

## CD4<sup>+</sup>CD25<sup>+</sup> T Regulatory Cells and MMP-9 as Diagnostic Salivary Biomarkers in Oral Lichen Planus

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Oral lichen planus (OLP) is a chronic inflammatory disease characterized by a dense subepithelial lymphocytic infiltrate, degeneration of basal keratinocytes and basement membrane disruption. Non-specific mechanisms include mast cell degranulation and matrix metalloproteinase (MMP) activation in OLP lesions. These mechanisms may combine to cause T-cell accumulation in the superficial lamina propria, basement membrane disruption, intra-epithelial T-cell migration, and keratinocyte apoptosis in OLP. Obviously, abnormality of immunological regulation may contribute to its pathogenesis. *This study aimed at evaluating the role of salivary CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells & Matrix Metalloproteinase-9 (MMP.9) as salivary biomarkers in patients with OLP. Twenty patients with various forms of OLP were investigated: 8 with bullous erosive form, 7 with atrophic and 5 with reticular forms. From each patient saliva was collected, and used for assessment of salivary T regulatory cells by immunofluorescent and MMP9 expression by quantitative RT-PCR. Nine out of the twenty patients showed CD4<sup>+</sup>CD25<sup>+</sup>Tregs in saliva by immunofluorescent staining with sensitivity (90%), specificity (83.3%) and diagnostic accuracy (87.5%). molecular detection of salivary MMP-9 levels in CD4<sup>+</sup>CD25<sup>+</sup> Tregs +ve cases showed a significant increase in CD4<sup>+</sup>CD25<sup>+</sup>Tregs +ve cases. In conclusion, salivary CD4<sup>+</sup>CD25<sup>+</sup> T regs and MMP-9 could be considered as sensitive and specific diagnostic & prognostic biomarkers in the detection of OLP.*

Lichen Planus (LP) is a chronic inflammatory muco-cutaneous condition, which most commonly affects the skin, genitalia and oral mucous membrane (McCartan & Healy, 2008). It is one of the most common dermatological conditions involving the oral cavity (Fernando *et al.*, 2008), the name "lichen" refers to the lichen plant, which grows on rocks or trees, and "planus" means flat. It was first described by Sir Erasmus Wilson in 1869 (Flavia *et al.*, 2011). The prevalence of Oral Lichen Planus (OLP) is 1-2% (Gorsky, *et al.*, 2004) while cutaneous lesions occur in 0.4%. Ten to twenty percent of patients with LP demonstrate both oral and cutaneous lesions (Anuradha *et al.*, 2008). OLP is a T-cell mediated autoimmune disease in which cytotoxic CD8<sup>+</sup> T cells trigger the apoptosis of oral epithelial cells. The nature of the antigen is uncertain. However, several predisposing factors have been implicated in the

pathogenesis of OLP and oral lichenoid reaction (OLR) (Sumairi *et al.*, 2007).

Typical oral lesions are striations (striae of Wickham), papules, plaques, mucosal atrophy, erosions (shallow ulcers) or blisters affecting the buccal mucosa, tongue, and gingiva.

Erythematous and erosive lesions are often sensitive or painful and there is ongoing concern that OLP may be premalignant (Eisen *et al.*, 2005). Many autoimmune features of OLP were reported including disease chronicity, adult onset, female predilection, association with other autoimmune diseases, depressed immune suppressor activity in OLP patients, and the presence of auto-cytotoxic T-cell clones in lichen planus lesions. The lichen planus antigen is unknown, although the antigen may be a self-peptide, thus defining lichen planus as a true autoimmune disease, Keratinocytes (KC) express the LP antigen only at the lesion site, so that the clinical

distribution of LP lesions is determined by the distribution of the LP antigen (Sugerman & Savage, 2002).

Oral lichenoid lesion (OLL) might also be triggered by mechanical trauma (Koebner's phenomenon) due to calculus deposits, sharp teeth, rough surfaces of dental restorations or prostheses, cheek or tongue biting and oral surgical procedures. Mechanical trauma may exacerbate lichenoid lesions, especially when it affects the midline of the buccal mucosa or the lateral margin of the tongue (Sugerman *et al.*, 2005).

Regulatory T cells (T regs) play a central role in inducing and maintaining immunologic tolerance and in the termination of immune responses. Deficiency or dysfunction of these cells may lead to autoimmunity or aggravated pathogen-induced inflammation (Sakaguchi *et al.*, 2005). Two main subsets of professional Treg have been proposed that differ in terms of development, specificity, and effector mechanisms: naturally occurring Treg (nTreg) cells' and adaptive Treg (iTreg) cells (Bluestone & Abbas, 2003). The latter develop from mature T cells in peripheral tissues under certain conditions of antigen-specific stimulation, such as suboptimal antigen concentrations and/or costimulation. Naturally arising Treg, which are induced in the thymus, display a constitutive high expression of CD25<sup>+</sup>, the alpha chain of the interleukin-2 receptor, and are generally referred to as CD4<sup>+</sup>CD25<sup>+</sup>Treg. These CD4<sup>+</sup>CD25<sup>+</sup>Treg suppress other T cells by cell-cell contact in a cytokine-independent fashion and are thought to play a pivotal role in the central tolerance to self-antigens (Onno *et al.*, 2007). The oral cavity has a full complement of immune cells, both Intra-epithelially and submucosally, and these include cells bearing CD4<sup>+</sup> and chemokine receptors type 5 (CCR5). Intra-epithelial CD4<sup>+</sup> and CD8<sup>+</sup> cells can be found in uninfamed oral mucosa up to the

permeability barrier, approximately one-third of the cell depth from the surface (Challacombe *et al.*, 2006). Among the several mechanisms that play role in maintaining peripheral self-tolerance is the existence of a unique CD4<sup>+</sup>CD25<sup>+</sup> population of naturally occurring regulatory T cells (Treg) that actively prevent both the activation and the effector function of autoreactive T- cells that have escaped different mechanisms of tolerance. Many studies, both in mice and humans, have confirmed the importance of Treg, and CD4<sup>+</sup> cell subset in the pathogenesis of many autoimmune diseases. Tregs with a distinct phenotype in tumor-infiltrating lymphocytes contribute to local immune suppression (Toubi, 2008).

