DISCUSSION
6. Discussion

Oral submucous fibrosis (OSMF) is a chronic fibrotic condition of the oral cavity. It is characterized by a generalized submucosal fibrosis. A number of factors trigger the disease process by causing a juxtaepithelial inflammatory reaction in the oral mucosa. Alkaloids and flavonoids present in areca nut cause constant irritation to oral tissues. In addition to this chemical irritation, the coarse fibers of areca nut also cause mechanical irritation to the oral mucosa which results in microtrauma and more diffusion of the constituents of areca nut. This serves as a chronic source of irritation to the oral mucosa resulting in chronic wound. Fibrosis, the extreme accumulation of extracellular matrix proteins such as collagen, in OSMF could be the result of the wound-healing response of the oral mucosa to this recurring injury. Thus, OSMF can be considered as a chronic wound where there is impaired healing resulting in fibrosis and therefore to study the pathogenesis of OSMF we chose to study the factors related to wound healing.

The development of fibrosis in OSF reflects an alteration of the wound healing process resulting from normally balanced process of extracellular matrix (ECM) production and its degradation. In physiological wound healing process, Factor XIIIa (FXIIIa) is considered to be a central molecule responsible for formation of provisional matrix which serves as a nidus for collagen fibrillogenesis. It causes cross linking of collagen which gives extra rigidity to collagen and a resistance against proteolytic degradation\textsuperscript{109}. In OSMF an important mechanism playing role in fibrosis is the formation of more stable and non-soluble collagen structure. Therefore, whether FXIIIa plays role in fibrosis in OSMF required an evaluation. Degradation of ECM occurs as a part of physiologic process in tissue repair and remodeling.
However, deregulated ECM turnover leads to fibrosis. Matrix metalloproteinases (MMPs) are the main enzymes implicated in ECM degradation. Matrix metalloproteinase-9 (MMP-9) is a gelatinase that can cleave a variety of ECM and non-ECM proteins. Pro- and active forms of MMP-9 have masses of 92 kDa and 83 kDa, respectively. In adults, MMP-9 is normally expressed by neutrophils and eosinophils. In inflammation, its expression is increased as it is expressed by endothelial cells, macrophages, fibroblasts, and other connective tissue cells. In OSMF there is increased deposition of extracellular matrix. As regulators of ECM degradation, gelatinases are able to modulate this process, and tissue remodeling has been associated with abnormal activation or inhibition of gelatinases in fibrosis. However, the role of MMP-9 in OSMF is not clear as very few studies have reported its expression in OSMF. OSMF is a long-term dynamic and progressive disease, and therefore the investigation of changing levels of the MMP-9 along different grades could have significance in understanding the real pathophysiology of this condition. Another reason for studying MMP-9 expression in OSMF is because MMP-9 promotes angiogenesis by causing liberation of VEGF. So to study the interrelation between MMP-9 and VEGF in OSMF, MMP-9 expression was studied.

OSMF can be considered a chronic wound where apart from inflammation and deposition of ECM, neovascularization plays a role in its pathogenesis. However, there is controversial data on vascularity in OSMF. There is little clarity whether vascularity is increased or decreased or occurs prior to or sets in with fibrosis. Vascular endothelial growth factor (VEGF), a potent angiogenic inducer, also plays an important role in regulating monocyte recruitment and infiltration. Apart from the usual role of angiogenesis, fibrogenic aspect of VEGF is unexplored in OSMF.
Therefore, to know the relation between angiogenesis and fibrogenesis in pathogenesis of OSMF, VEGF was studied.

In the present study, expression of FXIIIa, MMP-9 and VEGF was studied immunohistochemically in OSMF (Study group, **Group II**, n-60) and was compared with that of normal oral mucosal tissues (Control group, **Group I**, n-20). Group II was divided into 3 subgroups, **Group A**- Early, **Group B**- Moderately Advanced and **Group C**- Advanced. Expression of these markers was studied and compared with these histopathological grades of OSMF.

