

An Evaluation of the Prognosticative Value of Hyalinization in the Biological Behaviour of Oral Lesions Using Image Analysis

S Samyukta*, Harini Priya AHR, Sathish Muthu Kumar, Premika Sri V L

Abstract

Objective: To evaluate the efficacy of artificial intelligence-based analysis of polychromatic staining in oral premalignant and malignant lesions and to predict their biological behavior. The study also aims to evaluate the prognostic value of collagen in these oral lesions. **Methods:** In this study, a total of 45 histopathologically diagnosed normal (15), Premalignant lesions (n=15), and oral squamous cell carcinoma (n=15) were included. The tissue sections were subjected to routine Hematoxylin and Eosin (H and E) staining and a differential staining technique- Herovici's stain. The stained slides were viewed under 10x magnification in the microscope and images of these sections were captured. The images were labelled, transferred and stored in the computer for image analysis. The collagen content in the photomicrographs were analysed using Image J software. The results were tabulated and subjected to Kruskal- Wallis test using the SPSS software. **Results:** A significant increase in the amount of type III (blue stained) collagen fibers, compared to type I collagen fibers, was seen as the lesion progressed from premalignant disorders to oral squamous cell carcinoma. Normal mucosa showed predominantly type I (red stained) collagen fibers. The difference in the ratio of type I and III collagen fibers between the three groups was found to be statistically significant (P= 0.00). **Conclusion:** The study concluded that a significant change in stromal collagen composition exists, with an increase in the amount of type III collagen, that can be correlated to the lesion's progression from premalignant to oral squamous cell carcinoma. Differential staining is an inexpensive and highly reproducible method of evaluating the composition of the stroma and using Image analysis to carry out this analysis makes the process more objective and renders it bias free.

Keywords: Dysplasia- image analysis- collagen- differential stain

Asian Pac J Cancer Prev, 23 (8), 2829-2834

Introduction

In India, oral cancer is a major public health concern, where it is one of the country's top three cancers (Elango et al., 2006). Amongst the various types of oral cancer, oral squamous cell carcinoma (OSCC) is the most common making up almost 80% of all cancers in the oral cavity. OSCC has a high age-adjusted rate in India, at 20 per 100,000 people, and accounts for more than 30% of all cancers in the country (Sankaranarayanan et al., 2005). Early detection of OSCC decreases the mortality rate for the disease to 80%-90% (Wang et al., 2014). As most of the OSCC cases are asymptomatic in the early stages, timely identification of these lesions requires vigilant screening of high-risk individuals such as tobacco users, alcoholics, immunocompromised individuals, for the presence and progression of any potentially malignant disorders (PMD). Interventions provided to individuals at the stage of PMDs, prevents the development of OSCC and decreases the morbidity and mortality rates of the individual. In line with the principals of early detection, several studies

have been focused on identifying features that predict the transformation of a lesion from dysplasia to neoplasia. It is established that the development and differentiation of neoplastic tissue requires coordinated cross-talk between the cells of the epithelium and mesenchyme (Desmoulière et al., 2004). The stromal components not only act as a nutrient source for the tumor cells but also deter the progression of malignant cells. Proteolytic remodelling of the extracellular matrix promotes tumour growth by facilitating tumour cell invasion, which leads to changes in the collagenous stroma (Rich et al., 2005).

Histopathology is the gold standard for obtaining important prognostic information, such as, dysplasia and cancer grade, that can help clinicians make treatment decisions. The lack of agreement among various observing pathologists regarding the subjective aspects of histological grading, and may not provide adequate risk stratification or management advice (Warnakulasuriya et al., 2008; Lumerman et al., 1995; Kujan et al., 2007). This coupled with the limited availability of skilled pathologists in the more rural regions of the country has

further emphasized the importance of utilizing newer methods and technologies for a more quantitative, consistent, and accurate diagnosis in order to aid clinical decision-making, improving head and neck carcinoma (HNC) patient survival and reducing the disease burden on the health care system.