Santoro *et al.*, 2003 observed that nuclear factor (NF- KB) is expressed on basal and suprabasal keratinocytes in all cases of OLP, while normal epithelium is consistently negative. NF- KB expression by epithelial cells is correlated with the amount of cytotoxic cell infiltration. In the same context, Rhodus *et al.*, 2005 reported that the activation of NF- KB is essential for the survival of T-cells during the period immediately following activation and before the first cell division. The activation of NF- KB may be responsible for long-lasting inflammatory processes mediated by T-cells

Matrix metalloproteinase-9 (MMP-9) is a secreted multi-domain enzyme that regulates cell-matrix composition. It belongs to the gelatinase subfamily of the MMPs and therefore its main substrate is gelatin (a denatured collagen). MMP-9 is produced by selected cell types, including keratinocytes, monocytes, tissue macrophages, polymorphonuclear leukocytes, and by a variety of malignant cells. The main function of MMP-9 is the regulation of cell matrix composition (1, 6, 7 and 9). MMP-9 cleaves denatured collagen (gelatins) and type IV

collagen, which is the major component of the basement membranes. This cleavage helps lymphocytes, and other leukocytes to enter and leave the blood and lymph circulations. MMP-9 also cleaves myelin compounds such as myelin basic protein (MBP) and type II gelatins, leading to remnant epitopes that can generate autoimmunity, the so-called (REGA) model of autoimmunity (Maya & Yaniv *et al.*, 2006).

Regulation of MMP-9 expression and secretion by activated lymphocytes and monocytes is tightly regulated by cytokines, chemokines, eicosanoids and peptidoglycans. MMP-9 is under strict control at various levels: gene transcription, synthesis, secretion, activation, inhibition and glycosylation (Maya & Yaniv *et al.*, 2006).

T cells in OLP may be stimulated by TNF- $\alpha$  to secrete MMP-9. The antigen specificity of these T cells is unknown, although non-specific T-cells may be activated in this manner, thereby amplifying the MMP-9 produced in OLP lesions. T-cell-secreted MMP-9 may disrupt the epithelial basement membrane in OLP lesions. The disrupted basement membrane in OLP no longer delivers the keratinocyte survival signal, which may trigger keratinocyte apoptosis. In addition, MMP-9 induced basement membrane disruption may facilitate the passage of antigen-specific CD8<sup>+</sup> cytotoxic T-cells into the OLP epithelium, where they trigger further keratinocyte apoptosis (Sugerman *et al.*, 1992).

This study aimed at evaluating the role of salivary CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells & Matrix Metalloproteinase-9 (MMP.9) as salivary biomarkers in patients with oral lichen planus.

## Subjects and Methods

Twenty patients suffering from OLP (15 female and 5 male, age range 36 - 58 years) and 10 healthy controls (3 female and 7 male, age range 30 - 53 years), were

selected from the out-patient clinics of both; Department of Oral medicine and Periodontology, Faculty of Dentistry, and the Department of Dermatology and Andrology, Faculty of Medicine, Ain Shams University. The purpose of the study was explained to all participants and an informed consent was signed by each patient prior to conduction of research (Medical ethics committee approval)

### Patients Grouping

Group (1): twenty patients suffering from Oral Lichen Planus (OLP) clinically diagnosed according to modified World Health Organization (WHO) clinico-pathological diagnostic criteria for LP (Rad *et al.*, 2009).

Group (2): Ten apparently healthy individuals with no evidence of any mucosal pathology were considered as a control group.

### Inclusion Criteria

Patients free from any systemic diseases as evidenced by the health questionnaire, using modified Cornell medical index (Pendleton *et al.*, 2004) & patients not receiving any systemic medication.

### Exclusion Criteria

History of steroids, retinoids, tacrolimus, cyclosporine, and azathioprine, administration at least six months prior to the initiation of the study; either topical or systemic, Patients with lichenoid reaction, Smokers, Pregnant or lactating females or Active, recurrent infections. All patients and controls were subjected to: history taking, clinical examination & salivary sample collection.

### Clinical examination

It was carried out according to standard clinical criteria: All patients were examined clinically using spot light and magnifying mirror. The presence of Wickham's striae confirmed the clinical diagnosis. These striae were accentuated on stretching of the surface mucosa and were not eliminated by rubbing. The distribution of the lesions whether unilateral or bilateral was recorded. The different affected areas were recorded: buccal mucosa, tongue, lips, gingival or palate and the OLP type was also recorded.

### Types of OLP

- Reticular type; Hyperkeratosis patterns were presented surrounded by Wickham's striae, such feature may not be evident in certain sites, such as the dorsum of the tongue, where lesions present as keratotic plaques.

- Atrophic OLP lesions; exhibited erythematous areas. Symptoms ranged from discomfort to intensely painful episodes that interfered with chewing, surrounded by radiating thin striae.
- Erosive OLP lesions; the epithelium separated and erosions or ulcers were sometimes severe.