**Part I Discussion: Demographic details**

Fibrosis in OSMF leads to restricted mouth opening resulting in restriction of food consumption, difficulty in maintaining oral health as well as an impairment of the ability to speak. There is strong epidemiological evidence to associate this disorder with the habit of chewing areca nut. The present retrospective study comprised of 60 clinically and histopathologically diagnosed cases of oral submucous fibrosis (OSMF) (Study group, **Group I**) and 20 healthy individuals without any habit (Control group, **Group II**).

In our study group we found that 96.67% of the OMSF cases were having areca nut habit with tobacco in the form of gutkha and mawa while 3.33% cases were pan masala chewers. 80% OSMF cases were males in the age range of 21-40 while 20% were females suffering from OSMF were in the age range of 21-40.
Part II Discussion: FXIIIa in OSMF

As found through standard electronic databases, ours is the first study to report expression of FXIIIa in OSMF. Positive and more expression of FXIIIa was found within connective tissue interstitial cells of all cases of OSMF when compared with normal oral mucosa tissues. The difference was highly significant ($p$ value $0.000$). In normal oral mucosal tissues some expression of FXIIIa was noted and the cells expressing FXIIIa were spindle shaped. In OSMF, FXIIIa positive cells were more, they were numerous juxtaepithelially and in close proximity to the blood vessels (Fig. 3a). Expression of FXIIIa was also studied in early, moderate and advanced grades of OSMF.

FXIIIa is a transglutaminase which plays an important role in stabilization of the provisional matrix by causing cross-linking between proteins. It also mediates cell-matrix interactions during wound repair$^{201}$. It acts on fibronectin-fibronectin and fibrin-fibronectin cross-linking. This makes collagen strong and resistant against proteolytic degradation by MMPs. FXIIIa is known to cause stimulatory effect on fibroblasts and formation of stable bonds between various chains of collagen molecules, generating a different polymerization process$^8$. It was also shown to be a modulator of the amount and type of collagen synthesized by fibroblasts$^{202}$. Ueyama M et al has reported that FXIIIa favors fibroblast viability, and directly influences fibroblast functions$^{199}$. It stimulates attachment, morphological changes, and proliferation of fibroblasts. The enhancement of fibroblast proliferation by cross-linking fibrin matrix as well as by direct proliferation-inducing effect of FXIII has been reported$^{203}$. It also causes inhibition of apoptosis of ECM components and inhibition of proteolytic actions of MMPs by ECM stabilization at the wound site$^{204,205}$. FXIIIa expressing cells are found to be increased in various human tissues associated with fibrosis, such as in
scleroderma\textsuperscript{206}, liver cirrhosis\textsuperscript{207}, granulation of gastric ulcers\textsuperscript{208,209}, systemic nodular panniculitis\textsuperscript{210}, and fibrous stroma of salivary gland tumors\textsuperscript{211}. Parsons AC et al reported that dermal fibroblasts and histiocytes in nephrogenic systemic fibrosis (NSF) express factor XIIIa, indicating increased expression and/or activation of transglutaminases which contribute to fibrosis in NSF\textsuperscript{100}. M Toida et al in their study on pulmonary fibrotic tissues demonstrated that cells containing FXIIIaplay an important role in the development of pulmonary fibrosis\textsuperscript{89}. Authors have also reported distribution of FXIIIa-containing cells in association with collagenous component of oral and maxillofacial fibrotic lesions such as gingival hyperplasia, peripheral fibroma and chronic sclerosing submandibular adenitis. Based on the findings, they suggested role of FXIIIa in laying down of collagen in these fibrotic lesions\textsuperscript{10}. Mendoza FA et al studied cases of nephrogenic fibrosing dermopathy and postulated the hypothesis that CD68+/Factor XIIIa+ dendritic cells and transforming growth factor- beta (TGF-\(\beta\)) are closely related. The findings of their study suggested that TGF-\(\beta\) is released by FXIIIa dermal dendrocytes (DD) which can set in a vicious cycle as TGF beta regulates dendritic cell maturation leading to further activation of DD and more production of FXIIIa and TGF beta and more fibrosis\textsuperscript{212}. Our study shows increased expression of FXIIIa positive cells in connective tissue in OSMF tissues. A close relation between distribution of FXIIIa positive cells and that of collagenous components was also noted. These findings may suggest the role of FXIIIa in fibrosis in OSMF.

