

## Estimation of Serum Connective Tissue Growth Factor in Oral Submucous Fibrosis Patients and its Clinico-Pathologic Correlation

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### Abstract:

**Background:** Oral submucous fibrosis (OSMF) is a common potentially malignant disease characterized by epithelial atrophy and progressive accumulation of collagen fibers in the underlying submucosal layer. Pathogenesis involved in the progression of OSMF is mostly increased collagen synthesis and/or reduced collagen degradation. Mediators such as growth factors, hormones, cytokines, and lymphokines mostly influence the synthesis of extracellular matrix and collagens in such fibrotic diseases. These mediators can mostly used as a biomarker for detection on disease progression. Among these, a serum level of connective tissue growth factor (CTGF) is mostly associated with the onset and progression of fibrosis in many human diseases such as liver fibrosis, myocardial fibrosis, and gingival fibrosis. Therefore, the aim of present study was to estimate serum CTGF level in OSMF patients and to correlate the serum CTGF levels with different clinical stages and histopathological grades of OSMF.

**Materials and Methods:** Blood samples were collected by 40 patients with histologically proven OSMF patients and 40 healthy control subjects. Serum CTGF levels were measured by enzyme-linked immunosorbent assay test.

**Results:** The mean serum CTGF level was significantly elevated ( $P < 0.0001$ ) in OSMF group ( $27.85 \pm 4.306$ ) compared to control group ( $15.00 \pm 2.025$ ). Serum CTGF level was increased progressively from clinical Stage I to Stage III and the difference between different clinical stages was statistically significant ( $P < 0.0001$ ). Serum CTGF level was gradually increased from histological Grade I to Grade III and the difference between different histological grades was statistically significant ( $P < 0.0001$ ).

**Conclusion:** Present study highlighted that the mean serum CTGF level was significantly elevated in OSMF patients. It was also indicated that the mean serum CTGF levels were positively correlating with clinical stages and histopathological grades of OSMF. So, serum CTGF level CTGF can be helpful in monitoring the fibrosis and predicting the progression of OSMF. Thus, serum CTGF level can be used as an adjunctive serological biomarker for monitoring the fibrosis and disease severity of OSMF.

**Key Words:** Connective tissue growth factor, extracellular matrix, fibrosis, oral submucous fibrosis

### Introduction

Oral submucous fibrosis (OSMF) is a chronic oral mucosal disease characterized by epithelial atrophy and progressive accumulation of collagen fibers in the lamina propria and the underlying submucosal layer.<sup>1</sup> The abnormal fibrosis always leads to stiffness of the mouth, with eventual immobility of the lips, cheek, tongue, and soft palate. The patients may experience impaired ability to eat, speak, and dental care. Many studies have been made on the mechanisms of fibrosis in OSMF, and the results suggest that increased collagen synthesis and/or reduced collagen degradation may involve in the development of the disease.<sup>2</sup>

Epidemiological studies have clearly shown that the regular use of areca nut is the major etiological factor of OSMF. Alkaloids and flavonoids present in areca nut namely arecoline, arecaine, guvacine, and guvacoline can cause collagen overproduction and collagenase inhibition resulting in increased collagen deposition in oral tissue, leading to fibrosis.<sup>3</sup> This increase in fibrosis results in stiffness of oral mucosa, limitation of mouth opening, and reduced mobility of tongue. It also causes reduction of vascularity resulting in subsequent hypoxia of surface epithelium which predisposes it to atrophy. Atrophied epithelium in OSMF is more susceptible to effects of carcinogenic agents and is at a greater risk for development of oral cancer.<sup>4</sup> Hence, the collagen production and degradation has been extensively studied at the molecular level in elucidating the pathogenesis of OSMF.

