EVALUATION OF COLLAGEN FIBERS IN ORAL LEUKOPLAKIA, ORAL SUBMUCOUS FIBROSIS AND ORAL SQUAMOUS CELL CARCINOMA USING PICROSIRIUS RED BY POLARIZING MICROSCOPY

ABSTRACT

Background: In India oral squamous cell carcinoma is a common malignancy accounting for 50-70% of total cancer mortality. Oral squamous cell carcinoma arise either de novo or from oral potentially malignant disorders mainly leukoplakia. The aim of the study is to evaluate collagen fibers in oral leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma using picrosirius red by polarizing microscopy.

Materials and method: The study included 15 cases of well differentiated squamous cell carcinoma, 15 cases of oral submucous fibrosis without malignant transformation, and 15 cases of Leukoplakia (clinically) with mild to severe dysplasia. The birefringence color of collagen is evaluated using picrosirius red & polarizing microscopy.

Results: In case of well differentiated squamous cell carcinoma, greenish yellow color is observed in 10 cases (66.7%), greenish orange color is observed in 3 cases (20%), and no significant color in 2 cases (13.3%). In case of oral submucous fibrosis, greenish yellow color is observed in 5 cases (33.3%), green/green-orange color is observed in 4 cases (26.6%), and greenish orange color is observed in 6 cases (40%). In case of Leukoplakia, greenish yellow is observed in 11 cases (73.3%), reddish orange is observed in one case (6.6%), greenish orange in one case (6.6%), and no significant color is observed in 2 cases (13.3%).

Conclusion: In India tobacco chewing habit is more prevalent causing oral squamous cell carcinoma and potentially malignant disorders. Most of the oral squamous cell carcinoma will clinically appear as leukoplakia or erythroplakia. None of the treatment is effective in preventing the malignant transformation from oral potentially malignant disorders.

Epithelial dysplasia is one the main indicators for malignant transformation. Before dysplastic changes there are few evidence of change in the extracellular matrix mainly collagen. Even though many traditional stains are available, picrosirius red is the best one for collagen.

Picrosirius red is the special stain for collagen as it stains even the thin fibers. Under polarizing microscopy green or greenish yellow color indicates loosely packed fibers, whereas reddish orange, greenish orange, and yellowish orange indicates tightly packed collagen fibers. In case of well differentiated squamous cell carcinoma and leukoplakia greenish yellow color is predominant [loosely packed fibers]. In case of oral submucous fibrosis greenish orange was predominant [tightly packed fibers]. Further studies on large sample size with follow up of the patient are necessary to find the biological behavior of the lesion.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) constitutes 90% of all oral cancers. Oral squamous cell carcinoma arise either de novo or from oral potentially malignant disorders like leukoplakia and oral submucous fibrosis. Traditional risk factors for oral squamous cell carcinoma and oral potentially malignant disorders are consumption of alcohol, tobacco, and betel quid. Those with the above said habits have 38 times higher risk of developing cancer.[1]

Oral squamous cell carcinoma is a malignant neoplasm of stratified squamous epithelium that is capable of locally destructive growth and distant metastasis. The most common early presentation of intraoral squamous cell carcinoma is leukoplakia and erythroplakia.[20] Oral leukoplakia is the second most common oral potentially malignant disorder after oral submucous fibrosis. Leukoplakia is a clinical term that simply means "white patch". The World Health Organization (WHO) defines leukoplakia as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer". Leukoplakia is six times more prevalent in smokers.[1]The rate of malignant transformation is unlikely to exceed 1% of cases.[4]

Oral submucous fibrosis is a chronic debilitating oral mucosal disease caused by habitual chewing of areca nut/betel quid. The alkaloids in the arecanut namely arecoline, arecadine and tannins are the key factors which initiate the disease process. Most of the clinical features in oral submucous fibrosis are due to fibrosis and hyalinization of the sub-epithelial tissues. The possible mechanism in the development of disease process is increase in collagen synthesis or reduction in collagen degradation.[3]The study of collagen

has highlighted the knowledge of collagen biosynthesis and degradation in oral submucous fibrosis.[1]The rate of malignant transformation is 7.6%.

The spread of oral squamous cell carcinoma is through direct invasion and lymph route. During the course of invasion, tumor cells penetrate the connective tissue by break through the basement membrane and interact with extracellular matrix.[2]

In spite of advanced treatment strategies the five year survival rate for oral cancer is 50-55%.[5]Early detection of Oral potentially malignant disorders will have improved prognosis. The epithelial component of oral squamous cell carcinoma and oral potentially malignant lesions like dysplastic features are extensively explored, but the stroma is still less explored area. The stromal component like the collagen, elastic and reticulin fibers still await recognition as an important factor in the progression of the disease.[6]

In every organs of the body, collagen is the fundamental part of connective tissue. It constitutes 30% of body protein. The connective tissue undergoes significant alterations as lesions progressed from pre-malignancy to malignancy. During the transformation of tissue from pre neoplastic into carcinomas there will be an increase in collagenolytic enzyme activity.[7]

In case of oral cancer, collagen is involved in tumor progression in two different ways. In the one way collagen shows desmoplastic response to a tumor, and in the other way collagen degradation and decreased synthesis.[7]The correlation between the changes in polarization color of collagen with the epithelial changes implicate that the connective tissue change may be indicative of neoplastic transformation.[8]

In case of oral submucous fibrosis, the increase in collagen bundle thickness results in diminished vascularity leading to atrophy of the overlying epithelium. The atrophic epithelium is more susceptible to carcinogenic agents.[7]The change in polarization color of collagen was seen in the connective tissue of oral submucous fibrosis with different degrees of epithelial dysplasia.[8]

Various types of special stains have been used to detect collagen fibers. Collagen has natural birefringence which is attributed to the arrangement of its fibers. The arrangement of the collagen fibers is enhanced by special stains like van Gieson, Masson's trichrome and picrosirius red. van Gieson and Masson's trichrome will lead to under estimation of collagen content as it fails to reveal the thin collagen fibers. Thereby, van Gieson and Masson's trichrome are not ideal for detection of collagen fibers.[9]

Puchtler and colleagues demonstrated a better method in which Sirius red dissolved in saturated picric acid solution consistently stained thin collagen fibers. Picrosirius red stains did not fade and was appropriate for use with polarized light microscopy.[9]

Picrosirius red is an elongated birefringent molecule. Picrosirius red is an acidic dye that binds to a variety of molecules, not only collagens. When binds to collagen, it reacts with basic amino group in the collagen. The enhanced birefringence of collagen is due to attachment of the dye molecules parallel to its long axis. Thus, the complex of Sirius red and collagen is more birefringent than Sirius red with other proteins.[10]

Polarizing microscopy can mean any of a number of optical microscopy techniques involving polarized light. The techniques include illumination of the sample with polarized light. Directly transmitted light can, optionally, be blocked with a polarizer orientated at 90 degrees to the illumination. These illumination techniques are most commonly used on birefringent samples where the polarized light interacts strongly with the sample and so generating contrast with the background.[10]

Using polarizing microscopy to identify collagen in picrosirius red stained material is not only responsible for specificity, but also provides sensitivity and resolution.[11]According to Junqueira et al., different color of birefringence was produced by type I and type III collagen fibers.[11]Not only the fiber thickness determine the polarization color, and also the packing of collagen molecules determine the polarization colors with picrosirius red stained collagens.[12]Demonstration of collagen fibers using picrosirius red in conjunction with polarizing microscope can serve as a procedure to differentiate procollagens, intermediate and pathological collagen fibers, which are not tightly packed when compared to normal collagen fibers.[9]

Under polarized light, collagen fibers appear bright and in sharp contrast with the rest of the tissue that remains dark/black.[10, 13]. In a more mature stage, the birefringence color of collagen appears yellow, orange or red. In case of poorly formed collagen fibers, the birefringence color produced was greenish.[3]Thus, picrosirius red-polarizing microscopy is more economical, rapid and easy procedure to evaluate the connective tissue changes.

Thus, the aim of the study is to evaluate collagen fibers in oral leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma using picrosirius red and polarizing microscopy.

AIM AND OBJECTIVE

Aim:

The aim is to evaluate collagen fibers in oral leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma using picrosirius red by polarizing microscopy.

Objective:

The objective is to determine the predictive value of collagen in oral leukoplakia, oral submucous fibrosis, and oral squamous cell carcinoma.

REVIEW OF LITERATURE

Picrosirius red with polarizing microscopy

- In 1978, Junqueria et al.[11] conducted a study, where in vertebrates of different species like Tilapia melanopleura, and Geophagus brasiliensis (fish), Bufo ictericus (amphibian), Xenodon merremii and Phylodrias schotii (reptiles), Gallus gallus (bird) and mouse, rat, guinea pig, rabbit and dog were selected. At least three specimens from each species were selected. Picrosirius red stain produced different color of birefringence for type I, type II & type III collagen. In the present study type I collagen produced yellow, red or orange color, while type II present in cartilage and chondrosarcoma showed a variable color and type III collagen produces green color. The color and morphology of type II collagen is always distinct from type I & II collagen.
- In 1979, Junqueria et al.[14] conducted a study, wherein 15 species of vertebrates are studied. Tissue sections of 15 cases were stained with picrosirius red. Sirius red, a strong anionic dye reacts via its sulphonic acid groups with basic group presents in the collagen molecule. The elongated dye molecules are attached to the collagen fiber in such a way that their long axes are parallel. Enhanced birefringence is produced by parallel relationship between dye and collagen. Picrosirius red polarization method showed a different color for type I and type III collagen fibers. The authors concluded that, the use of Polarizing microscopy for picrosirius red stained sections to identify collagen is not only responsible for its specificity, but also provides sensitivity and resolution. The resolution property is

due to appreciation of very thin fibers, which are undetectable in normal microscopy becomes visible as a source of light against a dark background.

- In 1989, Dayan et al.[12] conducted a study, wherein 12 purified collagens were used in the study. Polarization color was determined for both thin and thick fibers. Most of the thin fibers showed green to yellowish-green polarization color. The thick fibers showed yellow-orange to red. The polarization colors of various purified collagens were studied in fibers of similar thickness. The fiber thickness was not the only factor involved in determining the polarization color. Better aligned and tightly packed collagen molecules showed polarization color of longer wavelength. Not only the fiber thickness determines the polarization color of picrosirius red-stained collagens, but the packing of collagen molecules determine the polarization colors.
- In 2011, Coleman[15] published an article about picrosirius red stain. Picric acid is used as both fixative and in dye. Picric acid will explode when it is dry; therefore it is supplied as 25-35% hydrated powder. Picrosirius red is used in histopathology for grading of fibrosis, capsular invasion in thyroid neoplasms, salivary gland neoplasms, and healing and repair of bone and dental tissues.
- In 2014, Lattouf R et al.[16] conducted a study, wherein three patients with clinically proven Ehlers-Danlos type IV and three control patients were studied. Picrosirius red is used as a tool to appraise collagen network in normal and pathological tissues. A skin lesion of type IV Ehlers-Danlos syndrome, which is clinically and histopathologically proved, was sectioned. Collagen in Ehlers-Danlos syndrome type IV was thinner and shorter fiber than normal collagen.

According to Junqueira and colleagues, picrosirius red is used to study collagen in different tissues. He stated that picrosirius red reveal the type of collagen fibers and in the other studies it was declared polarizing color reveal about fiber thickness and packing. The authors concluded that, in the present study polarizing color depends on the orientation of the collagen bundles and not about the type of collagen.

Oral submucous fibrosis

- In 1995, Murti et al.[17] reviewed the etiology of Oral submucous fibrosis in relation to areca nut consumption. Oral submucous fibrosis is a high risk precancerous condition, predominantly affecting Indians. The epidemiological study that included case-series reports, large cross-sectional surveys, case-control studies, cohort and intervention studies have identified areca nut as the major etiological agents. Tissue-culture studies were done involving fibroblast using areca nut extracts and areca nut alkaloids, which showed fibroblast proliferation and increased collagen formation. The authors conclude that, the use of areca nut in oral submucous fibrosis patient results in fibroblast proliferation and increased collagen formation.
- In 1995, Ma et al.[18] reviewed the lysyl oxidase activity in fibroblasts cultured from Oral submucous fibrosis associated with betel nut chewing. Growth characteristics and lysyl oxidase activity of fibroblasts in normal human mucosa and oral submucous fibrosis associated with betel nut chewing were compared in cell culture. Fibroblast from normal human mucosa and Oral submucous fibrosis showed difference in proliferation rates and lysyl oxidase activity. The authors

concluded that, collagen deposition in oral submucous fibrosis tissue may be attributed to increased lysyl oxidase activity.

- In 1966, Pindborg and Sirsat[19] published an article regarding oral submucous fibrosis. Where the clinically affected areas of oral submucous fibrosis were atrophic as compared with the thickness of normal epithelium. The atrophic epithelium first becomes hyperkeratotic (clinically leukoplakia), and later intercellular edema and basal cell hyperplasia develop. Followed by epithelial atypia with moderate epithelial hyperplasia were noted. From then on, carcinoma could develop at any time. The atrophy of oral epithelium is secondary to connective tissue changes. Occasionally, fiery red erythroplakia areas develop.
- In 2006, Tilakaratne et al.[21] reviewed the etiology and pathogenesis of oral submucous fibrosis. Arecanut is the main etiological factor for oral submucous fibrosis. Commercially available freeze dried products such as mawa, pan masala, gutkha have higher concentration of arecanut than concventional betel quid. The hypothesize states that the increased synthesis of collagen and reduced degradation of collagen is the possible mechanism in the development of oral submucous fibrosis. The alkaloids like tannin are released from arecanut when lime is added to it. In vitro studies on human fibroblast using arecanut extracts or chemically purified arecoline there was fibroblast proliferation and increased collagen formation. The copper content of arecanut is high and the copper acts as a mediator of fibrosis is demonstrated by upregulation of lysyl oxidase in oral submucous fibrosis. The increased amount of lysyl oxidase leads to increased

cross linking of collagen. The increased amount of cytokines in the lamina propria was also associated with cross linking of collagen.

- In 2009, Ceena et al.[22] conducted a study, wherein 50 cases were selected. Of which 40 were Oral submucous fibrosis and 10 cases were control group. Clinical and functional staging was done depending on definite criteria. Histopathological study was done using H & E and picrosirius red stain. Collagen fibers were analysed for thickness and polarizing color. Furthermore, clinical, functional, and histopathological stages were compared. When the severity of the disease increases clinically, there will be a definite progression in subjective and objective symptoms. Under polarizing microscopy there was a gradual decrease in the green-greenish yellow color of the fibers and a shift to orange red-red color with increase in severity of the disease. As the disease progress from early to advanced stage there is tight packing of collagen fibers. We observed that the comparison of functional staging with the histopathological staging was a more reliable indicator of the severity of the disease. The authors concluded that, the mouth opening was restricted with advancing stages of oral submucous fibrosis. Assessment of functional and histological staging is done in order to plan the treatment.
- In 2011, Smitha and Donoghue[23] conducted a study, wherein 41 cases were selected. Of which 33 cases of oral submucous fibrosis and 8 cases of normal tissue were taken. Histologically most of the collagen fibers were parallel to the epithelium, and there was a statistically significant difference in orientation between oral submucous fibrosis and control groups in both buccal mucosa and labial mucosa. The authors concluded that, the reason for unidirectional alignment

of clinical fibrous bands could be due to chronic stimulation of oral mucosa by the irritants leading to change in the orientation of collagen fiber bundles, which might result in scar formation and that is similar to wound healing in which collagen fibers are oriented parallel to epidermis.