Earlier it was not clear whether FXIIIa expressing cells are fibroblasts or macrophages. It was believed that FXIIIa+ cells in human connective tissues are fibroblasts\textsuperscript{209,213}. However, later studies demonstrated that FXIIIa is expressed by human monocytes and macrophages\textsuperscript{214}. Studies have also shown that FXIIIa expression is retained during differentiation of monocytes to macrophages\textsuperscript{20,21}. Therefore, FXIIIa
has been recognized by as a useful marker for monocytes/macrophages\textsuperscript{215,216}. According to Toida M et al, FXIIIa + cells in normal, fibromatous and carcinomatous buccal tissues represent a subset of macrophages derived from monocytes\textsuperscript{102}. Töröcsika D et al considered FXIII-a expression as a marker of alternatively activated mobile or fixed macrophages\textsuperscript{217}. In our study we observed that FXIIIa expressing cells were more in number juxtaepithelially where there is inflammatory infiltrate in OSMF. We also noted their proximity to the blood vessels. Morphologically these cells were dendritic. Thus, the localization and morphology of these cells suggest that FXIIIa expressing cells are macrophages in OSMF. Chiang CP et al and T Pereira et al have reported presence of macrophages in chronic inflammatory infiltrate in connective tissue of OSMF\textsuperscript{218,219}. Macrophages are linked to fibrosis. Based on this we can speculate that macrophages may promote fibrosis in OSMF through the production of FXIIIa.

Another important finding in our study was that the expression of FXIIIa decreased with the increasing grades of OSMF. High statistically significant difference was found between FXIIIa expression in various grades of OSMF (\textit{p value 0.000}). We also observed change in morphology of these cells with increasing grades of fibrosis. In early grades of OSMF, FXIIIa expressing cells were bigger and dendritic while those in advanced grades they were slender and spindle shaped. Toida et al in their study on radicular cyst and some fibrotic oral lesions found that in densely collagenous areas the number of FXIIIa positive cells was less\textsuperscript{11}. In systemic sclerosis, preceding the appearance of intense fibrosis, a reduction in FXIII-a-positive cell numbers was found. Based on this, authors suggested that the presence of FXIII-a-positive cells varies according to different stages of disease pathogenesis and play role in initiation of fibrosis rather than its progression\textsuperscript{220}. Pierard et al based on their study on fibrotic and sclerotic diseases such as scars, keloids, fibromas, scleroderma and lichen
sclerosus et atrophicus concluded that there is an inverse relationship between the density of Factor XIIIa+ dendrocytes and fibrosis as these cells were almost absent in sclerotic diseases. Factor XIIIa+ dendrocytes were found to be numerous in early scaring. On the contrary, scars and keloids, where there is intense fibrosis, have very few Factor XIIIa+ dendrocytes. Our study results suggest that FXIIIa expressing cells increase in number and are bigger and dendritic in early grade that is before fibrosis develops and the expression goes down and they become quiescent in advanced grade which may suggest that the role of FXIIIa diminishes in advanced fibrosis. Thus, we can interpret that FXIIIa may play role in initiating fibrosis in OSMF.

In OSMF there is chronic trauma to oral mucosa due to areca nut habit which elicits a protective inflammatory process. Aberrant and persistent tissue inflammation is crucial for tissue fibrosis. In our study we noticed dendritic morphology of FXIIIa positive cells, they were numerous in juxtaepithelial inflammatory infiltrate and were close to the blood vessels in OSMF tissues which may suggest that FXIIIa positive cells are a subset of macrophages. From this we can also relate role of inflammation in fibrosis.

