Journal of the Formosan Medical Association (2015) 114, 813–819

Available online at www.sciencedirect.com

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journal homepage: www.jfma-online.com

ORIGINAL ARTICLE

High frequencies of vitamin B_{12} and folic acid deficiencies and gastric parietal cell antibody positivity in oral submucous fibrosis patients

Yi-Ping Wang [a,b], Yang-Che Wu [a,b], Shih-Jung Cheng [a,b], Hsin-Ming Chen [a,b,c], Andy Sun [a,b], Julia Yu-Fong Chang [a,b,*]

[a] *Graduate Institute of Clinical Dentistry, School of Dentistry, National Taiwan University, Taipei, Taiwan*
[b] *Department of Dentistry, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan*
[c] *Graduate Institute of Oral Biology, School of Dentistry, National Taiwan University, Taipei, Taiwan*

Received 15 March 2015; received in revised form 26 March 2015; accepted 8 May 2015

KEYWORDS
anemia;
folic acid deficiency;
gastric parietal cell antibody;
hemoglobin;
oral submucous fibrosis;
vitamin B_{12} deficiency

Background/Purpose: Oral submucous fibrosis (OSF) is a chronic progressive scaring oral disease associated with areca quid chewing. This study evaluated whether OSF patients had anemia, hematinic deficiencies, and serum gastric parietal cell antibody (GPCA) positivity.
Methods: The blood hemoglobin (Hb), iron, vitamin B_{12}, and folic acid concentrations, mean corpuscular volume, and serum GPCA in 68 male OSF patients were measured and compared with the corresponding data in 136 age-matched male healthy control individuals.
Results: We found that five (7.4%), 14 (20.6%), 34 (50.0%), 28 (41.2%), and nine (13.2%) of the 68 male OSF patients had Hb ($<$ 13 g/dL), iron ($\leq$ 70 μg/dL), vitamin B_{12} ($\leq$ 450 pg/mL), and folic acid ($\leq$ 6 ng/mL) deficiencies, and serum GPCA positivity, respectively. Furthermore, OSF patients had a significantly higher frequency of Hb ($p = 0.006$), vitamin B_{12} ($p < 0.001$), or folic acid ($p < 0.001$) deficiency and of serum GPCA positivity ($p = 0.011$) than healthy control participants. Of the five OSF patients with anemia, two had thalassemia trait, one had iron deficiency anemia, and two had macrocytic anemia (mean corpuscular volume $\geq$ 100 fL). In addition, of the nine OSF patients with serum GPCA positivity, six had vitamin B_{12} deficiency, five had folic acid deficiency, and two had iron deficiency. However, none of the nine GPCA-positive OSF patients had pernicious anemia based on the strict World Health Organization definition.

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

* Corresponding author. Department of Dentistry, National Taiwan University Hospital, Number 1, Chang-Te Street, Taipei 10048, Taiwan.
E-mail address: jyfchang@ntu.edu.tw (J.Y.-F. Chang).

http://dx.doi.org/10.1016/j.jfma.2015.05.011

Conclusion: We conclude that there are high frequencies of vitamin B_{12} and folic acid deficiencies and of serum GPCA positivity in our male OSF patients.

Introduction

Oral submucous fibrosis (OSF) is a chronic progressive scaring oral disease predominantly affecting people of South Asian origin.[1–3] It is characterized by juxtaepithelial inflammatory cell infiltration followed by fibrosis in the lamina propria, submucosa, and superficial muscle layer of the oral mucosa. Epidemiologic studies have suggested the habit of areca quid chewing as a major etiologic factor of OSF.[1–3] In Taiwan, there are 2 million people who have the areca quid chewing habit.[4] Previous studies have demonstrated that all the OSF patients in Taiwan have the areca quid chewing habit.[5,6] Although the pathogenesis of OSF has so far been unclear, previous studies have suggested that the occurrence of OSF may be attributable to increased production of collagen and reduced degradation of collagen in the subepithelial connective tissue of the oral mucosa.[7–30] The areca nut alkaloids can stimulate the fibroblast proliferation and collagen synthesis.[7] Cytokines and growth factors produced by inflammatory cells within the OSF tissues may promote fibrosis by inducing proliferation of fibroblasts, upregulating collagen synthesis, and downregulating collagenase production.[8–12] Copper in areca nut can upregulate collagen production in oral fibroblasts.[13,14] Colligin/heat shock protein 47 produced by arecoline-stimulated human buccal fibroblasts can increase the processing and/or secretion of procollagen.[15,16]

