

Serum vitamin B12 associated with vitamin D/25(OH)D in women with recurrent aphthous stomatitis.

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Abstract: Vitamin B12 and Vitamin D deficiency may contribute to recurrent aphthous stomatitis (RAS). Current studies have showed vitamin B12 to be associated with vitamin D in women, however no study has assessed vitamin B12 associated with vitamin D/25(OH)D in women with RAS. **Objective:** To investigate the association between serum vitamin B12 and vitamin D/25(OH)D in women with RAS. **Materials and Methods:** Forty one women with RAS who meet the inclusion criteria participated in this study. The inclusion criteria were women with RAS without other oral diseases. The exclusions criteria were those who have systemic diseases, taking medications or smoked. All subjects underwent venupuncture to draw blood to quantify serum vitamin B12 and vitamin D/25(OH)D. The characteristic of subjects, severity of RAS, serum Vitamin B12 and Vitamin D/25(OH)D were collected and presented descriptively. The correlation between vitamin B12 and Vitamin D/25(OH)D was analyzed using Pearson correlation test with 95% confidence interval. This study was approved by Medical and Health Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. **Results:** All RAS subjects have normal mean value of serum Vitamin B12 (453.97pg/ml + 154.44pg/ml) and have low mean value of serum vitamin D/25(OH)D (10.79ng/ml + 3.29ng/ml) categorized as vitamin D deficiency. The Pearson correlation test showed that there was a significant positive correlation between mean serum Vitamin B12 and Vitamin D/25(OH)D ($r= 0.313, p<0.05$). **Conclusion:** There is correlation between vitamin B12 and Vitamin D, and a low level of Vitamin D may contribute in RAS in women.

Keywords: Recurrence; stomatitis, aphthous; women; serum; vitamin B12, vitamin D.

Resumen: Antecedentes: la deficiencia de vitamina B12 y vitamina D puede contribuir a la estomatitis aftosa recurrente (EAR). Los estudios actuales han demostrado que la vitamina B12 está asociada con la vitamina D en mujeres, sin embargo, ningún estudio ha evaluado la vitamina B12 asociada con la vitamina D/25 (OH) D en mujeres con EAR. **Objetivo:** investigar la asociación entre la vitamina B12 y la vitamina D / 25 (OH) D sérica en mujeres con RAS. **Material y Métodos:** Cuarenta y una mujeres con RAS que cumplen con los criterios de inclusión participaron en este estudio. Los criterios de inclusión fueron mujeres con RAS y sin otras enfermedades orales. Los criterios de exclusión fueron aquellos que tenían enfermedades sistémicas, tomaban medicamentos o fumaban. Todos los sujetos se sometieron a una venupuntura para extraer sangre para cuantificar la vitamina B12 y la vitamina D/25 (OH) D en suero. Las características de los

sujetos, la severidad del EAR, la concentración de vitamina B12 y la vitamina D/25 (OH) D sérica fueron recolectadas y presentadas descriptivamente. La correlación entre la vitamina B12 y la vitamina D/25 (OH) D se analizó mediante la prueba de correlación de Pearson con un intervalo de confianza del 95%. Este estudio fue aprobado por el Comité de Ética Médica y de Salud, Facultad de Medicina, Universitas Gadjah Mada, Yogyakarta, Indonesia. **Resultado:** Todos los sujetos con EAR tienen un valor medio normal de vitamina B12 sérica (453,97pg/ml + 154,44pg/ml)

INTRODUCTION.

Nutritional factors have been suggested to be associated with recurrent aphthous stomatitis (RAS). One of these is Vitamin B12 deficiency,¹⁻⁴ which may be caused by low dietary intake of vitamin B12, malabsorption, gastrointestinal disease and intestine surgery.^{5,6,7}

Vitamin B12 contains cobalt and has many functions such as being a co-enzyme in folate metabolism involved in nucleic acid synthesis, in the synthesis of certain amino acids, and fatty acid catabolism. Vitamin B12 is essential in red blood cell and myelin nerve synthesis.⁸ Studies have revealed that RAS patients tend to have low serum Vitamin B12 compared with healthy controls.⁹⁻¹³

The role of vitamin B12 deficiency in RAS is also supported by studies that show supplementation of vitamin B12 had positive effects on the duration of RAS and on the number of oral ulcers.^{14,15} However, one study reported opposite results, where Vitamin B12 therapy did not improve RAS.¹³ Furthermore, therapy with multivitamins containing vitamin B12 also did not reduce the number of ulcers, duration of illness and pain in RAS patients.¹⁶