**Part II Discussion: MMP-9 in OSMF**

Main features of OSMF include chronic irritation to epithelium, resulting inflammation, fibroblastic proliferation, and increased collagen production which eventually leads to fibrosis. Progressive fibrosis in OSMF results from deregulated extracellular matrix turnover, where matrix metalloproteinase (MMPs) are believed to play a crucial role. However, the extent to which MMPs contribute to initiation and progression of tissue fibrosis is not clearly understood. In the present study we found
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statistically significant increase in expression of MMP-9 in OSMF tissues when compared with normal oral mucosal tissues \( p \text{ value (epithelium)} = 0.009, p \text{ value (connective tissue)} = 0.000 \). Ours is the pioneer study where MMP-9 expression was studied in different grades of OSMF. We found increased expression of MMP-9 with the increasing grades of OSMF though the difference was not statistically significant. MMP-9 is found to be expressed by epithelial cells, inflammatory cells, fibroblasts and endothelial cells. Expression was very minimal in epithelium and connective tissue of normal oral mucosa. In early grade MMP-9 expression in epithelium was minimal, patchy and was present only in basal cell layers. While in advanced grades it was noticed in basal, parabasal and spinous cell layers. Increased connective tissue expression of MMP-9 was found with the increasing grades of OSMF (Early- \( 1.67 \pm 0.778 \), Moderate- \( 1.69 \pm 1.251 \), Advanced- \( 1.92 \pm 0.669 \)). With the advancing grades we could notice more number of MMP-9 positive cells in the connective tissue amongst total number of cells.

Increased MMP-9 expression in OSMF in our study is in accordance with the study by Rajendran et al. However in contrary to the studies by Mishra G et al who have reported decreased expression of MMP-1 in OSMF and Chang YC et al, who found minimal amounts MMP-2 and MMP-9 secreted by buccal mucosal fibroblasts in diseased tissues. Mishra G et al have studied expression of MMP-1 which has its activity on different substrate of ECM when compared with MMP-9 which is a gelatinase. In the study by Chang YC et al, authors have studied the effect of arecoline on human buccal mucosal fibroblasts in vitro where the conditions may not mimic exactly as in studies on humans subjects. In vivo studies have reported increased levels of MMP-9 in areca quid chewers and in oral cancer patients.
who are betel quid users\textsuperscript{225,226}. This may justify increased MMP-9 expression in OSMF in our study.

