

## DETECTION OF SERUM AND SALIVARY VEGF AMONG PATIENTS WITH DIFFERENT CLINICAL FORMS OF ORAL LICHEN PLANUS

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### ABSTRACT

The present study was designed to compare the prevalence of Vascular endothelial growth factor (VEGF) in saliva of oral lichen planus (OLP) patients with its serum levels, in order to verify the effectiveness of salivary VEGF in monitoring OLP lesions. Thirty eight individuals were included and subdivided into 4 groups, 8 patients suffering from papular OLP, 11 patients with atrophic OLP, 11 patients with erosive OLP and 8 individuals having age and gender matched with previous groups acting as a control group. Serum and whole unstimulated saliva samples were collected from all the included individuals to determine VEGF level in both saliva and serum utilizing the ELISA technique. The results showed that the control group had the lowest level of VEGF in both saliva and serum and the papular group had slightly higher values than that of the control. As for the erosive group, it showed the highest levels of VEGF in saliva as well as in serum followed by the atrophic group. Thus, it could be concluded that both serum and salivary VEGF levels correlate perfectly with the clinical severity of different OLP lesions and that VEGF seems to play an important role in pathogenesis, activity, and severity of OLP lesions also that the analysis of salivary VEGF level is a non-invasive reliable way for the diagnosis and the monitoring of disease activity and a measure of the effectiveness of new therapeutic modalities.

**Aim:** The current study compared the VEGF expression in both serum and saliva between various forms of OLP patients and control subjects in order to determine the effectiveness of salivary VEGF to reflect the disease activity.

### INTRODUCTION

Oral lichen planus (OLP) is an autoimmune chronic inflammatory disease affecting the oral mucous membrane in approximately 0.1-4% of the adult population worldwide with an incidence greater in women aged from 30 to 60 years. OLP

lesions usually have a symmetrical bilateral pattern located mainly on the buccal mucosa in about 80 – 90% of cases. OLP appears clinically as keratotic (reticular/papular or plaque like), erythematous/atrophic or erosive/ulcerative that may be accompanied by cutaneous lesions<sup>(1-4)</sup>.

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OLP cause is still unidentifiable with immunologic mechanisms contributing to its pathogenesis where there is an inflammatory response against the antigen on the keratinocytes of the epithelial basal cell layer, by activated T-lymphocytes, leading to their apoptosis accompanied by increased local cytokines expression and altered expression of adhesion molecules<sup>(3,4)</sup>.

Angiogenesis is a vital process that has a principle effect in physiological as well as pathological conditions where new blood vessels formation takes place from existing vascular elements to eliminate metabolic residues and supply O<sub>2</sub> and nutritive substances required for physiological conditions like normal tissue growth, embryonic development and wound healing<sup>(2)</sup>.

Angiogenesis is inactive and does not occur in the normal states in the adult vasculature. It plays an important role in the pathogenesis of chronic inflammatory diseases as rheumatoid arthritis, psoriasis, atherosclerosis and bronchial asthma and is also implicated in tumor development and metastasis<sup>(2,5)</sup>.

Vascular-endothelial growth factor (VEGF) being one of the main initiators of angiogenesis, it regulates directly and indirectly the differentiation and proliferation of endothelial cells. VEGF also referred to as vascular permeability factor (VPF) is secreted by various cells as tumor cells, macrophages, platelets and keratinocytes<sup>(2,6)</sup>.

A previous study showed that angiogenesis and VEGF expression were strongly associated with the different clinical forms of OLP lesions, which may reflect some insight into the mechanisms and treatment modalities of OLP<sup>(7)</sup>.

It was believed that the pathogenesis and progression of OLP was a result of the regulatory effect of VEGF on the endothelial cells<sup>(8)</sup>. Consequently, the current study was performed to screen the prevalence of VEGF in the saliva of OLP patients with various clinical presentations, compared to their serum samples, in order to

verify the effectiveness of salivary VEGF level for monitoring OLP lesions.

## Methodology

This study was performed on a total of 38 subjects where they were all assessed medically according to the modified "Cornell Medical index"<sup>(9)</sup> and were not under any current medical treatment, suffering from any systemic condition or having any other oral mucosal disease. The OLP patients were diagnosed based on clinical findings and confirmed by histopathological examination.

