WILEY ORAL DISEASES

World Workshop on Oral Medicine VII: Prognostic biomarkers in oral leukoplakia: A systematic review of longitudinal studies

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Abstract

Objective: To identify the prognostic biomarker candidates for stratification and long-term surveillance of oral leukoplakia progressing to cancer via a systematic literature review.

Materials and Methods: Systematic searches with no date restrictions were conducted on March 29, 2018, targeting the databases PubMed (Ovid), EMBASE (Ovid), EBM (Ovid), and Web of Science (ISI). Bias was assessed using the Quality in Prognosis Studies tool. Biomarkers were stratified based on hallmarks of cancer.

Results: Inclusion criteria were met by 25 of 3,415 studies. A range of biomarkers were evaluated experimentally for risk stratification, prognosis, and surveillance of oral leukoplakia in tissue, blood, and saliva. However, the studies were highly heterogeneous and require further validation. Biomarkers reported in these studies included inflammatory or oxidative markers, growth factors, ion channels, genetic and cellular regulatory factors, and epigenetic biomarkers. Studies tended to include small sample sizes, under-reported or variably reported histopathological data, did not address potential confounding, reported limited/variable follow-up data, or lacked a control group. Inclusion of subsets from chemoprevention trials may have introduced bias regarding reported malignant transformation rates and accuracy of prognostic biomarkers.

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Conclusions: This review identified insufficient longitudinal evidence to support validated prognostic biomarkers for oral leukoplakia. Further studies are needed to identify molecular targets with the potential to mitigate risk of malignant transformation.

KEYWORDS

biomarkers, cancer progression, leukoplakia, neoplastic cell transformation, prognosis, tumor

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) continues to have significant clinical and economic impact at the international level. It is associated with a high rate of mortality, which often is a consequence of delayed diagnosis due to lack of screening programs, low health literacy relative to OSCC, and lack of access to care. The World Health Organization (WHO) projected 354,864 new cases of oral cavity and lip cancer in 2018 (Bray et al., 2018).

Oral potentially malignant disorders (OPMDs), of which leukoplakia is one of the several phenotypes, represent a group of conditions and lesions with variable propensity for oncogenic potential (Warnakulasuriya, Johnson, & van der Waal, 2007). In the era of precision medicine, there is burgeoning interest in defining and characterizing relative risk of oral cancer emergence in association with OPMD. As the understanding of the molecular pathogenesis of oral cancer continues to expand, there is active interest in identifying biomarkers that could provide ability for clinicians to longitudinally track key molecular signals associated with OPMD, and intervene prior to neoplastic transformation. The overarching aims of the systematic reviews performed by the Precision Medicine Group of World Workshop on Oral Medicine VII were to:

- Assess whether prognostic biomarkers could accurately stratify the risk of malignant transformation of oral leukoplakia.
- Assess the relationship between prognostic biomarkers and the patient's risk profile including lesion clinicopathologic characteristics in addition to patient's risk factors.
- Evaluate whether biomarkers could independently predict malignant transformation of oral leukoplakia.
- Establish the minimum follow-up intervals required for biomarkers to predict malignant transformation.
- Assess the efficacy of the investigated biomarkers and management protocol.
- Formulate an algorithm that would help clinicians to provide the best supported evidence-based management protocol to patients with oral leukoplakia.

After oral submucous fibrosis, oral leukoplakia is the most common OPMD, with a worldwide prevalence of 4.11% (95% CI: 1.98–6.97) (Mello et al., 2018). Leukoplakia has been defined by the WHO as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" (Warnakulasuriya et al., 2007). Risk factors for oral leukoplakia are similar to those observed in oral cancer and include tobacco smoking, heavy alcohol consumption, areca nut chewing (especially in South Asian countries), immunosuppression (e.g., HIV/AIDS, post-organ transplantation), personal or family history of cancer (60%–70%), ultraviolet light exposure (for lip lesions only), and selected syndromes (e.g., dyskeratosis congenita) (Villa & Woo, 2017; Warnakulasuriya, 2018).

Leukoplakia is a clinical diagnosis, most commonly presenting in two main phenotypes: *homogeneous* and *non-homogeneous* leukoplakia. *Proliferative verrucous leukoplakia* represents a third, rarer, high-risk subtype (Warnakulasuriya, 2018). Irrespective of type of oral leukoplakia, the gold standard for final diagnosis remains incisional biopsy. Risk of malignant transformation depends on the clinical form and the grade of dysplasia, although other clinical and histopathological parameters have been reported as drivers (Speight, Khurram, & Kujan, 2018). Non-homogeneous leukoplakias carry a 20%–25% risk of cancer progression *versus* 0.6%–5% in homogeneous cases (Napier & Speight, 2008; Reibel, 2003; van der Waal & Axell, 2002).

