VEGF-A mRNA is induced in vivo in wounds by PDGF in fibroblasts and by keratinocyte growth factor in epidermal keratinocytes. Tumor necrosis factor-alpha (TNF-α) is an inflammatory cytokine with a wide spectrum of biological activity, including angiogenesis. It influences the formation of new vessels indirectly, rather than by directly promoting the sprout of endothelial cells and their growth. TNF-α triggers the release of angiogenic molecules (e.g., bFGF, PAF, VEGF-A, and VEGFC), and up-regulation of proteolytic systems (e.g., uPA). Moreover, it has been proven that
TNF-α also increases the transcription of the VEGFR-2 gene in vascular endothelial cells.

Cyclooxygenase (COX)-1 and -2 enzymes convert arachidonic acid to prostaglandins and thromboxanes. The COX-2 enzyme can be induced by a variety of proinflammatory cytokines (e.g., IL-1), growth factors (e.g., EGF), TGF-β, inducible nitric oxide synthetase, and ultraviolet B radiation. It stimulates endothelial motility and tube formation by increasing the production of proangiogenic factors such as VEGF-A.

Role of the matrix metalloproteases (MMP) in the induction of the angiogenic process has been described. MMPs, which are produced by microvascular endothelial cells, break down the extracellular matrix. This is one of the earliest and sustained events in the process of new capillary formation. VEGF-A has been shown to modulate the production and activity of these proteins. Bergers et al. (2000) showed that MMP-9 is a functional component of the angiogenic switch during multistage pancreatic angiogenesis. Activation of MMP-9 induces an up-regulation of VEGF-A.

4. Hormonal Regulation

Estrogen stimulates VEGF-A gene transcription and stabilizes VEGF-A mRNA, prolonging the half-life of the transcripts. The effect of testosterone on VEGF-A expression has been studied in the androgen-dependent S115 mouse breast cancer cell line and human prostatic tissue. Transcriptional activation has been shown to cause an increase in VEGF-A expression, as well as stabilization of the mRNA.

Cell differentiation has also been shown to play an important role in the regulation of VEGF gene expression.
Studies

**MM Abdo** et al\(^{190}\) immunohistochemically studied expression of VEGF-C in oral premalignant lesions and oral squamous cell carcinoma. Authors found highly significant correlation between expression of VEGF-C and degree of dysplasia in OPLs and concluded that VEGF-C may play an important role in the process of carcinogenesis.

**M Asteakar** et al\(^{191}\) studied VEGF expression immunohistochemically in 60 archival specimens, including 10 normal oral mucosa (NOM), 7 mild epithelial dysplasia (Mild ED), 8 moderate epithelial dysplasia (Mod ED), 5 severe epithelial dysplasia (SED), 14 well-differentiated SCC, 11 moderately-differentiated SCC, and 5 poorly-differentiated SCC. Authors found that VEGF and MVD is increased with disease progression and concluded that VEGF expression is upregulated during head and neck tumorigenesis.

**Anji Anura** et al\(^{192}\) evaluated c-Myc, HIF-1α, VEGF, VEGFRII and CD105 in 58 biopsies of Oral submucous fibrosis using computer aided quantification. Authors found that expression of VEGF in epithelium increased from OSMF without dysplasia to OSMF with dysplasia. In normal mucosa transitive faint to intense expression from basal layer to granular layer was observed. In OSMF without dysplasia faint expression in basal to spinal layer and significant expression in the granular layer was observed. In OSMF with dysplasia with increased degree of dysplasia, the VEGF expression increased in epithelium, micro blood vessels near basement membrane exhibited positivity and expression was observed throughout the epithelial thickness. Authors suggested that VEGF diffusion towards basement membrane was indicative of subsequent augmentation of neoangiogenesis in sub-epithelium of OSF with dysplasia.
According to authors neo-angiogenesis is promoted via diffusion of VEGF from dysplastic epithelium towards sub-epithelium and to help in epithelial cancerization. From the results of the study, authors concluded that VEGF can be used for risk-stratification.

Desai R et al\textsuperscript{193} evaluated Immunohistochemical expression of VEGF in 30 paraffin-embedded tissue sections of diagnosed cases of OSF and 10 control samples of healthy volunteers. Out of 30 cases, 18 cases showed cytoplasmic expression of VEGF in the basal epithelial cells and fibroblasts, of which 17 were of mild and one of moderate intensity respectively. Remaining 12 cases of OSF and 10 controls showed no immunoreactivity for VEGF. VEGF immunoreactivity was found to be statistically increased in OSF compared to normal healthy controls suggesting that upregulation of VEGF may play an important role in tumor progression during malignant transformation of atrophic epithelium in OSF.

Anura A et al\textsuperscript{194} correlated expressional alteration in prime epithelial marker E-cadherin, with neo-angiogenic molecules, VEGF and CD105 for elucidation of malignant potentiality in different stages of oral submucous fibrosis. For the study, 10 normal oral mucosa, 18 non-dysplastic OSMF and 40 OSMF with different dysplastic grades were semi-quantitatively analyzed for immunohistochemical expressions of E-cadherin, VEGF and CD105. Authors found an increase in neo-angiogenic attributes of oral submucous fibrosis with increase in dysplastic grades.

Combined VEGF and MMP-9 studies

Mukherji S et al\textsuperscript{195} studied immunohistochemically antioxidant markers like NADP(H) quinone oxidoreductase, neoangiogenic markers like VEGF, MMP 2
& 9, and cellular proliferation markers like CCNslD1 and CyA in clinically diagnosed cases of 5 oral leukoplakia, 5 oral submucous fibrosis, 2 oral verrucous carcinoma and 10 oral squamous cell carcinoma. In OSMF moderately positive immunolocalisation of VEGF in the lower half of the atrophic stratified squamous epithelium with diffuse immunolocalised in the juxtaepithelial connective tissue was observed. A generalized strong positive extracellular MMP-9 expression in the epithelial layer was observed. Authors concluded that these markers can help in determining the status of oral carcinogenesis.

In OSF, the degree of vascularity/angiogenesis has always been a matter of debate. There is contradictory data on vascularity in OSMF. In order to have more clarity over expression of this angiogenic molecule in OSMF and its relation with the progression of this disorder, its expression needs to be evaluated.