Mediators such as growth factors, hormones, cytokines, and lymphokines influence the synthesis of extracellular matrix (ECM) and collagens. Among these, transforming growth factor beta (TGF- $\beta$ ) has been suggested as the trigger for both the increased collagen production and decreased matrix degradation pathways in OSMF. However, many of the

downstream effects of TGF- $\beta$  leading to deposition of ECM are mediated by connective tissue growth factor (CTGF).<sup>5</sup>

CTGF, or CCN2, is a 38-kDa secreted protein belonging to the CCN protein family, consists of cysteine-rich 61 (CCN1), nephroblastoma overexpressed (CCN3), Wnt-induced secreted proteins-1 (Wisp-1/CCN4), -2 (Wisp-2/CCN5), and -3 (Wisp-3/CCN6). It promotes fibroblast proliferation, migration, adhesion, various ECM productions and plays important roles in wound healing.<sup>6</sup> Overexpression of CTGF is associated with the onset and progression of fibrosis in many human tissues, including scleroderma, pulmonary, hepatic, renal, and gingival fibrosis. In fibroblasts, CTGF is a downstream target of TGF- $\beta$  and synergizes with TGF- $\beta$  to promote a sustained fibrotic response *in vivo*.<sup>7</sup>

In recent years, the emphasis has been placed on detecting molecular markers from a body fluid such as serum, saliva, urine, and others, for detecting the disease, predicting prognosis and monitoring the disease progression. Screening and following patients by blood-based tests is more appealing than other tests due to its ease, economic advantage, non-invasiveness and possibility of repeated sampling.<sup>8</sup>

CTGF is secreted into the extracellular space and thus can reach the systemic circulation directly. It is detectable in various human body fluids such as serum, plasma, and urine. Its serum level may be the representation of the molecular changes at the tissue level. Serum level of CTGF may provide more information about fibrogenic activity, fibrotic remodeling and progression of fibrosis in various fibrotic diseases. Thus, it is used as a surrogate biomarker for fibrotic disorders.<sup>7</sup>

Considering OSMF as a premalignant condition with simultaneous involvement of multiple sites in the oral cavity, the serum CTGF level is likely to be more representative of disease activity than a biopsy from a particular site. Thus, it can be a promising biomarker for monitoring the fibrosis and disease severity of OSMF. So, the present study aimed to correlate the serum CTGF levels with different clinical stages and histopathological grades of OSMF.

### Materials and Methods

This study was enrolled by 40 OSMF patients (36 males and 4 females; mean age 26.95 years) and 40 healthy control subjects (36 males and 4 females; mean age 26.75 years). All the study patients were selected from those who visited the outpatient Department of Oral and Maxillofacial Pathology, Aurangabad. The patients with other malignancies, inflammatory diseases, or infections were excluded. An equal number of age and sex matched healthy subjects without any tissue abuse habits and without any clinically obvious oral lesions or systemic diseases were selected as the control group. All participants were thoroughly informed about the research study and written informed consent was obtained.

After obtaining ethical clearance from Institutional Committee, a detailed clinical history of the patients was taken, along with written informed consent from the patients (Figures 1-3). The patients were grouped clinically into three stages based on the criteria of given by Pindborg (1989).<sup>9</sup> An incisional biopsy was performed to confirm the diagnosis and histopathological grading was done into three grades according to Utsunomiya *et al.*, (2005) (Figures 4-6).<sup>9</sup>

Under all aseptic precautions, about 4 ml fasting venous blood was drawn from the cubital vein of individuals in study population using vacutainer blood collection system. Blood was collected in vacutainer sterile tubes. It was then allowed to clot at room temperature for about 2 h and then centrifuged at 3000 rpm for 10 min for serum separation. The serum samples obtained were stored at -20°C until analyzed for CTGF levels. Serum CTGF concentrations were measured by enzyme-linked immunosorbent assay in accordance with the manufacturer's instructions (Aviscera Bioscience Inc., Ltd. Santa Clara, CA).

Unpaired *t*-test was performed to compare the results of serum CTGF concentrations between controls and study



**Figure 1:** Oral submucous fibrosis patient with restricted mouth opening measured with the help of the vernier caliper.