The subjects were subdivided into four groups:

Group I: included 8 patients suffering from papular/reticular OLP.

Group II: included 11 patients suffering from atrophic OLP.

Group III: included 11 patients suffering from erosive OLP.

Group IV: included 8 age and gender matched subjects with OLP patients and without having any oral mucosal lesions. They acted as a control group.

A total of 38 serum as well as 38 whole unstimulated salivary samples were obtained from all patients diagnosed with different clinical subtypes of OLP and from the healthy control group.

All the patients and controls were subjected to the following:

**Comprehensive oral diagnosis** was carried out for all participating individuals. Specimens were obtained from all OLP lesions (after patients' consent) where a surgical double wedge incisional biopsy was carried out to a depth of about 2mm<sup>(10)</sup>. Biopsy specimens were processed for histopathological examination in order to confirm the clinical diagnosis.

**Salivary sample collection:** Collection of whole unstimulated saliva (WUS) using standard techniques was done as described by *Navazesh* in 1993 (*11*). Concisely, individuals refrained from eating,

drinking, chewing gum etc., for at least ½ h prior to the evaluation. Samples were collected by asking subjects to swallow first, tilt their head forward and expectorate all saliva in a tube for 5 minutes without swallowing. After collection, all samples were immediately stored at -20°C until assayed.

• **Serum sample collection:** Using plain tubes, peripheral venous blood samples (5ml) were taken from all patient groups and controls by standard venipuncture. Samples were centrifuged, the clarifying supernatant was filtered and stored at -20°C till VEGF detection.

• **Detection of VEGF in all salivary and serum samples:** Saliva and serum samples were centrifuged for 2 min. at 10,000 xg and the clarified supernatant was filtered through a 0.45 µm low protein binding membrane, separated into 0.5 ml aliquots and frozen at -80°C until use for quantitation of salivary and serum VEGF by ELISA.

An ELISA kit provided by AviBion, Orgenium Laboratories Business Unit, Vantaa FINLAND was used for quantitation of VEGF in saliva and serum samples. VEGF ELISA kit is an in vitro enzyme-linked immunosorbent assay (ELISA) for quantitative measurement of human VEGF in cell culture supernatants, serum, plasma, CSF and urine.

This assay employs an antibody specific for human VEGF coated onto a 96-well plate. Standards, samples and biotinylated anti-human VEGF are pipetted into the wells and VEGF present in a sample is captured by the antibody immobilized to the wells and by the biotinylated VEGF-specific detection antibody. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted into the wells. The wells are again washed. Following this second wash step, TMB substrate solution is added to the wells, resulting in blue color development proportional to the amount of VEGF bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Statistical analysis was performed using analysis of variance to compare means of the three OLP types with the control healthy means using Tukey contrasts at 0.05 significance level. Correlation analysis were done to find the value of Pearson correlation and its probability between VEGF of saliva and serum. Data were calculated and presented from both saliva and blood serum samples as pg per ml (pg.ml<sup>-1</sup>). All statistical work were done using R statistical software version 3.3.3, <sup>(12)</sup> using 'Rcmdr' package <sup>(13,14)</sup>.

## RESULTS

Totally, the 30 OLP patients included 21 females and 9 males, the papular/reticular group were 3 females (38%) and 5 males (62%) with mean age of 53±6.8 years, the atrophic group were 9 females (82%) and 2 males (18%) with mean age of 47±11.7 years while the erosive group were 9 females (82%) and 2 males (18%) with mean age of 54±6.6 years.

Regarding VEGF levels, the control group and the popular type group had mean values and standard deviation in serum 68.8±7.3 pg/ml and 91.2±10.2 pg/ml respectively and in saliva were 142.4±8.2 pg/ml and 161.5±7.0 pg/ml respectively. As for the atrophic and the erosive groups, their mean values and standard deviation in serum were 335.9 ±24.6 pg/ml and 452.8±32.8 pg/ml respectively and in saliva 206.4±12.9 pg/ml and 282.4±21.5 pg/ml respectively, as shown in table (1) and figure (1).

