Chapter 4: Materials & Methods

MATERIAL & METHODS
4. Materials and Method

The present case control study was carried out at the Department of Oral and Maxillofacial Pathology and Microbiology, Dr. D. Y. Patil Dental College and Hospital, Pimpri Pune and Yerala Dental College and Hospital, Navi Mumbai, Maharashtra, which has Immunohistochemistry laboratory setting. This study was a retrospective audit for which the necessary Institutional Ethics Committee waiver/exemption was obtained. The study population was obtained from cases of oral submucous fibrosis (OSMF) from year 2013 to 2016 in these institutes. Eligibility criteria included patients with clinically and histopathologically diagnosed cases of OSMF, who were untreated. The cases were graded according to modified Pindborg’s grading system for OSMF\(^{37}\). OSMF cases with oral leukoplakia or Squamous cell carcinoma were excluded from the study. Patients on any medical treatment or with any local or systemic illness and patients with history of major surgeries in last six months were excluded. Demographic data including, detailed clinical presentation, detailed history of relevant habits and their duration and histopathological features were noted.

The study was carried out on neutral buffered formalin fixed paraffin embedded tissue to detect expression of Factor XIIIa (FXIIIa), Matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF).
4.1 Research Design

4.1.1 Type of Study: Retrospective

4.1.2 Variables Measures:

1. Histopathological grades of OSMF.
2. Expression of FXIIIa, MMP-9 and VEGF expression in OSMF and its different grades.

4.1.3 Sample Size Calculations:

For OSMF  \[ = X^2 \times N \times P \times Q/C^2 \times (N-1) + X^2 \times P \times Q \]

\[ = 3.84 \times 108 \times 0.5 \times 0.5/(0.05)^2 \times 299 + 3.84 \times 0.5 \times 0.5 \]

\[ = 103.68/0.74 + 0.96 \]

\[ = 103.68/1.70 \]

\[ = 60.98 \]

Where N=Total No. of patients sample in last three years visited to department; P is 50% proportion rate (0.5); Q = (1-P) = 0.5; C=Confidence interval of one’s choice= 0.05; \( X^2 \) = Chi square value for 1 degree of freedom at some desired probability level i.e. 3.84

(Actual Sample included in this study = 60)

4.2 Detail Study Plan

Sample size was decided based on the above formula. Demographic data including, detailed clinical presentation, detailed history of relevant habits and their duration and histopathological features were noted. Such patients constituted study group. Study group has 60 clinically and histopathologically diagnosed cases of OSMF. Study group patients were divided into three subgroups based on histopahtological grading.
system by Pindborg et al. Control group had biopsies taken from normal oral mucosa tissues obtained from 20 healthy individuals who had no habit and any oral lesion.

4.2.1 Groups

- **I. Control Group** - 20 healthy individuals without any habits. (Age and Site matched)

- **II. Study Group** - 60 Clinically and histopathologically diagnosed cases of OSMF.

**Group A**-Early, **Group B**-Moderately Advanced, **Group C**-Advanced

4.2.2 Inclusion and exclusion criteria for the entry of participants:

**Inclusion criteria**

- Participants in the age range of 20-50 years were chosen for the study.
- Untreated cases of OSMF
- Normal subjects were chosen who were undergoing minor surgical procedures without any local inflammatory lesion

**Exclusion criteria**

- In Group II participants should be free of any oral lesions, tobacco and alcohol habits.
- OSMF with oral leukoplakia or Squamous cell carcinoma were excluded.
- Patients on any medical treatment or with any local or systemic illness were excluded.
- Patients with history of major surgeries in last six months were excluded.

4.2.3 Evaluation parameters
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- Factor FXIIIA (FXIIIA),
- Matrix metalloproteinase- 9 (MMP-9) and
- Vascular endothelial growth factor (VEGF)

4.3 Study Method

4.3.1 Obtaining consent: After explaining the patient/subject about the nature of study and the procedure, written consent was obtained.

4.3.2 Biopsy: Oral biopsies form healthy individuals who were undergoing minor surgical procedures such as disimpaction or buccal frenectomy. Tissues were taken under local anaesthesia following all aseptic measures, preferably from oral mucosa after confirming that the mucosa is not inflamed.