From the 20 patients: 8 patients showed bullous erosive form OLP, 7 atrophic and 5 showed reticular form.

#### Saliva sample collection

Collection of whole unstimulated saliva (WUS) using standard techniques was done as described by (Navazesh, 1993). Briefly, subjects refrained from eating, drinking, using chewing gum etc., for at least 1.5 hours prior to the evaluation. Samples were obtained by requesting subjects to swallow first then tilt their head forward, saliva was collected by careful aspiration using sterile syringe. Collected saliva was divided into 2 tubes; the first tube was used for indirect immunofluorescence test for categorization of CD4<sup>+</sup> and CD25<sup>+</sup> T-regulatory cells. The second tube was used for detecting genetic expression (mRNA) of matrix metalloproteinase -9 (MMP-9) by Real-Time Reverse transcription- polymerase chain reaction (Real-Time RT- PCR) technique, this second tube was immediately stored at -20° C until assayed.

Categorization of CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells by indirect Immunofluorescence technique using Detection system (Fremont, USA) The system consists of a labeled streptavidin-biotin immunoenzymatic antigen detection system). This technique involves the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen. A biotinylated secondary antibody that reacts with the primary antibody is added followed by the addition of a streptavidin-enzyme conjugate that binds to the biotin present on the secondary antibody. The specific antibody, secondary antibody, and the streptavidin/enzyme complex were then visualized by an appropriate substrate/chromogen.

Samples were processed as the manufacturer's instructions; In brief saliva samples were centrifuged, cytopsin was added to make a monolayer then washed in phosphate buffer saline (PBS) 4 times. Ultra V Block was added to the tubes and incubated at room temperature for 5 minutes. Five µl of primary monoclonal antibody CD4<sup>+</sup> Mouse Anti-Human Monoclonal Antibody tagged by phycoerythrin were added sequentially. Slides were incubated at 37-39C° for 30minutes. A fixation step was done by acetone for 10 minutes. Slides were washed in (PBS) 4 times and

dried around edges of sections. One to two drops of CD25<sup>+</sup> Biotinylated Goat Anti-polyvalent Monoclonal Antibody (IL2R.1 tagged by FITC), were sequentially put on the slides which were then incubated horizontally in a humid chamber at room temperature for 10 minutes. After washing in (PBS) 4 times and drying slides around sections, Streptavidin Peroxidase was applied on slides then they were kept in a humid chamber at room temperature for 10 minutes. After washing slides in (PBS) 4 times and drying them around sections, the Diaminobenzidine (DAB) chromogen was added over sections and slides were incubated at room temperature for 10 minutes. Then slides were washed in distilled water. Finally, slides were covered with cover slips using Canada balsam. Positive control slides (provided in the kit) were processed as manufacturers' instructions to define the positive slides as a quality control standardization of the assay. (Cut off point indicator of positivity = >25% /HPF)

Indirect immunofluorescence staining of cells was examined by Fluorescence Research Microscope\* to assess the prevalence of Immunopositivity of CD4<sup>+</sup> CD25<sup>+</sup> markers in the studied cases.

#### MMP-9 RT-PCR

Detecting genetic expression (mRNA) of (MMP-9) was carried out by Real-Time Reverse transcription PCR (RT- PCR): In principle the procedure begins with reverse transcription of total RNA. The complementary-DNA (C-DNA) is then used as template for real-time PCR with gene specific primers (GenBank®Accession Number: NM\_004994 provided by Rosche \_research Germany). Extraction of RNA: MMP-9-RNA was extracted from isolated mononuclear cells and neutrophils. The method used to obtain SPMNs was as follows: patients were asked to perform rapid sequential rinsings by placing in their mouths 15 mL of Hanks' balanced salt solution [free of calcium or magnesium ions (HBSS-CMF)], which contained 0.1% gelatin, then swishing the solution for 30 s, and expectorating into a polypropylene receptacle containing 400 mL 4 degrees C HBSS-CMF. This sequence was repeated for 20 min. The collected solution was stirred for 10 min, the cells were washed, and the re-suspended pellet was passed sequentially through a 20-microns and a 10-microns nylon mesh. The cells consisted of 97.7 +/- 1.7% SPMNs, only 2.3% epithelial cells, and were almost free of oral debris (Ashkenazi & Dennison, 1989). Then the RNA was extracted using pure script total RNA extraction kit (Gentra-System USA). The cells were lysed in the presence of an RNA preservative using an anionic detergent which solubilizes the cellular component.

The RNA preservative works by limiting the activity of enzymes that are capable of digestion of RNA (RNase) that are contained elsewhere in the environment. Contaminating DNA and proteins are then removed by salt precipitation. Total RNA is isolated by precipitation with alcohol and dissolved in RNA free water.

**MMP-9 primers:** Two pairs of oligonucleotides primers were prepared for human MMP-9-specific sequences (sense: 5' CGCAGACATCGTCATCCAGT 3', antisense: 5' GGATTGGC CTTGGAAGATGA 3') and ( $\beta$ -actin-specific sequences (sense: 5' AACTGGGACGACATGGAGAA 3', antisense: 5' ATACCCCTCGTAGATGGGCA 3'). The expected product size was 186 base pair (bp). According to the number of samples a premix from all components except c DNA was done. Then 20  $\mu$ L of this premix was dispensed into PCR tube containing c DNA sample. All precautions were carried out to prevent contamination. According to *Chen et al., 2007* relative gene mRNA levels were calculated using  $\Delta\Delta$ Ct method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. The tubes were subjected to the following light cycles: 95°C for 15 seconds (denaturation), 60°C for 1 minute (Annealing and extension) for 50 cycles.