Computer assisted diagnostics is one of the major researches focuses in the field of histopathology recently, owing to the advances in high-throughput tissue bank and archiving of digitized histological slides. Of this, image analysis has been an area of extensive study in cancer diagnostics research (Reyes-Aldasoro et al., 2013). It is based on digital image analysis and processing, which entails extracting useful information from images in order to delineate clinically important characteristics (segmentation) or classify lesions (Litjens et al., 2017; Shen et al., 2017). By explicitly describing a prior set of features and processing processes, a number of ad hoc feature analysis-based machine learning (ML) techniques have been proved to be successful in various diagnostic applications (Panayides et al., 2020).

The value of quantitative analysis of pathology images has been acknowledged by researchers in both the image analysis and pathology domains. The aim of the study was to evaluate the efficacy of a novel image analysis-based method to predict the biological behavior of oral premalignant and malignant lesions based on the ratio of collagen staining in the oral mucosal lesions.

Materials and Methods

In this hospital based, retrospective study, a total of 45 histopathologically diagnosed sections under three categories- Normal tissues obtained from the pericoronal region associated with impacted third molars (n=15), Premalignant lesions (n=15), Oral squamous cell carcinoma (n=15) were included. The paraffin- embedded tissue blocks were retrieved from the archives. Sections of 5µm thickness were made and mounted on the glass slide. The slides were deparaffinized, rehydrated and subjected to routine H and E staining and a differential staining technique- Herovici's stain.

Herovici's staining technique

The stain was prepared as a mixture of 0.1% w/v acid fuchsin in picric acid and 0.05% w/v methyl blue (sigma- Aldrich) in 1% v/v acetic acid solutions at a 2:1 ratio. The slides were incubated in this solution for 5 minutes and then differentiated in 1% acetic acid for 2 minutes, followed by a 5-minute wash in 100% ethanol. The slides were then dehydrated through alcohol and mounted with DPX.

The stained slides were viewed under 10x magnification in the microscope and images were captured using 2MP camera attached to the microscope. The images were classified, transferred and stored in the computer for image analysis.

Image analysis

The Image J software was used for the quantification of collagen fibers in the histological slides. The image

analysis was carried out in 4 steps- Image acquisition, Scale setting, Deconvolution and Quantification. The stained slides were viewed under 10x magnification and images were captured using a 2MP camera attached to the microscope. All photos were saved in a 24-bit RGB tagged image file format (tiff) with a resolution of 640 480 pixels. To set the scale for the analysis, The known distance of the scale bar was first measured using the "straight line" tool in the "analyze" menu. The value was then entered into the "known distance" and corresponding unit of µm was set in the "Unit of length" box under the set scale menu. The images were split into the red, blue and green components by performing an orthonormal transformation of the it's RGB information (Katsuda et al., 1992). The uploaded images were converted to RGB by selected "RGB color" option in the "Type" box under the "Image" menu (Figure 1). Following this, the converted images can be separated into the differently stained components by entering the "Image" menu and selecting the "Color deconvolution2" command (Figures 2 and 3). To measure the area covered by the blue fibers the area covered by the fibers were first delineated using the "threshold" command in the "Adjust" box under the "Image" menu (Figure 4). The selected area was manually adjusted until the complete are covered by the blue fibers were selected. Following which the area to be measured was set under the "Analyze" menu in the "Set measurement" tool by clicking on "Area" and "Limit to threshold". The results would now be displayed in the results window by simply clicking on the "measurement" function under the "Analyze" menu (Figure 5). The same procedure is repeated the measure the area covered by the Type I collage fibers that are stained red in color (Chen et al., 2017).

Statistical analysis

Data was then statistically analyzed using Kruskal-Wallis test with the SPSS software. A value of $P < 0.05$ was considered statistically significant.