Tukey contrasts was used to compare between the mean values of VEGF level in pg/ml in the serum and saliva of each two groups, regarding VEGF level in the serum, the papular group was higher than the control but with no statistical significance where the t value was 1.949 and P = 0.227 while between the control and the atrophic groups, the t value was -25.028 and there was high statistically significant difference (P=0.001) in favor of the atrophic group where it was higher than that of the control group, and between the control and the erosive groups, the t value was 35.985 and there was

high statistically significant difference (P=0.001), in favor of the erosive group, as for the papular and atrophic groups there was a high statistically significant difference (P=0.001) in favor of the atrophic group where the t value was -22.930. Also, when papular group was compared to the erosive, there was a high statistically significant difference (P=0.001) in favor of the erosive group with t value -33.888, when comparing the atrophic group and the erosive groups, there was a high statistically significant difference (p<0.001) with the t value 11.940 denoting significantly higher level in the erosive type, as shown in table (1).

Regarding VEGF level in the saliva, for the control and the papular groups, the t value was 2.645 and the P value=0.0563 showing no statistical significant difference, while between the control and the atrophic groups, the t value was -9.524 and there was high statistically significant difference (P=0.001) where the atrophic group VEGF level in saliva was significantly higher than that of the control group, also for the control group and the erosive group, the t value was 20.835 and there was high statistically significant difference (P=0.001), when comparing the papular group and the atrophic

group there was a high statistically significant difference (P=0.001) in favor of the atrophic group and the t value was -6.677. Moreover, when papular group was compared to the erosive group, the erosive group values were higher with statistically significant difference (P=0.001) and the t value -17.989, also the atrophic and the erosive groups, there was statistically significant difference (P=0.001) with the t value 12.326 in the favor of the erosive group, as shown in table (1).

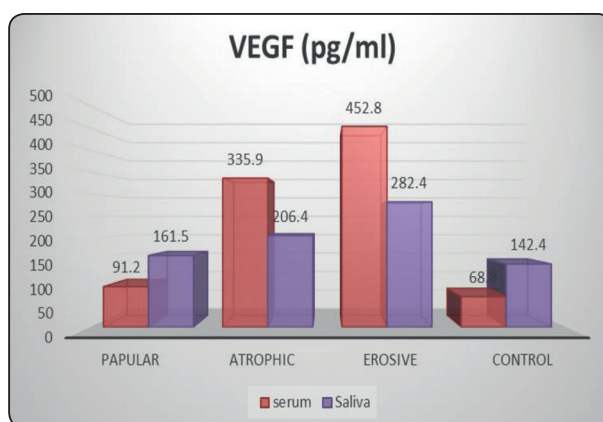


Fig. (1) Comparison of VEGF level in serum and saliva among all included groups

TABLE (1) Results of VEGF levels (pg/ml) in serum and saliva in all included patients' groups and the control group and statistical analysis comparing each two groups regarding VEGF level (pg/ml) in serum and saliva.

	Serum					Saliva				
	Mean ± SD	vs Control	vs papular	vs Atrophic	Vs Erosive	Mean ± SD	Vs Control	Vs papular	Vs Atrophic	Vs Erosive
Papular	91.2 ±10.2	t= 1.949 P<0.227	—	t= -22.930 P<0.001*	t= -33.888 P<0.001*	161.5 ±7.0	t=2.645 P<0.0563	—	t= -6.677 P<0.001 *	t= -17.989 P<0.001 *
Atrophic	335.9 ±24.6	t= -25.028 P<0.001 *	t= -22.930 P<0.001 *	—	t= 11.940 P<0.001 *	206.4 ±12.9	t= -9.524 P<0.001 *	t= -6.677 P<0.001 *	—	t=12.326 P<0.001 *
Erosive	452.8 ±32.8	t= 35.985 P<0.001 *	t= -33.888 P<0.001 *	t= 11.940 P<0.001*	—	282.4 ±21.5	t=20.835 P<0.001 *	t= -17.989 P<0.001 *	t=12.326 P<0.001 *	—
Control	68.8 ±7.3	—	t= 1.949 P<0.227	t= -25.028 P<0.001*	t= 35.985 P<0.001 *	142.4 ±8.2	—	t=2.645 P<0.0563	t= -9.524 P<0.001 *	t=20.835 P<0.001 *

## DISCUSSION

Angiogenesis plays an important role in the pathogenesis of a variety of chronic inflammatory disorders and intimately related to the disease activity as in rheumatoid arthritis, psoriasis, and osteoarthritis. Moreover a recent study stated that angiogenesis has a major effect on OLP chronicity and could be an indicator of its activity. OLP show apparent angiogenesis as a result of the hypoxic effect on the inflamed stromal area. The new vessels showed lymphocytes in-between endothelial cells indicating that angiogenesis in OLP has a critical role in the inflammatory elements turnover keeping the disease maintained as a consequence <sup>(2)</sup>.