The collagen deposition in the subepithelial connective tissue of oral mucosa may also contribute to the reduced collagen degradation. Previous studies showed a lower collagenase activity in OSF lesional mucosa than in normal oral mucosa and 3—5-fold lower levels of endogenous collagenase activities in OSF tissues compared with normal oral mucosa,[17] as well as more tissue inhibitor of metalloproteinase-1 protein production,[18–20] higher type I plasminogen activator inhibitor expression,[21,22] and less collagen phagocytosis activity in OSF than in normal oral mucosal fibroblasts.[23,24] Reduced collagen degradation can also be attributable to synthesis of structure-stable collagen type I trimer by OSF fibroblasts[25] and stabilization of collagen structure by areca nut polyphenolic compounds (catechin and tannin),[26,27] lysyl oxidase in OSF tissues,[28,29] or transglutaminase-2 produced by arecoline-stimulated fibroblasts.[30]

In our oral mucosal disease clinic, patients with OSF are sometimes encountered. Because the majority of OSF patients cannot tolerate spicy food, suffer from burning sensation of oral mucosa, and have a certain degree of mouth opening limitation, these functional impairments may affect normal food intake and lead to nutritional deficiencies. In this study, data on 68 male OSF patients were retrospectively collected from our oral mucosal disease clinic. The blood hemoglobin (Hb), mean corpuscular volume (MCV), and serum iron, vitamin B_{12}, folic acid, and gastric parietal cell antibody (GPCA) levels were examined and compared with the corresponding data in 136 age-matched male healthy control participants. We tried to find whether male OSF patients might have higher frequencies of anemia; of serum iron, vitamin B_{12}, and folic acid deficiencies; and of serum GPCA positivity than age-matched healthy male control individuals.

Materials and methods

Participants

Sixty-eight male OSF patients (mean age 38.7 ± 11.2 years, range 18—61 years) were enrolled in the study. For each patient, two age-matched (± 2 years of each patient's age) healthy male control individuals were selected. Thus, the normal control group consisted of 136 healthy male control individuals (mean age 38.2 ± 10.3 years, range 19—60 years). All the OSF patients and healthy control individuals were seen consecutively, diagnosed, treated, and selected in the oral mucosal disease clinic or dental clinic of National Taiwan University Hospital (NTUH; Taipei, Taiwan) from July 2007 to June 2014. Clinical diagnosis of OSF was made when patients showed characteristic features of OSF, including intolerance to spicy foods, blanching and stiffness of the oral mucosa, fibrous bands in the buccal mucosa, and progressive inability to open the mouth.[5,6] Some OSF patients might also have a burning sensation in the mouth, xerostomia, the presence of vesicles or ulcers on the oral mucosa, depapillation of the tongue, and impaired tongue mobility.[5,6] Incisional biopsy of buccal mucosa was taken from 20 of 68 OSF patients. Histological diagnosis was made based on the examination of hematoxylin and eosin-stained tissue sections. The histological criteria for a diagnosis of OSF were: (1) atrophic epithelium with either parakeratosis or hyperkeratosis, and (2) moderate to marked fibrosis or hyalinization in the lamina propria, submucosa, and superficial muscle layer.[5,6] The oral mucosal sites (soft palate, retromolar area, buccal mucosa, labial mucosa, floor of the mouth, and tongue) of involvement and the maximum mouth opening (MMO) of OSF patients were recorded. The severity of OSF was determined according to the sites of involvement or MMO; the more the sites involved and the less the MMO, the more severe the OSF. No patient with mild OSF was included in the study, because all our OSF patients had at least three sites of involvement. None of the OSF patients had any systemic diseases, autoimmune diseases (such as systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, pemphigus vulgaris, and cicatricial pemphigoid), inflammatory diseases,

malignancy, or recent surgery. Healthy control participants had dental caries, pulpal disease, malocclusion, or missing of teeth but did not have any oral mucosal or systemic diseases. None of our OSF patients had taken any prescription medication at least 3 months prior to entering the study.