- In 2012, Ganganna et al.[8] conducted a study, wherein 91 cases of Oral submucous fibrosis were studied for collagen using picrosirius red. The polarization colors of thin and thick collagen fibers were recorded. The birefringence of thin fibers showed no difference in both histopathological connective tissue stages and degrees of epithelial dysplasia. The polarization color of thick collagen fibers showed a gradual change from predominantly yellow-orange to greenish-yellow in advanced connective tissue changes and degrees of epithelial dysplasia. The authors concluded that, there was a significant change in birefringence of collagen between connective tissue stages and between mild, moderate to severe degree of epithelial dysplasia. This change in birefringence color and arrangement of collagen fibers may give an implication of impending neoplastic change in oral submucous fibrosis.
- In 2014, Ealla et al.[25] conducted a study, wherein 50 cases were selected. Of which 45 patients diagnosed with different functional and histopathological grades of oral submucous fibrosis and 5 in control group. Picrosirius red stain used to analyse collagen both histopathologically and qualitatively using polarizing microscopy. Collagen fibers showed mixed birefringence with a shift in polarizing color from yellow to red-orange in lamina propria, around the muscle and blood vessels, which was correlating with the conventional H & E

stain. The authors concluded that, the change in birefringence color and arrangement of collagen fibers might give an assumption of impending neoplastic change in oral submucous fibrosis.

- In 2015, Neha modak et al.[26] conducted a study, wherein 40 cases were taken. Of which 30 cases are Oral submucous fibrosis and 10 cases are in control group. Based on definite criteria clinical, functional and histopathological staging was done. Collagen fibers were analysed for polarizing color and thickness. Comparison of clinical, functional and histopathological staging was done. The correlation between clinical and functional staging was not significant, whereas the comparison of the functional staging with histopathological staging was more reliable. The authors concluded that, qualitative change in the collagen fibers of oral submucous fibrosis patients using polarized microscopy would help to assess its role in diagnostic evaluation and to determine the prognosis of the disease and to provide predictive treatments.
- In 2016, Radhika et al.[27] conducted a study, wherein 30 cases were selected. Of which group I have 20 oral submucous fibrosis samples and group II has 10 normal tissue samples. Clinical, functional and histopathological staging was performed for all oral submucous fibrosis samples. Using chi square test comparative analysis was done between clinical and functional stages with the histopathological staging. Qualitative changes in the collagen fibers of oral submucous fibrosis were analyzed using picrosirius red stain and polarizing microscopy. Comparative analysis between clinical and functional stages with the histopathological staging revealed a significant correlation between the functional

and histopathological stage. Different stages of oral submucous fibrosis showed enhanced birefringence of collagen fibers due to picrosirius red stain. The authors concluded that, correlating functional and histopathological staging is more reliable in determining the severity. Production of characteristic polarizing color in picrosirius red & polarizing microscopy is used as a tool in assessing the severity of this condition thereby aiding in better treatment planning.

- In 2016, Reshma et al.[28] conducted a study, wherein 10 cases of three different grades of oral submucous fibrosis are stained with Van-Gieson, Mallory's trichrome and Masson trichrome. The degree of fibrosis between muscle bundles could be detected in all the three special stains, but when compared the results were not statistically significant. The authors concluded that, pathogenesis of OSMF is related to fibro-elastic and muscle degenerative changes in the connective tissue followed by secondary changes in epithelium.
- In 2017, Arkeri et al.[29] reviewed the update on pathophysiology of oral submucous fibrosis and its malignant transformation. Oral submucous fibrosis is a potentially malignant disorder associated with arecanut chewing. The malignant transformation rate of oral submucous fibrosis was reported to be around 7.6% over a 17-year period. Arecanut acts as a carcinogen due to its active metabolite arecoline N–oxide.
- In 2017, Singh et al.[9] conducted a study, wherein 45 cases of Oral submucous fibrosis and 10 cases of normal mucosa were taken as control. The selection of clinical case was done by using classification of J. N Khanna and Andrade and it was correlated with histopathological grading by Pindborg J. J and Sirsat S. M.

The sections were stained with H & E and picrosirius red stains and assessment was done accordingly. In clinical grade I, 60% of cases showed histopathological grade II, while 53.4% cases in clinical grade II correlated with histopathological grade III and 33.4% of clinical grade III cases showed histopathological grade IV. The orientation pattern revealed a parallel orientation as oral submucous fibrosis advanced. There was a gradual shift from green-greenish yellow color of the fibers and a shift to orange red-red color. The authors concluded that, upon correlation of clinical grades with histopathological grades, no statistically significant difference was found. In case of clinical grades there was similar birefringence pattern but histopathological grades showed a contrast finding.

- In 2019, Thakkannavar and Naik[3] conducted a study, wherein 40 cases of Oral submucous fibrosis were analysed for color and orientation of collagen using picrosirius red. Image analysis on immunohistochemical stained sections for factor VIII-related antigen and analysed for microvascular density. As the severity of the oral submucous fibrosis increases, there was a shift in the color of collagen fibers from greenish yellow to orange red and red color. The collagen fibers showed mixed orientation in early oral submucous fibrosis and parallel orientation in advanced stages. From early to advanced oral submucous fibrosis there was a significant decrease in micro vascular density. The authors concluded that, the change in the color and orientation of collagen fibers in early and advanced oral submucous fibrosis could be attributed to the type of collagen, alignment, cross-linking and packing of collagen fibers. The section and fiber thickness also have the effect on color of collagen fibers. In advanced cases of oral submucous

fibrosis, the vascularity is reduced which may lead to epithelial atrophy and subsequent malignant changes.

- In 2019, Shih et al.[30] reviewed the etiology, diagnosis, and therapy of oral submucous fibrosis. In Asians, oral submucous fibrosis is prevalent among betel nut chewing habits. The pathogenesis is disruption of collagen homeostasis. In which there is increased production of collagen and decreased degradation of collagen. Abnormal collagen production is due to inflammation, ROS production, and mutations. The molecular pathology techniques focus on biomarkers that induce abnormal collagen deposition. Biomarkers are detected by both invasive and noninvasive methods. The authors concluded that, individuals susceptible to oral submucous fibrosis and malignant transformation should quite betel nut chewing and tobacco smoking. Those patients should have natural diet and anti-inflammatory with antioxidant properties.
- In 2019, Sharma and Radhakrishnan[31] revisited and redefined the definition of oral submucous fibrosis, in which the definition by Pindborg and Sirsat's (1966) was modified as “an insidious, chronic potentially malignant fibrotic disorder affecting the entire oral cavity and sometimes the pharynx and oesophagus. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria with epithelial atrophy leading to stiffness of the oral mucosa, progressive decrement in mouth opening and inability to eat’’.

- In 2019, Tejasvi et al.[32] conducted a study, wherein a cross-sectional study was done on 150 cases, all were having quid-chewing habit and oral mucosal lesions. The correlation between oral mucosal lesions and quid-chewing habits were compared. Quid-chewing habit is a common and old tradition in India. Detailed habit history was taken, clinical examination was done. Biopsy was done for the oral mucosal lesion and confirmed histopathologically. Middle aged men were more commonly involved. The authors concluded that, the present study confirmed association between betel, tobacco, and oral mucosal lesions like oral submucous fibrosis, leukoplakia, chewer's mucosa, lichenoid reaction, and chemical burn.
- In 2019, Nishat and Kumar[33] conducted a study, wherein 73 cases of oral submucous fibrosis with different clinical and histopathological staging were studied. All the cases were stained with hematoxylin and eosin, Van Gieson and picrosirius red. Collagen fibers were analyzed for polarization colors, distribution, and orientation. Reddish orange and yellowish orange were the most predominant colors. Parallel fibers were demonstrated in van Gieson but in case of picrosirius red both parallel and perpendicular fibers were demonstrated. In the lamina propria, Yellowish orange and greenish yellow fibers were predominant. While in the submucosa reddish orange fibers were predominant. The authors concluded that, picrosirius red is the special stain for collagen. Histopathological grading and polarization colors showed no association with each other. Due to fibrosis collagen fiber were more thick and tightly packed in the submucosa.

Leukoplakia

- In 1984, Silverman et al[34]. conducted a study, wherein 275 patients were studied for an average period of 7.2 years. 73% of the patients used tobacco, among these 45 patients developed squamous carcinomas in an average time of 8.1 years. High risks for malignant transformation were seen in non-smoking patients, the clinical presence of erythroplakia (erythroleukoplakia), and a clinical verrucous-papillary hyperkeratotic pattern. The authors concluded that, duration of leukoplakia increased the number of malignant transformation. Oral leukoplakia is a precancerous lesion and certain characteristics indicate greater risks and need more aggressive management.
- In 1995, Lumerman et al.[35] conducted a study, wherein clinical and microscopic features of 308 cases of oral epithelial dysplasia were studied. 44 cases were evaluated retrospectively, with follow-up for malignant transformation. Oral epithelial dysplasia, the histopathologic marker of a premalignant disorder may present clinically as leukoplakia, erythroplakia, or leukoerythroplakia. These premalignant disorders have chance for transformation to invasive squamous cell carcinoma.
- In 1997, van der Waal et al.[36] reviewed the clinicopathological features of oral leukoplakia. The term leukoplakia is only a clinical term. The annual percentage of malignant transformation varies depending on the tobacco and dietary habits. Epithelial dysplasia is an important predictor for malignant transformation, not all the dysplastic epithelium becomes malignant. Even the non-dysplastic epithelium

may become malignant. The tongue and floor of the mouth can be considered as high risk sites with regards to malignant transformation from leukoplakia.

- In 2001, Sciubba et al.[37] reviewed the importance of early diagnosis and treatment in oral cancer. Oral leukoplakia carries an increased risk of cancer development either in the area of the leukoplakia or elsewhere in the oral cavity or the head-and-neck region. Particularly in the case of asymptomatic lesion, 4-8 weeks of observation is done after removing the cause. Even the period of 4-8 weeks is longer in case of squamous cell carcinoma, carcinoma in situ or severe epithelial dysplasia. If the lesion doesn't resolve it may be considered as squamous cell carcinoma. If the lesion is symptomatic biopsy is strongly recommended before removing the causative factor.
- In 2002, Neville and Day[5] reviewed the clinical features of oral potentially malignant disorders and oral cancer with an attempt for early diagnosis. Invasive squamous cell carcinoma is often preceded by either white or red patches known as leukoplakia and erythroplakia.
- In 2003, Scheifele and Reichart[4] reviewed the annual transformation rate of leukoplakia. The cumulative risk of 38% of leukoplakia to transform into oral squamous cell carcinoma was estimated. The annual transformation rate of oral leukoplakia into oral squamous cell carcinoma amount up to 6.3% was estimated.
- In 2003, Petti[38] conducted a systematic review regarding pooled estimate of world leukoplakia prevalence. Estimate of pooled leukoplakia prevalence was lower than expected. Estimated incidence of oral cancer rate due to malignant

transformation from leukoplakia was high, which implies that the global number of oral cancer cases is underreported.

- In 2006, Holmstrup et al.[39] conducted a study, wherein 269 lesions in 236 patients were included. Among 269 cases, surgical intervention was done for 94 cases and in 175 cases no surgical intervention was done. In the 175 cases, biopsy was done in case of changes with malignant transformation. All the patients were encouraged to quite the habit of tobacco. The authors concluded that, there was seven times increased risk of non-homogenous leukoplakia for malignant development as compared with homogenous leukoplakia. For lesions greater than $200mm^{2,}$ the risk of malignant transformation is greater.
- In 2007, Warnakulasuriya et al.[40] reviewed the nomenclature and classification of oral potentially disorders. The World Health Organization (WHO) defines leukoplakia as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer". Leukoplakia have different behavioral pattern but with assessable tendency for malignant transformation. Leukoplakia has the similar risk factor of oral squamous cell carcinoma like tobacco, alcohol use, and specific viral infections are considered. Leukoplakia is more frequently seen in men of age 40-70 years.
- In 2007, Lodi and Porter[41] reviewed the management of potentially malignant disorders. Theoretically, the development of potentially malignant disorders and malignant transformation after cessation of habits like tobacco was stated. But there was no good study to prove the significant reduction of malignant transformation after cessation of habits.

- In 2008, Lodi et al.[42] reviewed the interventions for treating oral leukoplakia. Oral leukoplakia is a thickened white patch in the oral cavity that can't be rubbed off. Oral leukoplakia can become malignant. Preventing the malignant transformation from leukoplakia is critical, as the survival rate is very low, less than 5 years. No treatment proved to be effective in preventing malignant transformation.
- In 2012, Liu et al.[43] conducted a study, wherein a total of 320 patients with biopsy proven oral leukoplakia were followed for 5 years. The cancer development in oral leukoplakia was studied based on clinicopathological factors. Multivariate analysis revealed that the 4 factors including patient aged >60 years, lesion located at lateral/ventral tongue, non-homogenous lesion, high-grade dysplasia were significant indicators for malignant transformation. Elderly patients with lesion located at lateral/ventral tongue, non-homogenous lesion with high-grade dysplasia correlated much higher risk of transformation.
- In 2012, Ho et al.[44] conducted a study, wherein 91 patients meeting the criteria gave consent for inclusion to the cohort; follow up was done for 48 months. The clinical determinant of malignant transformation in oral epithelial dysplasia was studied. Patients with a histopathological diagnosis of oral epithelial dysplasia were selected. Within 5 years, there was 22% malignant transformation. The significant predictors for malignant transformation were non-smoking status, site, non-homogeneous appearance, size of lesion >200 mm2. The authors concluded that, although a number of these clinical determinants have associated with malignant transformation, the high-risk nature of lesions in non-smokers is of particular importance.

- In 2014, Van Der Waal[45] reviewed whether the malignant transformation of oral leukoplakia is predictable and preventable. The prevalence of oral leukoplakia is 1% and the annual rate of malignant transformation is 2 to 3 %. There are no reliable predicting factors for malignant transformation (clinicopathological or molecular factors) that can be used in an individual patient and such event cannot truly be prevented. Cessation of smoking habits may result in regression or even disappearance of the leukoplakia and will diminish the risk of cancer development either at the site of the leukoplakia or elsewhere in the mouth or the upper aerodigestive tract.
- In 2016, Warnakulasuriya and Ariyawardana[46] reviewed the malignant transformation rate of oral leukoplakia and the associated risk factors. Leukoplakia with moderate to severe oral epithelial dysplasia have higher rate of malignant transformation. The important risk factor for malignant transformation of Leukoplakia include grade of dysplasia, advanced age, female gender, lesion size greater than 200 mm^2, and non-homogeneous type.
- In 2017, Bewley and Farwell[47] published an article regarding oral leukoplakia and oral cavity squamous cell carcinoma was reviewed. Oral leukoplakia is a disease of clinical exclusion, which does not have a clear causal etiology rather than tobacco. Biopsy should be done for persistent lesions in order to rule out dysplasia. Lesions with dysplasia should be excised in an attempt to reduce the risk of malignant transformation. The authors concluded that, oral squamous cell carcinoma has a high rate of recurrence and low rate of overall survival, even after aggressive multimodal treatment. Therefore, all the cases of oral leukoplakia

should be followed closely for any clinical changes and repeat biopsy should be taken. An isolated lesion with mild dysplasia in non-smokers should be treated. While multi-focal lesions in smokers with mild dysplasia, can be simply followed. In general moderate to severe dysplasia should be treated.