The exact mechanism of Vitamin B 12 deficiency predisposing RAS is still not fully understood. It is suggested that vitamin B12 deficiency may affect red blood cell production and affect other dividing cells then result in the thinning of oral epithelium, making it more vulnerable to trauma or for pathogens to penetrate the thinned epithelium.¹³ The increased permeability caused by vitamin B12 deficiency may facilitate any pathogen-associated molecular patterns (PAMPs) (Toll-like receptor/TLR ligand) of oral microbes or other antigens to initiate a TLR-mediated response.^{17,18}

This is supported by studies that showed polymorphisms of TLR4¹⁹ and abnormal activity of TLR2 in RAS.²⁰ As such, RAS may be caused by an abnormality of the immune response in the oral mucosal and result in tissue damage mediated by CD8 T cell lymphocyte.^{1,3,21}

y un valor medio bajo de vitamina D sérica/25 (OH) D (10,79ng/ml + 3,29ng/ml) clasificado como deficiencia de vitamina D. La prueba de correlación de Pearson mostró que había una correlación positiva significativa entre la vitamina B12 media y la vitamina D/25 (OH) D en suero ($r=0.313$, $p<0.05$). **Conclusión:** Existe una correlación entre la vitamina B12 y la vitamina D, y un bajo nivel de vitamina D puede contribuir al RAS en las mujeres.

Palabras Clave: Recurrencia, estomatitis aftosa; mujeres; suero; vitamina B 12; vitamina D.

It is also suggested that vitamin B12 deficiency is involved in the suppression of cell-mediated immunity in the oral epithelium.²² Vitamin B12 deficiency may alter the balance of Th1/Th2 immune response and epithelial barriers, then causing functional impairment. This is supported by evidence that vitamin B12 deficiency may result in an increase in TNF α , a Th1-pro-inflammatory cytokine.²³ Tumor necrosis factor (TNF) α is a crucial mediator of immune system produced by Th1 cells, with a predominance in RAS and which has a direct toxic effect in causing oral mucosal damage.²⁴

Recently there was evidence that vitamin D deficiency may also be involved in the development of RAS. However, there have been inconsistent results regarding the role of vitamin D in the pathogenesis in RAS. Studies have revealed that RAS patients have low levels of vitamin D compared with healthy control.^{25,26} Another study revealed RAS patients tend to have low-level serum vitamin D compared with healthy control, but, there was not a significant difference in vitamin D status between both groups.²⁷

None of those studies showed that vitamin D status is associated with clinical parameters of RAS. Several studies have shown that vitamin D has an essential role in the immune response. Vitamin D is known not only to be involved in both calcium and phosphate metabolism but also to have an important role as the immunomodulatory activity in the human innate and cell-mediated immune response.²⁸⁻³⁰ Vitamin D is not only synthesized in the skin but also can be acquired from food intake. In the human body, vitamin D is metabolized in the liver to be hydroxylated into 25(OH)D which is the major circulating form and an indicator of Vitamin D status.

Finally, it is converted to the active form in the kidney.³¹ Vitamin D3/1,25(OH)2D3 or calcitriol is the active form of vitamin D which acts as an immunomodulator and down regulates TLR2 and TNF α in monocytes.^{32,33} This has been reported by a systematic review that revealed that

vitamin D has anti-inflammatory activity via decreased TLR expression in peripheral blood monocyte cell.³⁴

A current study showed an association between vitamin D with vitamin B12 in healthy women, that vitamin D may affect vitamin B12 absorption in the gastrointestinal tract. It also reported that obesity indirectly may affect that association³⁵ indicating the concentration of Vitamin D may affect Vitamin B12 concentration independently.

With the evidence that both vitamin B12 deficiency and vitamin D deficiency may be involved in the pathogenesis of RAS, it is not clear yet, whether Vitamin B12 is associated with Vitamin D in women with RAS and if there is a correlation between both vitamins and RAS severity. Hence, the aim of this study is to investigate the association between the concentration of serum of vitamin B12 and vitamin D/25(OH)D in women with RAS.

MATERIALS AND METHODS.

The participants of this study were women who visited The Department of Oral Medicine Clinic of the Dental Hospital of the Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia, and had been diagnosed with RAS. Those who agreed to participate in this study were informed about it.

The inclusion criteria were patients with RAS, age 18 to 30 years old and suffering oral ulcers by not more than three days. The exclusion criteria were patients who had systemic conditions or diseases, who were taking medications topically or orally including multivitamins, how had a smoking habit. This study was approved by the Ethical Committee for Research of the Faculty of Medicine of Universitas Gadjah Mada, Yogyakarta, Indonesia. Informed consent was obtained from all participants.