All 68 OSF patients had areca quid chewing habit; they chewed 1–120 (mean, 23) quids/d for 1–38 (mean, 14) years. Fifty (73.5%) OSF patients claimed that they swallowed some of the "juice" of areca quid during the chewing process. Sixty-four (94.1%) of the 68 OSF patients were smokers; they smoked from five to 60 (mean, 22) cigarettes/d for 2–38 (mean, 18) years. Forty-five (66.2%) of the 68 OSF patients were drinkers; they drank 45–6528 (mean, 938) g of pure alcohol per week for 1–32 (mean, 13) years. Of the 136 healthy control participants, none were areca quid chewers (1 quid or more daily for at least 1 year), 34 (25.0%) were smokers (1 cigarette(s) or more per day for at least 1 year), and 23 (16.9%) were drinkers (drinking $>$ 3 days a week).

The blood samples were drawn from all OSF patients and healthy control participants for measurement of complete blood count, blood iron, vitamin B_{12}, and folic acid concentrations. In addition, the presence of GPCA in sera of OSF patients and healthy control participants was also checked. All OSF patients and healthy control participants signed the informed consents prior to entering the study. This study was reviewed and approved by the Institutional Review Board at the NTUH.

Determination of complete blood count and blood iron, vitamin B_{12}, and folic acid concentrations

The complete blood count and blood iron, vitamin B_{12}, and folic acid concentrations were determined using routine tests performed in the Department of Laboratory Medicine of NTUH.[31–37]

Determination of serum GPCA level

The serum GPCA level was detected using the indirect immunofluorescence technique with rat stomach as a substrate as described previously.[36,37] In brief, 5-μm-thick cryostat sections of substrate tissues on slides were reacted with serially diluted patients' and control participants' sera in a moist chamber at room temperature for 30 minutes. The initial dilution of the patients' and control participants' sera was 1:20 with phosphate-buffered saline. After washing, the sections were incubated with fluorescein isothiocyanate-labeled goat antihuman immunoglobulin G antiserum (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA), which had been prediluted and kept in dropper vial by the manufacturer and was ready to use for another 30 minutes. The sections were washed again, mounted with buffered glycerine, and examined with an Olympus fluorescence microscope (Olympus, Tokyo, Japan). Sera were scored as positive when they produced fluorescence at a dilution of $\geq$ 20-fold.

Statistical analysis

Comparisons of the MCV and mean blood levels of Hb, iron, vitamin B_{12}, and folic acid between 68 OSF patients and 136 age-matched healthy control participants were performed using Student t test. The differences in frequency of Hb, iron, vitamin B_{12}, or folic acid deficiency or of serum GPCA positivity between 68 OSF patients and 136 age-matched healthy control participants were compared using the Chi-square test. The result was considered to be significant if $p < 0.05$.

Results

The mean MMO of 68 OSF patients was 30.7 $\pm$ 8.0 mm; 33 had an MMO of $\leq$ 31 mm and 35 had an MMO of $>$ 31 mm. The soft palate, retromolar area, and buccal mucosa were the three sites involved by OSF in every patient, with extra involvement of labial mucosa in 41 patients (60.3%), of floor of the mouth in 33 patients (48.5%), and of tongue in 27 patients (39.7%). Of the 68 OSF patients, 16 had three-site involvement, 18 had four-site involvement, 19 had five-site involvement, and 15 had six-site involvement by OSF.

The MCV and mean blood concentrations of Hb, iron, vitamin B_{12}, and folic acid in 68 male OSF patients and in 136 age-matched male healthy control individuals are shown in Table 1. We found that OSF patients had a significantly lower mean serum vitamin B_{12} level ($p < 0.001$) and a significantly lower mean serum folic acid level ($p < 0.001$) than healthy control participants (Table 1). However, there were no differences in the MCV and mean Hb and serum iron levels between 68 OSF patients and 136 age-matched healthy control participants (Table 1).