- In 2017, Greenslade[48] conducted a Cochrane review, regarding the interventions for treating leukoplakia to prevent oral cancer. Global prevalence of oral leukoplakia is 1-4% and the rate of malignant transformation exceeds 1%. Six groups of interventions were included in the study. None of the treatment has reduced the occurrence of oral cancer from leukoplakia.
- In 2018, Ganesh et al.[49] reviewed the potentially malignant disorders and its malignant transformation. Leukoplakia is most commonly caused by consumption of tobacco, alcohol and betel quid. Few cases of leukoplakia occur genetically and named as idiopathic leukoplakia. Based on the clinical presentation leukoplakia was classified as homogenous and non-homogenous leukoplakia. Among the non-homogenous leukoplakia, proliferative verrucous leukoplakia has highest rate of malignant transformation. The histopathological features per se are not sufficient to establish the diagnosis. The histopathological features combined with clinical features provide diagnosis for leukoplakia.
- In 2019, Chaturvedi et al.[50] conducted a study, wherein retrospective cohort study (1996-2012) was done using electronic medical records within Kaiser Permanente Northern California (KPNC). In which 4,886 cases of oral leukoplakia was selected. Of which biopsy was taken for 1,888 cases of leukoplakia and for those cases, case-cohort study was done to investigate histopathological predictors of

progression. The previous literature suggested high-risk lesions for biopsy which included lesion appearance (non-homogenous leukoplakia), size (>200 mm^2), anatomic location (tongue and floor of mouth), age (older individuals), and gender (females). The above said data is needed to estimate relative and absolute risk of oral cancer in relation to oral leukoplakia. The authors concluded that, regardless of clinical impression routine biopsy should be done for all cases of oral leukoplakia.

- In 2019, Farah and Fox[51]conducted a study, wherein molecular analysis of leukoplakia with or without dysplasia was studied. In which 25 excisional biopsy specimen were taken. Of which 13 were with oral epithelial dysplasia and 12 were without oral epithelial dysplasia. Differential expression analysis showed difference in both groups. Hierarchical clustering readily distinguished oral leukoplakia with dysplasia from oral leukoplakia without dysplasia. The down regulation of extracellular matrix (ECM) pathways was a feature of dysplastic lesions, as provided by ontology enrichment analysis provided. There were changes in the molecular profile including down regulated ECM of oral leukoplakia exhibiting dysplasia. The authors concluded that, reactive changes in the stroma may be an early manifestation of dysplastic development.

Squamous cell carcinoma

- In 1988, Van den Hooff[52] published an article regarding stromal involvement in malignant growth. He suggested that difference in birefringence of collagen fibers around the tumor islands may be due to the secretion of collagenases by the tumor cells in the immediate vicinity, the dedifferentiated tumor cells secreting an

abnormal matrix, formation of disorganized stroma around the tumor islands, and abnormal disintegration of the matrix by tumor cells.

- In 2012, George et al.[53] conducted a study, wherein 39 cases of oral squamous cell carcinoma and 6 cases of control were stained with 7 different special histochemical stains. The staining intensity of acid mucins, reticulin, glycoprotein, fibrin, sulfated mucins, elastic fibers, and collagen around the tumor islands and in the connective tissue is analysed. The authors concluded that, the tumor cells grow slowly in the host stroma. Where the tumor cells have the ability to prevent and disrupt the response of host towards tumor cells.
- In 2015, Gopinath PA et al.[54] conducted a study, wherein 50 cases of different grades of Oral squamous cell carcinoma and 10 cases of normal mucosa were selected. Of the 50 cases, 20 cases were well differentiated squamous cell carcinoma, 20 cases were moderately differentiated squamous cell carcinoma, and 10 were poorly differentiated squamous cell carcinoma. With the dedifferentiation of Oral squamous cell carcinoma, thin collagen fibers were increased and thick collagen fibers were decreased. There was a change in polarization color of thick collagen fibers from yellowish orange to greenish yellow with dedifferentiation of Oral squamous cell carcinoma indicating loosely packed fibers. The authors concluded that, there was a gradual change of birefringence of collagen from yellowish orange to greenish yellow from well to poorly differentiated squamous cell carcinoma, indicating that there is a change from mature form of collagen to immature form as tumor progresses. Study of collagen around tumor island using

picrosirius red along with routine staining will help in predicting the prognosis of tumor.

- In 2015, Manjunatha et al.[55] conducted a study, wherein 30 cases of different grades of oral squamous cell carcinoma were evaluated using picrosirius red and polarizing microscopy to analyze collagen fibers. In case of moderately differentiated to poorly differentiated Oral squamous cell carcinoma there was a gradual change in polarizing color from yellowish orange to greenish yellow particularly in the vicinity of invading tumor islands. Around the neoplastic area, thick collagen fibers forming parallel bundles are arranged in a discontinuous fashion. The authors concluded that, an observable stromal change was noted with a significant change in the arrangement from the early stage to the advanced stage, with the progression of neoplasm was indicated by picrosirius red in different thickness of collagen.
- In 2016, Patankar et al.[2] conducted a study, wherein 30 cases were selected. Of the 30 cases, 5 cases of normal mucosa, 10 cases of well-differentiated oral squamous cell carcinoma, 10 cases of moderately differentiated oral squamous cell carcinoma, and 5 cases of poorly differentiated oral squamous cell carcinoma were examined for the presence of any extra cellular matrix changes by using special stains. Van Gieson stain showed abundant thick collagen fibers, dispersed collagen fibers, thin few dispersed collagen fibers in well-, moderately- and poorly-differentiated oral squamous cell carcinoma respectively. PAS stain showed moderate staining in well differentiated squamous cell carcinoma cases, negative staining in moderate and poorly differentiated squamous cell carcinoma.

Verhoeff's van-Gieson showed negative staining for elastic fibers around tumor islands in different grades of oral squamous cell carcinoma. Picrosirius red stain showed type I collagen fibers in well and moderately differentiated Oral squamous cell carcinoma cases and type III collagen fibers in poorly differentiated cases. The authors conclude that, altered staining reactions of the collagenous stroma suggest that tumor cells may release certain enzymes that play a role in the manipulation of extra cellular matrix to enhance their own survival.

- In 2016 Kardam et al.[6] conducted a study, wherein 50 samples of varying grades of oral squamous cell carcinoma was stained with H & E, picrosirius red, and Verhoeff-van Gieson. Qualitative and quantitative analysis of collagen and elastic fibers were done. In case of well differentiated to poorly differentiated oral squamous cell carcinoma, change in color of collagen fibers was seen. As the grade of oral squamous cell carcinoma progressed, the collagen fibers were loosely packed and haphazardly arranged. Thin collagen fibers predominantly exhibited greenish yellow, but the thick fibers exhibited a variety of color. The authors concluded that, in the stroma of varying grades of oral squamous cell carcinoma, the collagen fiber undergoes a change in color, orientation and packing. The author also explored elastic fibers in oral squamous cell carcinoma which is the uniqueness of the study.
- In 2016, Kumari et al.[13] conducted a study, wherein 30 cases of histologically diagnosed, well-, moderately-, and poorly-differentiated oral squamous cell carcinoma were retrieved from the archives. Collagen was evaluated using picrosirius red stain and immunohistochemical analysis of the antibody to type III

collagen. Correlation between the collagen using picrosirius red and immunohistochemistry to type III collagen was evaluated. Collagen fibers showed a change in birefringence ranging from reddish-orange to greenish-yellow in well- to poorly-differentiated oral squamous cell carcinoma. Immunohistochemistry staining intensity of type III collagen changed from weak to strong as grade increased for oral squamous cell carcinoma. The authors concluded that, tumor progression reflected a change in collagen from type I to type III.

- In 2017, Kullage et al.[24] conducted a study, wherein 30 cases of different grades of histologically diagnosed cases of Oral squamous cell carcinoma and ten cases of normal buccal mucosa as a control. Nature of collagen was studied using picrosirius red and polarizing microscopy method, intensity of inflammatory cell infiltrate was recorded using software. Normal mucosa showed predominantly reddish birefringence. All cases of well differentiated squamous cell carcinoma showed reddish-orange color. 70% of moderately differentiated cases showed yellowish-orange. 60% of poorly differentiated cases showed greenish-yellow. The mean inflammatory cell count was highest in well differentiated cases. In case of moderately and poorly differentiated squamous cell carcinoma cases there was shift to yellow orange and green yellow and inflammatory cell count was decreased. The authors concluded that, both inflammatory cells and tumor cells have a role in determining the nature of collagen fibers in tumor stroma of oral squamous cell carcinoma. Both inflammatory cells and tumor cells have opposing effects on stromal behavior and helpful in predicting the prognosis.

- In 2017, Devendra et al.[56] conducted a study, wherein 29 cases of oral squamous cell carcinoma with invasive tumor front were stained with Hematoxylin-Eosin and picrosirius red for evaluation under polarizing microscopy. Tumors with a cohesive front had a thick collagen fiber, organized, well packed, red-yellow in color, and show strong birefringence. Gradual changes in the nature of collagen fibers were observed. In case of discohesive tumor front, where the collagen fibers were thin, disorganized, loosely packed, yellow-orange to green-yellow in color, with weak birefringence. The authors concluded that, a cohesive tumor front with organized collagen fibers prevents tumor invasion and metastasis. It inhibits an increase in tumor size. In discohesive tumor front, the fibers might enhance the movement of tumor cells, resulting in invasion and metastasis.
- In 2018, Arora et al.[7] conducted a study, wherein 70 slides were prepared and divided into three groups. Group I was 10 normal gingival tissue slides, Group II was 40 slides of oral potentially malignant disorders, Group III was 20 slides of well differentiated squamous cell carcinoma. Two sections were made from each group; one is stained with picrosirius red and the other with Matrix metalloproteinase-13. In group II, Matrix metalloproteinase-13 connective tissue expression was greater in oral submucous fibrosis than leukoplakia, group III showed elevated expression among 70% cases. Picrosirius red staining in group II shows higher staining of yellow-orange and green-yellow fibers in oral submucous fibrosis than leukoplakia, 50% cases of oral squamous cell carcinoma in group III showed green-yellow stained immature thin fibers. The authors

concluded that, picrosirius red with polarizing microscopy is an easy and reliable method to determine the state of stroma.

Picrosirius red in other lesions

- In 1986, Junqueria et al.[57] conducted a study, wherein 9 patients with the diagnosis of osteosarcoma. Collagen of the human osteosarcoma was studied using picrosirius red-polarisation microscopy. Using picrosirius red, there was a sharp distinction between osteoid and the other tissue components. In picrosirius red stain, osteoid from both normal osteogenesis and osteosarcoma showed randomly arranged, thin, short, weakly birefringent collagen fibers against a dark background. Type I & III collagen in the fibroblastic areas of tumors, type III in anaplastic areas, type II in chondroblastic areas of osteosarcoma. The distribution of different types of collagen in osteosarcoma indicates that the tumor arise from a common progenitor cell.
- In 1996, Hirshberg et al.[58] conducted a study, 5 cases of central odontogenic fibroma and 13 cases of hyperplastic dental follicle were studied. All the cases were stained with picrosirius red and viewed under polarising microscopy. Central odontogenic fibroma is a destructive lesion with persistent growth. There was a different pattern of collagen fibers in case of central odontogenic fibroma compared to hyperplastic dental follicle. Thick fibers in case of central odontogenic fibroma are green, or greenish-yellow to yellow. In case of hyperplastic dental follicle the collagen fibers were yellow, yellowish-orange and orange. The authors concluded that, green to greenish-yellow indicates normal

thin fibers. Whereas, birefringence color of yellowish-orange through orange to red indicates thick fibers. The green to greenish-yellow color indicates loosely packed collagen fibers in central odontogenic fibroma.

- In 1998, Kauppila et al.[59] conducted a study, wherein 16 cases with breast lesion were obtained. Of the 16 cases, one is a benign lesion and the remaining 15 were malignant lesions. The type of collagen gene expression in human breast cancer is studied. In the development of breast cancer development, there was increased synthesis and degradation of extracellular matrix components. Type I and type III procollagen mRNA expression was studied in benign and malignant breast lesions. Increased synthesis of fibrillar type I & type III procollagens serves as the pathway for tumor invasion. The enhanced synthesis is associated with the formation of aberrant collagen bundles, which may be more readily degradable and may thus facilitate breast tumour invasion.
- In 1999, Stenback et al.[60] conducted a study, wherein hairy and hairless mice of 10-12 week old is selected. The extracellular matrix in the development of tumor in the skin of mice was studied. The development of cancer involves epithelial-stromal interactions. Based on tumor morphology there is alterations in the synthesis and deposition of type I and III collagens. In case of benign lesions, there is increased synthesis and deposition of type I and III collagens. In case of well differentiated squamous cell carcinoma, a similar induction of collagen synthesis and deposition was observed. In case of moderately and poorly differentiated squamous cell carcinoma, the destruction of fibrillary structure was

more pronounced. The underlying stroma reacts to the development of epithelial tumors in a reproducible way, based on the carcinogenic agents involved.

- In 1999, Hirshberg et al.[61] conducted a study, wherein 15 cases of odontogenic keratocyst, 15 cases of dentigerous cyst and 15 cases of radicular cyst were studied for collagen fibers using picrosirius red and polarizing microscopy. The thick fibers in odontogenic keratocyst were significantly more greenish-yellow, when compared with those of detigerous cysts and radicular cysts. The authors concluded that, the staining of the collagen fibers in keratocyst is similar to that of odontogenic neoplasms, thus the stroma acts not only acts as a structural support, but also as a part in neoplastic behavior of the cyst.
- In 2001, Koren et al.[62] conducted a study, wherein capsules of 10 cases of widely invasive carcinoma, 10 cases of minimally invasive carcinoma and 28 cases of adenoma was studied using picrosirius red and polarisisng microscopy. The key criteria in the diagnosis of follicular thyroid carcinoma are capsular invasion. Carcinomas were assessed for site of definite invasion, minimal invasion, no evidence of invasion along the capsule. All foci were assessed for the color and color intensity of collagen fibers. At the site of invasion yellow-green collagen fibers were predominantly distributed. In case of non-invaded site orange red fibers were predominantly distributed. Thick capsules of adenomas were predominantly stained by orange-red color, although yellow-green color is also noted in some areas. The authors concluded that, picrosirius red staining provides diagnostically useful information in case of capsular invasion.

- In 2006, Allon et al.[63] conducted a study, wherein ten cases of pleomorphic adenoma, polymorphous low grade adenocarcinoma, adenoid cystic carcinoma, and mucus extravasation phenomenon (control) were selected. The stromal differences in salivary gland tumor of common histopathogenesis but with different biological behavior were studied. The stroma was studied with picrosirius red and polarizing microscopy.. 50 thin and thick fibers were counted in all the cases and classified as green-yellow or yellow-orange collagen fibers. Similar distributions of thin fibers were noted in all tumor types and controls. Different distribution of thick fibers in polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma compared to pleomorphic adenoma and mucous extravasation phenomenon. The distribution of thick fibers showed similar distribution in polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma. Thus similar distribution of fibers between polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma indicate that these tumors represent a single entity with a broad spectrum of biological behavior.
- In 2012 Singh et al.[64] conducted a study, wherein 50 cases were studied. Of which 15 cases are odontogenic keratocysts, 15 cases are dentigerous cyst, 15 are radicular cyst and 5 cases are progressive cases of odontogenesis. Histochemical study was done using picrosirius red staining and examined under polarizing microscope. In odontogenic keratocyst the collagen fibers are oriented parallel in a loosely packed stroma, in contrast to dentigerous cyst and radicular cyst. The authors concluded that, in the above three lesions the quality, organization and

packing of collagen fibers is different, which accounts for difference in biological behaviour of the lesions.

- In 2014, Datar et al.[65] conducted a study, wherein 7 cases of giant cell fibroma and fibroma were studied using picrosirius red and Van Geison stain. Yellow, yellow-orange and green in case of fibroma, whereas in case of giant cell fibroma yellow and orange colors. The authors concluded that, collagen in giant cell fibroma is more mature and dense. There were observable changes in the stroma of giant cell fibroma and fibroma.
- In 2015, Jahagirdar et al.[66] conducted a study, wherein 30 cases of odontogenic cyst and odontogenic tumor were studied with picrosirius red and polarizing microscope. Greenish-yellow birefringence indicating procollagen, intermediate, or pathologic collagen fibers were suggestive of loosely packed collagen fibers in 7 cases of inflammatory cyst. In case of developmental cyst, predominant yellowish-orange birefringence was noted. In case of odontogenic tumor, yellowish-orange and orangish-red to red birefringence indicates tightly packed fibers. The authors concluded that, picrosirius red with polarizing microscope helps to distinguish collagen fibers in odontogenic cyst and odontogenic tumor.
- In 2015, Yukti Raj et al.[67] conducted a study, wherein 5 cases of keratocystic odontogenic tumour (KCOT), dentigerous cyst (DC), unicystic ameloblastoma (UA) and solid/multicystic ameloblastoma (SMA) were studied using picrosirius red and polarisisng microscopy. Studies by polarizing microscopy have also shown that there is a difference in collagen and probably these differences may play a role in their biologic behavior. Collagen is the major component of the

extracellular matrix and possibly there is an alteration in the nature and structure of collagen in various pathological conditions. The authors concluded that, Collagen fibers in dentigerous cysts showed predominant yellowish-red birefringence and fibers in KCOT and ameloblastomas showed a predominantly greenish-yellow birefringence. Hence, our study suggests that the nature and character of collagen fibers may influence the clinical behavior of the lesion.

