DETERMINATION OF PLASMA HOMOCYSTEINE LEVELS IN ORAL SUBMUCOUS FIBROSIS & ORAL SQUAMOUS CELL CARCINOMA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS PLAUSIBILITY AS A POTENTIAL BIOMARKER.

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ABSTRACT

Homocysteine is a non essential amino acid, and the altered levels are noted in various diseases, including a plethora of cancers. A thorough literature review of various cancers like that of breast, lung, prostate, colorectal region, ovaries and head and neck, revealed inconsistent plasma homocysteine levels. Only a handful of studies on amino acids exist in oral potentially malignant disorders and oral carcinomas. Thus, we aimed at evaluating plasma Homocysteine levels in healthy controls, OSF and OSCC (Oral Squamous Cell Carcinoma) patients; and determining its plausibility as a potential biomarker in these conditions. Plasma homocysteine was evaluated in controls (n=10), OSF (n=20) and OSCC patients (n=20) using Reverse phase High Performance Liquid Chromatography. The results obtained were statistically analyzed using Non parametric tests such as Mann Whitney U and Kruskal Wallis tests. The plasma homocysteine levels were elevated in OSF and OSCC patients as compared to healthy controls. Conversely, levels in OSCC patients were lower compared to OSF patients. These levels were correlated amongst the three groups and with their clinical parameters. Although statistically insignificant, the altered plasma homocysteine level plays a vital role in the pathogenesis of these conditions as it brings about oxidative DNA damage, initiating carcinogenesis. It is an
indicator of folate and Vitamin B12 levels, and supplementation with these vitamins could act as chemopreventive agents in combating hyperhomocysteinemia, arresting the disease progression in OSF and aiding in and treatment of OSCC. Concerted efforts would help in early detection, management and monitoring the efficacy of treatment.

**KEYWORDS:** Homocysteine, OSF, OSCC, plasma.

**INTRODUCTION**

Oral cancer is on a perpetual rise in the Indian subcontinent, owing to an increase in chewing tobacco and allied products like betel nut, betel quid, pan-masala etc. Oral Squamous Cell Carcinoma (OSCC) develops through a multi-step process of genetic, epigenetic and metabolic changes resulting from exposure to carcinogens. Oral Submucous Fibrosis (OSF), a potentially malignant disorder (PMD) acts as a precursor to OSCC.

Biomarkers like plasma free amino acids may help to categorize PMDs as having a high risk for malignant transformation and may aid in its early detection and management. Amino acids and their derivatives can be useful ‘molecular/disease markers’ as they reflect the protein metabolism, problems related to dietary uptake and aid in understanding the metabolic derangements that occur during the pathological processes induced by the PMDs and cancer.[1] Plasma homocysteine, a non-essential amino acid, is considered a helpful indicator of vitamin status for its strong correlation with folic acid, Vitamin B12 and Vitamin B6.[2]

Only a handful of studies have shown an association of plasma homocysteine in PMDs of the oral cavity and elsewhere, with a few reports showing elevated homocysteine levels. The current study is only a third of its kind, to evaluate the plasma homocysteine levels in OSF and is the first to compare and analyze the plasma homocysteine levels between OSF and OSCC. Thus this study aimed to assess the plasma homocysteine levels in controls, OSF and OSCC patients; to compare the levels between the different groups and assess the levels during the progression of OSF to OSCC. This study also intended to study the plausibility of using plasma homocysteine as a biomarker.

**MATERIALS AND METHODS**

The study was conducted in the Department Of Oral Pathology and Microbiology, SDM College of Dental Sciences and Hospital, Dharwad. 50 patients, comprising of 20 cases each
of OSF and OSCC and 10 healthy controls visiting the OPD were included in the study after obtaining permission from the institutional review board. Age and sex matched subjects, who had no habit history and had no systemic or infectious diseases were included in the control group. A detailed case history of patients diagnosed with OSF and OSCC was recorded using a pre designed proforma. Clinical examination was performed and the clinical staging was done according to the criteria given by Ranganathan et al.,(2001) for OSF;(Fig. 1).