According to the World Health Organization (WHO) criteria, men with Hb $<$ 13 g/dL were defined as having Hb deficiency or anemia.[38] For serum iron, vitamin B_{12}, and folic acid levels, this study chose the serum iron level $\leq$ 70 μg/dL for men as the iron deficiency level, the serum vitamin B_{12} level $\leq$ 450 pg/mL as the vitamin B_{12} deficiency level, and the folic acid level $\leq$ 6 ng/mL as the folic acid deficiency level.[39,40] These iron, vitamin B_{12}, and folic acid concentrations were chosen because they were the cutoff point concentrations for giving the iron, vitamin B_{12}, and folic acid supplement treatment to oral mucosal disease patients with iron, vitamin B_{12}, or folic acid deficiency.[39,40] By the above-mentioned definitions, five (7.4%), 14 (20.6%), 34 (50.0%), and 28 (41.2%) of the 68 OSF patients had Hb, iron, vitamin B_{12}, and folic acid deficiencies, respectively. Moreover, nine (13.2%) OSF patients had serum GPCA positivity (Table 2). However, none (0%), 20 (14.7%), 30 (22.1%), eight (5.9%), and four (2.9%) of normal control participants had Hb, iron, vitamin B_{12}, and folic acid deficiencies and serum GPCA positivity by the aforementioned criteria, respectively. Furthermore, OSF patients had a significantly higher frequency of Hb ($p = 0.006$), vitamin B_{12} ($p < 0.001$), or folic acid ($p < 0.001$) deficiency and of serum GPCA positivity ($p = 0.011$) than healthy control participants (Table 2). Furthermore, 19 of the 68 OSF patients had no hematinic deficiency. Of the resting 49 OSF patients, 26 had single hematinic deficiency (iron, 3 patients; vitamin B_{12}, 13

Table 1 The mean corpuscular volume (MCV) and mean blood concentrations of hemoglobin (Hb), iron, vitamin B_{12}, and folic acid in 68 male patients with oral submucous fibrosis (OSF) and in 136 age-matched male healthy control participants.

	OSF patients ($n = 68$)		Healthy control participants ($n = 136$)		p (Student t test)
	Mean ± SD	Range	Mean ± SD	Range	
Hb (g/dL)	15.2 ± 1.7	6.8–18.5	15.3 ± 0.9	13.8–16.9	0.583
MCV (fl)	91.4 ± 8.0	63.0–114.3	90.8 ± 3.2	82.4–98.6	0.447
Iron (μg/dL)	114.2 ± 49.9	21.0–279.0	115.3 ± 24.8	65.0–151.0	0.833
Vitamin B_{12} (pg/mL)	510.9 ± 219.0	107.5–1000.0	652.8 ± 225.3	259.0–1000.0	< 0.001*
Folic acid (ng/mL)	7.0 ± 2.8	2.0–15.4	13.8 ± 6.2	4.1–24.0	< 0.001*

SD = standard deviation.
* Comparisons of MCV and blood concentrations of Hb, iron, vitamin B_{12}, and folic acid between 68 male OSF patients and 136 age-matched male healthy control participants by Student t test with $p < 0.001$.

Table 2 Number and percentage of individuals with hemoglobin (Hb), iron, vitamin B_{12}, or folic acid deficiency or with serum gastric parietal cell antibody (GPCA) positivity in 68 male patients with oral submucous fibrosis (OSF) and in 136 age-matched male healthy control participants.

Factor	Participant number (%)		p (Chi-square test)
	OSF patients ($n = 68$)	Healthy control participants ($n = 136$)	
Hb deficiency (< 13 g/dL)	5 (7.4)	0 (0)	0.006*
Iron deficiency (≤ 70 μg/dL)	14 (20.6)	20 (14.7)	0.388
Vitamin B_{12} deficiency (≤ 450 pg/mL)	34 (50.0)	30 (22.1)	<0.001*
Folic acid deficiency (≤ 6 ng/mL)	28 (41.2)	8 (5.9)	<0.001*
GPCA positivity	9 (13.2)	4 (2.9)	0.011*

* Comparison of frequency of Hb, iron, vitamin B_{12}, or folic acid deficiency or of serum GPCA positivity between 68 male patients with oral submucous fibrosis and in 136 age-matched male healthy control participants by chi-square test with $p < 0.05$.

patients; and folic acid, 10 patients), 19 had double hematinic deficiencies (iron and vitamin B_{12}, 5 patients; iron and folic acid, 2 patients; and vitamin B_{12} and folic acid, 12 patients), and four had triple hematinic (iron, vitamin B_{12}, and folic acid) deficiencies.