VEGF significantly controls the process of angiogenesis and new formation of vessels. Hypoxia as that occurring in OLP as well as various inflammatory mediators like TNF- $\alpha$ , IL-6, IL-1, and IL-8 may upregulate VEGF expression. Previous studies had investigated the level of VEGF in serum among different clinical subtypes of OLP (5).

The current study compared the VEGF expression in both serum and saliva between various forms of OLP patients and control subjects in order to determine the effectiveness of salivary VEGF to reflect the disease activity.

In the present study both the atrophic and erosive forms of OLP showed higher levels of VEGF in serum and saliva, whereas the papular pattern revealed levels much lower compared to them, but higher than the control group yet with no statistical significance. These results could be, in a way, partially in accordance with those of *Mardani et al.* (5), where these authors considered "atrophic/erosive" OLP lesions as one group, where they found elevated levels of serum VEGF, significantly higher than the papular OLP and control groups. However, they reported significant difference between papular OLP cases and normal controls which is in contrast to what was registered in our present study.

Such elevated levels of VEGF could be due to its massively increased production throughout the cascade of events taking place in OLP lesions where VEGF expression took place within keratinocytes, fibroblasts, inflammatory cells and endothelial cells of OLP tissues <sup>(7)</sup> and consequently they act as the source from where VEGF spillover to serum and more logically to saliva given its direct contact with the OLP tissues thus, explaining its elevated level in both serum and saliva.

The current study showed that serum and salivary VEGF was highly correlated to the clinical form of OLP with the erosive OLP group showing the highest values followed by the atrophic group and finally the papular group with statistical significance between each two groups which was in accordance with previous studies showing that VEGF expression and consequently angiogenesis were related to and altered according to various clinical subtypes of OLP <sup>(5,7)</sup>.

The atrophic and erosive patients suffer burning sensation and pain exacerbated with acidic and spicy foods that may cause difficulty with eating, swallowing and speech while keratotic lesions are usually painless <sup>(1,3)</sup>.

According to the results of the present study, the increased VEGF level showed in the erosive group compared to other groups could explain that it is the most severe clinical form among the three groups with the patients suffering the most whereas the reticular/papular form with the least expression of VEGF, only slightly higher than what is expressed in control subjects, being the mild less severe cases.

Based on the findings of several previous studies that angiogenesis was significantly increased in OLP various forms compared to normal control, angiogenesis and particularly VEGF expression was used as a possible therapeutic target where they revealed huge significant reduction of VEGF immunoreactivity after the intralesional injection of bevacizumab compared to the control group causing a decreased angiogenesis and thus reversing the disease course <sup>(8)</sup>.

The critical role of angiogenesis in OLP has led to the concept of using anti-angiogenic therapy in the resistant patients to conventional corticosteroid treatment and also when using systemic corticosteroids is contraindicated (2).

The significantly increased serum and salivary VEGF level among the different patients included in this study, may give another prove that angiogenesis in OLP contributes to the disease pathogenesis, activity and severity. Moreover, serum and salivary VEGF could be utilized as a diagnostic marker and also as a monitor for patient's response to new therapeutic modalities with the saliva having the advantage of being easily collected with no need to special tools or trained personal for its collection giving it the superiority over analyses of the cellular and chemical constituents of blood and other biologic fluids used for the diagnosis of diseases

## CONCLUSION

Both serum and salivary VEGF levels correlate perfectly with the clinical severity of OLP lesions where erosive type register the highest values followed by the atrophic form. VEGF seems to play a major role in the pathogenesis, activity and severity of OLP lesions. Also the analysis of salivary VEGF level correlates well with its serum content making saliva a reliable tool for the diagnosis and the monitoring of disease activity and a measure of the effectiveness of new therapeutic modalities.

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