![Fig. 1: Clinical photograph showing reduced mouth opening and the blanching of the soft palate and retromolar trigone in an OSF patient.](image)

While for OSCC patients (Fig. 2), clinical staging was done according to the 7th TNM Revision given by AJCC and UICC (2010).

![Fig. 2: Clinical photograph of patient with Squamous cell carcinoma of the right buccal mucosa and retromolar trigone, involving the gingivobuccal sulcus, alveolus, maxillary tuberosity and the posterior hard palate.](image)
5ml of blood was collected from study and control groups in EDTA coated tubes and centrifuged immediately to collect plasma and stored at -20 degrees centigrade. The plasma was subjected to Reverse Phase HPLC analysis at Prajna Biosciences, Hubli, to determine the homocysteine levels. For the determination of total thiols, 200µL of plasma was added to 100 µL of 1mol/L TRIS buffer (pH 7.0) and 10µL of 0.25mol/L tris(2-carboxyethyl)phosphine(TCEP) hydrochloride. After 15minutes of incubation at room temperature, 30µL of 0.1mol/L 1-benzyl-2-chloropyridinium bromide (BCPB) was added, vortex-mixed and kept at room temperature for 10min, followed by addition of 20µL of Perchloric acid (70-72%, ρ=1.67g/mL).(Fig. 3).

Fig. 3: TRIS buffer, TCEP [tris(2-carboxyethylphosphine hydrochloride)], DL-Homocysteine, Perchloric acid, Trichloroacetic acid, Lithium Hydroxide monohydrate, Acetonitrile used for derivatization of homocysteine.

The mixture was centrifuged at 12,000rpm for 10min and the supernatant thus obtained was transferred to a vial, followed by injection (20µL) into the chromatographic system for Reverse Phase HPLC analysis(Fig. 4).
Fig. 4: Reverse Phase- High Performance Liquid Chromatography (RP-HPLC) analysis unit.

The values obtained from the aforementioned three groups were subjected to statistical analysis using Non parametric tests like Mann Whitney U Test and Kruskal Wallis test with the aid of SPSS software (IBM).

RESULTS

In the control group, 9 were males and 1 was female. Among 20 OSF cases, all were males. Among 20 OSCC cases, 14 were males; 6 were females. The mean plasma homocysteine level in males and females were 30.22±22.46 and 17.82±7.76 at the mean age of 45.45±16.90 and 40.79±18.83 respectively. Thus, the plasma homocysteine concentration was higher in males with advancing age. (Table 1).

Table-1: Comparison of mean plasma homocysteine levels with mean age in males and females in the control and study groups.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number</th>
<th>Mean Concentration of Homocysteine ± SD</th>
<th>Mean Age ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>43</td>
<td>30.22 ± 22.46</td>
<td>40.79 ± 18.83</td>
</tr>
<tr>
<td>Females</td>
<td>7</td>
<td>17.82 ± 7.76</td>
<td>45.85 ± 16.90</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>28.48 ± 21.42</td>
<td>41.50 ± 18.49</td>
</tr>
</tbody>
</table>

The OSF and OSCC patients were divided into various groups based on the duration (in yrs) and frequency of habits (in times/day) and were compared with the plasma Homocysteine
levels patients. Most of the OSF patients gave the history of using tobacco products for about 5 times/day for about 5 yrs, while the OSCC were habituated with these products for about 5-10 times/day for over 15 years. (Table 2) No statistically significant differences were noted when the plasma homocysteine levels were correlated with the duration and frequency of habits.

