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ORIGINAL RESEARCH

SALIVARY VITAMIN D3 AS A NON-INVASIVE BIOMARKER IN RECURRENT APHTHOUS STOMATITIS- A CASE- CONTROL STUDY

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ABSTRACT

Background: Recurrent aphthous stomatitis (RAS) is a common, painful oral ulcerative disorder with a multifactorial etiology involving immune dysregulation and nutritional deficiencies. Vitamin D, known for its immunomodulatory and anti-inflammatory effects, has been increasingly studied for its role in RAS. While serum vitamin D levels have been explored, salivary vitamin D3 measurement offers a non-invasive alternative.

Objective: To evaluate salivary vitamin D3 levels in patients with RAS compared to healthy controls and assess its potential as a biomarker in disease pathogenesis.

Methods: A case-control study was conducted with 24 patients (12 clinically diagnosed RAS patients and 12 matched healthy individuals). Salivary samples were analyzed for vitamin D3 concentration using delayed competitive ELISA. A paired t test was done used using SPSS software for statistical analysis.

Results: RAS patients showed significantly lower salivary vitamin D3 concentrations (40.96–55 ng/mL) than controls (51.05–60.91 ng/mL), suggesting an association between vitamin D deficiency and RAS ($p < 0.05$). These results support vitamin D's role in modulating immune responses linked to ulcer formation. Saliva proved to be a reliable, non-invasive medium for vitamin D3 assessment.

Conclusion:The findings revealed vitamin D deficiency in patients with RAS and highlight salivary vitamin D3 as a practical biomarker. Incorporating vitamin D screening in clinical evaluation of RAS patients may improve diagnosis and management. Further research, including larger, multicentric studies with genetic and environmental considerations, is needed to clarify causality and optimize vitamin D-based therapeutic strategies.

Keywords: Recurrent aphthous stomatitis, Vitamin D3, Salivary biomarkers, Immune modulation, Oral ulcers

1. INTRODUCTION

Recurrent Aphthous Stomatitis (RAS), commonly referred to as canker sores, is one of the most prevalent ulcerative conditions affecting the oral mucosa. It is characterized by recurrent episodes of painful ulcerations primarily localized to the non-keratinized mucosal surfaces of the mouth, including the inner aspects of the lips, cheeks, floor of the mouth, ventral surface of the tongue, and soft palate. These lesions typically present as small, round or oval ulcers with a sharply demarcated erythematous halo surrounding a yellowish-gray pseudomembranous base. Patients frequently experience prodromal symptoms such as burning, itching, or tingling sensations 1 to 2 days prior to ulcer appearance, which can significantly impair daily functions including eating, speaking, and swallowing. Although RAS predominantly affects younger populations—children, adolescents, and young adults—it can occur at any age and is often characterized by its chronic relapsing nature. Involvement of keratinized tissues like the gingiva and hard palate is less common but has been documented in certain cases, sometimes complicating differential diagnosis with other mucosal disorders [1, 2.] .

The global prevalence of RAS varies widely, ranging from approximately 5% to 25% in different populations, with some studies reporting higher frequencies in females and individuals with a family history, suggesting a multifactorial and possibly hereditary component. Despite decades of research, the precise etiology and pathogenesis of RAS remain incompletely understood, primarily due to its complex interplay of multiple contributing factors. Trauma, whether mechanical or chemical, remains one of the most frequently reported local triggers, often precipitating ulcer formation at sites of mucosal injury. Infectious agents such as Helicobacter pylori and herpes simplex virus have also been proposed as potential microbial triggers, although their definitive role remains controversial [3].

Nutritional deficiencies, particularly in essential vitamins and minerals such as folate, vitamin B12, iron, and zinc, have been consistently implicated in RAS pathophysiology. Deficiencies in these nutrients may impair mucosal integrity, reduce immune competence, and disrupt cellular metabolism, thereby facilitating ulcer formation and delayed healing. Psychological factors including stress and anxiety have been identified as exacerbating agents, potentially through modulation of systemic inflammatory responses and neuroimmunological pathways[4]. Among these factors, immune dysregulation has garnered significant attention, given the clear evidence of altered cellular and humoral immune responses in RAS patients. Studies have demonstrated aberrations in T-cell subsets, increased activation of cytotoxic CD8+ lymphocytes, and elevated pro-inflammatory cytokines such as interleukin (IL)-2, IL-6, and tumor necrosis factor-alpha (TNF-α), pointing towards a localized immune-mediated mucosal injury [5–7].