Detection of PCR product by cyber green: Due to the optimized Reaction Mix of the Light Cycler®,

primer combinations the PCR is efficient and specific without any MgCl<sub>2</sub> Adaption with, addition of MgCl (not included in the kit) for final concentration of up to 1.0 M (titrate in 0.25 M steps).

#### Statistical Analysis

All data were collected coded, and statistically analyzed using IBM SPSS Statistics Version 20 for Windows. Quantitative data were presented as mean and standard deviation (SD) values. Student's t-test was used for comparisons between LP and control groups. Qualitative data were presented as frequencies and percentages. Chi-square ( $\chi^2$ ) test was used for studying the comparisons and associations between different qualitative variables. Mann-Whitney U test was used to compare between MMP-9 levels in (CD4<sup>+</sup>CD25<sup>+</sup> Tregs) +ve and -ve cases. Using ANOVA for significance level which was set at  $P \leq 0.05$

#### Results

The current study included twenty patients, it was noticed that females have statistically significantly higher prevalence in OLP group than control group, There was no statistically significant difference between marital status as well as the mean age values in the two groups (table1).

Table 1. Demographic data of patients with Oral Lichen Planus and controls.

| Demographic data    | OLP            |    | Control      |   | *P-value |       |
|---------------------|----------------|----|--------------|---|----------|-------|
|                     | n              | %  | n            | % |          |       |
| Gender              | Males          | 4  | (20)         | 8 | (80)     | 0.002 |
|                     | Females        | 16 | (80)         | 2 | (20)     |       |
| Marital status      | Married        | 15 | (75)         | 8 | (80)     | NS    |
|                     | Single         | 2  | (10)         | 1 | (10)     |       |
|                     | Widowed        | 3  | (15)         | 1 | (10)     |       |
| Age (Mean $\pm$ SD) | 48.5 $\pm$ 9.5 |    | 43 $\pm$ 9.2 |   | NS       |       |

\*P > 0.05 is not significant(NS) /OLP(Oral Lichen Planus)

Furthermore, this study clarified that there is a statistically significant association between lesion types and (CD4<sup>+</sup>CD25<sup>+</sup>) T regs. All

types of OLP showed higher prevalence of positive (CD4<sup>+</sup>CD25<sup>+</sup>) T regs than control group (table2).

Table 2. Frequency of patients with detectable salivary T regs according to types of Oral Lichen Planus.

| Type       | CD4 <sup>+</sup> CD25 <sup>+</sup> positive |        | CD4 <sup>+</sup> CD25 <sup>+</sup> negative |        | *P-value |
|------------|---|--------|---|--------|----------|
|            | n   | %      | n   | %      |          |
| Atrophic   | 3   | (100)  | 0   | (0)    | 0.029    |
| Ulcerative | 4   | (80)   | 1   | (20)   |          |
| Reticular  | 2   | (100)  | 0   | (0)    |          |
| Controls   | 1   | (16.7) | 5   | (83.3) |          |

\*P ≤ 0.05 is significant

The Immunofluorescence results in OLP and control group analysed using the Chi-square test compared the frequency and percentage of CD4<sup>+</sup> and CD25<sup>+</sup> in both OLP and control groups, results showed no statistically

significant difference between CD25<sup>+</sup> in both groups but CD4<sup>+</sup> showed statistically significant higher prevalence in OLP group (figure 1).

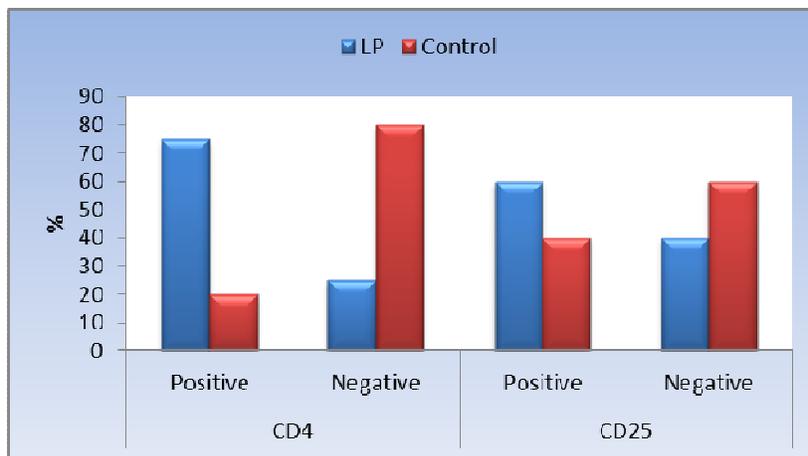


Figure 1. Bar chart representing Immunofluorescence results in OLP and control group.