Results

In the present study, diagnosis based on the routine H and E-stained slides revealed that of the 15 cases categorized as premalignant lesions, 6 cases were diagnosed as mild dysplasia, 5 cases as moderate dysplasia, 4 cases were severe dysplasia. Under the oral squamous cell carcinoma category, 9, 4 and 2 cases were categorized as well differentiated squamous cell carcinoma, moderately differentiated squamous cell carcinoma and poorly differentiated squamous cell

Table 1. Kruskal- Wallis Test of Significance for the amount of Type I and Type III Collagen in Premalignant and OSCC Group

	Test Statistics ^{a,b}	
	Collagen Type I	Collagen Type III
Chi-Square	23.978	30.428
df	2	2
Asymp. Sig.	0	0

† Kruskal-Wallis ANOVA, two sided P.value<0.05

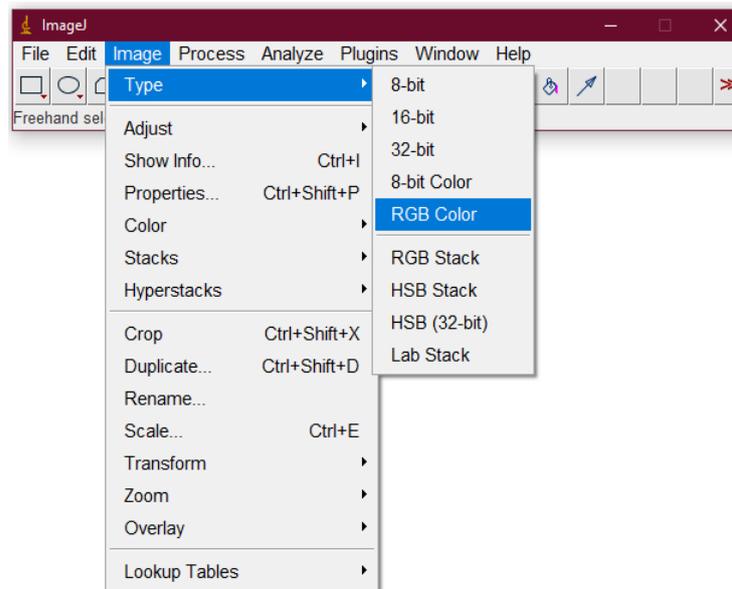


Figure 1. RGB Colour Space Conversion of Images on Image J Software

carcinoma respectively, based on their H and E slides. 15 normal H and E slides were taken as control.

The control tissues showed predominantly red staining of the collagen fibers indicative of type I collagen. All the grades of dysplasia in the premalignant group showed a slight shift in the ratio between the type I (red stain) and

type III (blue stain) collagen fibers, with type I still being more in quantity than type III. Analysis of the OSCC group showed a marked change in the distribution of the collagen fibers with the sections exhibiting predominantly blue staining of the collagen fibers (Figure 6). The quantification of the type I and type III collagen fibers