Vitamin D, a fat-soluble secosteroid hormone traditionally associated with bone metabolism and calcium homeostasis, has emerged as a pivotal modulator of immune function and inflammation in recent years. Synthesized in the skin upon exposure to ultraviolet B radiation and obtained from dietary sources, vitamin D undergoes hydroxylation in the liver and kidneys to form its biologically active form, 1,25-dihydroxyvitamin D3 (calcitriol). Beyond its classical endocrine functions, vitamin D exerts autocrine and paracrine effects on various immune cells by binding to the vitamin D receptor (VDR), a nuclear transcription factor expressed on macrophages, dendritic cells, B and T lymphocytes. These interactions regulate gene expression involved in cytokine production, cell proliferation, and immune tolerance, making vitamin D a crucial factor in maintaining immune homeostasis and preventing excessive inflammatory responses [8, 9.]

Emerging epidemiological and experimental evidence links vitamin D deficiency to an increased risk of several autoimmune and inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, autoimmune thyroid disorders, and inflammatory bowel disease. Vitamin D influences the immune system by enhancing innate immunity via upregulation of antimicrobial peptides like cathelicidin and defensins, while concurrently suppressing the adaptive immune system by inhibiting dendritic cell maturation and promoting the development of regulatory T cells (Tregs), which maintain peripheral tolerance and prevent autoimmunity. Its ability to downregulate pro-inflammatory cytokines such as IL-17, IL-6, and TNF-α further supports its anti-inflammatory role, making vitamin D deficiency a plausible risk factor or disease modifier in conditions involving immune dysregulation [10–12].

Given the immune-mediated nature of RAS, it is reasonable to hypothesize that insufficient vitamin D levels may contribute to the development, severity, or recurrence of aphthous ulcers. However, current literature examining the relationship between vitamin D status and RAS remains limited and somewhat inconclusive, with most studies relying on serum measurements. Saliva, an easily accessible and non-invasive biological fluid, has recently gained attention for its diagnostic potential in reflecting systemic health conditions. Saliva contains a range of biomolecules including hormones, antibodies, enzymes, and cytokines that mirror serum constituents, allowing for real-time monitoring of physiological and pathological states without the discomfort or risk associated with venipuncture [13].

Recent studies have demonstrated that salivary vitamin D

levels correlate reasonably well with serum concentrations, suggesting saliva as a reliable alternative matrix for assessing vitamin D status, particularly in pediatric, geriatric, or medically compromised populations where blood collection may be challenging. Furthermore, salivary diagnostics offer the potential for repeated measurements, enhancing disease monitoring and management. Despite these advantages, there is a paucity of research focusing on salivary vitamin D levels in oral mucosal diseases such as RAS, leaving a significant knowledge gap regarding the feasibility and clinical relevance of salivary biomarkers in this context [14, 15].

To date, no comprehensive studies have simultaneously assessed and correlated serum and salivary vitamin D levels in patients suffering from RAS. This study aims to address this gap by evaluating these parameters in individuals with recurrent aphthous ulcers compared to healthy controls, thereby elucidating the possible role of vitamin D deficiency in RAS pathogenesis. Moreover, it seeks to explore the potential of salivary vitamin D as a non-invasive biomarker for diagnosing systemic deficiency and monitoring inflammatory status in RAS patients. Understanding this relationship could offer new insights into the immunopathology of RAS and inform more targeted therapeutic interventions, including vitamin D supplementation, which may improve clinical outcomes and quality of life for affected individuals.

2. MATERIAL AND METHODS

The case control study was conducted at a private Dental College and Hospital in Chennai, Tamil Nadu, India. A total of 24 participants were enrolled based on prior statistical sample size estimation using G*Power analysis to ensure adequate study power. The participants were divided into two groups: Group 1 consisted of 12 patients clinically diagnosed with Recurrent Aphthous Stomatitis (RAS), while Group 2 included 12 healthy individuals matched for age and sex, serving as controls. All participants were within the age range of 20 to 40 years.

Ethical approval for the study was obtained from the Institutional Human Ethics Committee. Written informed consent was obtained from all participants prior to enrolment in the study, in accordance with the Declaration of Helsinki.

Each subject underwent a detailed clinical examination by a single trained investigator to reduce inter-examiner variability. The examination recorded data on the size, number, duration, location, recurrence frequency, and morphological characteristics of the ulcers. For consistency and accuracy, all lesions were documented using clinical photographs and standardized assessment criteria.