Noteworthy that the salivary MMP-9 level in the present study, showed an increase in OLP patients (2.15 ± 0.78) compared to control (0.48 ± 0.15) the difference was statistically significant at P ≤ 0.05, different letters are

statistically significantly different according to Turkey's test (a: highest mean Matrix metalloproteinase 9 (MMP 9) level, b: lowest mean MMP-9 levels) (table 3)

Table 3. Frequency of Salivary MMP-9 levels in different types of Oral Lichen planus

| OLP Types | MMP-9<br>Mean $\pm$ SD       | *P-value |
|-----------|------------------------------|----------|
| Atrophic  | 1.06 $\pm$ 0.70 <sup>b</sup> | 0.020    |
| Erosive   | 0.99 $\pm$ 0.24 <sup>b</sup> |          |
| Reticular | 4.21 $\pm$ 1.51 <sup>a</sup> |          |

\*P  $\leq$  0.05 is significant

In addition, it was noticed that CD4<sup>+</sup>CD25<sup>+</sup>Tregs showed high sensitivity (90%), high specificity (83.3%) and high diagnostic accuracy (87.5%). The ROC curve analysis showed moderate sensitivity and perfect specificity. The Area under the curve (AUC) was high (0.800) and the results were statistically significant (P-value = 0.005) (table 4, 5) (fig2)

Table 4. Sensitivity, specificity and diagnostic accuracy of CD4<sup>+</sup> CD25<sup>+</sup>Tregs in detecting Oral Lichen Planus.

| (CD4 <sup>+</sup> CD25 <sup>+</sup> )Tregs | OLP              |                  | Total |
|--|------------------|------------------|-------|
|  | OLP              | Control          |       |
| + ve                                       | 9<br>(True +ve)  | 1<br>(False +ve) | 10    |
| - ve                                       | 1<br>(False -ve) | 5<br>(True -ve)  | 6     |
| Total                                      | 10               | 6                | 16    |

$$\text{Sensitivity (\%)} = \frac{9}{9+1} \times 100 = 90\%$$

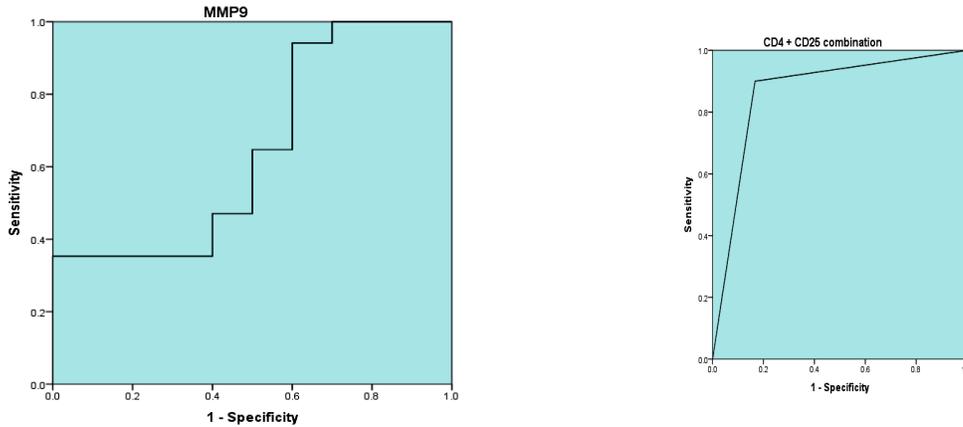
$$\text{Specificity (\%)} = \frac{5}{1+5} \times 100 = 83.3\%$$

$$\text{Diagnostic accuracy (\%)} = \frac{9+5}{16} \times 100 = 87.5\%$$

Table 5. ROC curve analysis for CD4<sup>+</sup>CD25<sup>+</sup>T regs

|                                |                |
|--------------------------------|----------------|
| Area under the ROC curve (AUC) | 0.800          |
| Sensitivity (%)                | 60             |
| Specificity (%)                | 100            |
| Standard error                 | 0.106          |
| 95% Confidence interval        | 0.548 to 0.946 |
| P-value                        | 0.005*         |

\*Significant at P  $\leq$  0.05



**Figure 2.** ROC curve for MMP-9. Compare between MMP-9 and CD4<sup>+</sup>CD25<sup>+</sup>Tregs in positive and negative cases

As regards the cut-off point in MMP9, it was recorded more than 1.25 meaning that cases with MMP9 > 1.25 are diagnosed as having LP while cases below 1.25 are diagnosed as negative cases (table 6).

Table 6. Shows the ROC curve analysis for MMP-9

|                                |                |
|--------------------------------|----------------|
| Area under the ROC curve (AUC) | 0.647          |
| Cut-off point                  | >1.25          |
| Sensitivity (%)                | 35.3           |
| Specificity (%)                | 100            |
| Standard error                 | 0.108          |
| 95% Confidence interval        | 0.441 to 0.819 |
| P-value                        | NS             |

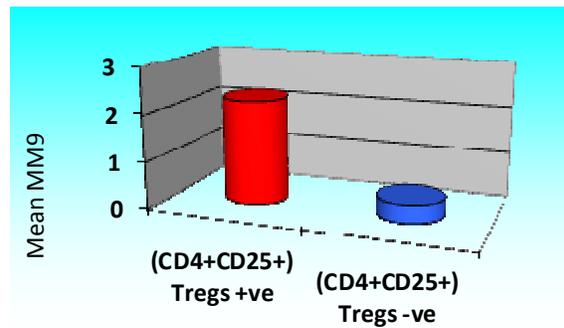
\*P > 0.05 is not significant(NS)

ROC curve analysis showed low sensitivity and perfect specificity of MMP9. Figure 3 shows that the Area under the curve (AUC) was moderate (0.647) and the results were not statistically significant (P-value = 0.174). On the other hand, the salivary level of CD4<sup>+</sup>CD25<sup>+</sup> T regs as well as MMP-9 were significantly higher in OLP lesion group compared to control group, (P ≤ 0.05) (table 7 & figure 3).