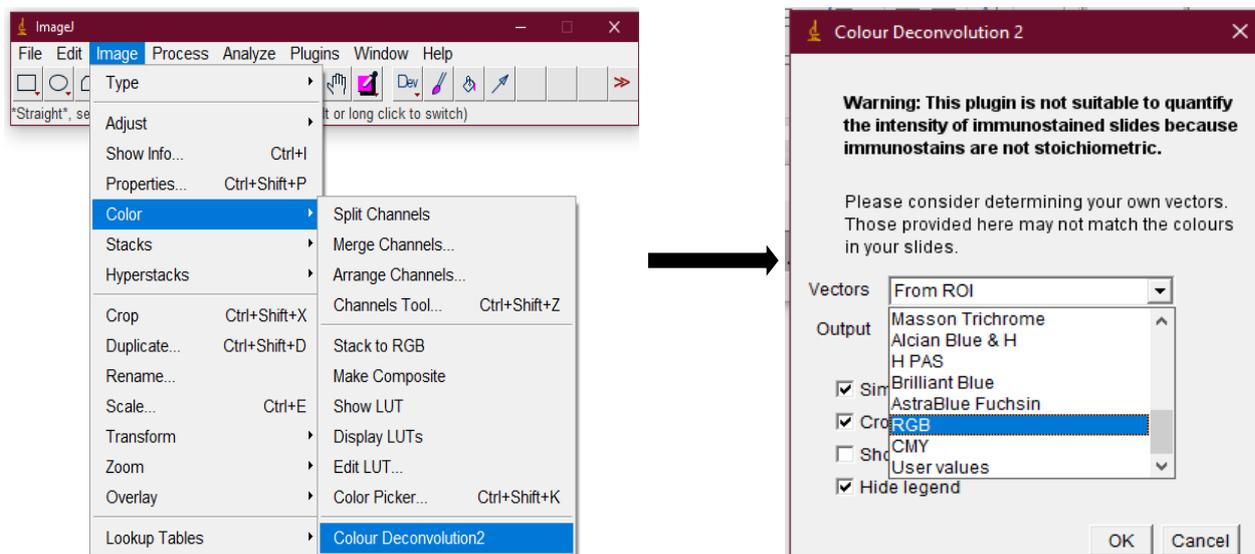


Figure 2. Colour Deconvolution Plugin

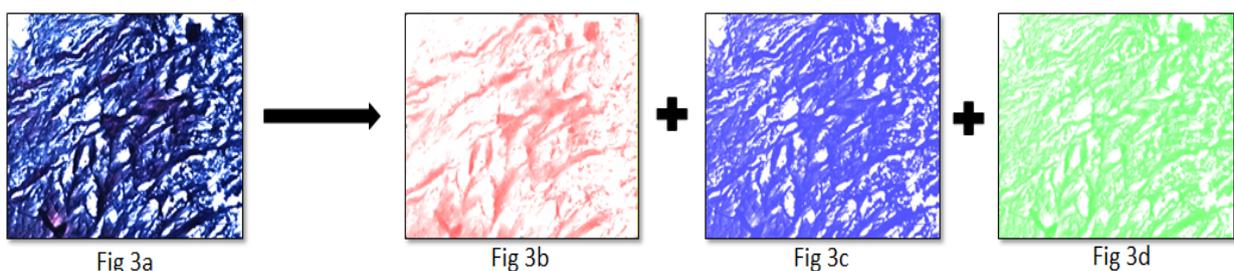


Figure 3. a, Polychrome stained section of moderately differentiated SCC showing differential staining of collagen fibres; b,c&d, Red, blue and green components of 3a following colour deconvolution

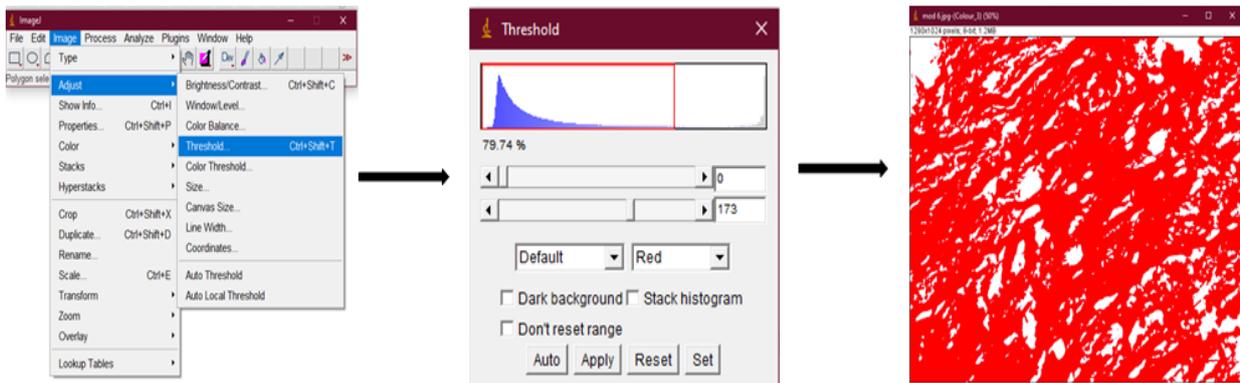


Figure 4. Threshold Value Adjustment for the Blue Stained Collagen Fibres in MDSCC 3a

present in both the groups and subsequent statistical analysis via Kruskal- Wallis test, a significant difference was noted in the distribution of type I and type III collagen fibers between the premalignant and the oral squamous cell carcinoma group ($P < 0.05$) (Table 1).