In OSMF the activities of proteinases (MMPs), that can degrade matrix, might be expected to be under-expressed. However, MMPs have more diverse role to play in pathogenesis of tissue fibrosis. It is found that though some MMPs are anti-fibrotic, others can be profibrotic. Duarte S et al found MMP-9 expression in early stages of hepatic fibrogenesis and suggested that MMP-9 may release/activate TGF-β from ECM reservoirs and thus can contribute to fibrosis\textsuperscript{227}. MMP-9 is found to be increased in idiopathic pulmonary fibrosis lungs where it was found to be expressed by alveolar epithelial cells, macrophages, neutrophils and fibroblasts in fibroblastic foci\textsuperscript{228}. The spatial expression analysis of MMP-9 by Tsai J-P et al in nephrectomized specimens of patients suffering from renal fibrosis indicated an unusual role for MMP-9 in development of fibrosis\textsuperscript{229}. They postulated that increased intranuclear MMP-9 expression may reflect intranuclear gelatinase proteolysis, which plays role in oxidative DNA damage by cleaving nuclear matrix proteins (PARP-1 and/or XRCC1), and contribute to cell death and fibrosis. Thian Kui et al demonstrated an important role of MMP-9 in renal fibrosis. Their results suggested that MMP-9 of both tubular epithelial cells and macrophage origin may contribute to the pathogenesis of renal fibrosis by causing tubular cell epithelial -mesenchymal transition and osteopontin cleavage, which in turn further recruit macrophage and lead to fibrosis\textsuperscript{230}. Elevated level of MMP-9 has been associated with liver fibrosis in chronic hepatitis C\textsuperscript{231}. Ducharme A, et al postulated a profibrotic role of MMP-9 by showing that MMP-9-deficient mice had reduced fibrosis as well as decreased neutrophil and macrophage infiltration in resolving cardiac infarcts\textsuperscript{232}. Yu Q et al reported that MMP-9 is able to activate TGF-β1\textsuperscript{233} and thus participates in the initiation of hepatic fibrosis.
as TGF-β leads to differentiation of hepatic stellate cells into myofibroblast. Ye Zhao et al and Tan TK et al suggested a strong profibrotic role of MMP9 in kidney fibrosis through its role in EMT\textsuperscript{234, 235}. Zhao H et al reported important contribution of MMP-9 in kidney fibrosis by casing EMT and secretion by activated macrophages which ultimately leads to fibrosis in chronic kidney disease\textsuperscript{235}. Wang et al suggested that MMP-9 plays role in myofibroblast activation or survival\textsuperscript{236}. Apart from the main role in ECM remodeling and homeostasis, MMPs also function in immunoregulation. MMP-9 is expressed by inflammatory cells such as neutrophils, eosinophils, macrophages and T cells\textsuperscript{13}. MMP-9 may play role in fibrosis by recruiting these inflammatory and other connective tissue cells which suggests the link between inflammation and fibrosis. Thus, MMP-9 can have pro-fibrotic role which could be indirect, by recruiting inflammatory cells that more directly orchestrate the fibrotic response or could be direct through release of TGF beta, its involvement in EMT and activation of myofibroblasts. Increased expression of MMP-9 in OSMF in our study also supports profibrotic role of MMP-9 in pathogenesis of OSMF.

**Part III Discussion: VEGF in OSMF**

OSMF can be considered a chronic wound where apart from inflammation, deposition of ECM, and its defective degradation, neovascularization plays an important role in its pathogenesis. Vascularity in OSMF has always been a matter of dispute. There is controversial data on vascularity in OSMF. VEGF is one of the most effective angiogenic inducer. It is a key regulator of angiogenesis and has been known to play role in pathogenesis of fibrotic disorders such as lung fibrosis, liver and kidney\textsuperscript{237, 238}. However, its relation to fibrosis in OSMF has not been studied yet. In literature, studies done on VEGF in OSMF are in relation to predict the malignant potential of OSMF where the difference in its expression was studied between
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dysplastic and non-dysplastic OSMF\textsuperscript{194,239}. However, here we studied VEGF expression in epithelium and connective tissue with increasing grades of OSMF to know the relation between this angiogenic cytokine and fibrosis.

In our study we got increased expression of VEGF in epithelium and connective tissue of OSMF tissues as compared to normal oral mucosal tissues and the difference was statistically significant \((p \text{ value (epithelium and connective tissue)} 0.000)\). Expression of VEGF was observed in the endothelial cells, inflammatory cells and epithelium. In normal oral mucosal tissues VEGF expression was seen in basalmost cell layer. While in OSMF immunolocalization of VEGF was seen in basal and parabasal cell layers. There was an increase in VEGF expression with increasing grades of OSMF though the difference was not statistically significant.

In the present study there was an increase in VEGF expression in OSMF as compared to normal oral mucosa and the difference was statistically significant \((p \text{ value (epithelium)} 0.000, p \text{ value-(connective tissue)}-0.000)\). However, Madhavannirmal R found no significant difference in expression of VEGF between normal oral mucosa and OSMF, and also among different grades of OSMF\textsuperscript{239}. AnjiAnura et al. in their study on OSMF reported a drop in VEGF expression as compared to normal oral mucosa. According to them reduction in vascularity due to progressive fibrosis and atrophy of epithelium in OSMF reasons the reduced VEGF expression in OSMF\textsuperscript{194}. Garg N and Mehrotra R reported reduced mean vascular area (MVA), mean vascular luminal diameter (MVLD) and vessel perimeter in OSMF when compared with normal oral mucosa\textsuperscript{240}. Rajendran R et al in their study reported same mean vascular density (MVD) in control and study group\textsuperscript{16}. However, Fang CY et al\textsuperscript{241}, Desai SR et al\textsuperscript{16}, Sabarinath B et al\textsuperscript{242}, Murgod VN et al\textsuperscript{243}, Tekade et al\textsuperscript{244} reported increased MVD in OSMF as compared to normal. Present study supports the findings
of these studies. Expression of angiogenic factors such as VEGF increase progressively from normal mucosa to OSMF.

