RESEARCH ARTICLE

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To Determine the Immune Check Point Protein Expression CTLA-4 and VISTA With Clinical and Pathological Staging of Potential Oral Malignant Lesions by Immunohistochemistry Analysis

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Abstract

Introduction: The immune microenvironment is essential in the development of leukoplakia. Which is a potentially precancerous lesion with increased chances of evolving into oral squamous cell carcinoma (OSCC). One of the most significant co-stimulatory molecules involved in the negative regulation of T cells during carcinogenesis is the immunological check point protein V-domain Ig Suppressor of T-cell Activation (VISTA), also known as CTLA-4. The VISTA and CTLA-4 serves as a emerging new biomarker in early detection and to determine malignant potential of leucoplakia. **Material and method:** Tissue samples from patients with suspected oral malignant lesions were analyzed using immunohistochemistry (IHC). We used established scoring methods to analyze the expression levels of CTLA-4 and VISTA. Clinical finding and histopathological grading were established. The association between the patterns of protein expression and the clinicopathological features was discovered by statistical analysis. **Result:** VISTA expression varied among the different dysplasia grade with a non-linear relationship observed between VISTA expression and the severity of dysplasia. There was a progressive rise in CTLA 4 positivity that paralleled the severity of dysplasia. **Conclusion:** CTLA 4 expression could serve as a helpful indicator for determining the risk of malignant transformation in oral potentially malignant disorders.

Keywords: CTLA-4- VISTA- leukoplakia- immune checkpoint proteins- inflammatory check points

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Introduction

The statistical report from the ICMR Globocan 2012 states that males are more likely to get oral cancer than females. Odds of developing oral cavity and oropharyngeal cancer vary between 1 in 141 women and 1 in 60 males. Each year, over 77,000 Americans receive a diagnosis of oral cancer, In India, oral cancer is often not detected until it has progressed, which leads to less favorable results and a reduced chance of survival [1].

The Immune checkpoints binds with the partner proteins bind and send an "off" signal to the T cells. Thereby preventing the immune system from destroying the cancer cells. Check point protein have shown clinical efficacy in several solid malignancies including head and neck squamous cell carcinoma1. OSCC is frequently preceded by the emergence of precancer lesions that have the potential to progress to invasive carcinoma. Theoretically, identifying and eliminating cancer precursors would eradicate the majority of human cancers [2].

Cancer is a two-step process that starts with a potentially malignant precursor lesion and progresses to cancer. Among the precancerous conditions, oral leukoplakia is recognized as the most common potentially malignant oral disorders (PMODs). Notably, oral leukoplakia is distinguished as the most prevalent PMOD, exhibiting a considerable likelihood of progression to oral squamous cell carcinoma (OSCC) [3]. While current clinical practices predominantly depend on histological evaluations and assessments of severity of dysplasia, recent studies indicate the promise of alternative prognostic indicators. DNA ploidy analysis has been proposed as a potential surrogate marker for forecasting

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the behavior of PMODs. Nevertheless, the expression of immune checkpoint proteins in OSCC remains largely unexamined, especially within our demography. These molecular pathways may have diagnostic, prognostic, and recurrence-related consequences, even if they have not gotten much notice in the scientific literature.

Our present study aims to evaluate the expression patterns of immune checkpoint proteins CTLA-4 and VISTA in possibly cancerous tumour of oral cavity and Correlate immune checkpoint protein expression with clinical and pathological staging thereby exploring their potential as diagnostic and prognostic markers in OSCC. There by giving us a insights into the immunological processes that underlie the development of oral cancer and contribute to precise early detection and management strategies.

Materials and Methods

Ethical consideration

Retrospective study tissue samples was collected between March 2022 to December 2024 from Department of Oral Pathology, Karpaga Vinayaga Institute of Dental Sciences, Tamil Nadu. The study started after getting approved by the Institutional Ethical Committee with the reference number KIDS/IEC/2023/III/015. Prior inform consent was obtained in accordance with the principles outlined in the Helsinki Declaration.

Study participants

Oral pathologists examined 60 specimens; and categorized as group I&II those from healthy, normal subjects without history of any forms of tobacco, made up Group 1. The group II consisted of histopathological diagnosed case of oral leukoplakia with mild, moderate, or severe dysplasia [4]. Additional analyses were conducted on the samples based on demographic, clinical data.