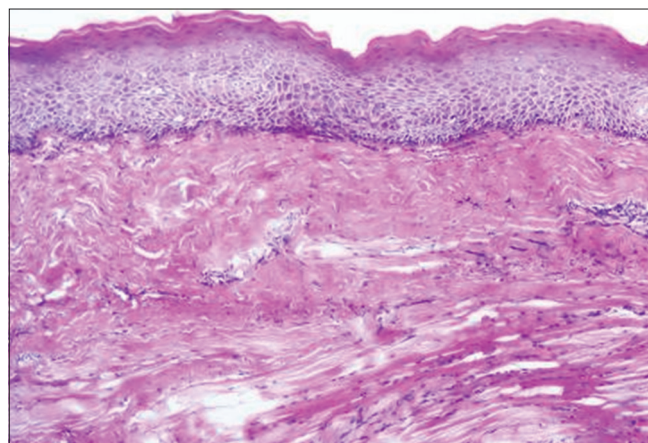


**Figure 2:** Intraoral photograph of oral submucous fibrosis patient showing blanching of the mucosa at the floor of the mouth.

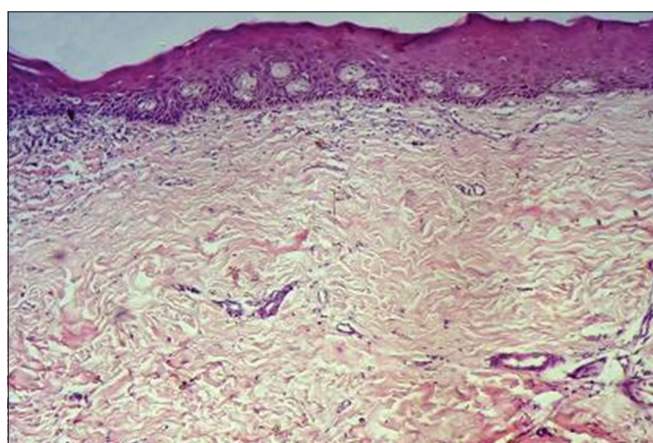




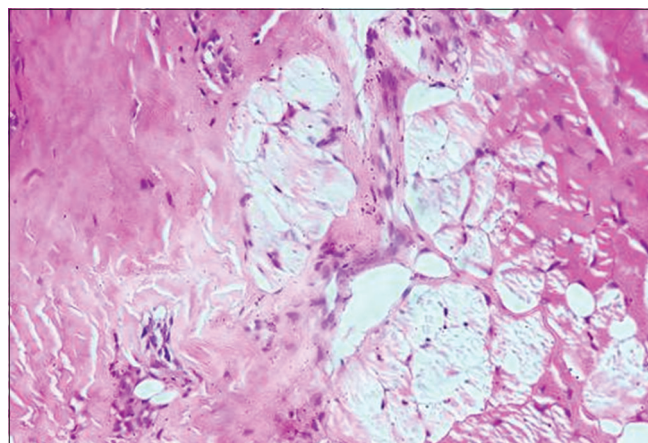
**Figure 3:** Intraoral photograph of oral submucous fibrosis patient showing blanching of the mucosa at the soft palate and also showing shrunken uvula.



**Figure 5:** Histopathological picture of the slide of oral submucous fibrosis patient showing Grade II changes in the epithelium and the connective tissue (H and E staining, ×100).



**Figure 4:** Histopathological picture of the slide of oral submucous fibrosis patient showing Grade I changes in the epithelium and the connective tissue (H and E staining, ×100).



**Figure 6:** Histopathological picture of the slide of oral submucous fibrosis patient showing Grade III changes in the epithelium and the connective tissue (H and E staining, ×400).

participants. One-way ANOVA and unpaired *t*-test were used as appropriate to validate the serum CTGF level in relation with clinico-pathological features of OSMF. All statistical analyzes were performed with the software Graph Pad prism 5 for Windows, version 5.02 and  $P < 0.05$  was accepted as statistically significant.