Table-2: Duration and frequency of habits in OSF and OSCC patients.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Duration (in yrs)</th>
<th>Frequency(times/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OSF</td>
<td>OSCC</td>
</tr>
<tr>
<td></td>
<td>Duration No. Of patients</td>
<td>No. Of patients</td>
</tr>
<tr>
<td>GROUP I</td>
<td>0-5 13</td>
<td>0 0</td>
</tr>
<tr>
<td>GROUP II</td>
<td>5-10 2</td>
<td>1 5</td>
</tr>
<tr>
<td>GROUP III</td>
<td>10-15 2</td>
<td>3 10</td>
</tr>
<tr>
<td>GROUP IV</td>
<td>&gt;15 3</td>
<td>&gt;15 1</td>
</tr>
</tbody>
</table>

Non parametric test, such as Kruskal Wallis test was used to compare the plasma Homocysteine, levels with age, duration and clinical stages whereas, another non parametric test, Mann Whitney U Test was used to evaluate the frequency of the habit.

The mean plasma homocysteine levels in OSF patients complaining of burning sensation only were 31.4850±17.46504. While in patients complaining of reduced mouth opening, the mean was 26.7139±19.65777. The plasma homocysteine levels appeared higher in patients who had both burning sensation and reduced mouth opening with the mean being 45.4067±39.57513. No statistically significant correlation was noted in patients with the different chief complaints and the plasma homocysteine levels. (Table 3).

Table-3: Details of chief complaint and association with the plasma levels of homocysteine in OSF group.

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Chief complaint</th>
<th>Number of patients</th>
<th>Mean concentration (μmol/L)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Burning Sensation</td>
<td>7</td>
<td>31.4850</td>
<td>17.46504</td>
</tr>
<tr>
<td>2</td>
<td>Reduced mouth opening</td>
<td>3</td>
<td>26.7139</td>
<td>19.65777</td>
</tr>
<tr>
<td>3</td>
<td>Burning sensation &amp; reduced mouth opening</td>
<td>9</td>
<td>45.4067</td>
<td>39.57513</td>
</tr>
<tr>
<td>4</td>
<td>Others like vesiculation</td>
<td>1</td>
<td>12.2382</td>
<td></td>
</tr>
</tbody>
</table>

A majority of the OSF patients were in Stage III of this disease with a mean plasma homocysteine levels of 50.1463μMol/L. While the mean levels in Stage I, II and IV were
53.2186μMol/L, 38.5585μMol/L and 50.1463μMol/L respectively, displaying an inconsistency in the plasma homocysteine levels with the progression of the disease, albeit a decrease was noted in the advancing stages of the disease. A statistically significant difference was noted amongst the plasma homocysteine levels of Stage I and Stage II as the $P=0.033$ (Table 4).

Table-4: Comparison of the clinical staging and the mean concentration of plasma homocysteine level in OSF patients.

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>Mean conc of homocysteine ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>53.2186</td>
</tr>
<tr>
<td>Stage II</td>
<td>38.5585</td>
</tr>
<tr>
<td>Stage III</td>
<td>50.1463</td>
</tr>
<tr>
<td>Stage IV</td>
<td>6.3342</td>
</tr>
</tbody>
</table>

Most of the OSCC patients were in Stage III (n=16; mean=29.6965μMol/L) and Stage IV (n=4; mean=30.0136μMol/L). However, the mean levels in stage I and II were 53.2186μMol/L and 22.7134μMol/L respectively. A statistically insignificant difference was observed between the disease progression and the concentrations of plasma homocysteine. This is probably because of uneven distribution of sample in the study. (Table 5).

Table-5: Comparison of mean concentration of plasma homocysteine amongst the clinical stages of OSCC.

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>Mean conc of homocysteine ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>53.21±25.30</td>
</tr>
<tr>
<td>Stage II</td>
<td>22.71±13.62</td>
</tr>
<tr>
<td>Stage III</td>
<td>29.69±26.17</td>
</tr>
<tr>
<td>Stage IV</td>
<td>30.01±20.80</td>
</tr>
<tr>
<td>Stage IVa</td>
<td>27.37±5.88</td>
</tr>
</tbody>
</table>

The mean concentration is higher in the OSF group as compared to the OSCC and the control group. The plasma homocysteine levels were compared between the three groups using Mann Whitney U test to qualitatively analyze which revealed statistical insignificance. (Table 6).