Criteria for inclusion and exclusion

Patients with 20-60 years, clinically suspicious oral lesions requiring biopsy, histopathological grading of mild, moderate, severe dysplasia and confirmed leukoplakia were included in the study. Inadequate tissue samples, Samples with significant processing artifacts immunosuppressive medications, autoimmune disorders that might affect immune checkpoint expression Prior systemic or topical treatment that might affect immune checkpoint expression were excluded.

Immunohistochemistry

The tissue samples are fixed with 10% n formalin for 24-48 hours followed by embedding the tissue in paraffin , section in 4-5 μm thickness and placed positively charged slides. Allow the sections to dry overnight at room temperature. Deparaffinization by heating the slides at 60°C for 1 hour in xylene followed by rehydration with 100% ,95%& 70% ethanol and rinse in distilled water. Antigen Retrieval done using citrate buffer (pH 6.0) for 20 minutes at 95-100°C.Allow the slides to cool to room temperature for 20 minutes. Endogenous peroxidase blocking with 3% hydrogen peroxide for 10 minutes. Apply primary antibody Monoclonal antibody against CTLA4 AND VISTA from BioGenex, USA (Ready-To-Use, New & Improved Super Sensitive™ Polymer-HRP Detection Kit, HRP/DAB) was used and Incubate for 30 min. Wash in phosphate buffer solution (PBS). BioGenex Super Sensitive™ Polymer-HRP Detection Systems use a non-streptavidin-biotin proprietary micropolymer-complex technology to minimize background staining wherein an antibody enhancer/amplifier and a polymer-HRP(Horseradish Peroxidase).Reagent are bound to the primary antibody and visualized by diaminobenzidine (DAB) Counterstain with Mayer's hematoxylin for 1-2 minutes. Rinse in running tap water until clear Dehydrate through graded ethanol series (reverse of rehydration),Clear in xylene Mount with permanent mounting medium and coverslip.

Analysis of CTLA4 and VISTA Expression

CTLA4 at cellular level Primarily expressed in cytoplasmic and membranous staining in T cells, particularly activated T cells and regulatory T cells (Tregs). VISTA IN cytoplasmic expression. In tissues CTLA4 Predominantly present in lymphoid tissues and tumour-infiltrating lymphocytes (TILs) VISTA is expressed in Myeloid cells (macrophages, dendritic cells),T lymphocytes.

Interpretation of CTLA4 and VISTA Expression

Immunoreactivity score was calculated by evaluating both the intensity of staining and the percentage of positive cells. Therefore, we classified the staining intensity of tumor cells into the groups: 0 (negative), 1+, 2+, and 3+ and assessed the percentage of tumor cells in each of the groups. Intensity 0: No reaction on cell membrane, or in cytoplasm. Intensity 1+: A weak intensity and/or incomplete circumferential cell membrane staining, or a low number of cytoplasmic granules had the reactions. Intensity 2+; Complete circumferential cell membrane staining, or a moderate number of cytoplasmic granules had the reaction. Intensity 3+; A strong circumferential cell membrane staining, or a high number of cytoplasmic granules had the reactin [4, 5] . H-score = 3 × the percentage of strong staining + 2 × the percentage of moderate staining + the percentage of weak staining. The following criteria was used to observe the intensity of CTLA AND VISTA stain.

Results

Utilizing IBM SPSS Statistics v26, we conducted all statistical analyses (IBM, Armonk, NY, USA), including standard frequency, descriptive assessments as well as inferential statistics was done and Significance was determined with the Chi-square test. Table 1 displays the study's demographic and clinical data. Histopathological grading and expression of CTLA -4 and VISTA shown in Table 1.