Discussion

Epithelial mesenchymal transition (EMT) has also been implicated as a major player in the progression

and metastatic transformation of oral squamous cell carcinoma. The extracellular matrix is important for tissue growth, regulation, differentiation, and structure. The stromal cells can retain control over cell growth, function, and response to wounds and other pathologic situations by modifying the ECM (Shetty et al., 2015). The mechanical quality of the stroma is largely determined by its collagen concentration, and this is considered a major barrier to be crossed during invasion, allowing infiltrating cell mass to enter (van den Hooff et al., 1988). The content of the

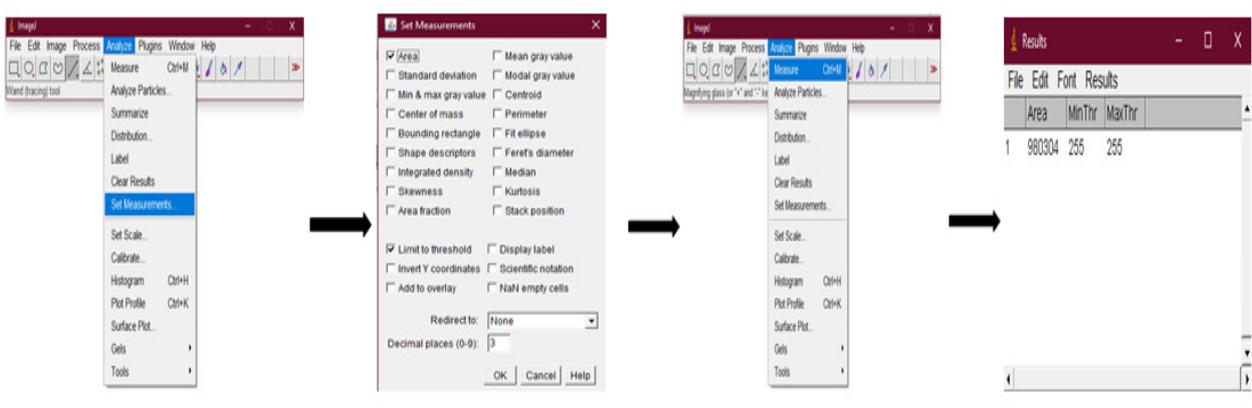


Figure 5. Measurement of Area Covered by Fibres in the Region of Interest.

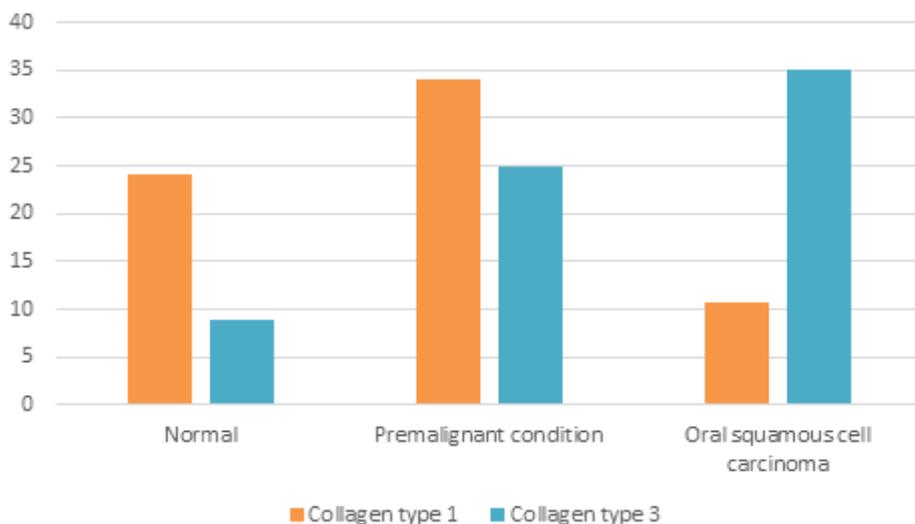


Figure 6. Distribution of Collagen Type I and Type III in Normal, PMD and OSCC Groups.

stroma varies from one condition to another. Under normal conditions; collagen type I, composed of mature, thick and closely packed fibers makes up 90% of the stroma whereas type III collagen, a more immature, thin fibrillar variant occupies 8-10% (Berkovitz et al., 2009; Montes et al., 1991). Inflammation is promoted by dysplastic epithelial cells, which produce growth factors, hormones, cytokines, and proteinases such as MMP leading to a change in the stromal characteristics (Aggarwal et al., 2006). This study aims to quantitatively analyze the change in the stroma, particularly the collagen content, in both dysplastic and carcinomatous lesions.