A key step to better understanding oral leukoplakia outcomes is to identify the molecular factors that drive malignant progression, as these factors may also represent attractive candidates for targeted therapies. With the advent of precision medicine, a growing evidence base has explored predictive and prognostic biomarkers for oral leukoplakia. This paper systematically reviewed longitudinal studies which specifically aimed to: (a) assess whether prognostic biomarkers could accurately stratify the risk of progression of oral leukoplakia to cancer, and (b) evaluate the reliability of biomarkers in long-term surveillance of oral leukoplakia. Future studies will focus on the other overarching aims as mentioned above.

2 | MATERIALS AND METHODS

This study was conducted by the Precision Medicine Work Group within the World Workshop on Oral Medicine VII (WWOM VII). Results are reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher, Liberati, Tetzlaff, & Altman, 2009). PICO (**P**atients, **I**ntervention/exposure/prognostic factor, **C**omparison group and **O**utcome) was used to formulate the research question.

- **P**atients: patients with oral leukoplakia
- **I**ntervention/exposure/prognostic factor: biomarkers in human specimens (saliva, blood, gingival crevicular fluid, oral tissues)
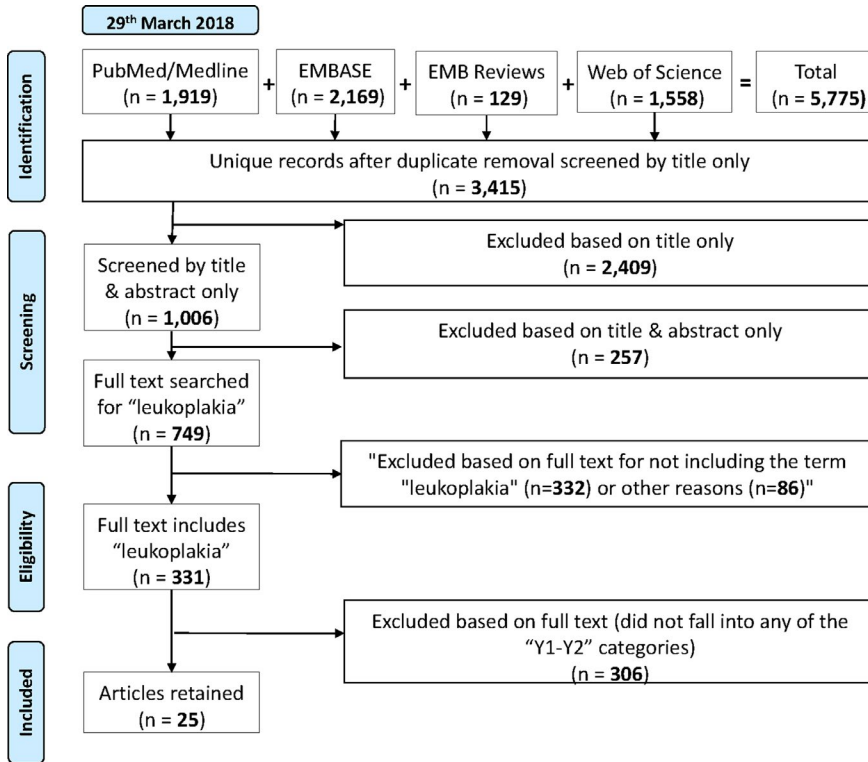
FIGURE 1 Selection of studies for systematic review of prognostic biomarkers for oral leukoplakia

- **C**omparison: healthy controls or patients with oral squamous cell carcinoma
- **O**utcome: squamous cell carcinoma
- **S**tudies: longitudinal (prospective) studies

2.1 | Study selection

Studies that were potentially eligible for inclusion evaluated biomarker expression in: (a) human specimens (saliva, blood, gingival crevicular fluid, oral tissues) with follow-up data (over time); (b) patients with oral leukoplakia compared to healthy controls; or (c) patients with oral leukoplakia compared to patients with oral squamous cell carcinoma. Studies were excluded if they were: (a) studies investigating only non-human tissues, (b) withdrawn/retracted studies, (c) reviews, (d) case reports, (e) commentaries, (f) opinion articles, (g) letters to the Editor, or (h) congress abstracts.