Table-6: Comparison of the mean concentration of plasma homocysteine and mean age amongst the study and control groups:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of Samples</th>
<th>Mean conc ± SD</th>
<th>Mean age ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSF</td>
<td>20</td>
<td>36.07 ± 29.81</td>
<td>30.90 ± 14.20</td>
</tr>
<tr>
<td>OSCC</td>
<td>20</td>
<td>23.52 ± 11.18</td>
<td>58 ± 12.64</td>
</tr>
<tr>
<td>CONTROLS</td>
<td>10</td>
<td>23.24 ± 11.98</td>
<td>29.70 ± 9.46</td>
</tr>
</tbody>
</table>
DISCUSSION

Oral Submucous Fibrosis, a PMD has a multi-factorial etiology although, chewing of areca nut and tobacco are chiefly associated with this disorder in the south East Asian populations. It causes significant morbidity and has a malignant transformation rate of about 7-13%. The pathogenesis of this PMD has not been exactly elucidated. The nutritional deficiencies like iron, folates, Vitamin B12 and Vitamin B6 might not play a primary role in the etiopathogenesis, but it could synergize the symptomatology by contributing to epithelial atrophy. It is still contentious if nutritional deficiency is the primary etiology of OSF or if it occurs secondary to this disease process.

The total amino acids content of plasma reflects the nutritional/metabolic status, whilst, the individual amino acids profile of biological fluids are of importance in confirming or to rule out suspected pathological conditions such as liver and renal failure, diabetes, muscle dysfunction, aminoacidemia, cervical dysplasia, oral PMDs such as OSF, laryngeal leukoplakia, oral lichen planus and PMDs elsewhere in the body and various cancers. Hence these ‘biomarkers’ hold great promise in early detection, screening, disease progression monitoring, or in evaluation of the efficiency of therapeutic measures.

Homocysteine is a non-essential, sulfur containing amino acid. Numerous nutritional, hormonal and genetic factors are characterized by elevations in circulating homocysteine concentrations and they are also associated with specific pathological conditions, including cancer development, autoimmune diseases, vascular dysfunction and neurodegenerative disease. There is a paucity of studies that evaluate alterations of homocysteine in the PMDs of the oral cavity.

In the current study, in the OSF group, all the 20 subjects were males. About 70% of these patients chewed gutkha alone or in combination with other habits. Hegde K et al., found a similar male preponderance for this condition in their study, as they were addicted to gutkha and other related products which were easily available.

Most of the patients of the OSF study group belonged to the second and third decades of life with the mean age being 30.9yrs±14.20. Interestingly, habitual gutkha users alone, or in combination with betel quid, present with OSF at earlier ages, compared with traditional betel quid users.
Most of the patients with OSF, present to the clinicians either with the complaint of reduced mouth opening or with burning sensation which renders a phase of difficulty in consumption of normal diet leading to poor nutrition. A wide body of work done on OSF, substantiates the association of symptomatology with that of nutrition. Deficiency of iron and vitamin B complex, other trace elements and lipids, could possibly initiate anemia, alter the cell-mediated immunity and generate free radicals and reactive oxygen species (ROS) from the peroxidation of lipids, which acts as a promoting factor to this pre-existing pathologic response of the lamina propria and induce DNA damage.\[^{12,13}\] After a frank establishment of the lesion, anemia may further perpetuate by inadequate intake of food due to fibrosis and trismus.\[^{14}\] In the present study, 16 out of 20 patients complained of burning sensation either alone or in combination with reduced mouth opening and vesiculation. Although, hyperhomocysteinemia was noted in patients complaining of both burning sensation and reduced mouth opening, we found no statistically significant differences amongst the various presentations. The psychological stress due to burning, pain and reduced mouth opening could have lead to reduced intake of food, leading to the nutritional deficiency, consequently leading to an increase in homocysteine levels.\[^{15}\]