## Results

In the present study, the levels of serum CTGF were compared between the OSMF group and control group. The mean serum CTGF level in OSMF group was found to be  $27.85 \pm 4.306$  while in the control group it was  $15.00 \pm 2.025$ . Statistical analysis using an unpaired *t*-test showed a statistically highly significant difference in these two groups with  $P < 0.0001$  (Table 1 and Graph 1).

The levels of serum CTGF within different clinical stages were compared (Table 2). The mean serum CTGF level in clinical Stages I, II, and III was  $22.40 \pm 1.647$  ng/ml,  $28.92 \pm 2.985$  ng/ml, and  $33.40 \pm 1.140$  ng/ml, respectively.

This indicates that the levels increased progressively from Stage I to Stage III. The statistical evaluation of comparison was done using the one-way ANOVA test and it was observed that the difference in levels of serum CTGF between the clinical stages of OSMF was statistically highly significant ( $P < 0.0001$ ). It was observed that the difference in the serum CTGF levels between paired means of clinical stages were statically highly significant using unpaired *t*-test (Table 3 and Graph 2).

The levels of serum CTGF within different histopathological grades of OSMF were compared (Table 4). The mean serum CTGF level in histopathological Grades I, II, and III was  $21.429 \pm 0.787$  ng/ml,  $28.500 \pm 3.156$  ng/ml, and  $33.200 \pm 1.304$  ng/ml, respectively. This indicates that the levels increased progressively from Grade I to Grade III. From the statistical analysis using one-way ANOVA, it was observed that the difference in levels of serum CTGF between different histopathological grades of OSMF was statistically highly significant ( $P < 0.0001$ ). It was found that the difference in the serum CTGF levels between paired means of histopathological

grades were statically highly significant using unpaired *t*-test (Table 5 and Graph 3).

## Discussion

Fibrosis is the formation of excessive fibrous connective tissue in an organ or tissue in a reparative or reactive process. In the pathological state, it acts to deposit an excess of fibrous connective tissue, which can obliterate the structure and function of the underlying tissue or organ. Fibrotic disorders

are characterized by excessive formation, deposition and contraction of extracellular substances. This chronic process usually lead to organ or tissue dysfunction.<sup>10</sup>

OSMF is a form of pathological fibrosis affecting the oral mucosa. It is a chronic, insidious oral mucosal condition that occurs predominantly among Indians. Once initiated, the disease is ever-progressive even after cessation of the putative causative factor. It appears that fibrosis and hyalinization of the subepithelial connective tissue in OSMF accounts for most of the clinical features such as difficulty in opening the mouth, difficulty in protruding the tongue and pallor of the mucosa.<sup>11</sup> In addition, some of the epithelial changes such as atrophy, ulceration, and dysplastic changes have been proven to be associated with the advancement of fibrosis.

**Table 1: Comparison of mean serum CTGF level in controls and OSMF cases by unpaired *t*-test.**

Groups	Number of cases	Mean±SD	" <i>t</i> " value	P value
Serum CTGF (ng/ml)				
Controls	40	15.00±2.025	17.08	P<0.0001 HS
Cases	40	27.85±4.306		

HS: Highly significant, S: Significant, CTGF: Connective tissue growth factor, OSMF: Oral submucous fibrosis, SD: Standard deviation

**Table 2: Comparison of mean serum CTGF level in controls and various clinical stages of OSMF by one-way ANOVA test.**

Groups	Mean CTGF (ng/ml)	SD	F value	P value
Controls	15.00	2.025	237.5	P<0.0001 HS
Stage I	22.400	1.647		
Stage II	28.920	2.985		
Stage III	33.400	1.140		

HS: Highly significant, S: Significant, CTGF: Connective tissue growth factor, OSMF: Oral submucous fibrosis, SD: Standard deviation

**Table 3: Comparison of mean serum CTGF level among controls and various clinical stages of OSMF by unpaired *t*-test.**

Stages	Controls	Stage I	Stage II	Stage III
Stage I	P<0.0001 HS			
Stage II	P<0.0001 HS	P<0.0001 HS		
Stage III	P<0.0001 HS	P<0.0001 HS	P<0.01 S	