- In 2015, Soma Susan Varghese et al.[68] conducted a study, wherein 24 cases of mild, moderate and severe grades of oral epithelial dysplasia, 8 cases of inflammatory fibrous hyperplasia, and 8 cases of normal mucosa were selected. The collagen fibers in all these cases were analysed using picrosirius red and polarising microscopy. Greenish yellow birefringence was observed in all the grades of epithelial dysplasia confirming the presence of loosely arranged pathological collagen in the presence of moderate inflammation. Statistically significant differences were observed in the packing and orientation of collagen between dysplasia and inflammatory fibrous hyperplasia. The authors concluded that, even in mild epithelial dysplasia there was loosely packed thin disoriented collagen. Thus the loosely packed collagen is due to the release of tumourigenic factors into the connective tissue stroma.
- In 2017, Charan Gowda et al.[69] conducted a study, wherein labial mucosa of 30 deceased individuals (18 male and 12 female) were fixed in 10% formalin at 12 hour. Tissue was processed, sectioned and stained using picrosirius red stain and the birefringence pattern of collagen was studied. The ratio of thick and thin fibers was compared among males and females. Thick fibers in males were more than in

females, whereas thin fibers in females were more than male. The authors concluded that, the picrosirius red and polarizing microscopy may be used as a tool in gender differentiation.

- In 2019, Peddapelli et al.[70] conducted a study, wherein 15 cases of keratocystic odontogenic tumor and 15 cases of ameloblastoma were studied using picrosirius red and polarising microscopy. The thickness, color, and orientation of collagen fibers were evaluated. Significant difference was observed between yellowish-orange collagen fiber bundles. There was no significant difference between greenish-yellow and orange-red collagen bundles. The authors concluded that, the connective tissue stroma in case of keratocystic odontogenic tumor acts as both structural and functional support. Thin, parallel, and loosely arranged fibers in keratocystic odontogenic tumor were attributed to its high recurrence rate and biological aggressiveness.

MATERIALS AND METHODS

Materials

- Formalin fixed, paraffin embedded tissue blocks of 45 cases
- Microtome [Fig 1] [Thermo fisher scientific HM340E with automatic section transfer system]
- Leica blade
- Slide
- Hot air oven for dewaxing
- Xylene as clearing agent
- Coplin jars
- Grades of alcohol
- Harris hematoxylin & Eosin [For routine H & E]
- Picrosirius red (Direct Red 80, Sigma-Aldrich)
- Weigert's hematoxylin for nuclear counter stain
- Wash solution (1% acetic acid)
- DPX mountant (Dibutylpthalate polystyrene xylene)
- Coverslip
- Light microscope
- Polarizing microscope (Olympus microscope BX43 with polariser & analyser) [Fig 2]
- Camera for photomicrography

Staining protocol for picrosirius red

1. De-wax and hydrate paraffin sections.
2. Stain nuclei with weigert's hematoxylin [Hematoxylin & Ferric chloride is mixed just before staining] for 5-8 minutes.
3. Wash in running tap water for 5 minutes.
4. Add 1 ml of picrosirius red solution on the section and keep it for one hour.
5. Wash with two changes of 1% acetic acid.
6. Wash in running tap water.
7. Dehydrate in three changes of alcohol.
8. Clear in xylene and mount with Dpx mountant.

Principles of Polarising microscope [Fig 3]

- To detect birefringence, white light from the microscope illuminator (path is left to right) is polarized before interacting with the sample.
- Upon crossing collagen molecules (anisotropic), the polarized light is refracted and divided into two separate rays.
- The two rays of light initially vibrate perpendicular to each other, until an analyzer (often a quarter-wave plate) "flips" one ray so that it becomes parallel to the other ray to allow its detection.
- The relative retardation of one ray with respect to the other is indicated by an equation (thickness multiplied by refractive index difference) and governs intensity of detection. Because refractive index difference is constant for a given material (i.e., collagen fibers)

- The intensity of detection is thus directly proportional to the thickness of the material (if the tissue sections are kept at a constant thickness).

Methods

The study comprises formalin fixed, paraffin embedded tissue blocks of 45 cases. The 45 blocks consists 15 cases each of well differentiated squamous cell carcinoma, oral submucous fibrosis without malignant transformation, and leukoplakia (clinically) with mild to severe dysplasia were selected. All the case blocks were retrieved from the archives of department of oral pathology, TNGDC & H, Chennai. Ethical clearance was obtained from the institutional review board, Tamil Nadu government dental college and hospital, Chennai, **With IRB Reference No: 7/IRB/2017.**

From each tissue blocks, 2 sections of thickness 3.5µ were made. One section is stained with Harris Hematoxylin & Eosin and viewed under light microscope. The other one with picrosirius red (Direct Red 80, Sigma-Aldrich) and viewed under both light microscope & Polarizing microscope.

In both Harris Hematoxylin & Eosin and picrosirius red stained sections collagen fibers seen around tumor islands in well differentiated squamous cell carcinoma were evaluated. In case of oral submucous fibrosis and leukoplakia, collagen fibers in lamina propria were evaluated. The picrosirius red stained sections under light microscope shows red colour for collagen and yellow color in all other region. Under polarizing microscopy, the birefringence colors produced by collagen are red, orange, yellow, and green.[3]The photomicrographs were taken under 40X magnification.

Fig 1 Microtome (Thermofisher scientific HM340E with automatic section transfer system)

Fig 2 Olympus microscope with polarizer (right) and analyser (left)

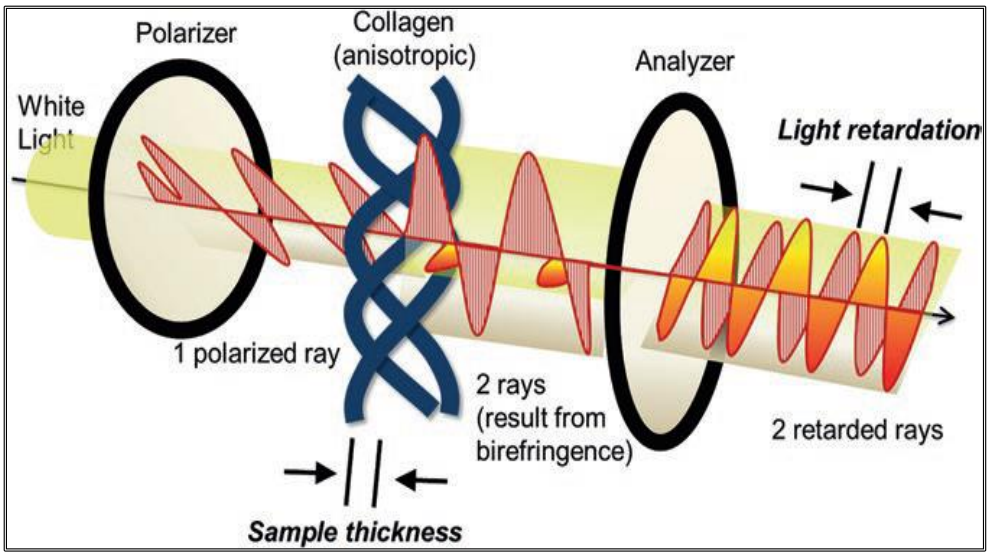


Fig 3 Principles of Polarizing microscopy

RESULTS

The study included 45 tissue blocks. Two sections are made from each block. One is stained with H & E and the other one with picrosirius red. Picrosirius red stained sections were viewed under polarizing microscopy for all 45 cases. Picrosirius red stained sections produce red color for collagen and yellow for muscle fibers and epithelium, under light microscope. When viewed under polarizing microscopy it produces birefringent colors. Collagen under polarizing microscope produces green, yellow, red, and orange colors. In a more mature stage the collagen appears yellow, orange or red. In case of poorly formed collagen fibers are greenish in color.[3]

In case of well differentiated squamous cell carcinoma (WDSCC) the collagen fibers around the tumor islands were examined. In all the 15 cases of well differentiated squamous cell carcinoma, the collagen fibers around tumor islands were examined under both light microscope and polarizing microscope. Images were taken under 40X magnification. Under polarizing microscopy the collagen fiber produces greenish yellow in 10 cases (66.7%) [Fig 4-a, 4-b, 4-c], greenish orange in 3 cases (20%) [Fig 5-a, 5-b, 5-c], and there was no significant color in 2 cases (13.3%). [Table 1]

In case of oral submucous fibrosis (OSMF) without malignant transformation the subepithelial collagen fibers in the lamina propria is analyzed. In all the 15 cases of oral submucous fibrosis, the subepithelial collagen fibers in the lamina propria were examined under both light microscope and polarizing microscope. Images were taken under 40X magnification. Under polarizing microscopy the collagen fiber produces greenish orange

in 6 cases (40%) [Fig 6-a, 6-b, 6-c], greenish yellow in 5 cases (33.3%) [Fig 7-a, 7-b, 7-c], green/green-orange in 4 cases (26.6%) [Fig 8-a, 8-b, 8-c]. [Table 1]

In case of leukoplakia (clinically) with the mild to severe dysplasia the collagen fibers in the lamina propria is analyzed. In all the 15 cases of Leukoplakia, the subepithelial collagen fibers in the lamina propria were examined under both light microscope and polarizing microscope. Images were taken under 40X magnification. Under polarizing microscopy the collagen fiber produces greenish yellow in 11 cases (73.3%) [Fig 9-a, 9-b, 9-c], greenish orange in 1 case (6.6%) [Fig 10-a, 10-b, 10-c], reddish orange in 1 case (6.6%) [Fig 11-a, 11-b, 11-c], no significant color in 2 cases (13.3%). [Table 1]

The frequency distribution of the polarization color of collagen fibers in well differentiated squamous cell carcinoma [Fig 12], oral submucous fibrosis [Fig 13], and leukoplakia [Fig 14] is represented using pie chart.

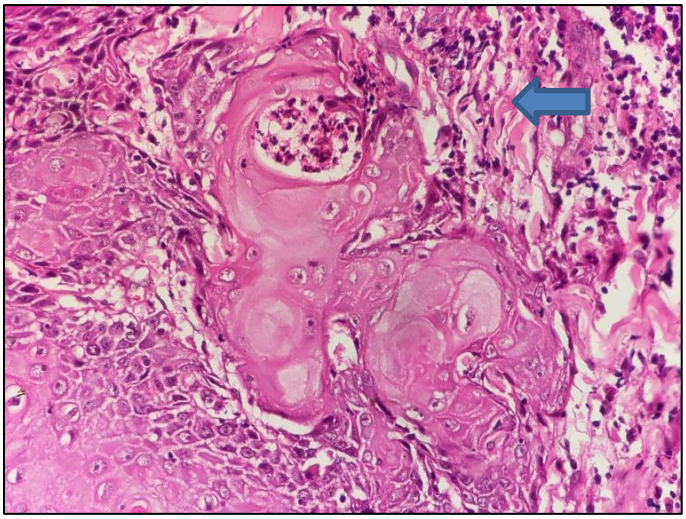


Fig 4-a H & E stain of WDSCC, with collagen fibers around tumor islands [40X].

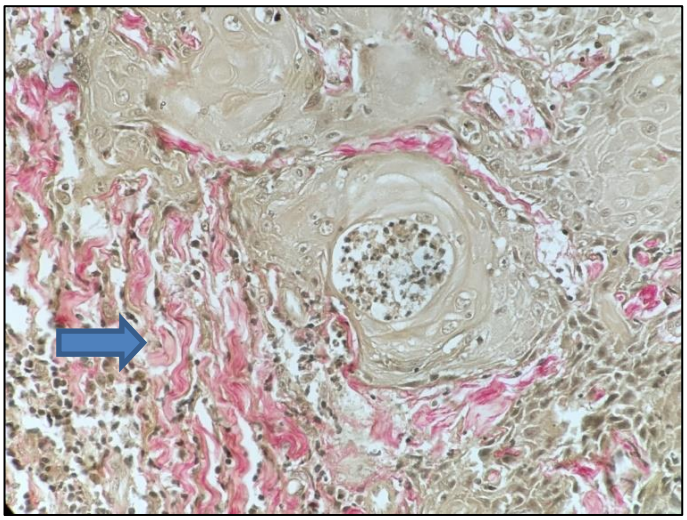


Fig 4-b Picrosirius red stain of WDSCC, under light microscopy with Collagen fibers around tumor islands are stained red and all other areas stained yellow [40X].

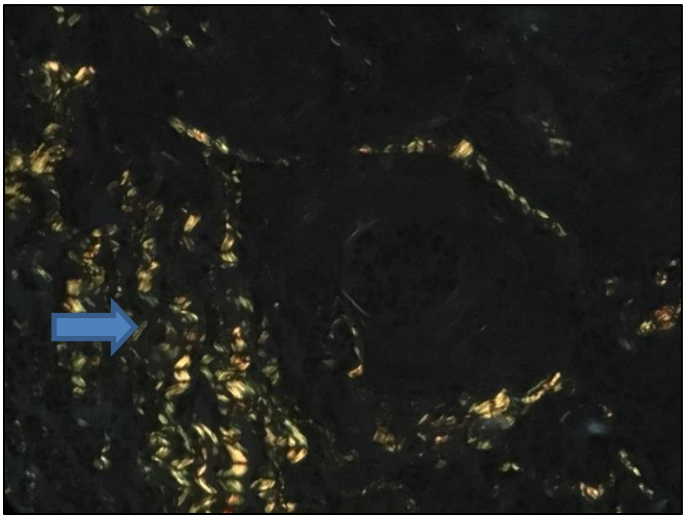


Fig 4-c Picrosirius red stain of WDSCC, under polarized microscopy with collagen fibers around tumor islands appears greenish yellow [40X].

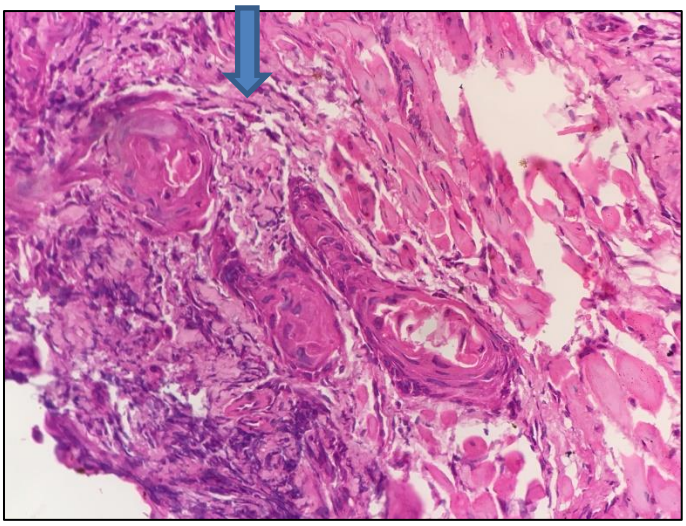


Fig 5-a H & E stain of WDSCC, with collagen fibers around tumor islands [40X].