The metabolism of homocysteine involves the activity of folates (belonging to B complex vitamins) as co-factors. The deficiency of micronutrients such as folate leading to hyperhomocysteinemia, consequently leads to impaired methyl transfer in the methionine cycle, disrupting the stability of normal structure of DNA and inducing chromosomal breaks and abnormalities. There is evidence that homocysteine levels greater than 20μmol/L, noted in our cases as well, brings about a copper-dependant oxidative damage to cellular and isolated DNA. In the presence of copper, homocysteine causes cleavage of thymine residues located at 5' and/or 3' to guanine. In patients with betel quid chewing habit, the betel nut within the quid, acts as a source of high levels of soluble copper, which gets acted upon by Lysyl oxidase, a copper dependant enzyme, which is vital for collagen synthesis and cross linkage of fibers in OSF. Thus, it can be postulated that, the increased availability of copper and homocysteine doubles the chance of DNA damage.\[^{16}\] However, the mechanism of chromosomal damage by folate deficiency might be due to reduced methylation of uracil to thymine, leading to subsequent incorporation of uracil into human DNA.\[^{17}\] Thus, elevated homocysteine is now considered an important risk factor for genetic instability as it induced direct damage to cellular and isolated DNA through the generation of reactive oxygen species.\[^{18}\] It can be inferred that, the pre-existing nutritional deficiency alleviates DNA
damage caused by gutkha chewing habit, thus predisposing an individual for malignant transformation into OSCC.

We studied and analyzed the association of the alterations in plasma homocysteine levels with the various stages of OSF, in order to understand the disease progression and the pathological rise in homocysteine levels. A statistically significant difference was noted in the plasma levels of homocysteine in Stage I and II as the p=0.033. A gradual increase in plasma homocysteine levels from Stage II to Stage III of the disease has been noted. These findings are similar to those of Bias PS et al., who estimated serum homocysteine and concluded it as a biological marker, in OSF patients of western Uttar Pradesh.[19] Irrespective of the inconsistencies in plasma homocysteine levels with the progression of the disease, it is imperative to note that the altered levels could reflect the pathological processes of OSF, aiding in the diagnosis and prognosis of the disease.

The mean plasma homocysteine levels in OSF patients were increased compared to that of controls and similar results were noted in the study conducted by Narang et al., who carried out a study in OSF patients to determine if serum homocysteine could aid in the diagnosis of OSF. It was inferred that the homocysteine levels were directly associated with the disease progression; and that it could be used as a prognostic marker.[20]

Various essential and non essential amino acids have been studied extensively in the head and neck squamous cell carcinomas (HNSCC); albeit only a handful of studies have analyzed plasma homocysteine in OSCC.

The current study consisted of 20 OSCC patients with the mean age of 58±12.64 years with a predilection for males (14/20) and the buccal mucosa (due to betel quid chewing habit) being the most commonly affected site. These findings are well in accordance with that found in the literature.[21-23]

The mean plasma homocysteine levels of patients with OSCC were slightly higher compared to the control group, though no statistically significant association was noted amidst the two. As staging is paramount in assessing the prognosis of the patient, we attempted to study the association of plasma homocysteine with the progression of the malignancy. A gradual reduction in the levels of homocysteine was noted from Stage I to IV, although statistically insignificant; which was attributed to the utilization of homocysteine by the tumoral cells.
These findings were in consonance with that of Eleftheriadou A et al., who studied serum folate and homocysteine levels in HNSCC patients and its association with smoking. They found no correlations between the levels of homocysteine and folate with that of tumor stage and other clinical parameters and hence did not consider hyperhomocysteinemia and hypofolatemia to be markers of tumor progression.[24] Due to confounding reports obtained from various studies, it is imperative to study the association of the plasma homocysteine levels and the tumor progression.