Clinical and Demographic Correlations

According to our demographic analysis, the biggest group of participants (45%) were between the ages of

Table 1. Demographic and Clinical Date

Variable	Number of Patients	Percent (%)
Age		
30-40	5	8.33
40-50	27	45
50-60	20	33.33
60-70	8	13.33
Gender		
Male	30	50
Female	30	50
Duration		
Less Thsn 6months	21	35
Less Than1 Year	13	21.67
Less Than 3 Year	9	15
More Than 5 Year	17	28.33
Tobacco		
Smoking		0
Cigratte	9	15
Bidi	6	10
Smokeless		
Chewing	18	30
Quiding	17	28.33
Alcohol	19	31.67
Site		
Buccal Mucosa	18	30
Floor of the Mouth	1	1.67
Gingiva	1	1.67
Hard Palate	2	3.33
Labial Mucosa	4	6.67
Tongue	3	5
Retromolar Area	1	1.67
Size		
Less The 2 Cm	12	20
Greater Than 2 Cm	18	30
Surface		
Homogeneous	20	33.33
Non Homogeneous	10	16.67
Symptoms		
Burning Sensation	8	13.33
No Symptoms	12	20.2

40 and 50, followed by those between the ages of 50 and 60 (33.33%). This age distribution is common for oral illnesses that might be malignant. The equal gender distribution in our study (50% male, 50% female) varies from several earlier reports that suggest a male predominance in oral leukoplakia, possibly reflecting changing patterns of risk factor exposure.

The anatomical site distribution showed a clear predilection for the buccal mucosa (30%), which correlates with common sites of tobacco placement in users of smokeless tobacco. This is further supported by our risk factor analysis, which identified tobacco chewing (30%), quid use (28.33%), and alcohol consumption (31.67%) as the most prevalent risk factors among the study participants.

Table 2. Pattern of Expression of CTLA- 4 and VISTA in Oral Leukoplakia

	Degree of Dysplasia	Total (n%)	Mean
CTLA-4 in Leukoplakia	Mild	VISTA positive	0
		VISTA negative	10 (100%)
	Moderate	VISTA positive	1 (10%)
		VISTA negative	9 (90%)
	Severe	CTLA positive	4 (40%)
		CTLA negative	8 (80%)
Chi-square value: 5.00 and P-value: 0.082085 (not statistically significant)			
	Degree of Dysplasia		Total(n%)
VISTA in Leukoplakia	Mild	CTLA- 4 positive	0
		CTLA- 4 negative	10 (100%)
	Moderate	CTLA- 4 positive	4 (20%)
		CTLA- 4 negative	6 (80%)
	Severe	CTLA- 4 positive	8 (20%)
		CTLA- 4 negative	2 (60%)
Chi-square value: 13.33 and P-value: 0.0012726 (statistically zignificant)			

Pattern of expression of CTLA-4 in histopathological leukoplakia

The primary outcome of our research highlights a string correlation between CTLA 4 expression and the extent of dysplasia in oral leukoplakia. A distinct progressive rise in CTLA 4 positivity that paralleled the severity of dysplasia, with 0% expression observed in mild dysplasia, 40% in moderate dysplasia, and 80% in severe dysplasia(figure e &f). The statistical significance of this link (p=0.001273) suggests that CTLA 4 expression may be used as a biomarker to assess the likelihood of malignant progression in oral diseases with cancerous potential. CTLA 4 negative expression in healthy control tissues further reinforces its potential as a specific marker for dysplastic alterations. These results are in line with emerging research in other cancers, where CTLA 4 has been connected to mechanisms of immune evasion, enabling neoplastic cells to evade immune detection (Table 2).

Pattern of expression of VISTA in histopathological leukoplakia

The result analysis of 30 oral leukoplakia samples, there was an equal distribution of 10 cases across each degree of dysplasia. Although VISTA expression varied among the different dysplasia grades (figure g &h) -recording 0% in mild cases, 20% in moderate cases, and 40% in severe cases this correlation did not achieve statistical significance (p=0.00127726). The non-linear relationship observed between VISTA expression and the severity of dysplasia (Table 2).