Many differential staining techniques are available to evaluate, detect, and measure stromal tissue and collagen. Traditional stains for detecting collagen, such as the Masson trichrome, Van Gieson techniques and Picrosirius red, have their own drawbacks. They have inherent flaws, such as a lack of specificity for thin collagen strands or require specialized equipment such as polarized microscopes for their viewing. In the present study, we used the Herovici's polychrome staining technique, that stains both mature and immature collagen fibers red and blue respectively. This eliminates the need for any expensive, specialized technique or equipment for the purpose of collagen fiber analysis.

Though histopathology remains the gold standard for the diagnosis of lesions, it is wrought with inter and intra- observer variability. Artificial intelligence in the form of image analysis helps us quantify the subjective components in histopathologic diagnosis. In the present study, we quantified the amount of type I and type III collagen in the stroma using Image J software and compared the contents in both premalignant lesions and oral squamous cell carcinoma lesions. The variation of stromal composition in regards to the type of collagen was statistically significant with oral squamous cell carcinoma showing predominantly type III collagen. Premalignant lesions show comparatively lesser amount of type I collagen but still more than type III, than the normal tissues. The results obtained in the current study has been able to quantitatively prove the subjective conclusions drawn in several previous studies regarding the change in stromal collagen composition (Fuentes et al., 2012; Venigella et al., 2010; Yokoyama et al., 2003; Arora et al., 2018; Varghese et al., 2015).

The mechanism behind this change in collagen formation is still unclear. A few studies suggest that the state of hypoxia seen during the transition from epithelial dysplasia to oral squamous cell carcinoma causes genetic instability and increases angiogenesis, thereby making the stroma edematous and unstable. Following this the carcinoma cells change collagen by producing Carcinoma Associated Fibroblasts (CAFs) and increasing collagenolytic enzyme activity as the cancer advances. The generation of altered collagen is aided by the changed fibroblast phenotype (Daley et al., 2008). Another study stated that chronic inflammation linked to dysplasia could be one of the causes of collagen fiber disorganization and loose packing. The anaplastic cells in the lesion secrete cytokine that attract innate immune cells, which produce MMPs and reactive oxygen species that can result in

deterioration of extracellular matrix collagen (Varghese SS et al., 2015).

In conclusion, the findings of our study suggest that there is a significant difference in the collagen composition of the stroma as the lesion evolves from dysplasia into oral squamous cell carcinoma. The prognosticative value of collagen in the aggressiveness of oral lesions has been studied recently. This study has utilized a simple differential staining technique and image analysis to quantify the amount of collagen present without the need for any special training, Expensive molecular techniques or elaborate time- consuming procedures that ultimately delays the diagnosis for the patient. Artificial intelligence makes a rapid, quantitative and bias free pathologic diagnosis a reality.

Author Contribution Statement

S Samyukta- Study idea, Data collection and Article writing, Harini Priya A H- Study idea, Statistical analysis, R Sathish Muthukumar- Study idea and design, Premika Sri V L- Article writing

Acknowledgements

Approval

Study was approved by the Institutional human ethics committee. Reference number: IHEC-I/0264/21. Study approved as part of student's short term project.

Ethical Declaration

Study was approved by the Institutional Human Ethics Committee (IHEC) of Chettinad dental college and research institute.

Conflict of Interest

There is no conflict of interest.