In the present study, about 5/20 patients were smokers for over 15 years. Chronic smoking is associated with a lowered systemic status of several B vitamins, reduced oral folate and changes in folate form and its distribution in the mouth. Therefore, cigarette smoking alters the systemic uptake or metabolism of folate either directly or by modifying other components of one-carbon metabolism. This fact is exemplified by depletion of Vitamin B6, which serves as a cofactor for the inter-conversion of different forms of folate, in smokers.[25] Besides the direct genotoxic effect of tobacco smoke on buccal mucosal cells, a second pro-carcinogenic pathway initiated by smoking may involve the depletion of folate and thereby, the reduction of cofactors necessary to support efficient DNA synthesis, repair and methylation. Folate depletion causes various genetic and epigenetic aberrations in mammalian cells such as genomic and p53-specific DNA breaks and genomic and p53-specific hypomethylation; and resultant hyperhomocysteinemia. Thus, in all likelihood, folate deficiency might attenuate the genotoxic effects of smoking and thus predispose and individual to the development of cancer.[26]

Alcohol consumption is associated with deficient availability of folate, due to the blockade in its intestinal absorption. Thus, causing hyperhomocystenemia and bringing about numerous cellular and metabolic aberrations. Alcohol also can affect levels of homocysteine and its metabolites, S-adenosine Methionine (SAM) and S-adenosine Homocysteine (SAH), by reducing MTR activity, which in turn, results in decreased SAM levels and enhanced generation of homocysteine and SAH. A decrease in the MTHFR and Betaine homocysteine methyltransferase (BHMT) activity is induced after alcohol ingestion. However, after extended periods of alcohol exposure, this alternate pathway cannot be maintained, resulting in a decrease in the hepatocyte level of SAM, increase in SAH and homocysteine levels and a reduced SAM-to-SAH ratio.[25] In the current study, 5/20 patients in the OSCC study group were chronic alcoholics who consumed alcohol daily for over 15 years. Thus, it can be
hypothesized that alcohol causes hyperhomocysteinemia in the deficiency of folate leading to predisposition to cancer. Hence, the synergistic activity of the habits and the nutritional deficiency can amplify the development of oral cancer.

Nutrition is an important factor required to assess the mortality and morbidity of OSCC. The patients with OSCC show signs and symptoms of ulceration, pain and swelling. Dietary intake reduces due to the associated increased morbidity. Thus, the nutritional role has been postulated to play either a primary role or to secondarily contribute to the disease process and its progression. The mechanism for the lowered levels of folate being a risk factor for OSCC, has not been fully exemplified. The lowered folate levels could be due to change in physiology of oral cancer patients, leading to the methylation levels to become highly folate dependent. The tumor is made up of a rapidly dividing population of cells, having a high nutritional demand, indicating an increase in protein synthesis and DNA replication and consequently, an elevated demand for methionine, folate and homocysteine is noted to aid in this chain reaction. This demand causes disarray in the ratio of cofactors that are paramount for balance and proper functioning of the methylation process, particularly in dividing cells. At the bottom line of it all, there is an increased demand for folate in cancer patients; but their dietary patterns will not increase to effectuate that requirement and may rather lessen due to the disease. Due to the intense demand for folate, there is an ensuing struggle that the cells undergo to function with lowered levels of folate. Thus, the methylation status of these individuals becomes even more perturbed. Research has shown that whereas folate supplementation in healthy cells is generally protective against the development of tumors, in epigenetically disrupted cancerous cells, supplementation in fact may increase tumor growth.\(^{[27]}\)

The genetic factors play a momentous role in the metabolic pathway of homocysteine. Among genes involved in the methionine cycle, genes coding for MTHFR and Cystathionine β-synthase (CBS) enzyme, play a pivotal role in the synthesis of purines and pyrimidines of the DNA and have been studied extensively in relation to carcinogenesis.\(^{[28]}\) The polymorphisms associated with the MTHFR gene like \((677\; C\; to\; T;\; Ala\; to\; Val)\) have been studied extensively in relation with cancer risk.