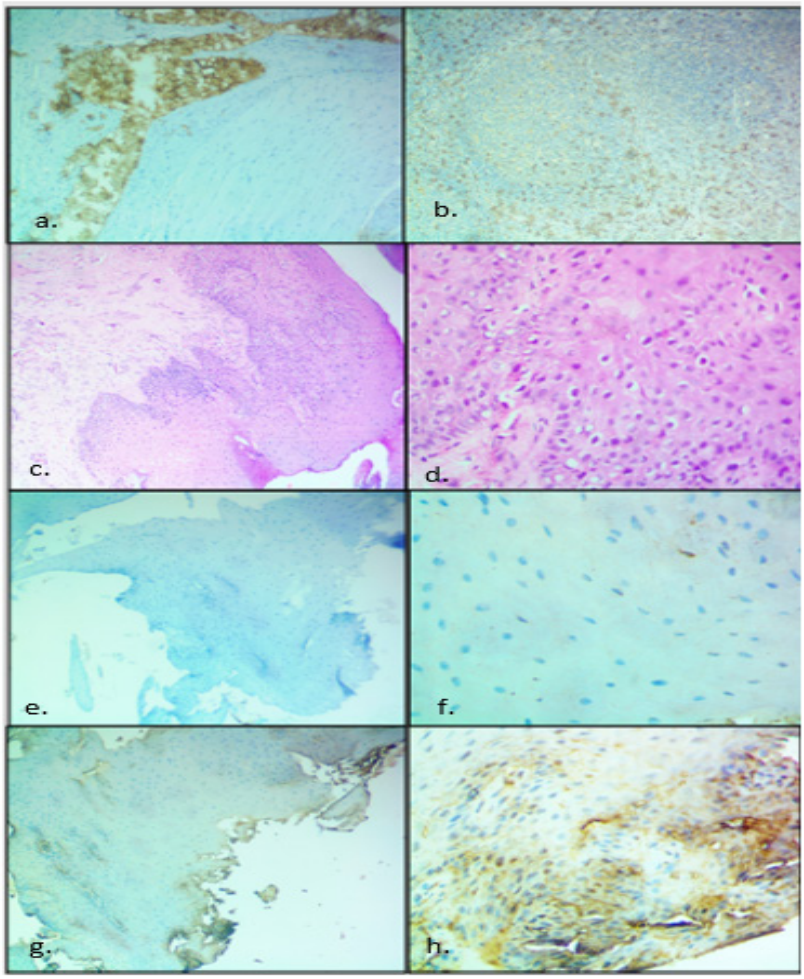

Figure 1. Micrographic Immunohistochemical Expression of (a) tonsillar epithelium as positive control of VISTA (b) tonsillar epithelium as positive control CTLA-4,(c &d) Immunohistochemistry of hematoxylin and eosin stain of oral leukoplakia low power & 10x high power 40x, (e &f)Illustrative Immunohistochemistry staining expression of VISTA (e) low power10x (f) high power40x (g& h) Illustruative Immunohistochemistry expression pattern of CTLA -4 (g) low power10x (h) high power 40x

Discussion

Demographic and Risk Factor Considerations

The epidemiological age distribution patterns of oral potentially malignant disorders (OPMDs) with predominance in the 40-50 year (45%) and 50-60 year (33.33%) age groups, this finding is consistent with Warnakulasuriya (2018), who reported peak incidence of leukoplakia in the fifth and sixth decades of life [5, 6].

The equal gender distribution observed (50% male, 50% female) is a significant change from the earlier reported male predominance by Petti6. In particular, this could represent an increase in risk and health hazards, particularly tobacco smoking among women, as noted by Sridharan [7].

The anatomical predilection for buccal mucosa strongly correlates with 30% of habitual placement of smokeless tobacco products. This site specific association of tobacco consumption and lesion site was established by Hashibe et al. [8]. Further analyzing the prevalence of chewing tobacco (30%), quid (28.33%), and alcohol (31.67%), corroborates the finding of Mello et al (2018) which attributed the increased rates of chewing, dribling and smoking to OPMDs [9].

CTLA-4 Expression pattern and Dysplasia Progression

Gradual increase of CTLA-4 expression corresponding with ($p = 0.001273$) the severity of dysplasia (0% in mild, 40% in moderate, and 80% in severe) with p shows that dysplastic lesions are malignant. This pattern leads to the suggestion that CTLA-4 might serve as a biomarker in the risk of malignant transformation of OPMDs. The same correlations has been done in the CTLA-4 over expression in younger cases of esophageal dysplasia by Zhang et al. [10].

The negative result of CTLA-4 expression in control tissues strengthens its specificity for marker of dysplastic transformation. This aligns with the findings on in vitro investigations done by Stasikowska-Kanicka et al. (2018) who showed that ordinary oral epithelium doesn't express CTLA-4, This is consistent with the results on manifests considerable expression of this molecule [11].