The selection of studies for review was conducted in the following five steps as illustrated in Figure 1.

Step 1: Electronic literature searches were conducted on March 29, 2018, using the PubMed (Ovid), EMBASE (Ovid), Evidence Based Medicine (EBM) Reviews (Ovid), and Web of Science (ISI) databases with no publication year restrictions. The search strategies according to the syntax rules of each database are displayed in Supporting Information Table S1. The identified citations were imported into the electronic database EndNote X8 (Clarivate Analytics, Philadelphia, PA, USA). Deduplication was achieved by the EndNote software and manually by the two reviewers (AVil and AC).

Each subsequent step was conducted by two blinded reviewers (AVil and AC) to exclude records ineligible for inclusion, based on sequential review of title only, title and abstract, and finally full text (Step 4). The applied exclusion categories are shown in Table 1. When more than one exclusion category would pertain to a study, the most important was selected. At each step, review discordance between the reviewers was resolved by discussion between the two reviewers (AVil and AC) under supervision of the senior reviewer (CSF).

Step 2: Among unique records screened by title, only 1,006 out of 3,145 were retained. Cohen's kappa statistic for inter-reviewer agreement was 0.95 (95% CI: 0.94–0.96), and the absolute agreement between the two reviewers was 96.7%.

Step 3: Screening by both title and abstract resulted in retention of 749 of 1,006 for full-text review. The absolute agreement between the two reviewers was 96.7%, and the kappa statistic was 0.92 (95% CI: 0.91–0.94).

Step 4: Based on review of the full text, 331 of 749 were retained based on identification of "leukoplakia" as the definitive diagnosis. The reasons for excluding the 418 studies are shown in Table 1.

Step 5: The remaining 331 papers were allocated to one or more of the following eligibility categories, which respectively assessed the efficacy of biomarkers in:

- Y1: stratifying the risk of progression of oral leukoplakia to cancer (prospective longitudinal studies only).
- Y2: long-term surveillance of oral leukoplakia (longitudinal studies only).

TABLE 1 Reason for exclusion after full-text assessment

Exclusion category	Reason for exclusion	Number of studies
N0	Not English language	17
N1	Not original study (review, guideline, editorial, conference abstract)	2
N2	No human tissue or only immortalized human cell lines in vitro	3
N3	No clinical diagnosis of oral leukoplakia, only dysplasia	332
N4	Cancer patients only: resection margins/perilesional tissue in oral/oropharynx squamous cell carcinoma; history of cancer prior to or during study; undergoing chemotherapy or radiation therapy	6
N5	Predatory journals	2
N6	Retracted articles, or title includes "expression of concern"	3
N7	Other reason for not meeting inclusion criteria	53
All (N1-N7)	Reports ineligible for inclusion	418

- Y3: diagnosis of oral leukoplakia as an adjunct to oral examination (prospective case–control/cross-sectional studies only)
- Y4: progression of oral leukoplakia in a retrospective data set (case–control/cross-sectional studies only)
- Y5: differentiating oral leukoplakia from controls; correlation of differential biomarker expression with diverse clinicopathologic parameters (case–control/cross-sectional studies only).

Inter-reviewer absolute agreement for Step 5 was 85.47%, and the kappa statistic was 0.59 (95% CI: 0.50–0.68), but 100% absolute agreement was reached upon a second revision.

As per title, the current report was limited to the 25 longitudinal studies included in the categories Y1 regarding progression risk and Y2 on long-term surveillance. Consequently, 306 of 331 studies classified by Y categories that did not meet eligibility for inclusion in Y1 or Y2 categories either due to lack of eligibility for any of the Y category or because they were included into categories Y3–Y5 were excluded from further review in the current report. Of note, all papers included in the Y2 category were also included in the Y1 category and the results will therefore be reported collectively.

The reviewer (AVil) and the consultant (CSF) extracted and entered into specifically developed forms (Microsoft Excel 2010, Redmond, Washington, USA) all relevant data from the selected papers. Risk of bias for the 25 included studies was assessed independently by one reviewer (AVil) and the consultant (CSF) using the "**Qu**ality **i**n **P**rognosis **S**tudies" (QUIPS) tool (Hayden, van der Windt, Cartwright, Cote, & Bombardier, 2013), which evaluates the six domains "study participation," "study attrition," "prognostic factor measurement," "outcome measurement," "study confounding," and "statistical analysis and reporting." Discord was resolved by consensus.