Additionally, all demographic and clinical information including age, ethnicity, education level, marriage status, sex/gender, and predisposing factors were obtained by using a questionnaire. A visual analog scale (VAS) with a 0-10 scale was used to examine the severity of RAS. The participants were asked to rate the severity of pain on a 10 points scale, ranging from 0 (none) to 10 (most severe).

A venipuncture was performed to obtain a blood sample from all participants to determine serum Vitamin B12 and Vitamin D/25(OH)D after physical examination. Vitamin D/25(OH) was determined by ECLIA method (electro-chemiluminescence, Cobas, Roche Diagnostic

International Ltd). Vitamin D deficiency was considered when the vitamin D (25-OH)D concentration was <20ng/ml, vitamin D insufficiency 21-29ng/mL, normal serum vitamin D (25-OH) >30ng/mL,²⁸ Vitamin B12 was determined by a chemiluminescent method (Roche Diagnostic International Ltd). The normal range considered for serum Vitamin B12 was 211-911pg/ml.

Statistical analysis

The demographics of participants was analyzed using descriptive statistics. The Shapiro-Wilk test was used in order to test whether the data had a normal distribution.

The association between serum Vitamin D/25(OH) D, Vitamin B12 and clinical parameters of RAS were analyzed using the Pearson correlation test when data normally distributed or analyzed using the Spearman correlation test when the data was not normally distributed. All statistical analysis was performed using a 95% Confidence Interval (CI) in SPSS 17.00.

RESULTS.

There were forty one RAS participants in this study, all female. The mean age was 25.24 years old. Most of the subjects were college students (80.5%), Javanese (71%), and were not married (100%). Most subjects had less than 10 ulcers (82.9%), and the size of oral ulcers was generally less than 10mm (95%).

Hormonal variation was reported to be the precipitating factor for most of the subjects. Buccal and labial mucosa were the most common locations of the oral ulcers. All subjects reported oral ulcers accompanied by pain according to VAS, and indicated moderate pain (Table 1).

All RAS patients had low serum Vitamin D/25(OH) D levels, the mean value of Vitamin D/25(OH)D concentration was 10.79ng/ml + 3.29ng/ml, thus categorized as deficiency (<20ng/ml). The mean serum level of Vitamin B12 was 453.97pg/ml + 154.44pg/ml, categorized as normal. Both serum vitamin D/25(OH) D and vitamin B12 data had a normal distribution but the RAS parameters were not normally distributed after assessment using the Shapiro-Wilk test. When analysis with the Pearson correlation test, a positive significant correlation between serum vitamin D/25(OH)D and Vitamin B12 was found (Table 2).

However there was not a significant correlation between the mean value of serum Vitamin D and Vitamin B12 and RAS parameters (Table 3 and Table 4), only the number of ulcers showed a positive correlation with pain score (Table 5).

Table 1. The characteristic of the study participants.

Variable		RAS patients (n =41)	
Age, in years:	mean (SD)		25.24 (6.07)
Education level:	n (%)	High education level	33 (80.5)
Ethnicity :	n (%)	Javanese	29 (70.7)
Marriage status :	n (%)	Single	41 (100)
Predisposing factor* :	n (%)	Stress	11 (26.8%)
		Hormonal	11 (26.8%)
		Hormonal and stress	5 (12.1%)
		Unknown	14 (34.3%)
Oral ulcer characteristic			
Ulcer numbers :	n (%)	<10mm	34 (82.9)
		>10mm	7 (17.1)
Ulcer size : mm	n (%)	>10mm	2 (5)
		<10mm	39 (95)
Pain scale (VAS) score :	mean (SD)		4.80 (2.2)
Ulcer locations:	n (%)	Floor of the mouth	4 (5.5)
		Tongue	9 (12.5)
		Gingiva	10 (13.8)
		Labial mucosa	30 (41.6)
		Buccal mucosa	16 (22.2)
		Soft palate	2 (2.7)
		Vestibulum	1 (1.4)

mm: millimeter; n: number of subjects. RAS: Recurrent aphthous stomatitis. SD: standard deviation. VAS: visual analog scale. *: Based on subjective examination of the patients.

Table 2. The correlation between serum vitamin B12 and vitamin D/25(OH)D in females with RAS.

Serum Markers	Mean (SD)	r-value	p-value
Vitamin D/25(OH)D	10.79 (3.29) ng/ml	0.313	0.047
Vitamin B12	453.97 (154.44) pg/ml		

ml: milliliter. p-value: significancy with $p < 0.05$; r-value: correlation coefficients of Pearson correlation test. RAS: recurrent aphthous stomatitis; SD: standard deviation. ng: nanogram. pg/ml: picogram per milliliter.