Increased CTLA-4 expression in severe forms of dysplasia has strong immunologic consequences that suggest tumor associated immune suppression. Teng et al. [12] noted that upregulation of immune checkpoint

molecules CTLA-4 creates an immune suppressive microenvironment and enhances tumor progression. This fact adds to the concept that CTLA-4 is not a simple biomarker, but might contribute to carcinogenesis by suppressing anti-tumor immunity.

The significant association between CTLA-4 expression and dysplasia severity has potential clinical applications. Sieviläinen et al [19] demonstrated that immune checkpoint expression profiles could stratify risk of malignant transformation in oral leukoplakia [13]. Our findings support the potential utility of CTLA-4 as a prognostic biomarker for Finding high-risk lesions that need greater aggression management.

VISTA Expression Patterns and Dysplasia Progression

The lack of statistical significance (p=0.00127726) suggests that correlation between VISTA expression and the severity of dysplasia is a phenomenon with much intricacy. Clare et al revealed comparable results that demonstrated variable VISTA expression across stages of colorectal neoplasia [14]. This variation might capture the different potential roles of VISTA in immunological regulation. As explained by ElTanbouly and colleagues (2020), VISTA can function in both co-inhibition and co-stimulation depending on surrounding microenvironment [15]. The non-significant associations with grade of dysplasia may be capturing the variations of VISTA expression that are influenced by other factors beyond mere histopathological progression.

Furthermore, the differential expression patterns between CTLA-4 and VISTA highlight the complexity of immune checkpoint regulation in OPMDs. Callahan et al. [16] emphasized the importance of comprehensive profiling of multiple immune checkpoints to fully understand the immunological landscape of premalignant lesions [16]. These insights may inform potential immunotherapeutic approaches. The established efficacy of anti-CTLA-4 therapies in various malignancies, as reviewed by Callahan et al. [16], raises the possibility of exploring immune checkpoint inhibition as a preventive strategy in high-risk OPMDs showing elevated CTLA-4 expression. Future research should explore the functional consequences of CTLA-4 upregulation in oral dysplasia, potentially through in vitro models as employed by Li et al. [17].

The PD-L1 positivity of subepithelial TAFs ($p < 0.001$) increased with increasing grades of oral leukoplakia. The PD-L1 labeling index of tumor cells and the PD-1 labeling index of lymphocytes infiltrating the tumor were shown to have a strong correlation with one another in OSCC, according to Pearson's correlation (p value: 0.005) [17]. According to Feng et al and Kujan etal, a more complete immunological profile might be obtained by integrating various immune checkpoint markers, such as PD-1/PD-L1 with CTLA-4 and VISTA [15]..

In conclusion, our research finding contribute to understand the immune evasion mechanisms in oral carcinogenesis and suggest that immunohistochemical evaluation of CTLA-4 expression could potentially enhance the histopathological assessment of oral leukoplakia, helping to identify high-risk lesions that may be improved by more aggressive management or closer surveillance. Further larger-scale studies and follow up is necessary to definitively correlate CTLA-4 expression with malignant transformation rates and their clinical applications.

Author Contribution Statement

Dr Karthick Sigamani- analysis, data collection. Dr. Kalaiselvi Santhosh - manuscript review, conceptualization, formal analysis, manuscript writing, Dr. Santhosh kumar soft ware. Dr Shakila Ramalingam - manuscript review. Dr. Saravanan Thalaimalai -manuscript review. Dr. Aravindh Swamy. software, resources, review.