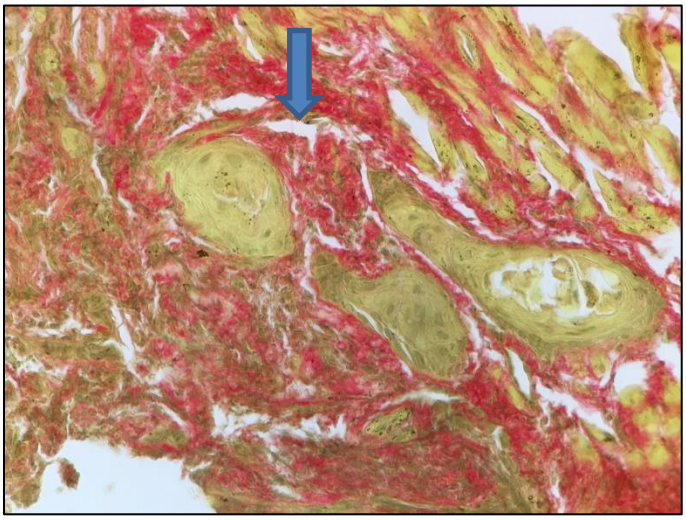


Fig 5-b Picrosirius red stain of WDSCC, under light microscopy with Collagen fibers around tumor islands are stained red and all other areas stained yellow [40X].

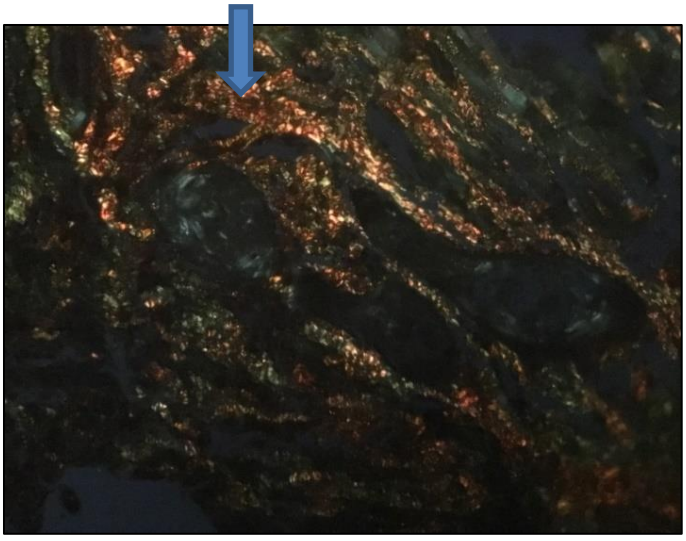


Fig 5-c Picrosirius red stain of WDSCC, under polarized microscopy with collagen fibers around tumor islands appears greenish orange [40X].

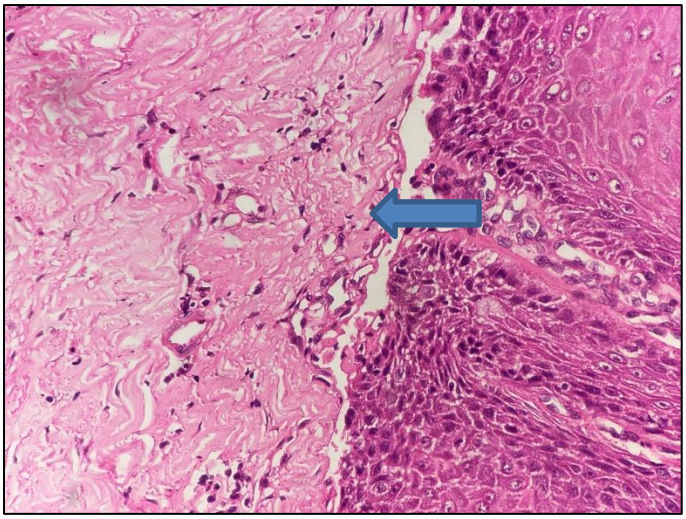


Fig 6-a H & E stain of OSMF, with densely packed collagen fibers subepithelially in the lamina propria [40X].

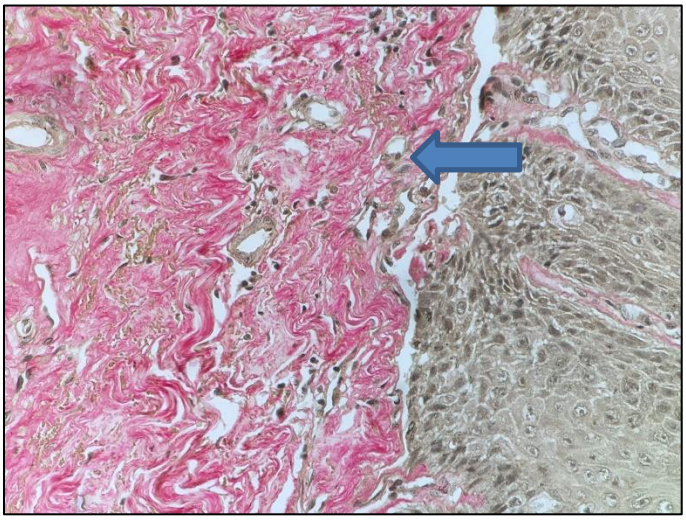


Fig 6-b Picrosirius red stain of OSMF, under light microscopy with densely packed collagen fibers subepithelially in the lamina propria appears red [40X].

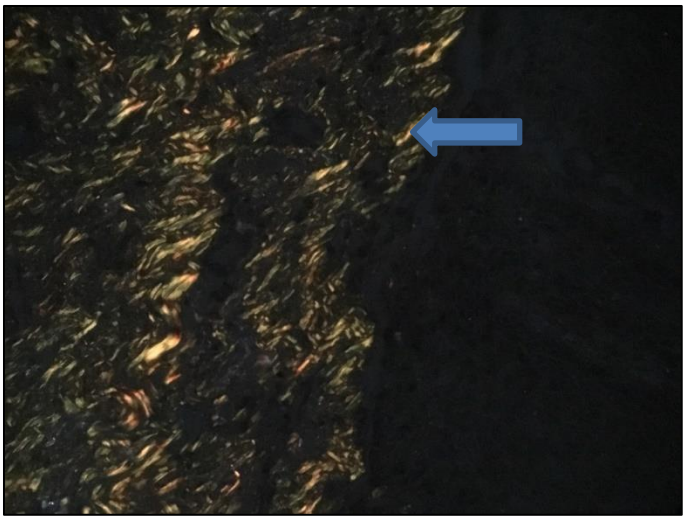


Fig 6-c Picrosirius red stain of OSMF, underpolarised microscopy with densely packed collagen fibers subepithelially in the lamina propria appears greenish yellow [40X].

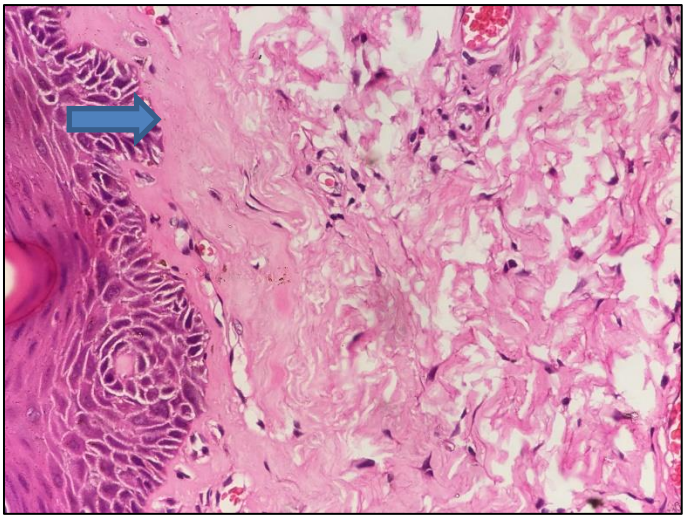


Fig 7-a H & E stain of OSMF, with densely packed collagen fibers subepithelially in the lamina propria [40X].

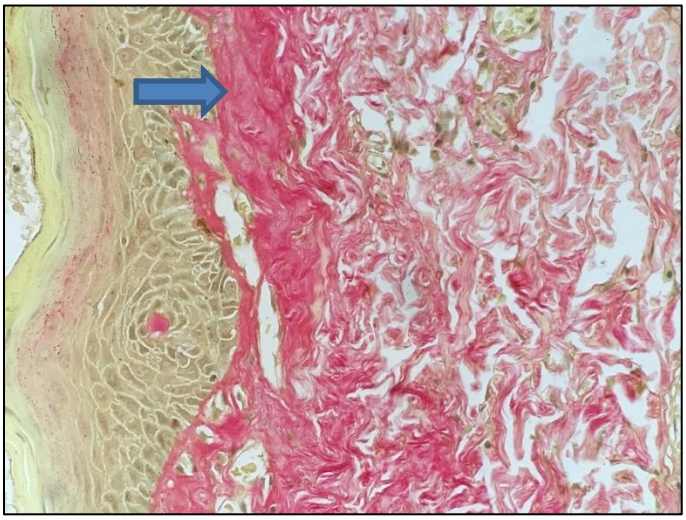


Fig 7-b Picrosirius red stain of OSMF, under light microscopy with densely packed collagen fibers subepithelially in the lamina propria appears red [40X].

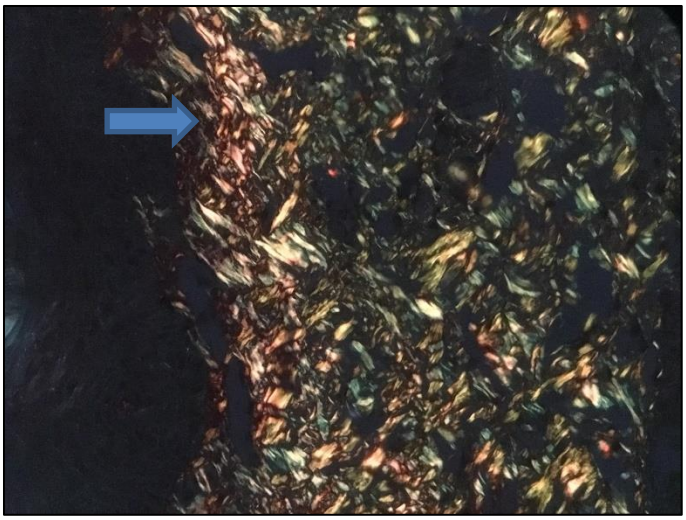


Fig 7-c Picrosirius red stain of OSMF, under polarised microscopy with densely packed collagen fibers subepithelially in the lamina propria appears greenish orange

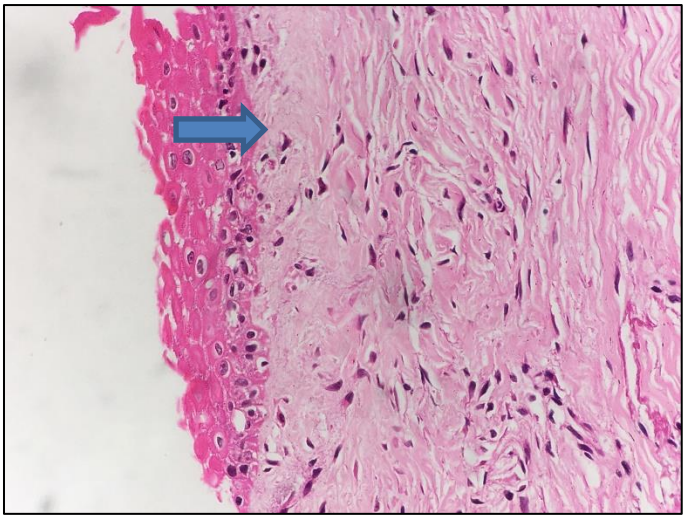


Fig 8-a H & E stain of OSMF, with densely packed collagen fibers subepithelially in the lamina propria [40X].

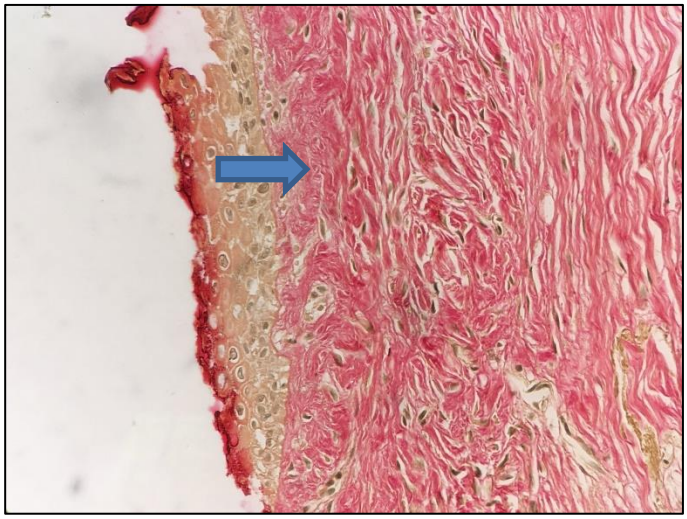


Fig 8-b Picrosirius red stain of OSMF, under light microscopy with densely packed collagen fibers subepithelially in the lamina propria appears red [40X].

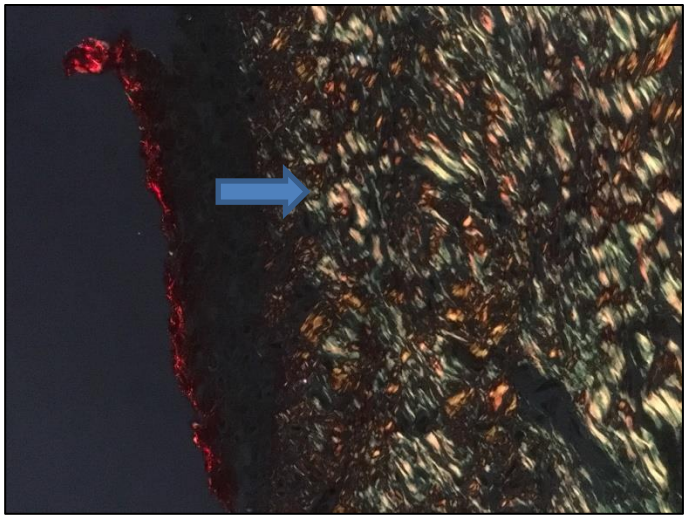


Fig 8-c Picrosirius red stain of OSMF, under polarised microscopy with densely packed collagen fibers subepithelially in the lamina propria appears Green/ Green-orange [40X].

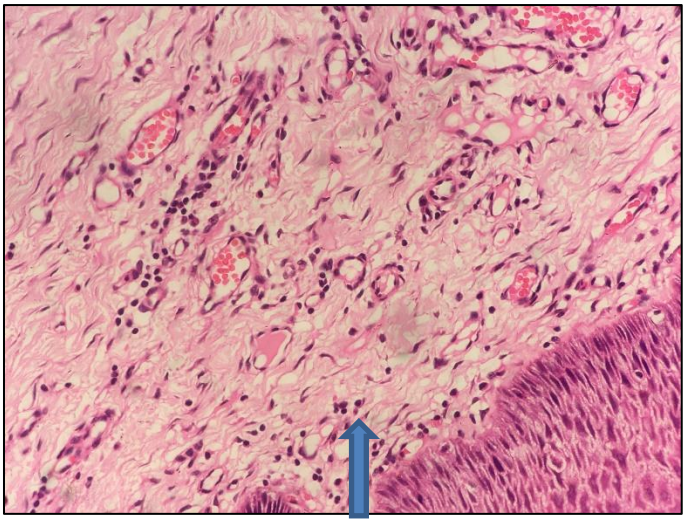


Fig 9-a H & E stain of leukoplakia, with collagen fibers subepithelially in the lamina propria [40X].