We found increased VEGF expression in early grade of OSMF (epithelium-1.54±1.07, connective tissue-1.33±0.888). In OSMF, there is chronic tissue injury to oral mucosa due to areca nut which sets in chronic inflammation. Early grade of OSMF is characterized by juxtaepithelial inflammatory infiltration. Marked angiogenesis and inflammatory response is observed during the development of fibrosis in OSMF. New vessel formation or neoangiogenesis is a central mechanism associated with inflammation where VEGF plays an important role. Thus increase in VEGF (an angiogenic factor) in early grade of OSMF could be inflammation induced vascular response. Many inflammatory mediators such as interleukins 1 and 6 and prostaglandin E2 have been shown to increase VEGF mRNA levels. Inflammatory cells are involved in the early induction of angiogenesis by producing VEGF. Hence, inflammation could be the reason for increased VEGF expression in early grade of OSMF. Moreover, expression of VEGF can perpetuate the inflammatory response as newly formed vessels can transport inflammatory cells and can maintain supply of nutrients and oxygen to the injured regenerating tissue site. Thus VEGF may also contribute to the chronicity of inflammatory response in OSMF. Chronic inflammation is known to drive fibrosis. VEGF also regulates monocyte recruitment and their infiltration in tissue. Macrophages are known to play role in fibrogenesis. Thus increase in VEGF expression in epithelium and connective tissue in early grade could be related to inflammation which sets in a vicious cycle by perpetuating inflammation and driving fibrosis.

We found an increase in VEGF expression from early (epithelium-1.54±1.07, connective tissue-1.33±0.888) to moderate grade (epithelium-1.62±1.19, connective
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tissue-2.00±1.155) of OSMF. However, this is in contradiction to other studies where authors have reported decreased vascularity with the increasing stage or grade of OSMF\textsuperscript{240,241}. In OSMF accumulation of inflammatory cells along with fibrosis leads to tissue hypoxia. In tissue hypoxia, hypoxia inducible factor-1 is known to induce various angiogenic molecules including VEGF\textsuperscript{178}. Thus, progressive fibrosis and resulting tissue hypoxia in moderate grade could induce VEGF and this could be the reason for increased VEGF expression in moderate grade of OSMF.