A comprehensive exclusion protocol was implemented. Individuals with any known systemic illness (e.g., diabetes mellitus, autoimmune disorders), chronic inflammatory diseases, periodontitis, or a history of liver or renal impairment were excluded. In addition, subjects with poor oral hygiene, malnutrition, or those who had taken vitamin D supplements within the last three months were not considered for inclusion. Participants with a history of smoking, alcohol consumption, or ongoing corticosteroid or immunosuppressive therapy were also excluded to eliminate potential confounding variables.

To evaluate dietary influences on vitamin D levels, a structured dietary intake chart was used to record participants' routine food habits, with specific attention to foods rich in vitamin D such as dairy products, egg yolks, fatty fish, and fortified items. This helped to screen for any unintentional dietary supplementation.

Saliva and blood samples were collected under standardized conditions, preferably in the morning hours to account for circadian variations. The samples were stored and processed in accordance with established biosafety and clinical laboratory protocols to measure serum and salivary vitamin D levels using enzyme-linked immunosorbent assay (ELISA). This methodology was designed to ensure accurate comparison between systemic and salivary levels of vitamin D and to assess any correlation with the presence of RAS.

3. RESULTS

The study population included 24 participants, comprising 12 clinically confirmed cases of Recurrent Aphthous Stomatitis (RAS) and 12 healthy, age- and sex-matched controls. Salivary vitamin D_3 levels were quantitatively assessed in all participants using a standardized assay.

Among the RAS group, salivary vitamin D_3 concentrations ranged from 38.96 ng/mL to 55.00 ng/mL, whereas the control group exhibited higher levels, ranging from 51.05 ng/mL to 60.91 ng/mL. The mean salivary vitamin D_3 level was substantially lower in the RAS group compared to the controls.

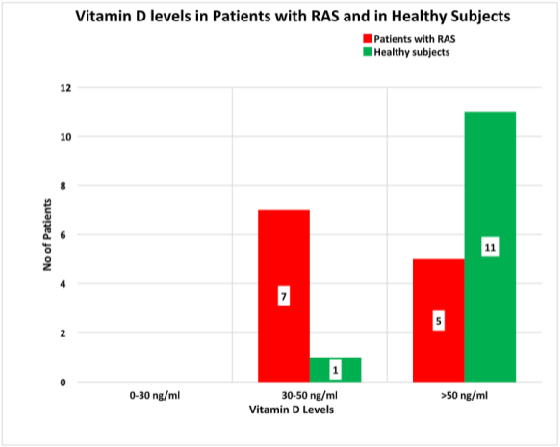

Fig 1 : Graph showing Salivary vitamin D3 levels in patients with RAS and in healthy subjects

A paired t-test was employed to determine the statistical significance of the difference in salivary vitamin D_3 levels between the two groups. The analysis revealed a highly significant difference, with a p-value of 0.001. These findings indicate a statistically and clinically significant reduction in salivary vitamin D_3 concentrations among patients with RAS in comparison to healthy individuals, suggesting a potential association between vitamin D deficiency and the occurrence of aphthous ulcers.(Table 1)

Table.1 Table showing the statistically significant result (p<0.05) Paired-t test done using SPSS software version 23.0

Paired t test		
Study group	**Mean**	**Significance (2-tailed)**
Case group	47.98 ng/ml	0.001
Control group	55.98 ng/ml	

DISCUSSION

Vitamin D, a fat-soluble secosteroid mainly produced in the skin under ultraviolet B exposure, is traditionally recognized for its critical role in calcium and phosphate homeostasis and bone integrity. Yet, over the past decade, its significance has broadened to encompass modulation of immune and inflammatory pathways, raising intriguing possibilities about its involvement in various chronic inflammatory and autoimmune diseases, including recurrent aphthous stomatitis (RAS) [1,16-22]. RAS remains one of the most common oral mucosal disorders, afflicting nearly one-fifth of the population, predominantly adolescents and young adults [23]. Clinically, it manifests as painful, discrete ulcers with erythematous margins and a fibrinous base, preferentially affecting non-keratinized mucosa such as the buccal mucosa and soft palate [24].