4.3.3 Histopathological examination and grading: Formalin fixed tissues of OSMF were assessed for Grading of OSMF tissues. The grading was done as Early, Moderately Advanced and Advanced\(^1\). Following criteria are followed for grading\(^3\)

**Early (Group A)(Fig. 4.1a)**- Juxta-epithelial connective tissue shows early hyalinization, collagen in separate bundles, plump, young fibroblasts in moderate numbers, blood vessels dilated & congested, inflammatory cells, primarily lymphocytes, eosinophils and occasional plasma cells.

**Moderately advanced (Group B)(Fig. 4.1b)**- Collagen is moderately hyalinised, less fibroblastic response, more fibrocytes, normal or constricted blood vessels, inflammatory cells consists of lymphocytes and plasma cells.
Advanced (Group C) (Fig. 4.1c)- collagen is completely hyalinized, separate bundles of collagen are not seen, hyalinised areas of collagen are devoid of fibroblasts, blood vessels are completely obliterated or narrowed, inflammatory cells- lymphocytes & plasma cells.

4.3.4 Immunohistochemistry (IHC) staining: OSMF & Control group unstained tissue sections were subjected for this specialized staining technique which is based on antigen antibody reaction. To detect FXIIIa, MMP-9, VEGF in tissues, Anti-Factor XIII Subunit A [clone E980.1, Mouse Monoclonal Antibody], Anti-Human MMP-9, [clone-EP127, Rabbit Monoclonal Antibody] and Anti-VEGF [Human recombinant VEGF165, Polyclonal Rabbit] (all three antibodies from Biogenix, CA, USA) were used.

Positive control: Placenta was used as positive control for FXIIIa and VEGF and for MMP-9, breast carcinoma tissues were used as positive control.

4.3.4a Reagent Preparation:

Prior to staining the following reagents were prepared.

A. Phosphate Buffer Solution (PBS) pH – 7.4, 0.05M
   For 1 liter
   • Sodium dihydrogen phosphate - 3.4gms
   • Disodium hydrogen phosphate - 12.0gms
   • Sodium chloride - 8.5gms
   • Distilled water - 1000ml

B. Sodium Citrate Buffer solution, pH – 6.0, 10mM
   • Sodium citrate - 2.1gms
   • Distilled water – 1000ml
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Titrate to pH 6.0 with 1M HCL.

4.3.4b Coating of slides with section adhesive

To enhance section adhesion and minimize section loss due to pretreatment, slides were precoated with 3amino propyl triethoxy silane (Silane: USA product code: A-3648 SIGMA-ALDRICH). The slides were submerged in a jar containing acetone for 5 min, followed by 2% silane in acetone for 5 minutes and rinsed in double distilled water. Then slides were allowed to air dry.

From each block three sections (4μm thick) were prepared for staining with hematoxylin and eosin, and immunohistochemical staining of FXIIIa, MMP-9 and VEGF.

4.3.4b Immunohistochemical method for detection FXIIIa, MMP-9 and VEGF Antigen

For immunohistochemistry (IHC), Universal Immuno-enzyme Polymer method was employed. Sections of study and control group tissues along with positive control tissues taken on silane coated slides were subjected for IHC staining. The sections were deparaffinized with xylene and hydrated. Sections were first deparaffinized in two changes of xylene for 10 minutes each, and then were rehydrated through graded alcohols. To prevent nonspecific reactions, sections were incubated at room temperature in a humidifying chamber for 60 min. Endogenous peroxidase was blocked using freshly prepared 3% hydrogen peroxide in methanol at room temperature for 10 minutes. To prevent nonspecific reactions, sections were incubated with 10% serum for 10 min. Respective antigens were retrieved using Antigen retrieval system (Biogenix, USA, Fig. 4.3) in 0.01 mol/L sodium citrate buffer (pH 6) for 30 minutes at 90 C.

The sections were allowed to cool to room temperature and were stained with respective antibody at room temperature in a humidifying chamber for 60 min. One section from each positive control was used as the negative control by omitting the primary antibody and by incubating with serum. After being rinsed with phosphate buffered saline (PBS) three times for 10 min each, the slides were incubated with chromogen 3, 3’ Diaminobenzidine (DAB) to visualize the antigen-
antibody reaction. After chromogen development, slides were washed with two changes of water and counterstaining was done with Hematoxyline before dehydration in graded ethanol and mounting will be done. PBS was used in every washing procedure.

4.4.4 Assessment of immunohistochemically stained sections:

Sections stained with FXIIIa, MMP-9 and VEGF antibodies (Fig. 4.2) were examined under Leica DM LB2 (Leica microscope, Fig. 4.4) at x100 followed by x400 magnification.