In our study we got consistent expression of VEGF in advance grade of OSMF(epithelium- 1.67±0.89, connective tissue- 2.08±0.793) which is in accordance with the study by Rajendran R et al\textsuperscript{15} and Sabarinath B et al\textsuperscript{242}. However, result of our study are in contradiction to the studis by Fang CY et al(2002)\textsuperscript{241}, Singh M et al\textsuperscript{250}, Desai SR et al\textsuperscript{16}, DebnathS et al\textsuperscript{251}, Murgod VN et al\textsuperscript{243}, Pandiar D and Shameena PM\textsuperscript{252}, Garg N and Mehrotra R\textsuperscript{240} and Tekade et al\textsuperscript{63} who have reported decreased MVD in advanced stage or grade of OSMF. However, all these authors have specifically studied vascularity while we studied VEGF expression in relation to increasing grades of fibrosis in OSMF. In advanced grade of OSMF we got persistent VEGF expression in epithelium and connective tissue in spite of reduced vascularity. This could be interpreted here as VEGF expression in advance grade doesn’t result in angiogenesis/results in impaired angiogenesis that is VEGF may not be able to serve its primary role of angiogenesis during progressive fibrosis. Distinct from the traditional role of VEGF as an angiogenic factor, evidence also exists linking VEGF signaling directly to fibrogenesis. Studies have revealed direct fibrogenic effect of VEGF through release of fibrosis-enhancing molecules. Fehrenbach et al in a study on a model of pulmonary fibrosis reported an increase in VEGF positive cells in the absence of increased vascularization in areas of fibrosis. Based on the findings,
authors suggested role of VEGF in development of fibrosis\textsuperscript{237}. VEGF overexpression has been shown to accelerate liver fibrosis by increasing hepatic collagen deposition\textsuperscript{233}. It is proposed that VEGF upregulates the pro-fibrotic factor CTGF\textsuperscript{254}. Role of CTGF is well established in fibrosis in OSMF. More convincing evidence about the fibrogenic role of VEGF comes from the evidence of the therapies based on inhibition of VEGF to treat fibrosis\textsuperscript{255,256}. Thus in OSMF VEGF could contribute to fibrogenesis by causing release of profibrotic or fibrosis enhancing molecules. This phenomenon can be called as an angio-fibrotic switch that is when VEGF leaves its primary function of angiogenesis and contributes to active fibrogenesis. Result of our study supports this concept as we didn’t get decrease in VEGF expression in moderate and advanced grades of OSMF while vascularity decreases and degree of fibrosis increases with the increasing grades of OSMF. Hence in advancing grades of OSMF, there could be shift in function of VEGF from angiogenesis to fibrogenesis. In our study VEGF levels were significantly higher in early grade of OSMF when there is active neovascularization and less fibrosis. While its persistent expression with advancing grades could be related to its shift of function from angiogenesis to fibrogenesis (angio-fibrotic switch) as there is reduced vascularity reported in the advanced grade of OSMF. This concept needs further validation to make anti-VEGF treatment modality a possibility for treatment of OSMF.

Expression of VEGF in advancing grades of OSMF could also be a compensatory reflex mechanism in response to the persistent tissue hypoxia in severely fibrotic areas so as to support the viability of the tissue. Another reason for VEGF not been able to cause angiogenesis in advanced grade of OSMF could be increased matrix density which can act as a physical barrier resulting in restricted cell migration. Moreover, matrix stiffness can modify the response of endothelial cells to angiogenic inducers.
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including VEGF. As more and more collagen is deposited in submucosa, fibrosis can impede material exchange, signal communication and cell migration causing impaired angiogenesis\(^{257}\). Angiogenesis is regulated by a balance between angiogenic and angiostatic factors. Impaired angiogenesis in OSMF could be a consequence of fibrosis due to angiostatic signals derived from the continuous deposition of ECM. This can result in shift of balance towards angiostatic factors over angiogenic factors (such as VEGF) resulting in impaired angiogenesis. This also could be the reason for decreased vascularity in advancing grades of OSMF in spite of VEGF expression as we got in our study. However, to have a better clarity over vascularity in OSMF, angiostatic factors should be studied along with angiogenic factors in OSMF. Thus increase in VEGF expression in OSMF can be related to inflammation induced angiogenic response in early grade, a compensatory mechanism to counteract tissue hypoxia to support tissue viability and its contribution to fibrogenesis with the advancing grades of fibrosis. Here in our study we highlight fibrogenic role of VEGF in OSMF which needs to be substantiated with further research.

**Interrelationship between FXIIIa, MMP-9 and VEGF in pathogenesis of OSMF**

When studied the correlations of FXIIIa, MMP-9 and VEGF in OSMF by Pearson’s correlation analysis, FXIIIa was found to be positively correlated with VEGF in moderate grade of OSMF (0.554) with significance value of 0.049. Positive relation was found in early grade (0.518) as well though it was not statistically significant (0.084). However, this relation between FXIIIa and VEGF was not found in advanced grade of OSMF. Positive relation between FXIIIa and VEGF could be attributed to pro-angiogenic property of FXIIIa. FXIII is found to act on endothelial cell VEGFR-2 and integrin αvβ3 which downregulates antiangiogenic
protein thrombospondin-1. αvβ3 - VEGFR-2 interaction also lead to upregulation of transcription factors such as early growth response-1 and c-Jun, which may enhance endothelial cell proliferation and survival. This results in promotion of new blood vessel formation. This interrelation could result in active angiogenesis which is seen in early grades of OSMF. However, we couldn’t get this relation in advanced grade of OSMF. Reduced FXIIIa expression as found in our study and absence of active angiogenesis seen in advanced grade supports this.