Despite decades of research, the precise etiology of RAS remains elusive. Its multifactorial nature is widely accepted, encompassing genetic predispositions, immune dysregulation, microbial factors, hormonal fluctuations, local trauma, psychological stress, and nutritional deficiencies [25]. Among these, vitamin D's role has garnered growing attention, largely due to its immunomodulatory and anti-inflammatory properties that could influence the underlying pathogenic mechanisms of RAS [26]. Unlike classical vitamins, vitamin D acts as a hormone, modulating innate and adaptive immunity, especially through T-cell regulation and cytokine production [27]. The present study's observation of significantly reduced salivary vitamin D3 levels in individuals with RAS compared to healthy controls adds a valuable dimension to this narrative. Earlier investigations predominantly measured serum vitamin D levels, reporting a general trend towards deficiency or insufficiency in RAS patients[28,29]. Few studies have documented significantly lower serum 25(OH)D concentrations in RAS patients versus controls, suggesting systemic hypovitaminosis D may contribute to disease pathogenesis. However, serum sampling, while informative, is invasive and less convenient for repeated monitoring. Saliva, containing hormones, cytokines, antibodies, and enzymes reflective of systemic physiology, has emerged as an attractive alternative for non-invasive diagnostics [30-32]. The present study's use of salivary vitamin D3, quantified by a sensitive delayed competitive ELISA, thus represents a novel, patient-friendly approach, aligning with trends in biomarker research that seek to minimize discomfort and improve clinical applicability [33-36].

Interestingly, the mean salivary vitamin D3 concentration among RAS patients in this study (38.96–55 ng/mL) was consistently lower than in healthy subjects (51.05–60.91 ng/mL). This significant difference supports the hypothesis that insufficient vitamin D levels may skew the immune environment towards a pro-inflammatory state conducive to ulcer formation. This finding is in harmony with recent works, where also reported lower salivary vitamin D3 in RAS patients, further substantiating the utility of saliva as a diagnostic medium [37]. It seems likely that vitamin D insufficiency disrupts the delicate balance of cytokine regulation in oral mucosa. Immunologically, vitamin D's interaction with its receptor (VDR) on immune cells such as macrophages, dendritic cells, and T lymphocytes orchestrates a complex regulatory network. It downregulates pro-inflammatory cytokines including IL-2, IL-6, and TNF-α, while promoting anti-inflammatory cytokines like IL-10, thus maintaining mucosal homeostasis. A deficiency could therefore precipitate exaggerated inflammation and impaired healing characteristic of RAS lesions. These mechanistic insights demonstrated that vitamin D deficiency enhances susceptibility to oral mucosal damage through modulation of T-helper cell responses . Moreover, vitamin D's role in maintaining epithelial barrier integrity and promoting wound healing may be especially pertinent in the recurrent breakdown and slow repair seen in RAS [15].

Vitamin D deficiency has also been implicated in other inflammatory oral diseases, such as periodontitis and oral lichen planus, conditions sharing some immunopathological features with RAS [35-37]. This overlap lends credibility to the hypothesis that vitamin D insufficiency may represent a common denominator in oral mucosal inflammatory diseases. However, unlike periodontitis where bacterial biofilm plays a major role, RAS's etiopathogenesis remains less well-defined, making the identification of modifiable risk factors like vitamin D status particularly valuable [37].

Micronutrient deficiencies in general have been associated with RAS, as evidenced by studies, which

found prevalent deficiencies in B-complex vitamins in recurrent oral ulcer patients and noted clinical improvement with targeted supplementation[38]. Contrastingly, Lalla et al. (2021) reported that broad-spectrum multivitamin supplementation did not significantly reduce ulcer frequency or severity, raising doubts about the effectiveness of generalized supplementation without addressing specific deficiencies[39]. Our findings reinforce the notion that targeted assessment and correction of vitamin D deficiency might offer a more focused and effective therapeutic approach rather than indiscriminate multivitamin use.

Adding complexity, polymorphisms in the VDR gene have been linked to various autoimmune and inflammatory diseases, including RAS[40]. These genetic variations can alter receptor function and vitamin D signaling, influencing individual susceptibility to vitamin D deficiency and disease manifestation. Environmental factors, notably geographic location and cultural practices, further modulate vitamin D levels. Paradoxically, populations in sun-rich regions such as South Asia often exhibit high rates of hypovitaminosis D, due to factors like sun-avoidance behaviors, clothing customs, and indoor lifestyles. This cultural-environmental interplay underscores the multifactorial nature of vitamin D deficiency and its downstream effects in diseases like RAS.