HS: Highly significant, S: Significant, CTGF: Connective tissue growth factor, OSMF: Oral submucous fibrosis

**Table 4: Comparison of mean serum CTGF level in controls and various histopathological grades of OSMF by one-way ANOVA test.**

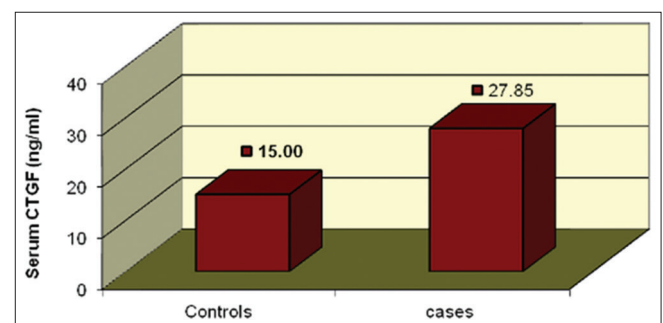
Groups	Mean CTGF (ng/ml)	SD	F value	P value
Controls	15.00	2.025	F=216.0	P<0.0001 HS
Grade I	21.429	0.787		
Grade II	28.500	3.156		
Grade III	33.200	1.304		

HS: Highly significant, S: Significant, CTGF: Connective tissue growth factor, OSMF: Oral submucous fibrosis

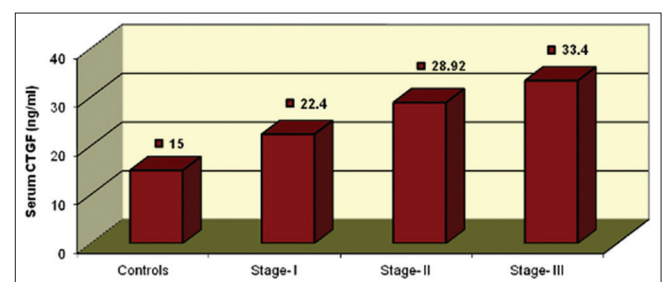
**Table 5: Comparison of mean serum CTGF level among controls and various histopathological grades of OSMF by unpaired *t*-test.**

Grades	Controls	Grade I	Grade II	Grade III
Grade I	P<0.0001 HS			
Grade II	P<0.0001 HS	P<0.0001 HS		
Grade III	P<0.0001 HS	P<0.0001 HS	P<0.01 S	

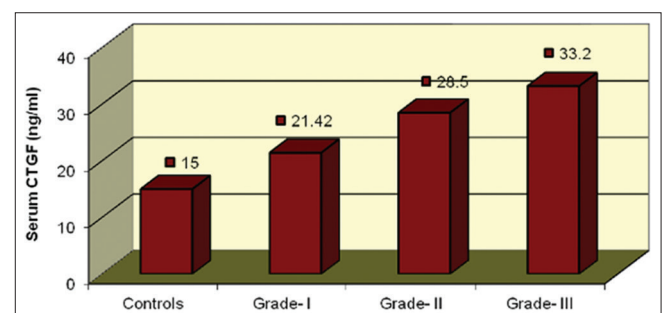
HS: Highly significant, S: Significant, CTGF: Connective tissue growth factor, OSMF: Oral submucous fibrosis, SD: Standard deviation



**Graph 1:** Comparison of mean serum connective tissue growth factor level in controls and oral submucous fibrosis cases.



**Graph 2:** Comparison of mean serum connective tissue growth factor level in controls and various clinical stages of oral submucous fibrosis.



**Graph 3:** Comparison of mean serum connective tissue growth factor level in controls and various histopathological grades of oral submucous fibrosis.