Table 7. Shows deviation (SD) values and results of Mann-Whitney U test for comparisons between MMP-9 level and Mean, standard CD4<sup>+</sup>CD25<sup>+</sup>Tregs.

| CD4 <sup>+</sup> CD25 <sup>+</sup> T regs |             | *P-value |
|---|-------------|----------|
| +ve                                       | -ve         |          |
| 2.23 ± 2.22                               | 0.37 ± 0.45 | 0.034    |

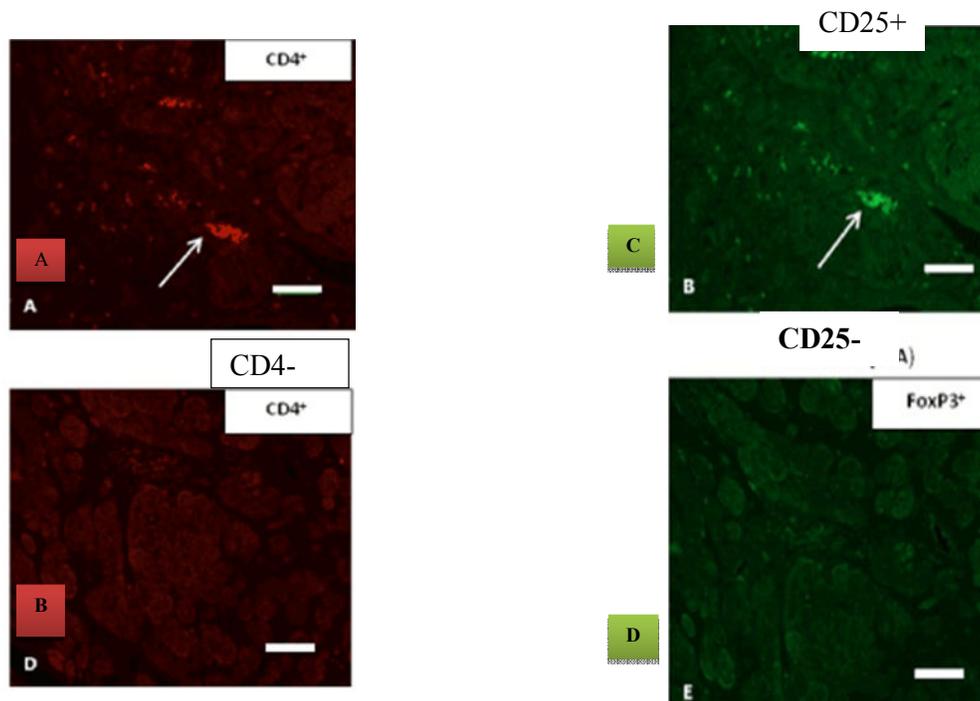
\*Significant at P ≤ 0.05



**Figure 3.** Showing salivary MMP-9 levels in the two group;.CD4<sup>+</sup>CD25<sup>+</sup> Tregs +ve cases showed a statistically significant higher level of salivaryMMP-9 (RNA) than that in CD4<sup>+</sup>CD25<sup>+</sup>Tregs –ve cases.

Noteworthy that the level of salivary CD4<sup>+</sup> and CD25<sup>+</sup> in OLP were measured by indirect immunofluorescence technique. Chi-square test was used to compare the frequency and percentage of CD4<sup>+</sup> and CD25<sup>+</sup> in both OLP and control groups, results showed no statistically significant difference between CD25<sup>+</sup> in both groups but CD4<sup>+</sup> showed statistically significant higher prevalence in OLP group. (figure 4).

The cut-off point in MMP9 was more than 1.25 meaning that cases with MMP9 > 1.25 are diagnosed as having LP while cases below 1.25 are diagnosed as negative cases. ROC curve analysis showed low sensitivity and perfect specificity of MMP9. The Area under the curve (AUC) was moderate (0.647) and the results were not statistically significant ( $P$ -value = 0.174).



**Figure 4.** (A) case of OLP showed positive staining of wall of CD4<sup>+</sup> cells ,(B) Controls with negative staining of CD4, (C) case of OLP showing positive staining by CD25<sup>+</sup>,(D) Controls with negative staining of CD25. By high power magnification lens x40 & total magnification power x400)

## Discussion

OLP is a relatively common inflammatory disease of buccal squamous epithelium. It is considered to be an autoimmune disorder mediated by T-cells and it is characterized by dense subepithelial lympho-histiocytic infiltrate band like-occupying the superficial lamina propria, increased numbers of intra-

epithelial lymphocytes, and hydropic degeneration of basal keratinocytes (Lodi *et al.*, 2005). In OLP, most lymphocytes of epithelium are CD8<sup>+</sup> and lymphocytes of lamina propria are CD4<sup>+</sup>) (Zohreh *et al.*, 2011). OLP pathogenesis is mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes. The basal layer disruption may be result of the cytotoxic

effects of the T cells in variable distribution at the sub-epithelial inflammatory infiltrate (Bascones *et al.*, 2007).

Suri *et al.*, 1998 noted that the IL-2 receptor expressing CD25<sup>+</sup> cells were present in high numbers in OLP. These cells most probably were T cells and could represent activated T cells or distinct populations of regulatory T cells.

T regulatory cells are a component of the immune system that suppress immune responses of other cells, Regulatory T cells come in many forms with the most well-understood being those that express CD4<sup>+</sup>, CD25<sup>+</sup>, and Foxp3 CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, or "Tregs"). These cells are involved in shutting down immune responses after they have successfully eliminated invading organisms, and also in preventing autoimmunity. In addition, there may be other more important mechanisms of immunological regulations contributing to OLP pathogenesis. In recent years, the crucial roles of CD4<sup>+</sup>CD25<sup>+</sup> FOXP3<sup>+</sup> T reg cell have been identified in a series of autoimmune and/or inflammatory disease (Zhou *et al.*, 2002).