The positive control was examined for the presence of a colored end product at the site of the target antigen (DAB chromogen brown end product). The presence of these colors was interpreted as positive staining result, indicating proper performance of kit reagents. The absence of nonspecific staining in the negative control confirmed the specificity of primary antibody.

4.4.4a Assessment of FXIIIa positive cells (Fig. 4.5a): Cells were considered positive for the FXIIIa if there was cytoplasmic DAB staining (brown color). A 10 x 10 ocular graticule outlining an area of 0.064 mm² was used at a magnification of 400x to assist in the counting of cells for sections stained for factor XIIIa. Dendritic and spindle-shaped positive cells were counted. The grid was used to count the number of positive cells against the number of negative cells in 5 high power fields. The number of positively stained cells was expressed as a percentage of the total number counted. This percentage of positive cells is used for comparisons.

4.4.4b Assessment of MMP-9 positive cells (Fig. 4.5b): MMP-9-stained slides were evaluated semiquantitatively according to cytoplasmic positivity of epithelial and
connective tissue cells in 5 high power fields (x 400). The percentage (P) of positive-stained cells was graded as 0: no positive staining; 1: less than 10% positive cells; 2: 11–50% positive cells; 3: 51–80% positive cells; 4: more than 80% positive cells. The intensity (I) of immunostaining was determined as 0 (negative), 1 (light yellow), 2 (yellow-brown), or 3 (dark brown). Staining intensity was compared with that of positive control. Finally, an immunoscore was calculated by multiplying the percentage of positive cells (P) by the staining intensity score (I). The immunoscores were classified into four groups: (0) score-0, (1) score 1–2, (2) score 3–5, (3) score 6–8, and (4) score 9–12 by two observers independently.

4.4.4c Assessment of VEGF positive cells(Fig. 4.5c): VEGF expression was assessed semiquantitatively according to (i) the presence of positive staining and (ii) staining intensity (0 = none, 1 = mild, 2 = moderate, 3 = intense) as performed by Moriyama et al. The intensity of VEGF expression was assessed according to the staining of DAB under light microscopy by comparing with the staining intensity of endothelial cells. Intensity of the stain was scored on the following scale: 0 = no staining, 1 = mild staining, 2 = moderate staining and 3 = intense staining. Slides with no positive staining were scored 0. The percentage of VEGF expression was counted on a semiquantitative scale ranging from 0 (no expression) to 4 (highest level of expression). It was scored as follows: 0 = no stained cells in any microscopic field, 1 = <25% of cells stained positively, 2 = 25%–50% of cells stained positively, 3 = 50%–75% of cells stained positively, and 4 = more than 75% of cells stained positively. The sum of the scores for area and intensity of staining were used for statistical analysis.
All the histopathology slides and IHC slides were blinded for analysis. Two independent experienced oral pathologists did the grading of OMSF. The degree of agreement between them was statistically significant. IHC scoring of FXIIIa, MMP-9 and VEGF was done without knowing the grading status of the OSMF cases. The IHC values obtained were then computed with the normal, and early, moderate and Moderately advanced grades.

4.4 Statistical analysis

The mean score was obtained for FXIIIa, MMP-9 and VEGF expression in all the groups. The obtained data was analyzed statistically using (SPSS 17.0 version software for Windows). The obtained score was compared in normal and study group by using one way ANOVA and Multiple comparisons by using Tukey HSD test. The obtained score were also compared and correlated with grades of OSMF. Pearson’s rank correlation test was used for correlation analysis as and when applicable. Differences among groups were assessed by the, unpaired t test. The Kruskal-Wallis test was also used for comparison between clinical grades of OSMF. Subsequent comparisons between groups were carried by Mann-Whitney U test and independent student T test as and when applicable. The level of statistical significance is at p<0.05.
Fig. 4.1: Grading of OSMF on H and E stained sections:
Early (a), moderate (b) and advanced (c)

Fig. 4.2: Anti-Factor XIII Subunit A, Anti-Human MMP-9, and Anti-VEGF antibodies

Fig. 4.3: Antigens retrieval system

Fig. 4.4a: Research microscope with attached camera

Fig. 4.4b: Imaging software
Fig. 4.5: Counting of IHC stained slides

Fig. 4.5a: Counting of FXIIIa

Fig. 4.5b: Counting of MMP-9

Fig. 4.5 c: Counting of VEGF