Patients with OSMF have a significant tendency to develop cancer with percentage ranging from 2% to 30%.<sup>12</sup> The pathogenesis of dysplastic lesions in OSMF is still not clearly understood. Jayasooriya *et al.*, found that the incidence of epithelial dysplasia was increased with the increase of the thickness of fibrosis, highlighting the fact that the advancement of fibrosis in OSMF increases the risk of occurrence of epithelial dysplasia. It has also been hypothesized that the reduction of blood supply allows carcinogens to accumulate on the surface epithelium for a longer duration, making the epithelium more susceptible to carcinogenesis. In addition, the hypoxic condition created due to the presence of dense fibrosis leads to the atrophic changes in the epithelium, making it more vulnerable to dysplastic changes.<sup>4</sup>

Various mechanisms have been suggested for increased fibrosis of OSMF. These include stimulation of fibroblast proliferation and collagen synthesis by areca nut alkaloids, stabilization of collagen structure by catechin and tannins from areca nut, decreased secretion of collagenase, increase in collagen cross-linking by up-regulation of lysyl oxidase, etc.<sup>13,14</sup> Others include deficiency in collagen phagocytosis and increased secretion of fibrogenic cytokines by activated macrophages, T lymphocytes, and fibroblasts.<sup>15</sup>

Among the fibrogenic cytokine released during the development of OSMF, TGF- $\beta$  is generally regarded as one of the key factor involved in fibrosis. However, many of the downstream effects of TGF- $\beta$ 1 leading to deposition of ECM are mediated by CTGF and ED-A form of the matrix protein fibronectin (FN).<sup>10</sup> Among these, CTGF has been shown to be up-regulated in various chronic fibrotic diseases.

In fibroblast, CTGF along with TGF- $\beta$  can promote a sustained fibrotic response *in vivo*. Neutralizing antibody to human CTGF inhibited TGF- $\beta$ -induced fibrosis, suggesting that CTGF acts as a secondary cytokine for TGF- $\beta$ . Biological effects on fibroblastic cells indicate that CTGF plays a key role in TGF- $\beta$  stimulated connective tissue formation in human fibroblastic diseases. CTGF alone can also stimulate a large increase in collagen synthesis over a range of low concentrations indicating that it can induce collagen synthesis in TGF- $\beta$ -dependent and -independent manners.<sup>5</sup>

As the clinical course of chronic fibrotic disorders varies between individual patients, there is a great need for non-invasive biomarkers that can help predict progression. CTGF seems to be an ideal candidate biomarker for monitoring ongoing fibrosis as it is a key mediator of fibrosis and is readily quantifiable in body fluids.<sup>7</sup>

In the present study, the mean serum level of CTGF was compared between control group and OSMF group. These levels were also correlated with clinical stages and histological grades of OSMF.

The mean level of CTGF was increased in the OSMF group ( $27.85 \pm 4.306$ ) compared to control group ( $15.00 \pm 2.025$ ). The statistical evaluation using unpaired *t*-test showed the difference in the levels of CTGF between the control group and OSMF group to be statistically highly significant. An increase in serum level of CTGF was noticed by Tamatani *et al.*,<sup>16</sup> Morikawa *et al.*,<sup>17</sup> Sato *et al.*,<sup>18</sup> Cheng *et al.*,<sup>19</sup> Koitabashi *et al.*, (2007)<sup>20</sup> in various chronic fibrotic disorders where they found an increase in serum CTGF levels compared to normal control groups. The biological functions of the CTGF such as proliferation, differentiation, ECM production and remodeling, FN production and cell-matrix interactions are mainly responsible for the development and maintenance of chronic fibrotic diseases.<sup>21</sup> As OSMF is a chronic fibrotic disease showing ECM production and remodeling, it is associated with an increase in the serum CTGF levels compared to control group.

Clinical stage wise analysis in OSMF cases showed that mean serum CTGF level gradually increased from Stage I to Stage III (Stage I  $22.40 \pm 1.64$ , Stage II  $28.92 \pm 2.98$ , and Stage III  $33.40 \pm 1.14$ ). The difference of mean serum CTGF level between different stages and controls was seen to be statistically highly significant using one-way ANOVA. There was a highly significant difference in the controls- Stage I, controls- Stage II, controls- Stage III, Stage I- Stage II, Stage I- Stage III, and Stage II- Stage III. So, the present study reported a positive correlation of serum CTGF levels with the clinical stages of OSMF.