The role of immune dysregulation in the pathogenesis of OLP is supported by a lot of evidence, especially involving the cellular part of the immune system. At a cellular level, lichen planus probably results from an immunologically induced degeneration of the basal layer. Whilst the basal cells are considered to be the prime target for a cell-mediated immunological reaction, the precise antigen remains unknown. OLP occupies an important place in dental practice because of their propensity for malignant development (Xue *et al.*, 2005).

Experimental *in vivo* studies have demonstrated that the absence of regulatory T cells allows organ and non-organ-specific autoimmune diseases such as thyroiditis, gastritis, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) to occur,

whereas the addition of this T cell population can prevent or delay these diseases by suppressing the proliferation of effector T cells, thus, maintaining self-tolerance (Toubi, 2008).

Matrix metalloproteinase-9 plays a role in pathological processes, a product of macrophages and a component of cytoplasmic granules of neutrophils. It is also secreted by stromal cells upon stimulation by inflammatory cytokines. The main function of MMP-9 is regulation of cell matrix composition. MMP-9 cleaves denatured collagen and type 4collagen, which is the major component of the basement membrane. Basement membrane, disruption is one of the major characteristics of OLP, expression and secretions of MMP-9 by activated lymphocytes and monocytes are tightly regulated by inflammatory cytokines (Paulusova *et al.*, 2012).

Saliva is an accessible fluid that can easily be collected by individuals with modest training, including patients. Testing salivary samples is easy and non-invasive no special equipment is needed for collection of saliva, neither painful nor traumatic. Whole saliva contains locally produced as well as serum derived markers. The components of saliva act as a "mirror of the body's health," (Richard & Esteban, 2008) thus, it is useful in the early diagnosis of a variety of systemic disorders, monitoring the disease course in conjunction with treatment and detection of addictive drugs. The most commonly used laboratory diagnostic procedures involve the analyses of cellular and chemical constituents of blood. Diagnosis of disease via the analysis of saliva is potentially valuable for children and older adults and non-collaborative subjects and in many circumstances in which blood and urine sampling is not available (Chiappin *et al.*, 2007). Further, analysis of saliva may provide a cost-effective approach

for the screening of large populations (Kaufman & Lamster, 2002).

Consequently, the aim of present study was to evaluate salivary CD4<sup>+</sup> CD25<sup>+</sup> T reg cells and MMP- 9 as biomarkers in the diagnosis of oral lichen planus.

The present study included two groups; Group (1): twenty patients suffering from oral lichen planus, clinically diagnosed according to modified World Health Organization (WHO) clinico -pathological diagnostic criteria for LP (Rad *et al.*, 2009). Group (2): Ten apparently healthy individuals with no evidence of any mucosal pathology was considered as a control group. Both patient & control groups were age and sex matched to avoid any changes in such variables.

The level of salivary CD4<sup>+</sup> and CD25<sup>+</sup> in OLP were measured by indirect immunofluorescence technique. This technique was chosen as it an integral component of clinical immunology laboratories. The indirect immunofluorescence has a greater sensitivity than direct immunofluorescence (Wong, 2006).

In this study the salivary MMP-9 level was measured by Real-Time PCR; the Real-Time PCR is identical to a simple PCR except that, the progress of the reaction is monitored by a camera or detector in "real-time". Each technique uses some kind of fluorescent marker, which binds to the DNA. Hence, as the number of gene copies increases during the reaction, so the fluorescence increases. This is advantageous because the efficiency and rate of the reaction can be seen. MMP-9 detection could be performed by several other techniques in contrast to PCR, immunohistochemistry, ELISA and zymography. These techniques were not selected in the present study, due to their disadvantages, being complicated techniques and less sensitive than the molecular technique provided by the PCR (Maggi *et al.*, 2005).

Among the studied groups, female showed a high a percentage of prevalence in OLP group (80%) than control group (20%). In addition, the age range of patients suffering from OLP was from 40-65 years, this goes in agreement with Eisen, (2002) and Rhodus *et al.*, (2005). Out of the 20 OLP patients, (75%) were married, (10%) single and (15%) widowed, compared to control group (80%) married, (10%) single and (10%) widowed. In agreement with our study, Bhattacharyya *et al.*, (2003) reported no significant correlation between marital status and the development of LP.

The OLP patients recruited for the present study represented most of the OLP forms; 6 of reticular type (30%), 8 of erosive type (40%) and 6 of atrophic type (30%), This goes in contrast with other authors Sumairi *et al.*, 2007 and Eisen *et al.* 2002, who reported that the most frequent clinical form is reticular lichen planus.

The results of the present study showed a highly statistical significant increase in salivary CD4<sup>+</sup> levels (75%) in oral lichen planus group when compared to the control group (20%). When comparing the CD4<sup>+</sup> level between the 3 types of OLP, there was no statistically significant difference.

These results are in accordance with Yamamoto & Osaka, (1995) who stated that OLP showed an increase in CD4<sup>+</sup> T- cells compared to the control group. Our results were also in agreement with Garca *et al.*, (2006), who reported that CD4<sup>+</sup> was significantly higher in OLP than normal control group. Moreover, Hofman *et al.*, (2001) registered higher numbers of CD4<sup>+</sup> positive cells in active lesions as compared to regressive lesions only in the OLP group. In contrast to our results, Alfouzan *et al.*, (1996) have found that the proportions of helper CD4<sup>+</sup> T-cells were significantly lower in patients with lichen planus than in healthy controls. Their results

indicated that this reduction increased slightly over time.