Of the five OSF patients with anemia (Table 3), two had thalassemia trait (Patient 1 also had vitamin B_{12} and folic acid deficiencies),[32] one had iron deficiency anemia,[34] and two had macrocytic anemia (MCV ≥ 100 fL; Patient 4 also had iron, vitamin B_{12}, and folic acid deficiencies).[37] All five OSF patients were serum GPCA-negative. Moreover, anemia was found in two of four OSF patients with macrocytosis (MCV ≥ 100 fL) and three of five OSF patients with microcytosis (MCV < 80 fL; Table 3).

Of the nine OSF patients with serum GPCA positivity, six had vitamin B_{12} deficiency, five had folic acid deficiency, and two had iron deficiency (Table 4). However, none of the nine GPCA-positive OSF patients had pernicious anemia based on the strict WHO definitions (Hb < 13 g/dL for men and < 12 g/dL for women, MCV ≥ 100 fL, serum vitamin B_{12} < 200 pg/mL, and serum GPCA-positive; Table 4).[36]

Discussion

This study found that five (7.4%), 14 (20.6%), 34 (50.0%), 28 (41.2%), and nine (13.2%) of the 68 male OSF patients had Hb, iron, vitamin B_{12}, and folic acid deficiencies, and serum

Table 3 Diagnosis, hemoglobin (Hb), mean corpuscular volume (MCV), red blood cell (RBC) number, serum iron level, serum vitamin B_{12} level, serum folic acid level, and serum gastric parietal cell antibody (GPCA) positivity in five male OSF patients with anemia (Hb < 13 g/dL).

Patient No.	Diagnosis	Hb (g/dL)	MCV (fL)	RBC ($\times 10^{12}$/L)	Mentzer index (MCV/RBC)	Iron (μg/dL)	Vitamin B_{12} (pg/mL)	Folic acid (ng/mL)	GPCA positivity
1	Thalassemia trait	12.4	70.2	5.57	12.6	130	449	6.0	—
2	Thalassemia trait	12.4	63.0	5.92	10.6	126	770	8.3	—
3	Iron deficiency anemia	6.8	69.2	3.70	18.7	21	452	6.2	—
4	Macrocytic anemia	10.0	114.3	2.59	44.1	37	228	4.8	—
5	Macrocytic anemia	12.5	101.1	3.74	27.0	109	678	15.4	—

Table 4 Hemoglobin (Hb), mean corpuscular volume (MCV), red blood cell (RBC) number, serum iron level, serum vitamin B_{12} level, and serum folic acid level in nine male OSF patients with serum gastric parietal cell antibody (GPCA) positivity.

Patient No.	Hb (g/dL)	MCV (fl)	RBC ($\times 10^{12}$/L)	Iron (μg/dL)	Vitamin B_{12} (pg/mL)	Folic acid (ng/mL)	GPCA positivity
1	15.4	93.9	5.06	95	486.1	8.3	+
2	14.5	94.0	4.64	95	107.5	5.7	+
3	14.8	93.5	4.64	174	429	8.1	+
4	15.8	99.4	4.75	58	291	4.1	+
5	14.8	90.6	4.91	85	222	6.8	+
6	13.6	97.1	4.13	54	812	3.3	+
7	15.0	94.7	4.86	111	274	3.1	+
8	15.9	95.9	4.84	128	295	14.1	+
9	14.6	89.1	4.85	119	775	3.6	+

GPCA positivity, respectively. Furthermore, OSF patients had a significantly higher frequency of Hb, vitamin B_{12}, or folic acid deficiency and of serum GPCA positivity than healthy control participants. In addition, single hematinic deficiency and multiple hematinic deficiencies were found in 26 (38.2%) and 23 (33.8%) of the 68 OSF patients, respectively. These findings suggest that hematinic deficiencies are frequently found in OSF patients. Because all of our OSF patients had moderate or severe OSF with the mean MMO being 30.7 mm, it was possible that the Hb, iron, vitamin B_{12}, and folic acid deficiencies were at least partially attributable to difficult and insufficient food intake secondary to the limited mouth opening and intolerance to spicy food in OSF patients. Wahi et al[41] also reported a significantly higher prevalence of malnutrition in 104 OSF patients than in 200 normal control participants. In their study, poor nutritional status was evident on clinical examination in 28% of the 70 male and 35% of the 34 female OSF patients. In addition, of 104 OSF patients, 40 (38.5%) had multiple vitamin deficiencies, 20 (19.2%) had vitamin A deficiency, 56 (53.8%) had vitamin B deficiency, and 54 (51.9%) had vitamin C deficiency.[41]