The methodological strengths of this study lie in its non-invasive salivary analysis and the use of delayed competitive ELISA, a robust and specific assay for steroid hormones, which improves reliability over earlier colorimetric methods[41]. Saliva sampling is painless, easily repeatable, and suitable for chairside screening, potentially enabling earlier detection and monitoring of vitamin D status in patients with oral ulcers.

Nonetheless, certain limitations temper the findings. The modest sample size restricts the generalizability of results and calls for larger multicentric studies to validate these observations. Additionally, while salivary vitamin D correlates with serum levels, it may not fully mirror systemic status due to factors such as salivary flow, circadian variation, and oral hygiene practices[42,43]. Moreover, confounders like dietary vitamin D intake, sun exposure habits, skin pigmentation, and seasonal effects were not fully controlled, which could influence vitamin D levels independently of RAS presence.

Furthermore, the absence of genetic profiling for VDR polymorphisms or enzymes involved in vitamin D metabolism constitutes a missed opportunity to dissect gene-environment interactions in this context. Incorporating such analyses in future research could elucidate underlying susceptibilities and refine personalized treatment strategies[24]. It would also be useful to investigate longitudinal changes in salivary vitamin D levels relative to RAS flare-ups and remission phases.

Therapeutically, the observed salivary vitamin D3 deficiency in RAS patients hints at a potentially modifiable risk factor. Pilot intervention studies exploring oral vitamin D supplementation have yielded encouraging results, showing reductions in ulcer frequency and severity, though sample sizes remain small and methodologies heterogeneous. Vitamin D's capacity to enhance mucosal healing and barrier function further supports its therapeutic relevance[34]. Nonetheless, standardized protocols regarding optimal dosage, duration, and formulations are lacking, necessitating rigorously designed randomized controlled trials before routine clinical recommendations can be made.

From a clinical perspective, these findings argue in favor of incorporating vitamin D screening—preferably non-invasive salivary assays alongside serum measures—into the routine diagnostic evaluation of patients with recurrent oral ulcers. Early identification and correction of vitamin D deficiency could reduce disease burden, improve patient quality of life, and potentially prevent progression to more severe mucosal damage. Clinicians should also counsel patients on safe sun exposure practices and encourage dietary sources rich in vitamin D, tailoring advice to individual lifestyles and cultural context.

In essence, this study contributes to a growing body of evidence implicating vitamin D deficiency in the pathogenesis of RAS. While the data are compelling, causality remains to be definitively established. It seems likely that vitamin D insufficiency acts synergistically with genetic, environmental, and immunological factors to promote mucosal vulnerability and ulceration. Further expansive, longitudinal, and mechanistic studies are essential to unravel these complex interactions and to translate findings into effective preventive and therapeutic strategies.

Ultimately, if vitamin D supplementation proves effective, it could offer a safe, low-cost, and accessible adjunct treatment for a condition that, despite being benign, significantly impairs oral comfort and patient wellbeing. Emphasizing patient-centered, individualized care that integrates nutritional, genetic, and lifestyle factors will likely be the key to advancing management of recurrent aphthous stomatitis.

CONCLUSION

This study demonstrates a significant association between lower salivary vitamin D_3 levels and recurrent aphthous stomatitis, suggesting a potential role of vitamin D deficiency in its pathogenesis. These findings support the need for further research on vitamin D supplementation as a preventive or therapeutic approach for managing RAS.

DECLARATIONS

Conflict Of Interest

All the authors declare that there was no conflict of interest in the present study.

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Ethics approval and consent to participate: Ethical Committee Clearance Number: (IHEC/SDC/UG - 2273/24/PHARM/096). "The study was approved by the institutional human ethical committee board of Saveetha Dental College and Hospitals. (IHEC/SDC/UG - 2273/24/PHARM/096). The protocol of the study was approved by the Scientific Review Board (IHEC/SDC/UG - 2273/24/PHARM/096) and it conforms to the provisions of the declaration of Helsinki. Informed consent was obtained from all the patients and their legal guardians by informing and clearly explaining the details of the study. All the methods in the study were performed in accordance with the relevant regulations and guidelines.

Consent to participate: Informed consent was obtained from all the patients and their legal guardians by informing and clearly explaining the details of the study.

Consent for publication: Informed consent was obtained from all the patients and their legal guardians by informing and clearly explaining the details of the study.

Data availability: All data and materials of the study are available and can be provided on request. The corresponding author Dr. Sandra Sagar can be contacted anytime to get the data of the study.

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