Table 3. The correlation between serum vitamin D/25(OH)D and RAS paramaters.

Variable		Mean (SD)	r-value	p-value
Vitamin D/25(OH)D		10.79 (3.29) ng/ml		
RAS parameters	Ulcer size	3.24 (2.05) mm	-0.172	0.281
	Number of oral ulcer	5.71 (2.94)	0.033	0.837
	VAS score	4.80 (2.2)	-0.050	0.755

ml: milliliter. p-value: significancy with $p < 0.05$; r-value: correlation coefficients of Spearman correlation test. RAS: recurrent aphthous stomatitis; SD: standard deviation. ng: nanogram. pg/ml: picogram per milliliter.

Table 4. The correlation between serum vitamin B12 and RAS paramaters.

Variable	Mean (SD)	r-value	p-value
Vitamin B12	53.97 (154.44) pg/ml		
RAS parameters	Ulcer size	3.24 (2.05) mm	-0.165
	Number of oral ulcer	5.71 (2.94)	0.168
	VAS score	4.80 (2.2)	-0.195

ml: milliliter. p-value: signficancy with $p < 0.05$; r-value: correlation coefficients of Spearman correlation test. RAS: recurrent aphthous stomatitis; SD: standard deviation. ng: nanogram. pg/ml: picogram per milliliter.

Table 5. The correlation between RAS parameters and pain (VAS) score

Variable	Mean (SD)	r-value	p-value
Pain (VAS) score	4.80 (2.2) pg/ml		
RAS parameters	Ulcer size	3.24 (2.05)	0.305
	Number of oral ulcer	5.71 (2.94)	0.363

ml: milliliter. p-value: signficancy with $p < 0.05$; r-value: correlation coefficients of Spearman correlation test. RAS: recurrent aphthous stomatitis; SD: standard deviation. ng: nanogram. pg/ml: picogram per milliliter.

DISCUSSION.

To the best our knowledge, this study was the first to show a correlation between serum Vitamin B12 and Vitamin D/25(OH)D in recurrent aphthous stomatitis (RAS) in women. Recurrent aphthous stomatitis is an oral mucosal disease with a high prevalence in the community. Studies have shown that the prevalence of RAS is higher in females. Nutritional factors have been suggested to be predisposing factors for RAS.²

Based on subjective examination, we found that both hormonal changes and stress were reported by the subjects to be predisposing factors for RAS. Most of our study participants did not know their nutritional status. The high prevalence of RAS in women may be caused by changes in hormone levels associated with the menstrual cycle. The fluctuation of estrogen and progesterone in the luteal phase of the menstrual cycle may contribute to inducing the dysregulation of oral mucosal immune response which causes oral mucosal inflammation and tissue destruction.²³

Another factor that has been suggested to contribute for the development of RAS in this study was stress. Both physical and psychological stress have been known to be predisposing factors for RAS.¹⁻³

It may be correlated with the education level in our participants, who were mostly college students who may face highly stressful conditions during their daily activities. A recent study has shown that RAS was

prevalent in college students and stress was indicated as the precipitating factor.³⁶ This study was in line with another study that showed both hormonal and stress to be the major precipitating factor for RAS.³⁷

The minor type of RAS was most the common type we found. Our results show that many subjects experienced oral ulcers less than 10mm. Furthermore, the participants also reported variations in the number of oral ulcers in each episode of RAS, with most subjects reporting less than 10 ulcers in each RAS episode.³⁸ However some subjects reported more than 10 ulcers, probably representing the herpetiform type of RAS, but not cause by herpes simplex virus (HSV) infection. HSV infection may not only be characterized by a number of oral ulcers greater than ten²¹ but also is characterized by multiple oral ulcers found in both non keratinized and keratinized mucosa of the oral cavity. In addition, HSV infection is more likely preceded by vesicles with prodromal symptoms, such as fever and malaise.³⁹

Based on subjective examination, most of the oral ulcers of this study were found in non keratinized oral mucosa (buccal and labial mucosa) without being preceded by systemic symptoms or vesicles. Since RAS is an acute oral mucosal disease, the oral ulcers are always accompanied by pain of different severity. The pain derived from oral ulcers is categorized as a nociceptive pain caused by the inflammation reaction.⁴⁰ The severity of RAS was assessed by the degree of pain reported by

the participants. The mean VAS score indicated moderate pain with a mean score of 4.80 (1-10 in range). This pain sensation may be influenced by the size and numbers of oral ulcers,^{24,38} but the pain score in our participants was relatively not too high, as most subjects may have similar size and numbers of oral ulcers. However, in the result of our study, the pain (VAS) score correlated with the number of oral ulcers.