Ramanathan[42] reported that five (63%) of the eight OSF patients have a depressed serum iron level below the normal value. Rajendran et al[43] found a significantly lower serum iron level in 50 OSF patients than in 50 age- and sex-matched control participants. Anuradha and Devi[44] attributed the deficiencies of vitamin C and iron in 36 OSF patients to the increased utilization of these two substances in collagen synthesis. Tadakamadla et al[45] discovered a significantly higher mean serum copper level and a significantly lower mean serum iron level in 50 OSF patients than in 50 age- and sex-matched control participants. However, there was no significant difference in the mean serum iron level between 68 male OSF patients and 136 age-matched male healthy control participants in the present study.

Ramanathan[42] reported that 10 (77%) of the 13 OSF patients in Malaysia have iron deficiency anemia. The present study showed anemia in five (7.4%) of the 68 OSF patients and a significantly greater frequency of anemia in 68 OSF patients than in 136 healthy control participants. Wahi et al[41] discovered that 6% of the 70 male OSF patients and 11% of the 34 female OSF patients had anemia, but the frequency of anemia did not differ significantly from that of the control participants. A South African study also found no significant difference in the prevalence of iron deficiency anemia between OSF patients and the general population.[46]

In a South African study, Seedat[46] reported that serum vitamin B_{12} and folate levels of OSF patients are within normal limits. However, Ramanathan[42] found folic acid deficiency in six of the six OSF patients. This study also discovered that 34 (50.0%) and 28 (41.2%) of OSF patients had vitamin B_{12} and folic acid deficiencies, respectively. Only six of the 34 vitamin B_{12}-deficient OSF patients were GPCA-positive. In these six GPCA-positive OSF patients, the vitamin B_{12} deficiency could be attributed to the presence of GPCA, because GPCA can induce destruction of parietal cells and in turn result in failure of intrinsic factor production.[47,48] Lack of intrinsic factor finally leads to failure of vitamin B_{12} absorption.[49,50] Furthermore, the vitamin B_{12} deficiency in the other 28 GPCA-negative OSF patients may be attributable to other causes including inadequate intake or malabsorption of vitamin B_{12}, the presence of anti-intrinsic factor antibodies, or transcobalamin II deficiency.[50] Folic acid deficiency may result from poor nutritional intake, malabsorption, hepatobiliary dysfunction, increased folate catabolism, and medication (e.g., methotrexate, 5-fluoro-uracil, phenytoin).[50] In the present study, we suggest that the folic acid deficiency is probably due to poor or insufficient intake of folic acid-containing diet because OSF patients usually have functional impairment in food intake and chewing. Further studies are necessary to explore the real causes resulting in vitamin B_{12} and folic acid deficiencies in OSF patients.

Our previous study demonstrated that 14.7% of 109 male OSF patients are GPCA-positive.[6] A similar result was found in this study, showing serum GPCA positivity in nine (13.2%) of 68 male OSF patients. Although six of the nine GPCA-positive OSF patients had vitamin B_{12} deficiency in this study, none of these six OSF patients had pernicious anemia by the strict WHO definitions.[36] Our previous studies also found that only 12.9% of 124 GPCA-positive,[36] 18.9% of 90 vitamin B_{12}-deficient (unpublished data), and 16.7% of 60 macrocytosis patients with oral mucosal diseases had pernicious anemia based on the strict WHO definitions.[37]

Our results demonstrated that five (7.4%), 14 (20.6%), 34 (50.0%), 28 (41.2%), and nine (13.2%) of the 68 male OSF patients had Hb, iron, vitamin B_{12}, and folic acid deficiencies, and serum GPCA positivity, respectively. Furthermore, OSF patients had a significantly higher frequency of Hb, vitamin B_{12}, or folic acid deficiency and of

serum GPCA positivity than healthy control participants. We conclude that there are high frequencies of vitamin B_{12} and folic acid deficiencies and of serum GPCA positivity in our OSF patients.

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