In previous studies, it is well documented that, in OSMF there is progressive increase in blanching and stiffness of mucosa, palpable fibrous bands, inability to open the mouth and restriction of tongue movements depending on the severity of the disease process.<sup>22,23</sup> Presence of palpable fibrous bands and blanching are the most distinct clinical features for staging of OSMF. Increase in the rigidity of the mucosa in OSMF is mainly due to the result of production and accumulation of collagen fibers in the oral tissues. So, the collagen and ECM production may be responsible for increasing severity of clinical staging of OSMF.<sup>24</sup> Present study demonstrated a gradual increase in the mean CTGF level from clinical Stage I to clinical Stage III of OSMF. This may be due to increasing role of CTGF in production and accumulation of collagen fibers and ECM at various oral sites, which is observed with increased clinical severity.

In the present study, mean CTGF level in relation to histopathological grade was found to be  $21.429 \pm 0.787$  in Grade I,  $28.500 \pm 3.156$  in Grade II, and  $33.200 \pm 1.304$  in Grade III. The difference of mean serum CTGF levels between controls and different grades of OSMF was seen to be statistically highly significant. The difference was highly significant among the various histopathological grades of OSMF. So, the present study reported a positive correlation

between serum CTGF levels and histopathological grades of OSMF.

Progressive accumulation of collagen fibers in lamina propria and the submucosa is the hallmark for OSMF. In OSMF, tissues have been shown to contain fibroblast with an elevated basal collagen synthesis. The increase in histopathological grades is associated with an increase in fibrotic changes in the connective tissue. There is a progressive increase in thickness, hyalinization and dense arrangement of collagen fibers with the advancement of the disease.<sup>22</sup> Experimental study in chronic fibrotic diseases suggested that serum CTGF expression increases over time and run parallel with the process of fibrosis.<sup>7</sup> Increase in the serum CTGF levels in OSMF from Grade I to Grade III may be due to the increase in the process of fibrosis and hyalinization. CTGF was found to be responsible for the sustained fibrotic response in the disease.<sup>10</sup> Hence, its higher expression was seen in advanced grade.

A positive correlation of serum CTGF with clinical staging and histopathological grading of OSMF also demonstrates that the levels of CTGF correlate with the process of fibrosis in OSMF. In OSMF, only clinical staging and histopathological grading may not be a strict criterion to measure the degree of fibrosis and/or disease severity. As OSMF causes simultaneous involvement of multiple sites and there is variability in symptoms, clinical staging has some limitations. Similarly, OSMF is well-recognized as a pre-cancerous condition affecting various parts of the oral cavity. Hence, for thorough histopathological grading, biopsies from different sites in the same patient may be required which is impractical. On the other hand, changes in blood though they are secondary to the tissue changes may be more representative of the disease than clinical staging or histopathological grading.

The current study revealed a highly significant difference in the serum CTGF levels between control subjects and OSMF cases. There was an increase in serum CTGF levels with an increase in the severity of clinical stages as well as with the increase in histopathological grades. This increase in serum CTGF levels in OSMF positively correlated with disease severity.

From the present study, it was evident that serum CTGF seems to be an ideal non-invasive biomarker for monitoring and predicting the progression of OSMF. This in turn may be helpful for proper management of this potentially malignant condition.

## Conclusion

From the present study, it was evident that the mean serum CTGF levels were found to be positively correlating with clinical stages and histopathological grades of OSMF. So, CTGF can be helpful in monitoring the fibrosis and predicting the progression of OSMF. Thus, it can be concluded from this

study that serum CTGF level can be used as an adjunctive serological biomarker for monitoring the fibrosis and disease severity of OSMF. At the same time, it may helpful in controlling the disease progression from premalignant condition to malignant condition.

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