Positive relation was found between VEGF epithelium and VEGF connective tissue (0.591), with the significance value (0.043) as well as between MMP-9 epithelium and MMP-9 connective tissue (0.565) with significance value-0.044 which suggest that when VEGF and MMP-9 expression increases they are co-expressed by epithelium as well as by connective tissue.

Oral submucous fibrosis is characterized by fibrosis of oral, pharyngeal and part of esophageal mucosa resulting in stiffness. In chronic fibrosis, tissue stiffness itself creates an environment which enhances the chronicity of fibrotic disease and can result in malignant transformation. Therefore it is important to elucidate the pathologic pathways which lead to fibrosis and increase the chronicity of this disorder. Present study reports expression of FXIIIa in OSMF. From the results of our study we can interpret that FXIIIa may have role in initiation of fibrosis in OSMF. FXIIIa expressing cells could be macrophages and as FXIIIa can have role in collagen deposition, we can relate chronic inflammation with fibrosis. We also got positive relation between FXIIIa and VEGF in our study which emphasizes the role of FXIIIa as proangiogenic factor. Thus role of FXIIIa in pathogenesis of fibrosis in OSMF could be through its transglutaminase and proangiogenic
Transglutaminases are targeted in anti-fibrotic treatment modalities. FXIII has been considered for anti-fibrotic treatment in systemic sclerosis. Inhibitors of FXIII can be tried for treatment of fibrosis in OSMF after substantiating its role further. MMPs are earlier linked with decreased degradation of matrix in OSMF. However, in our study we got increased expression of MMP-9 which could suggest pro-fibrotic role of MMP-9. To have a better clarity on role of MMP-9 in fibrosis, its fibrotic mechanisms in OSMF should be explored in detail. We got increased VEGF expression in OSMF. We noted presence of VEGF expression even in advanced grade of OSMF which can be understood as though this angiogenic factor is expressed in advanced grade of fibrosis it may not result in active angiogenesis. From the results of our study we can also interpret that VEGF may have role in fibrogenesis in OSMF apart from angiogenesis. VEGF may lead to angio-fibrotic switch which can contribute to fibrosis in progression of OSMF. This concept needs further research so as to develop anti-VEGF based antifibrotic treatment modalities in OSMF. Thus from the results of our study we can conjecture that FXIIIa may have role in initiation of fibrosis in OSMF while MMP-9 and VEGF may lead to progression of fibrosis in pathogenesis of this fibrotic disorder. Though significant efforts have been made in explicating the pathologic pathways in OSMF, currently the treatment modalities for OSMF are focused on cessation of habit initially and surgery in advanced cases. However, most of the times patients are resilient to leave the habit and in advanced cases resection of fibrotic tissues in fact can create deep wounds which in turn can promote fibrosis. Thus these conventional treatment modalities provide limited success in the restricting the progression of fibrosis in OSMF. The progression of fibrosis in OSMF is a critical factor as it can create more complications and also predisposes the patient to the development of oral
squamous cell carcinoma. Limited success in treating OSMF patient could be because the basic pathogenesis of fibrosis has been given little emphasis while exploring the definite protocols for treatment in OSMF. Present study attempts to provide a new insight in the pathogenesis of OSMF by exploring molecular factors such as FXIIIa, MMP-9 and VEGF which could pave way to make antifibrotic therapy as a possible treatment option for OSMF. This could be further validated by extending the research. Targeted therapies that would attenuate the deposition of fibrous tissue or reverse fibrosis would start a new era in treatment of OSMF and this research is one such humble effort in this direction.