Vitamin D/25(OH)D is an important nutrient not only for bone and calcium metabolism together with parathyroid hormone but also has an important role in the immune system.^{30,31,41-43} Few studies have investigated the role of vitamin D/25(OH)D in RAS.

It is hard to explain the causal and effect of vitamin D in RAS from those studies, although they concluded that vitamin D was the etiology of RAS, as most studies that reported on the role of vitamin D in RAS were cross-sectional studies and have different results. A study showed that RAS patients have lower serum vitamin D compared with healthy control.²⁵ However, another study reported that there was no difference of serum Vitamin D between RAS patients and healthy controls.²⁷ On the other hand, an intervention study showed that vitamin D supplementation had a beneficial effect in recurrent aphthous stomatitis.²⁶

Our study showed that all subjects have low/decreased serum levels of vitamin D/25(OH)D, which may contribute to the pathogenesis of RAS. The low level of serum vitamin D/25(OH)D in the participants indicates that RAS development in our subjects may be caused by role of vitamin D as an immunomodulator on cell-mediated immunity. RAS is a type of T cell-mediated disease with the involvement of the stimulation of TNF α , CD8 T cells, and results in acute inflammation of the oral mucosa.^{2,44}

Vitamin D may activate vitamin D receptors (VDR) expressed in neutrophils, macrophages, dendritic cells and in lymphocytes (both B and T cells). The active form of Vitamin D (1,25(OH)₂D) may have anti-inflammatory activity in cell-mediated immunity.

Furthermore, vitamin D may reduce the production of T helper type 1 (Th1) cells and lead to an increase in the proliferation of T helper type 2 cells (Th2) by increasing production of cytokines such as interleukin (IL)-4, IL-5, and IL-10.^{42,45} Vitamin D may inhibit B cell proliferation and reduce the production of immunoglobulins, as well as promoting the apoptosis of immunoglobulin-producing B cells.²⁸

This study revealed that serum vitamin B12 was associated with serum vitamin D/25(OH)D in RAS patients. This indicates that vitamin D may affect vitamin B12 or vice versa. We did not find any correlation between mean serum Vitamin B12 and Vitamin D/25(OH)D levels with RAS parameters. Interestingly, the participants in our study did not show a low level of serum vitamin B12, which mean vitamin B12 deficiency is not a requisite for the development of RAS.

The exact explanation of the correlation between Vitamin B12 and Vitamin D is still unclear, but a study has shown levels of vitamin B12 to be associated with levels of vitamin D in obese females, where vitamin D was proposed to be a possible mediator between obesity and vitamin B12 status, although it was stated that serum vitamin B12 levels did not depend on obesity and body volume.

Since our study did not collect data on body weight or obesity, it is impossible to state that the normal value of vitamin B12 of the participants was due to normal body mass indexes, and represents one limitation of our study. However, the evidence of the role of obesity on the levels of vitamin B12 is still lacking.

One possible mechanism that explains the correlation between vitamin B12 and vitamin D in the current study is that vitamin D/25(OH)D may affect vitamin B12 absorption, which means vitamin B12 has vitamin D-dependent absorption and transcytosis from terminal ileum. Vitamin D/25(OH)D could have a positive effect on the capacity and affinity of the cubilin and amnionless receptor system, essential for the absorption of vitamin B12. Vitamin D may affect endocytosis, intracellular recycling and translocation of cubilin and amnionless to the membrane of the enterocyte facing the lumen.

Low-level of vitamin D may cause lower transcellular absorption of calcium in the duodenum and higher intraluminal concentration of calcium. There is an increase of calcium binding affinity of the N-terminal part of the cubilin sequence and calcium-dependent internalization to the vitamin B12 and intrinsic factor complex.³⁵

Other limitations of our study include that a correlation between serum vitamin D/25(OH)D and vitamin B12 was not assessed in a healthy control group, and the small number of subjects in the study. Furthermore, we did not measure the concentration of other nutritional factors that may affect the development of RAS, such as iron/ferritin and micronutrients such as zinc.

Both ferritin and zinc may have a correlation with

vitamin D.^{46,47} Also we did not assess hormonal variations that may affect vitamin metabolism, and probably also affect the development of RAS.

Further studies are needed in order to reveal the associated between nutritional factors including vitamin D and clinical parameters of RAS in both sexes and using a higher number of subjects.

CONCLUSION.

It can be concluded that there is an association between serum vitamin B12 and Vitamin D/25(OH)D, and that low levels of vitamin D may contribute to RAS in females, with no association found between both vitamins and RAS clinical parameters.

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