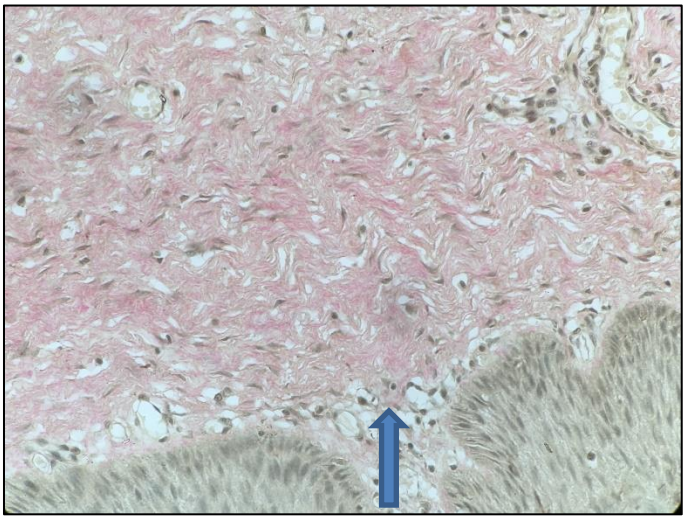


Fig 9-b Picrosirius red stain of Leukoplakia, under light microscopy with collagen fibers subepithelially in the lamina propria appears red [40X].

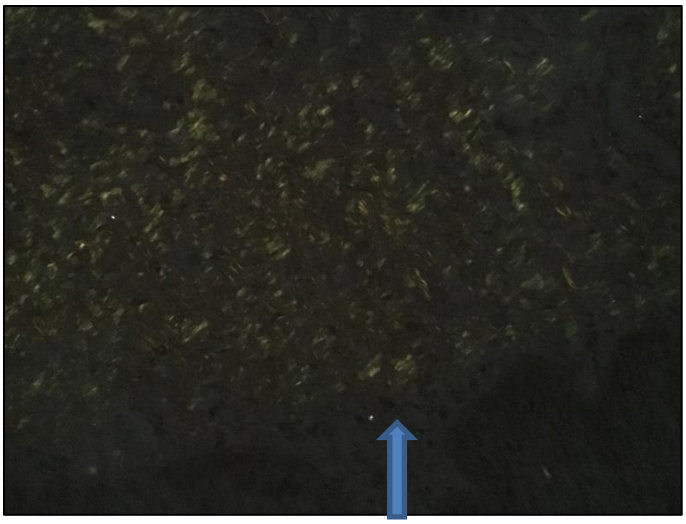


Fig 9-c Picrosirius red stain of Leukoplakia, under polarised microscopy with collagen fibers subepithelially in the lamina propria appears Greenish yellow [40X].

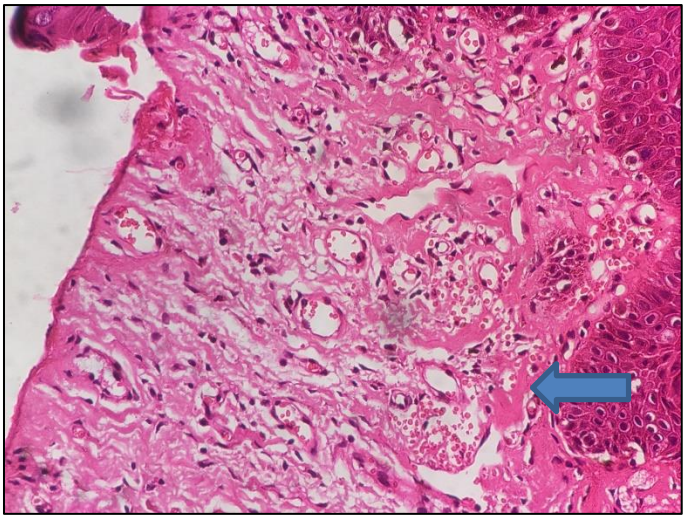


Fig 10-a H & E stain of leukoplakia, with collagen fibers subepithelially in the lamina propria[40X].

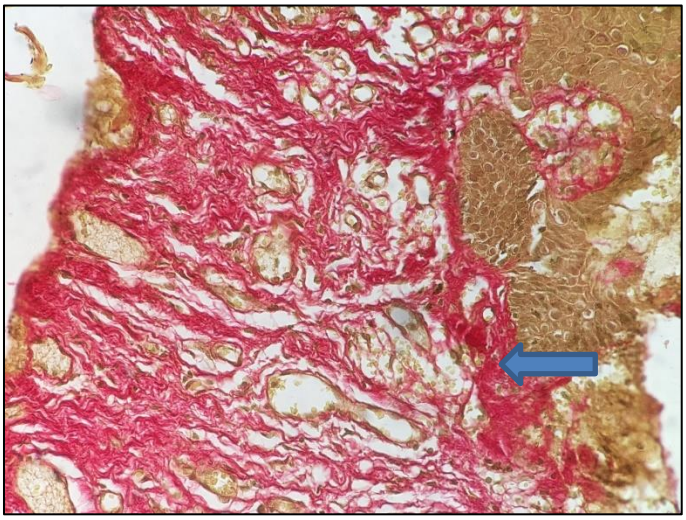


Fig 10-b Picrosirius red stain of Leukoplakia, under light microscopy with collagen fibers subepithelially in the lamina propria appears red [40X].

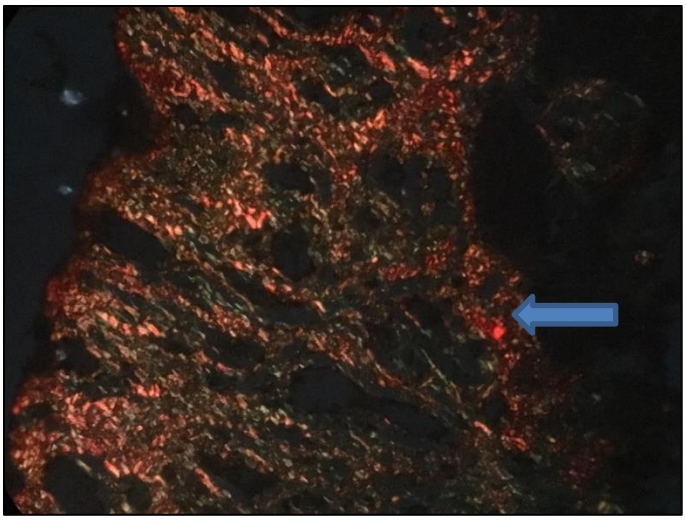


Fig 10-c Picrosirius red stain of Leukoplakia, under polarised microscopy with collagen fibers subepithelially in the lamina propria appears Reddish orange [40X].

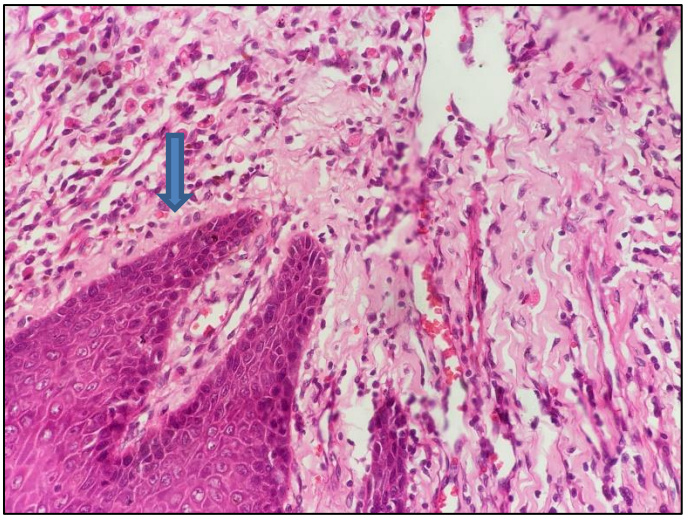


Fig 11-a H & E stain of leukoplakia, with collagen fibers subepithelially in the lamina propria [40X].

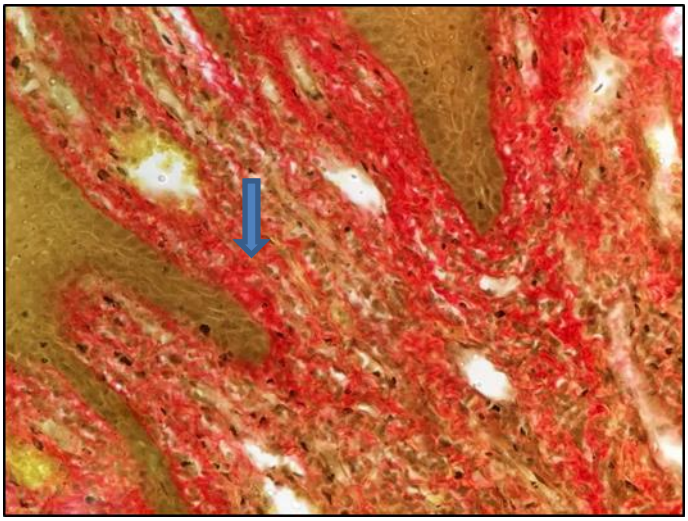


Fig 11-b Picrosirius red stain of Leukoplakia, under light microscopy with collagen fibers subepithelially in the lamina propria appears red [40X].

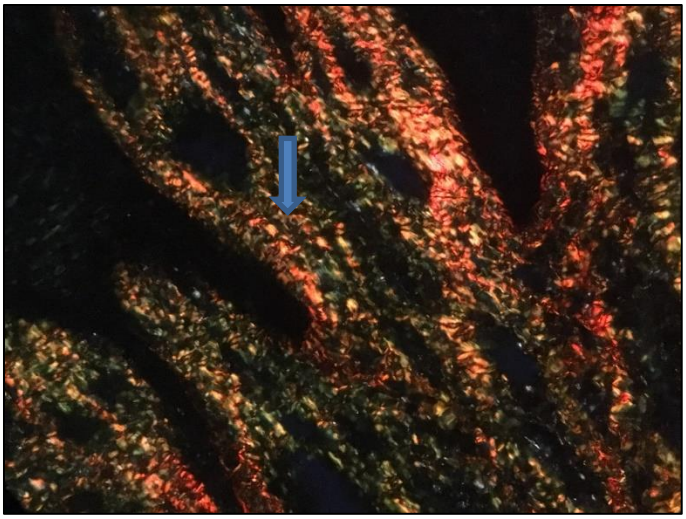


Fig 11-c Picrosirius red stain of Leukoplakia, under polarised microscopy with collagen fibers subepithelially in the lamina propria appears Greenish orange [40X].

Table 1: Under polarizing microscopy the color of picrosirius red stained collagen fibers

Frequency distribution of color

Group	Color	Frequency	Percent
WDSCC	No Color	2	13.3
	Greenish Yellow	10	66.7
	Greenish Orange	3	20
OSMF	Greenish Yellow	5	33.3
	Green /green-orange	4	26.6
	Greenish Orange	6	40
LEUKOPLAKIA	No Color	2	13.3
	Greenish Yellow	11	73.3
	Reddish orange	1	6.6
	Greenish Orange	1	6.6

Group	Color					Total
	No Color	Greenish Yellow	Greenish Orange	Green /green-orange	Yellow Orange	
WDSCC	2	10	1	0	2	15
OSMF	0	5	6	4	0	15
Leukoplakia	2	11	2	0	0	15