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Conflict of interest

The authors declare that they have no conflict of interest

References

1. Dutta S, Ganguly A, Chatterjee K, Spada S, Mukherjee S. Targets of immune escape mechanisms in cancer: Basis for development and evolution of cancer immune checkpoint inhibitors. Biology (Basel). 2023;12(2). https://doi.org/10.3390/biology12020218.
2. McCord C, Kiss A, Magalhaes MA, Leong IT, Jorden T, Bradley G. Oral squamous cell carcinoma associated with precursor lesions. Cancer Prev Res (Phila). 2021;14(9):873-84. https://doi.org/10.1158/1940-6207.Capr-21-0047.
3. Kumari P, Debta P, Dixit A. Oral potentially malignant disorders: Etiology, pathogenesis, and transformation into oral cancer. Front Pharmacol. 2022;13:825266. https://doi.org/10.3389/fphar.2022.825266.
4. Warnakulasuriya S. Clinical features and presentation of oral potentially malignant disorders. Oral Surg Oral Med Oral Pathol Oral Radiol. 2018;125(6):582-90. https://doi.org/10.1016/j.oooo.2018.03.011.
5. Saeed S, Rauf F, Iqbal F, Khan AS, Khan AH, Alamgeer R. Comparison of pd-l1 expression in oral squamous cell carcinoma and premalignant lesions of oral cavity. Asian Pac J Cancer Prev. 2022;23(12):4039-45. https://doi.org/10.31557/apjcp.2022.23.12.4039.
6. Petti S. Pooled estimate of world leukoplakia prevalence: A systematic review. Oral Oncol. 2003;39(8):770-80. https://doi.org/10.1016/s1368-8375(03)00102-7.
7. Sridharan G. Epidemiology, control and prevention of tobacco induced oral mucosal lesions in india. Indian J Cancer. 2014;51(1):80-5. https://doi.org/10.4103/0019-509x.134651.
8. Hashibe M, Mathew B, Kuruvilla B, Thomas G, Sankaranarayanan R, Parkin DM, et al. Chewing tobacco, alcohol, and the risk of erythroplakia. Cancer Epidemiol Biomarkers Prev. 2000;9(7):639-45.
9. Mello FW, Miguel AFP, Dutra KL, Porporatti AL, Warnakulasuriya S, Guerra ENS, et al. Prevalence of oral

potentially malignant disorders: A systematic review and meta-analysis. J Oral Pathol Med. 2018;47(7):633-40. https://doi.org/10.1111/jop.12726.
10. Zhang XF, Pan K, Weng DS, Chen CL, Wang QJ, Zhao JJ, et al. Cytotoxic t lymphocyte antigen-4 expression in esophageal carcinoma: Implications for prognosis. Oncotarget. 2016;7(18):26670-9. https://doi.org/10.18632/oncotarget.8476.
11. Stasikowska-Kanicka O, Wągrowska-Danilewicz M, Danilewicz M. Immunohistochemical analysis of foxp3(+), cd4(+), cd8(+) cell infiltrates and pd-l1 in oral squamous cell carcinoma. Pathol Oncol Res. 2018;24(3):497-505. https://doi.org/10.1007/s12253-017-0270-y.
12. Teng MW, Galon J, Fridman WH, Smyth MJ. From mice to humans: Developments in cancer immunoediting. J Clin Invest. 2015;125(9):3338-46. https://doi.org/10.1172/jci80004.
13. Sieviläinen M, Almahmoudi R, Al-Samadi A, Salo T, Pirinen M, Almangush A. The prognostic value of immune checkpoints in oral squamous cell carcinoma. Oral Dis. 2019;25(6):1435-45. https://doi.org/10.1111/odi.12991.
14. Clare P, Al-Fatyan F, Risheh B, Nellany K, Ward FJ, Abu-Eid R. A novel role for the soluble isoform of ctla-4 in normal, dysplastic and neoplastic oral and oropharyngeal epithelia. Cancers (Basel). 2023;15(6). https://doi.org/10.3390/cancers15061696.
15. Kujan O, van Schaijik B, Farah CS. Immune checkpoint inhibitors in oral cavity squamous cell carcinoma and oral potentially malignant disorders: A systematic review. Cancers (Basel). 2020;12(7). https://doi.org/10.3390/cancers12071937.
16. Callahan MK, Postow MA, Wolchok JD. Ctla-4 and pd-1 pathway blockade: Combinations in the clinic. Front Oncol. 2014;4:385. https://doi.org/10.3389/fonc.2014.00385.
17. Greeshma LR, Joseph AP, Sivakumar TT, Raghavan Pillai V, Vijayakumar G. Correlation of pd-1 and pd-l1 expression in oral leukoplakia and oral squamous cell carcinoma: An immunohistochemical study. Sci Rep. 2023;13(1):21698. https://doi.org/10.1038/s41598-023-48572-w.