In the present study salivary CD25<sup>+</sup> level in oral lichen planus group measured (60%), While in the control group it was (40%), however, the difference wasn't statistically significant. In addition, there was no statistically significant difference between the level of CD25<sup>+</sup> in any of the 3 types of OLP. In a study conducted by Garca *et al.*, (2006) they reported different prevalence of cells with inflammatory response membrane markers between OLP and cGVHD diseases, with a larger amount of CD1<sup>+</sup>, CD86<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and CD25<sup>+</sup> immunocompetent cells in lichen planus as compared to cGVHD, implying a different regulation of the inflammatory response in both conditions.

Hasséus, (2001) also noted that IL-2 receptors expressing CD25<sup>+</sup> cells were present in a high number in OLP than in cGVHD affected epithelium and connective tissue, these cells were activated T cells or a distinct population on regulatory cell.

Measuring the diagnostic accuracy (sensitivity and specificity) of both CD4<sup>+</sup> and CD25<sup>+</sup>. Our results showed that, CD4<sup>+</sup> have higher sensitivity (75%) than CD25<sup>+</sup> (60%). Regarding specificity, CD4<sup>+</sup> showed a higher specificity (80%) than CD25<sup>+</sup> (60%).

The results of our study showed that, there was a statistically significant association between lesion types and (CD4<sup>+</sup>CD25<sup>+</sup>)T regs. All types of OLP showed higher prevalence of positive (CD4<sup>+</sup>CD25<sup>+</sup>) T regs than control group. These results are in accordance with, X-a Tao *et al*, 2009, who noted that, the increased numbers of FOXP3<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup>Treg cells were detected in OLP lesions, and the transcription of FOXP3 was found to be significantly increased in OLP lesions. Therefore, these results indicated that FOXP3<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup> Treg cells were significantly correlated with the clinic forms and activity of OLP.

The variation in the present study results of CD4<sup>+</sup>CD25<sup>+</sup> T regs may be due to the technique used in X-a Tao *et al*, 2009 study, which was immune- histochemical and real time RT-PCR. However, in the present study we used indirect immunofluorescence technique. CD4<sup>+</sup>CD25<sup>+</sup> T regs showed high sensitivity (90%), high specificity (83.3%) and high diagnostic accuracy (87.5%). In addition, the ROC curve analysis showed the results were statistically significant ( $P < 0.001$ ). They also added that the density of FOXP3<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup>T regs cells in lesions was negatively correlated with the score of OLP disease activity, these results indicated that FOXP3<sup>+</sup> CD4<sup>+</sup>CD25<sup>+</sup>Treg cells were significantly correlated with the clinic forms and activity of OLP.

The salivary MMP-9 level in the present study, showed an increase in OLP patients ( $2.15 \pm 0.78$ ) compared to control ( $0.48 \pm 0.15$ ) the difference was statistically significant. Our results were in agreement with multiple studies conducted by Hofman (2001), Larrson *et al.*, (2005) & David *et al.*, (2010) they all concluded that, MMP-9 level was greater in OLP patients when compared to control groups. Moreover, Zhou *et al.*, (2001), noted an increase in MMP-9 level secretion by T cells in OLP using ELISA.

From the previous results we can conclude that, MMP-9 is a good salivary biomarker for OLP. Comparing the MMP-9 level between lesion types; Reticular type showed a statistically significant highest mean of MMP-9 level, whilst, there was no statistically significant difference between atrophic and erosive types, both showed a statistically significant low mean of MMP-9 levels. This was in accordance with Mazzarella *et al.*, (2006), who reported high levels of MMP-9 in reticular OLP than in erosive OLP. However, Chen *et al.*, (2008) stated that MMP-9 expression was significantly higher in oral

squamous cell carcinoma and atrophic OLP than in non-atrophic OLP and healthy tissue.

In addition, the variation in present study results for MMP-9 may be due to technique used in Chen *et al.*, 2008, study that was immunohistochemistry and ELISA technique. However, in the present study was used real time RT-PCR technique with notably higher sensitivity, specificity & diagnostic accuracy.

MMP-9 may be a useful predictive marker for determining malignant transformation potential in OLP. There are also several problems connected to the methodology of the research in the field. A plenty of studies used different methods of evaluating MMPs. Many quantitative protein expression studies confirmed the increased MMP -9 expression in OLP patients especially the reticular type. On the other hand, there are immunohistochemistry studies that described variable distributions of the enzymes. Both approaches usually confirm increased occurrence of MMPs in OLP patients. Such discrepancy can probably be explained by differences in the techniques of MMP-9 level assessment.

There was no statistically significant difference between +ve CD4<sup>+</sup>CD25<sup>+</sup> T regs and MMP-9 as salivary biomarkers in oral lichen planus therefore we conclude that in OLP patients the level of CD4<sup>+</sup>CD25<sup>+</sup>T regs is correlated with level of the MMP-9. The salivary level of CD4<sup>+</sup>CD25<sup>+</sup> T regs as well as MMP-9 were significantly higher in OLP lesion group compared to control group, therefore, both could be considered as a diagnostic biomarker for OLP and as a sensitive non-invasive, cost effective monitor for OLP disease activity, and therapeutic approaches.

In the future aspects, diagnosis of OLP histopathologically together with molecular biology and localization of their MMP-9 levels, can be used as an early biomarker for malignant transformation. OLP should be

carefully followed due to the possibility of malignant transformation and the definitive diagnosis should be established as early as possible. Finally the selective expansion of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells may stand as future strategy of OLP management.

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