FIG 12 POLARISATION COLOR OF COLLAGEN FIBERS IN WDSCC

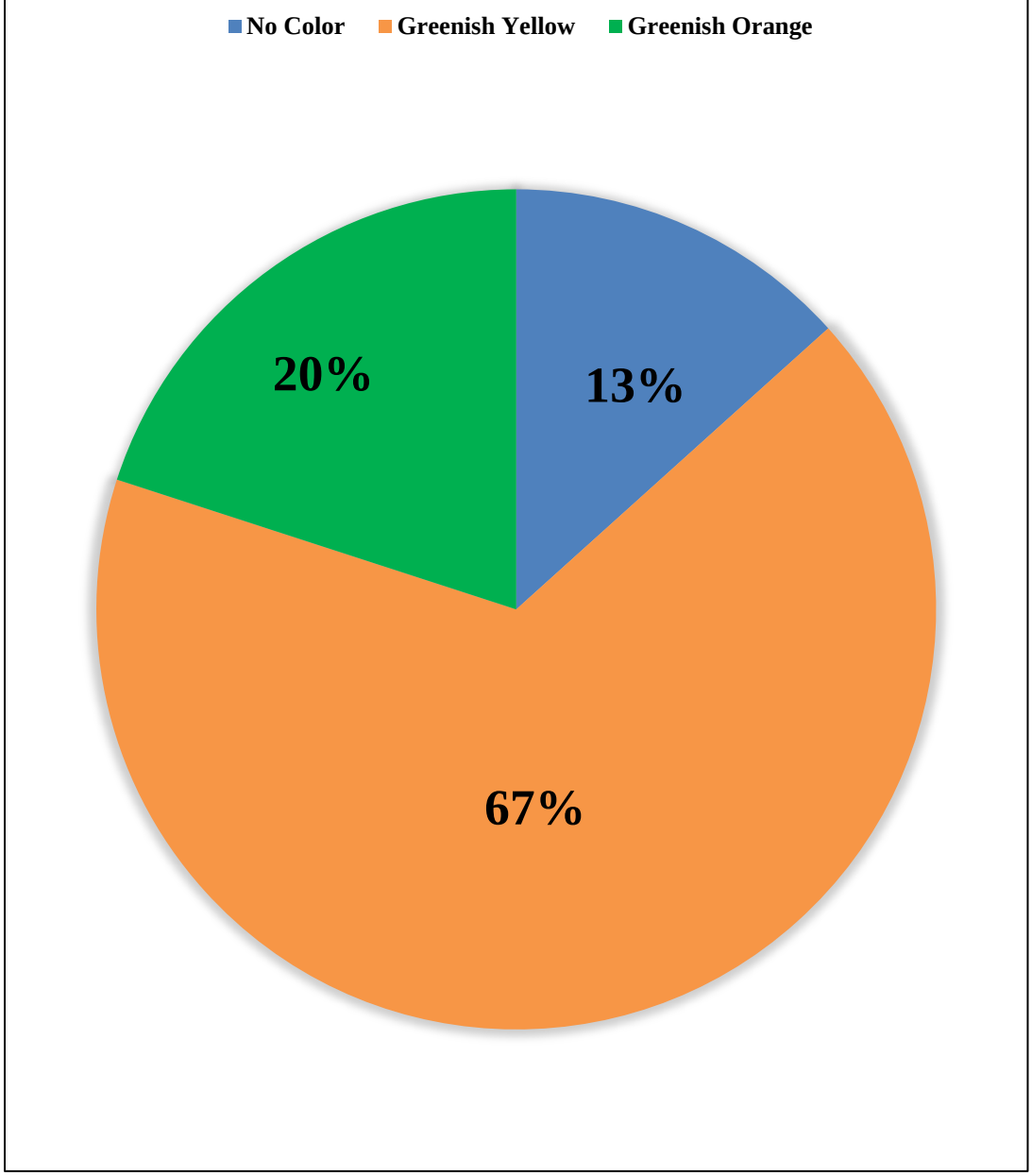


FIG 13 POLARISATION COLOR OF COLLAGEN FIBERS IN OSMF

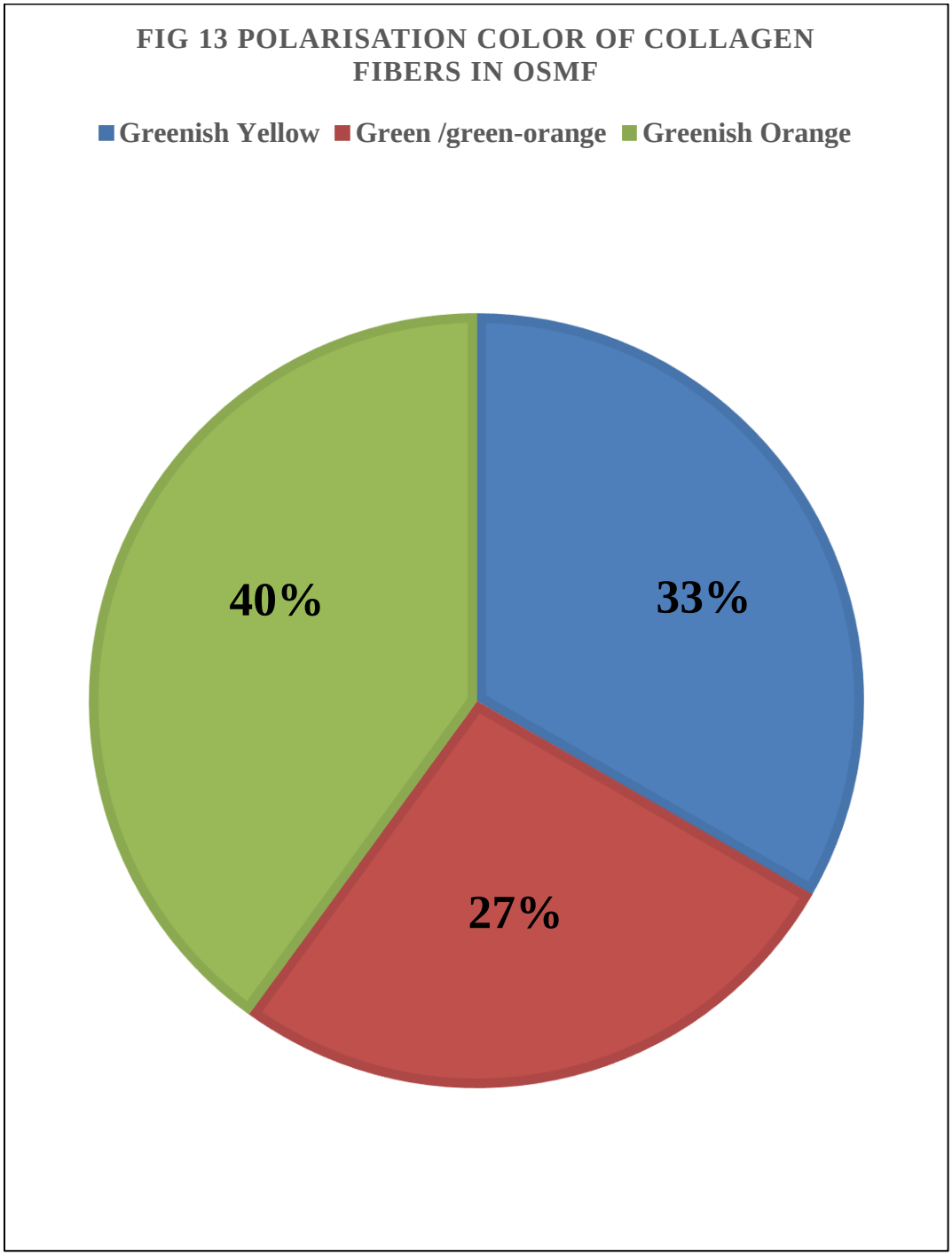
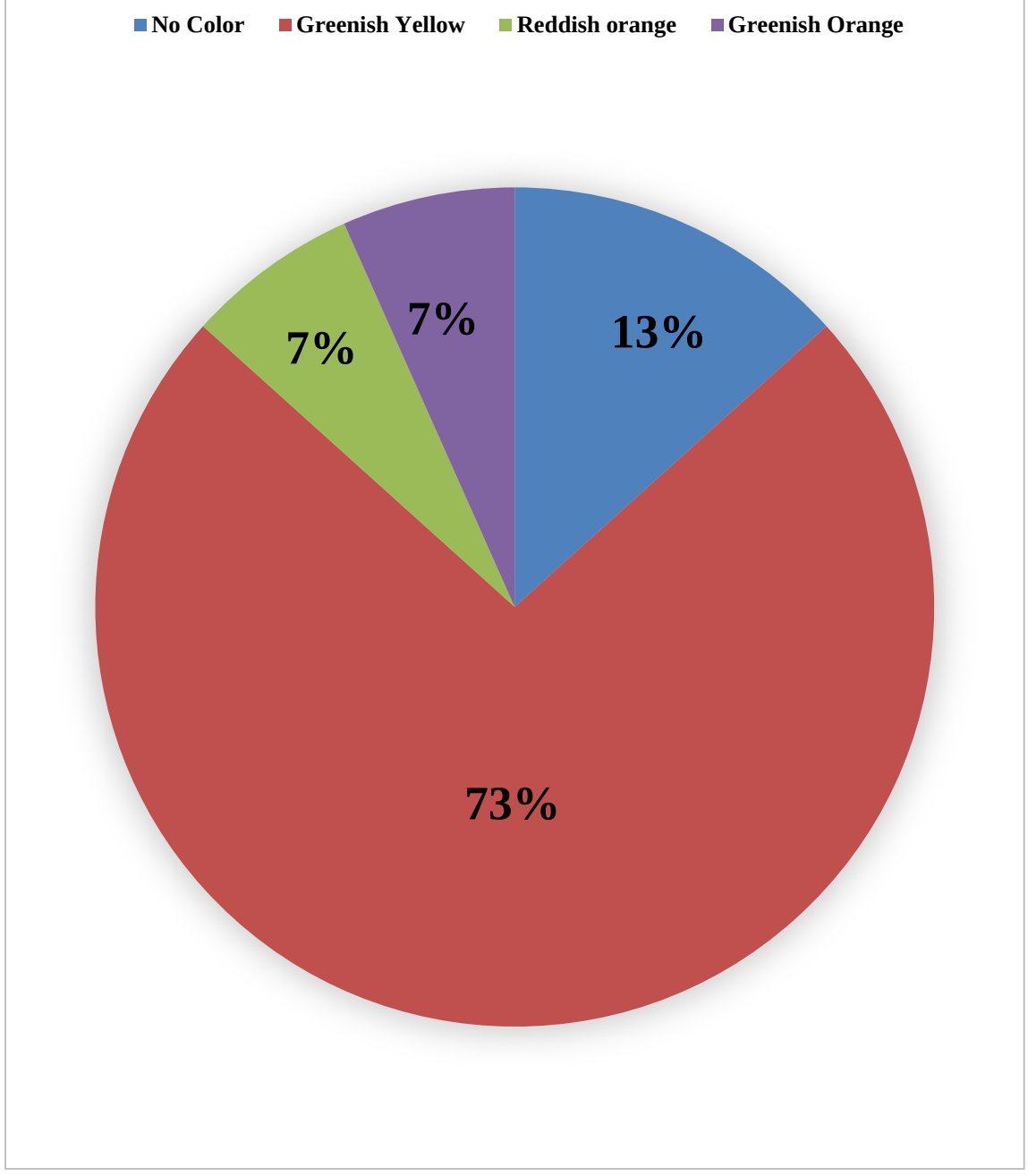


FIG 14 POLARISATION COLOR OF COLLAGEN FIBER IN LEUKOPLAKIA



DISCUSSION

The study comprises formalin fixed, paraffin embedded tissue blocks of 45 cases. The 45 blocks consists 15 cases each of well differentiated squamous cell carcinoma, Oral submucous fibrosis without malignant transformation, and Leukoplakia (clinically) with mild to severe dysplasia were selected. All the case blocks were retrieved from the archives of department of Oral Pathology, TNGDC & H, Chennai. Ethical clearance was obtained. The aim is to evaluate collagen fibers in oral leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma using picrosirius red by polarizing microscopy.

Oral submucous fibrosis is an insidious chronic, potentially malignant disorder affecting any part of oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with juxtaepithelial inflammatory reaction followed by a fibroelastic change of lamina propria with epithelial atrophy leading to stiffness of the oral mucosa, progressively limiting mouth opening and causing inability to eat".[31]The pathogenesis of Oral submucous fibrosis is due to areca nut constituents notably alkaloids, which results in the stimulation of fibroblasts causing increased collagen synthesis. Also the large quantities of tannins and the flavonoid catechin in the areca nut inhibit collagen degradation by collagenase, resulting in accumulation of collagen.[17]. Lysyl oxidase, an extracellular copper enzyme that initiates the cross-linking of collagen is secreted by fibroblasts. The activity of Lysyl oxidase is increased in fibroblast of oral submucous fibrosis.[18]

The increase in amount of collagen fiber bundles results in diminished vascularity leading to atrophy of overlying epithelium, which becomes susceptible to carcinogenic agents. The atrophy of oral epithelium is secondary to connective tissue changes. The

atrophic epithelium first become hyperkeratotic (clinically leukoplakic), later intercellular edema and basal cell hyperplasia develop, finally followed by epithelial atypia with moderate epithelial hyperplasia. Thereafter, carcinoma could develop at any times.[19]

Initial stages of Oral submucous fibrosis are characterized by excessive deposition of collagen fibers and in the advanced stages dense collagen fibers forms zone of hyalinization.[27]

Traditional stains for collagen such as Van Geison and Masson's trichrome, rely on binding of dye molecule to tissue components that lack accurate detection, leading to underestimation of collagen. But picrosirius red stain demonstrated superior results compared to other stains. Collagen fibers are stained intensely with striking birefringence. The stains are more stable and don't fade easily.

Acccording to Junqueira et al.[11] different birefringence color is produced by different types of collagen. According to Dayan et al.[12] not only the thickness of fibers determine the polarization colors, but also the packing of collagens determine the polarization colors of picrosirius red stained sections. While analyzing the collagen fibers in the lamina propria of oral submucous fibrosis under polarizing microscopy, the majority of the collagen showed green to greenish yellow color and there was a shift from orange red to red color. The polarization color of collagen fibers is determined not only by thickness, but also by packing of collagen fibers.[3]

In the present study, in case of oral submucous fibrosis the color of collagen fibers, subepithelially in the lamina propria is analysed. Under polarizing microscopy the color of collagen fibers were greenish yellow in 5 cases. The greenish yellow fibers indicate loosely packed fibers, which is consistent with Ceena et al, [22] Singh et al, [9]

Thakkannavar & Naik.[3] Greenish orange in 6 cases, the orange color indicates tightly packed fibers, which is consistent with Thakkannavar & Naik.[3] Green/ green-orange in 4 cases, the green color indicates loosely packed fibers, the color of collagens are consistent with Thakkannavar & Naik, [3] and Hirshberg et al.[61] According to Hirshberg et al.[61] the normal colors of loosely packed fibers were green to greenish yellow. In case of tightly packed fibers the birefringence is yellow orange through orange to red.

Leukoplakia is a clinical term that implies "white patch". The World Health Organization (WHO) defines leukoplakia as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer". Leukoplakia is diagnosed based on clinical and histopathological diagnosis. The global prevalence of leukoplakia is 1-4% and the rate of malignant transformation is not exceeding 1%.[48] Reactive changes in stroma is an early changes in dysplastic epithelium.[51] Prevention and early diagnosis of leukoplakia is difficult because of lack of clinical exposure among dental practitioner.[48]None of the treatment is proved to be effective in preventing the risk of malignant transformation from leukoplakia.[41]Cessation of habit reduces the risk of malignant transformation not only in case of isolated lesion but also the development of squamous cell carcinoma in the mouth or upper aerodigestive tract.[45]

In the present study, in case of Leukoplakia, the color of collagen fibers in the lamina propria is analysed. Under polarizing microscopy the color of collagen fibers were greenish yellow in 11 cases. The greenish yellow fibers indicate loosely packed fibers, which is consistent with Arora et al.[7] Greenish orange in one case, which is consistent with Arora et al.[7] Reddish orange color in one case which indicates tightly packed

collagen fibers, which is consistent with Kardam et al, [6] Kullage et al, [24] Davendra et al, [56] and Kumari et al.[13] No significant color was produced in 2 cases.

Squamous cell carcinoma is a malignant neoplasm of stratified squamous epithelium that is capable of locally destructive growth and distant metastasis. Oral squamous cell carcinoma is often preceded by presence of both white or red patch known as leukoplakia and eryhtroplakia.[20] Tumors cells exhibit squamous differentiation and varying degree of keratinization. Squamous cell carcinoma may arises de novo from the overlying stratified squamous epithelium or it begins as dysplasia and progress until the dysplasia breach the basement membrane and invades into the connective tissue.[1]

Collagen is one of the major extracellular matrix components and any change or alteration in collagen will alter the biological behavior of tumor.[61]Collagen in the extracellular matrix acts as both barrier and promotes cancer.[24] Hirshberg et al.[61] stated that thin collagen fibers appears green to greenish-yellow under picrosirius red and polarizing microscopy. And also in case of thick normal fibers it appears as yellow orange through orange to red.[61]Tumor cells are capable of degrading the extracellular matrix and modify the extracellular matrix and facilitate tumor cell migration.[2]Because of stromal destruction there is movement of tumor cell towards blood vessel or lymphatic vessels.[55] Different grades of squamous cell carcinoma produce varying birefringence of color around tumor islands. According to van Den Hoff, the difference in birefringrnce is due to action of collagenase secreted by tumor cells on the stroma, abnormal disintegration of matrix by the tumor cells, dedifferntiated tumor cells produce abnormal matrix.[52]

Gopinath et al.[54] and Hirshberg et al.[61] stated that when thickness of collagen fibers was kept constant, the difference in polarizing color is due to packing of the collagen fibers.

In the present study, in case of well differentiated squamous cell carcinoma, the color of collagen fibers around the tumor island is analysed. Under polarizing microscopy the color of collagen fibers were greenish yellow in 10 cases. The greenish yellow fibers indicate loosely packed fibers, which is consistent with Hirshberg et al,[61] Arora et al.[7] Greenish orange in 3 cases, the orange color indicates tightly packed fibers, which is consistent with Gopinath et al,[54] Manjunatha et al,[55] and Kumari et al,[13] Hirshberg et al[61] No significant color was produced in 2 cases.

The limitations of the study include small sample size. The recommendation from the study is to concentrate on the site of lesion, follow-up of the patients and large sample size.

SUMMARY & CONCLUSION

To summarize, oral squamous cell carcinoma is a common malignancy accounting for 50-70% of total cancer mortality in India. Oral squamous cell carcinoma arise either de novo or from oral potentially malignant disorders mainly leukoplakia. The aim of the study is to evaluate collagen fibers in oral leukoplakia, oral submucous fibrosis and oral squamous cell carcinoma using picrosirius red by polarizing microscopy. The study included 15 cases of well differentiated squamous cell carcinoma, 15 cases of oral submucous fibrosis without malignant transformation, and 15 cases of Leukoplakia (clinically) with mild to severe dysplasia. The birefringence color of collagen is evaluated using picrosirius red & polarizing microscopy. In case of well differentiated squamous cell carcinoma, greenish yellow color is observed in 10 cases (66.7%), greenish orange color is observed in 3 cases (20%), and no significant color in 2 cases (13.3%). In case of oral submucous fibrosis, greenish yellow color is observed in 5 cases (33.3%), green/green-orange color is observed in 4 cases (26.6%), and greenish orange color is observed in 6 cases (40%). In case of Leukoplakia, greenish yellow is observed in 11 cases (73.3%), reddish orange is observed in one case (6.6%), greenish orange in one case (6.6%), and no significant color is observed in 2 cases (13.3%).

To conclude**,** tobacco chewing habit is more prevalent in India causing oral squamous cell carcinoma and potentially malignant disorders. Most of the oral squamous cell carcinoma will clinically appear as leukoplakia or erythroplakia. None of the treatment is effective in preventing the malignant transformation from oral potentially malignant disorders. Epithelial dysplasia is one the main indicators for malignant transformation. Before dysplastic changes there are few evidence of change in the

extracellular matrix mainly collagen. Even though many traditional stains are available, picrosirius red is the best one for collagen.