References

- Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G (2006). Inflammation and cancer: how hot is the link?. *Biochem Pharmacol*, **72**, 1605-21.
- Arora KS, Nayyar A, Kaur P, et al (2018). Evaluation of collagen in leukoplakia, oral submucous fibrosis and oral squamous cell carcinomas using polarizing microscopy and immunohistochemistry. *Asian Pac J Cancer Prev*, **19**, 1075..
- Berkovitz BKB, Holland GR, Moxham BJ (2009), Oral mucosa, in *Oral Anatomy, Histology and Embryology*, chapter 14, Elsevier, Edinburg, UK, pp 223-52
- Chen Y, Yu Q, Xu CB (2017). A convenient method for quantifying collagen fibers in atherosclerotic lesions by ImageJ software. *Int J Clin Exp Med*, **10**, 14904-10.
- Daley WP, Peters SB, Larsen M (2008). Extracellular matrix dynamics in development and regenerative medicine. *J Cell Sci*, **121**, 255-64
- Desmoulière A, Guyot C, Gabbiani G (2004). The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. *Int J Dev Biol*, **48**, 509-17.
- Elango JK, Gangadharan P, Sumithra S, Kuriakose MA (2006). Trends of head and neck cancers in urban and rural India, *Asian Pac J Cancer Prev*, **7**, 108-12.
- Fuentes B, Duaso J, Droguett D, et al (2012). Progressive

- extracellular matrix disorganization in chemically induced murine oral squamous cell carcinoma. *ISRN Pathology*.
- Katsuda S, Okada Y, Minamoto T, et al (1992). Collagens in human atherosclerosis. Immunohistochemical analysis using collagen type-specific antibodies. *Arterioscler Thromb Vasc Biol*, **12**, 494-502.
- Kujan O, Khattab A, Oliver RJ, et al (2007). Why oral histopathology suffers inter-observer variability on grading oral epithelial dysplasia: an attempt to understand the sources of variation. *Oral Oncol*, **43**, 224-31.
- Litjens G, Kooi T, Bejnordi BE, et al (2017). A survey on deep learning in medical image analysis. *Med Image Anal*, **42**, 60–88.
- Lumerman H, Freedman P, Kerpel S (1995). Oral epithelial dysplasia and the development of invasive squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, **79**, 321-9.
- Montes GS, Junqueira LCU (1991). The use of the picrosirius-polarization method for the study of the biopathology of collagen. *Memórias do Instituto Oswaldo Cruz*, **86**, 1–11.
- Panayides AS, Amini A, Filipovic ND, et al (2020). AI and medical imaging informatics: current challenges and future directions. *IEEE J. Biomed. Health Inform*, **24**, 1837–57.
- Reyes-Aldasoro CC (2013). Cancer Image Analysis. *Oncol News*, **8**, 158-60.
- Rich L, Whittaker PC, Picrosirius RS (2005). A polarized light assessment of fibrillar hue and spatial distribution. *Braz J Morphol Sci*, **22**, 97–104.
- Shen D, Wu G, Suk HI (2017). Deep learning in medical image analysis. *Annu Rev Biomed Eng*, **19**, 221–48.
- Shetty A, Tamgadge A, Bhalerao S, et al (2015). Study of polarization colors in the connective tissue wall of odontogenic cysts using picrosirius red stain. *J Orofac Sci*, **7**, 119.
- Van den Hooff A (1988). Stromal involvement in malignant growth. *Adv Cancer Res*, **50**, 159–96. .
- Varghese SS, Sarojini SB, George GB, et al (2015). 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*, **20**, 275.
- Venigella A, Charu S (2010). Evaluation of collagen in different grades of oral squamous cell carcinoma by using the picrosirius red stain -Histochemical study. *J Clin Diagn Res*, **4**, 3444- 49.
- Wang Q, Gao P, Wang X et al (2014). The early diagnosis and monitoring of squamous cell carcinoma via saliva metabolomics. *Sci Rep*, **4**, 6802. .
- Warnakulasuriya S, Reibel J, Bouquot J, Dabelsteen E (2008). Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. *J Oral Pathol Med*, **37**, 127-33.
- Yokoyama M (2011). Alterations in stromal reaction during tumour progression in oral mucosa. *J Hard Tissue Biol*, **20**, 23-30.



This work is licensed under a Creative Commons Attribution-Non Commercial 4.0 International License.