The tumoral progression is associated with two principal mechanisms through which the alterations in the levels of folate and homocysteine may increase the risk of malignancy. Folate deficiency, by reducing intracellular SAM, can alter cytosine methylation in DNA,
leading to the inappropriate activation of proto-oncogenes, repression of tumor suppressor genes and induction of malignant transformation. Alterations in DNA methylation, particularly, global hypomethylation and a regional hypermethylation, especially of promoters of tumor suppressor genes, have been described in human tumors. In HNSCC, promoter hypermethylation of key genes in critical pathways is common and has recently been described. Since folic acid is fundamental in the normal DNA synthesis and repair, its deficiency may cause an imbalance in DNA precursors, uracil misincorporation.\(^{29}\)

One of the key determinants of homocysteine metabolism is cellular methylation demand. The over-production of methionine due to aberrations in the metabolic pathway, leads to formation of cystathionine via the trans-sulfuration pathway. However, when methionine levels are low, homocysteine is mainly metabolized via re-methylation pathway, generating methionine and, consequently, SAM, which is the exclusive source of methyl groups for all methylation reactions in cells. Thus, high levels of homocysteine are associated with reduced methylation capacity and, therefore, the re-methylation reaction of homocysteine to methionine is probably the most important reaction affecting plasma homocysteine concentration.\(^{30}\)

As described earlier, the over-production of the oxygen free radicals generated from the oxidation of homocysteine, is the major cause of DNA damage. As the reduced free homocysteine contains a free sulfhydryl group, free radicals including hydrogen peroxide can be generated upon oxidation of homocysteine, forming a disulfide linkage with free sulfhydryl group of albumin, cysteine or homocysteine. Almost certainly, it has been proven that the plasma level of reduced free homocysteine is the one that affects and enhances oxidative stress. The hydrogen peroxide and oxygen free radicals endogenously attack the DNA which leading to fabrication of many DNA adducts that can be detected in human cells. Oxidation of DNA may cause gene mutation such as p53 and ras gene, and eventually lead to carcinogenesis. Studies have also shown that oxidative DNA damage such as 8-hydroxyguanine accumulates in cancerous tissue.\(^{31}\)

The mean plasma homocysteine levels in OSCC patients (23.52±11.18) were higher when compared to the controls (23.24±11.98) but lower when compared to the OSF patients (36.07±29.81) The increase has been associated with the tumoral progression and is in accordance with the findings of Almadorri et al.\(^{29}\) While, the reduction in the levels is attributable to the increased uptake of homocysteine by the rapidly dividing tumoral cells or
due to the excessive production of ROS (Reactive Oxygen species) and NO (Reactive Nitrosative species) produced by increased oxidative stress in the tumor. This causes the over activity of metabolic pathways (trans-methylation and trans-sulfuration pathways) of homocysteine. This finding is in congruence with that of Campos et al., who studied the modulation of oxidative stress in Melanoma cell lines.\textsuperscript{[32]}

CONCLUSION
In the current study, no statistically significant differences were noted in the plasma homocysteine levels amongst the study and control groups. This might be attributed to the limited subjects studied in each group. Thus, this study needs to be conducted on larger population to derive promising results.

The plasma homocysteine level is a sensitive marker to screen for the subclinical deficiencies of micronutrients like folate and Vitamin B12. Since the deficiency of vitamin B12 and folates are shown to be causative of OSF and OSCC, homocysteine could be used as a plausible biomarker in detecting OSF and OSCC at the early stages of the disease.

Since hypofolatemia leads to hyperhomocysteinemia, folic acid, Vitamin B6 and B12 supplementation can be used as chemopreventive agents, which act as a primary mode of prevention and reduce the risk of cancer development in high risk patients. It might probably improve the prognosis and reduce the loco-regional recurrence of the primary disease as well as the development of the second primary tumors in the already treated cases of OSCC.

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REFERENCES