Picrosirius red is the special stain for collagen as it stains even the thin fibers. Under polarizing microscopy green or greenish yellow color indicates loosely packed fibers, whereas reddish orange, greenish orange, and yellowish orange indicates tightly packed collagen fibers. In case of well differentiated squamous cell carcinoma and leukoplakia greenish yellow color is predominant [loosely packed fibers]. In case of oral submucous fibrosis greenish orange color was predominant [tightly packed fibers]. Further studies on large sample size with follow up of the patient is necessary to find the biological behavior of the lesion.

BIBLIOGRAPHY

1. Farah CS, Kujan O, Prime S, and Zain RB. Contemporary Oral Medicine. Springer Nature Switzerland AG. 2019.
2. Patankar SR, Wankhedkar DP, Tripathi NS, Bhatia SN, Sridharan G. Extracellular matrix in oral squamous cell carcinoma: Friend or foe?. Indian J Dent Res 2016; 27(2): 184-189.
3. Thakkannavar SS, Naik VV. Histochemical and immunohistochemical analysis of collagen fibers and microvascular density in various grades of Oral submucous fibrosis. Iran J Pathol 2019; 14(2): 127-134.
4. Scheifele C, Reichart PA. Is there a natural limit of the transformation rate of oral leukoplakia?. Oral Oncology 2003; 39(5): 470–475
5. Neville BW, Day TA. Oral cancer and precancerous lesions. CA Cancer J Clin 2002; 52(4): 195-215
6. Kardam P, Mehendiratta M, Rehani S, Kumra M, Sahay K, Jain K. Stromal fibers in oral squamous cell carcinoma: A possible new prognostic indicator?. J Oral Maxillofac Pathol 2016; 20(3): 405-412.
7. Arora KS, Nayyar A, Kaur P, Arora KS, Goel A, Singh S. Evaluation of Collagen in Leukoplakia, Oral Submucous Fibrosis and Oral Squamous Cell Carcinomas Using Polarizing Microscopy and Immunohistochemistry. Asian Pac J Cancer Prev 2018; 19 (4): 1075-1080.
8. Ganganna K, Shetty P, Shroff SE. Collagen in histologic stages of oral submucous fibrosis: A polarizing microscopic study. J Oral Maxillofac Pathol 2012; 16(2): 162-166.
9. Singh A, Singh S, Saxenac S, Kumarb S, Bangaa A, Purohit N. Polarized microscope assisted study of collagen fibers in Oral Submucous Fibrosis and their clinicopathologic correlation: An in-vitro study. J Oral Biol Craniofac Res 2018; 8(3): 206-211.
10. Rittie L. Methods in molecular biology. Springer. 2017.

11. Junqueira LCU, Cossermelli W, Brentani R. Differential staining of collagens type I, II and III by Sirius Red and polarization microscopy. Arch Histol Jpn 1978; 41(3): 267-274.
12. Dayan D, Hiss Y, Hirshberg A, Bubis JJ, Woiman M. Are the polarization colors of Picrosirius red-stained collagen determined only by the diameter of the fibers?. Histochemistry 1989; 93(1): 27-29.
13. Kumari K, Ghosh S, Patil S, Augustine D, Venkatesiah SS, and Rao RS. Expression of type III collagen correlates with poor prognosis in oral squamous cell carcinoma. J Invest Clin Dent 2016; 8(4): 1–7.
14. Junqueira LCU, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. The Histochemical Journal 1979; 11(4): 447-455.
15. Coleman R. Picrosirius red staining revisited. Acta Histochemica 2011; 113(3): 231–233.
16. Lattouf R et al. Picrosirius red Staining. Journal of Histochemistry & Cytochemistry 2014; 62(10): 751–758.
17. Murti PR, Bhonsle RB, Gupta PC, Daftary DK, Pindborg JJ, Mehta FS. Etiology of oral submucous fibrosis with special reference to the role of areca nut chewing. J Oral Pathol Med 1995; 24(4): 145-52.
18. Ma RH, Tsai CC, Shieh TY. Increased lysyl oxidase activity in fibroblasts cultured from oral submucous fibrosis associated with betel nut chewing in Taiwan. J Oral Pathol Med 1995; 24(9): 407-412.
19. Pindborg JJ, Sirsat SM. Oral submucous fibrosis. Oral Surg Oral Med Oral Pathol 1966; 22(6): 764–779.
20. Sapp JP, Eversole LR, Wysocki GP. Contemporary oral and maxillofacial pathology. Second edition. Mosby. 2004.
21. Tilakaratne WM, Klinikowski MF, Saku T, Peters TJ, Warnakulasuriya S. Oral submucous fibrosis: review on aetiology and pathogenesis. Oral Oncol 2006; 42(6): 561-568.
22. Ceena DE, Bastian TS, Ashok L, Annigeri RG. Comparative study of clinicofunctional staging of oral submucous fibrosis with qualitative analysis of

collagen fibers under polarizing microscopy. Indian J Dent Res 2009; 20(3): 271-276.

23. Smitha BR and Donoghue M. Clinical and histopathological evaluation of collagen fiber orientation in patients with oral submucous fibrosis. J Oral Maxillofac Pathol 2011; 15(2): 154–160.
24. Kullage S, Jose M, Shanbhag VK, Abdulla R. Qualitative analysis of connective tissue stroma in different grades of oral squamous cell carcinoma: A histochemical study. Indian J Dent Res 2017; 28(4): 355-61.
25. Velidandla S, Gaikwad P, Ealla KKR, Bhorgonde KD, Hunsingi P, Kumar A. Histochemical analysis of polarizing colors of collagen using Picrosirius red staining in oral submucous fibrosis. J Int Oral Health 2014; 6(1): 33-38.
26. Modak N, Tamgadge S, Tamgadge A, Bhalerao S. Comparative Study of Clinical Staging of Oral Submucous Fibrosis with Qualitative Analysis of Collagen Fibers Under Polarized Microscopy. Iran J Pathol 2015; 10 (2): 111 – 119.
27. Radhika T, Sekaran P, Narasimhan M. Qualitative Analysis of Collagen Fibers in Oral Submucous Fibrosis using Picrosirius red Stain and Polarising Microscope. J Clin Diagn Res 2016; 10(2): 4-7.
28. Reshma V, Varsha BK, Rakesh P, Radhika MB, Soumya M, D'Mello S. Aggrandizing oral submucous fibrosis grading using an adjunct special stain: A pilot study. J Oral Maxillofac Pathol 2016; 20(1): 36-46.
29. Arkeri G et al. Oral submucous fibrosis: an update on pathophysiology of malignant transformation. J Oral Pathol Med 2017; 46(6): 413-417.
30. Shih YH, Wang TH, Shieh TM, Tseng YH. Oral Submucous Fibrosis: A Review on Etiopathogenesis, Diagnosis, and Therapy. Int J Mol Sci. 2019; 20(12): 1-22
31. Sharma M, Radhakrishnan R. Revisiting and revising the definition of oral submucous fibrosis. Oral Oncol 2019; 92: 94.
32. Avinash Tejasvi ML, Anulekha CK, Afroze MM, Shenai KP, Chatra L, Bhayya H. A correlation between oral mucosal lesions and various quid-chewing habit patterns: A cross-sectional study. J Can Res Ther 2019; 15(3): 620-624.

33. Nishat R, Kumar H. Collagen fibers in oral submucous fibrosis – A polarizing microscopy study using two special stains. Indian J Pathol Microbiol 2019; 62(4): 537-543.
34. Silverman JRS, Gorsky M, Lozada F. Leukoplakia and Malignant Transformation a Follow-Up Study of 257 Patients. Cancer. 1984; 53(3): 563-568.
35. Lumerman H, Freedman P, Kerpel S. Oral epithelial dysplasia and the development of invasive squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1995; 79(3): 321-329.
36. Van der Waal I, Schepman KP, Van der Meij EH, Smeele LE. Oral Leukoplakia: a Clinicopathological Review. Oral Oncol. 1997; 33(5): 291-301.
37. Sciubba JJ. Oral Cancer the Importance of Early Diagnosis and Treatment. Am J Clin Dermatol 2001; 2 (4): 239-251.
38. Petti S. Pooled estimate of world leukoplakia prevalence: a systematic review. Oral Oncology 2003; 39(8): 770–780.
39. Holmstrup P, Vedtofte P, Reibel J, Stoltze K. Long-term treatment outcome of oral premalignant lesions. Oral Oncol. 2006; 42(5): 461–474.
40. Warnakulasuriya S, Johnson NW, Van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med. 2007; 36(10): 575-580.
41. Lodi G, Porter S. Management of potentially malignant disorders: evidence and critique. J Oral Pathol Med 2008; 37(2): 63–69.
42. Lodi G, Sardella A, Bez C, Demarosi F, Carrassi A. Interventions for treating oral leukoplakia (Review). Cochrane Database Syst Rev. 2006; (4): 1-29.
43. Liu W, Shi L-J, Wu L, Feng J-Q, Yang X, Li J. oral cancer development in patients with leukoplakia-clinicopathological factors affecting outcome. PLos ONE 2012; 7(4): 1-7.
44. Ho et al. The clinical determinants of malignant transformation in oral epithelial dysplasia. Oral Oncol 2012; 48(10): 969–976.
45. Van der Waal I. Oral potentially malignant disorders: Is malignant transformation predictable and preventable?. Med Oral Patol Oral Cir Bucal. 2014; 19 (4): 386-390.

46. Warnakulasuriya S, Ariyawardana A. Malignant transformation of oral leukoplakia: a systematic review of observational studies. J Oral Pathol Med 2016; 45(3): 155–166.
47. Bewley AF, Farwell DG. Oral Leukoplakia and Oral Cavity Squamous Cell Carcinoma. Clin Dermatol. 2017; 35(5): 461-467.
48. Greenslade R. Interventions for treating oral leukoplakia to prevent oral cancer. Nurs Stand. 2017; 31(39): 42-43.
49. Ganesh et al. Potentially Malignant Oral Disorders and Cancer Transformation. Anticancer Res. 2018; 38(6): 3223-3229.
50. Chaturvedi AK et al. Oral leukoplakia and risk of progression to oral cancer: A population-based cohort study. J Natl Cancer Inst. 2019; 20:1-30.
51. Farah CS, Fox SA. Dysplastic oral leukoplakia is molecularly distinct from leukoplakia without dysplasia. Oral Dis. 2019; 25(7): 1715–1723.
52. Van den Hooff A. Stromal Involvement in Malignant Growth. Adv Cancer Res. 1988; 50: 159-196.
53. George J, Narang RS, Rao NN. Stromal response in different histological grades of oral squamous cell carcinoma: A histochemical study. Indian J Dent Res 2012; 23(6): 842.
54. Gopinathan PA, Kokila G, Jyothi M, Ananjan C, Pradeep L, Nazir SH. Study of Collagen Birefringence in Different Grades of Oral Squamous Cell Carcinoma Using Picrosirius red and Polarized Light Microscopy. Scientifica (Cairo). 2015; 2015: 1-7.
55. Manjunatha BS, Agrawal A, Shah V. Histopathological evaluation of collagen fibers using picrosirius red stain and polarizing microscopy in oral squamous cell carcinoma. J Can Res Ther 2015; 11(2): 272-276.
56. Devendra A, Niranjan KC, Swetha A, Kaveri H. Histochemical analysis of collagen reorganization at the invasive front of oral squamous cell carcinoma tumors. J Invest Clin Dent. 2018; 9(1): 1-8.
57. Junqueira LCU, Assls FIGUEIREDO MT, Torloni H, Montes GS. DIFFERENTIAL HISTOLOGIC DIAGNOSIS OF OSTEOID. A Study on

Human Osteosarcoma Collagen by the Histochemical Picrosirius-Polarization. Method. J Pathol. 1986; 148(2): 189-196.

58. Hirshberg A, Buchner A, Dayan D. The central odontogenic fibroma and the hyperplastic dental follicle: study with Picrosirius red and polarizing microscopy. J Oral Pathol Med. 1996; 25(3): 125-127.
59. Kauppila S, Stenbäck F, Risteli J, Jukkola A, RISTELI L. Aberrant type i and type iii collagen gene expression in human breast cancer in vivo. J Pathol. 1998; 186(3): 262-268.
60. Stenback F et al. The extracellular matrix in skin tumor development a morphological study. J Cutan Pathol. 1999; 26(7): 327-38.
61. Hirshberg A, Sherman S, Buchner A, Dayan D. Collagen fibres in the wall of odontogenic keratocysts: a study with picrosirius red and polarizing microscopy. J Oral Pathol Med 1999; 28(9): 410-412.
62. Koren et al. Capsular collagen staining of follicular thyroid neoplasm by Picrosirius red:role in differential diagnosis. Acta histochem 2001; 103(2):151-157.
63. Allon I, Vered M, Buchner A, Dayan D. Stromal difference in salivary gland tumors of a common histopathogenesis but with different biological behavior: A study with Picrosirius red and polarisisng microscopy. Acta histochemica 2006; 108: 259-264.
64. Singh HP, Shetty DC, Wadhwan V, Aggarwal P. A quantitative and qualitative comparative analysis of collagen fibers to determine the role of connective tissue stroma on biological behavior ofodontogenic cysts: A histochemical study. Natl J Maxillofac Surg. 2012; 3(1): 15–20.
65. Datar UV, Bhavna CM, Hallikerimath S, Angadi p, Kale A, Mane D. Clinicopathological study of a series of giant cell fibroma using Picrosirius red polarizing microscopy technique. Arch Iran Med. 2014; 17(11): 746 – 749.
66. Jahagirdar PB, Kale AD, Hallikerimath S. Stromal characterization and comparison of odontogenic cysts and odontogenic tumors using picrosirius red stain and polarizing microscopy: A retrospective and histochemical study. Indian J Cancer 2015; 52(3): 408-412.

67. Raj et al. Evaluation of the Nature of Collagen Fibers in KCOT, Dentigerous Cyst and Ameloblastoma using Picrosirius red Stain – A Comparative Study. JCDR 2015; 9(11): 1-4.
68. Varghese SS et al. Evaluation and Comparison of the Biopathology of Collagen and Inflammation in the Extracellular Matrix of Oral Epithelial Dysplasias and Inflammatory Fibrous Hyperplasia Using Picrosirius red Stain and Polarising Microscopy: A Preliminary Study. J Cancer Prev 2015; 20(4): 275-280.
69. Charan Gowda BK, Kokila G, Gopinathan PA, Praveen KS. Picrosirius red and Polarization Microscopy – A Tool for Gender Differentiation. JCDR 2017; 11(1): 107-109.
70. Peddapelli K, Rao V, Kumar MP, Sravya T, Rakesh D. Analysis of collagen fibers in keratocystic odontogenic tumor and ameloblastoma: A polarizing microscopic study. Indian J Dent Res 2019; 30(5): 731-735.

ANNEXURE

CHRONOLOGY OF THESIS ACTIVITY LOG

S. NO	DATE	WORK DONE
1	31/10/2017	Topic selected
2	31/10/2017	Topic approval by Dr. I. Ponniah and mailed the study protocol to IEC
3	01/12/2017	Received IRB clearance certificate
4	24/2/2018	Showed review of literature to Dr. I. Ponniah
5	19/4/2019	Showed pilot study slides to Dr. I. Ponniah
6	22/6/2019	Showed dissertation slides to Dr. I. Ponniah
7	26/6/2019	Discussed with Dr. I. Ponniah about photos of polarizing microscope
8	28/6/2019	Showed photomicrograph of dissertation slides to Dr. I. Ponniah
9	29/6/2019	Showed photomicrograph of dissertation slides to Dr. I. Ponniah
10	01/7/2019	Showed photomicrograph of dissertation slides to Dr. I. Ponniah
11	02/7/2019	Showed photomicrograph of dissertation slides to Dr. I. Ponniah
12	04/7/2019	Showed photomicrograph of dissertation slides to Dr. I. Ponniah
13	04/9/2019	Showed dissertation slides to Dr. I. Ponniah
14	10/1/2020	Submitted thesis dummy copy to Dr. I. Ponniah

Signature of the candidate Signature of the HOD