2.2 | Statistical analysis

Absolute percent inter-reviewer agreement and Cohen's kappa coefficient were calculated using IBM Statistics 23 (SPSS, Chicago, Illinois, USA). The heterogeneity of the studies and the high number of different biomarkers studied prevented any quantitative analysis of the results, so no meta-analysis was performed.

3 | RESULTS

Of the 331 publications eligible for the categories Y1–Y2 out of the originally identified 3,415 unique records, 25 studies were allocated to groups Y1 and Y2 and therefore included in this report (Figure 1). Results are reported by types of biomarkers stratified to the hallmarks of cancer (Hanahan & Weinberg, 2011). The main characteristics of the studies are presented in Table 2, and a detailed description of the biomarkers identified is reported in the Supporting Information Appendix S1.

3.1 | Types of biomarkers

Biomarkers reported in these studies (Table 2) included inflammatory or oxidative markers (Chang et al., 2013; Massarelli et al., 2005; Rai, Kaur, Jacobs, & Singh, 2010), growth factors (Beenken et al., 1994, 1999; Uehara, Ikeda, Nonaka, & Asahina, 2010; Wan, Meyskens, Armstrong, Taylor, & Kennedy, 1999), cell signaling biomarkers (Saintigny et al., 2011, 2018, 2018; Sakata et al., 2017), genetic and cellular regulatory factors (Lee et al., 2000; Mao et al., 1996; Nagao et al., 2017; Tanic, Tanic, Milasin, Vukadinovic, & Dimitrijevic, 2009; Visioli, Lauxen, Sant'ana Filho, & Rados, 2012),

TABLE 2 Findings from the 25 eligible studies using biomarkers with leukoplakia specimens

Author Year Country Study Design Duration	Biomarker[a]	Specimen type	Leukoplakia (n)	Risk factors: leukoplakia No. of cases/smoking or alcohol use	Age, (year) Mean (±SD)[b] (Range)	Sex #M/#F	Histopathology No. of cases/type	Site of lesion: No. of cases/location	Outcomes/Results Biomarker–Malignancy link
Tissue invasion, metastasis, and inflammatory markers									
Chang et al., 2013; China Follow-up: 28 months (only available for OSCC patients)	IL-6 M-CSF TGF-β1 ICAM-1 E-selectin CRP SAA MMP-2 MMP-9	Serum	46	NA	Leukoplakia Mean 46.7 Range: (26–72) Healthy: Mean 48.3 Range: (27–82)	44/2	NA	Leukoplakia samples: 4 tongue 30 buccal mucosa 2 hard palate 2 retromolar trigone 5 tongue + buccal mucosa 2 lip + buccal mucosa	Significant difference: TGF-β1 (AUC: 0.756, $p < 0.001$); CRP (AUC: 0.704, $p < 0.001$) MMP-9; (AUC: 0.729, $p < 0.001$) in leukoplakia vs. healthy controls. Sensitivity to detect leukoplakia or OSCC following evaluation of MMP-9, MMP-2, TGF-β1, CRP, and E-selectin: 67.4% and 90%, respectively.
Massarelli et al. (2005) USA Follow-up: median of 28 months	p27 & E-cadherin protein expression	Tissue (biopsy)	31	Smokers: E-cadherin leukoplakia group (laryngeal/oral): 26 former 11 current 20 never p27 leukoplakia group (laryngeal/oral) 23 former 8 current 9 never	E-cadherin group: Mean 58.3 p27 group: mean 57.3	E-cadherin group: 15/31 p27 group: 14/26	E-cadherin group: 5 hyperplasia/ hyperkeratosis Dysplasia: 13 mild 16 moderate 12 severe p27 group: 5 hyperplasia/ hyperkeratosis Dysplasia 13 mild 12 moderate 10 severe	NA	21 mo post-follow-up: Loss of E-cadherin expression 25/46 (54%) Development of OSCC: 14/23 (61%). No. who developed OSCC with: - leukoplakia (14/31 (45%)). - p27 loss (13/19 (68%)) -p27 retention: (7/21 (33%)). p27 loss was an independent predictor of malignant transformation (log-rank test; $p = 0.02$). E-cadherin and p27 loss: was associated with a decreased time to disease progression (log-rank test; $p = 0.04$).
Serum oxidative markers									
Rai et al. (2010) India Follow-up: 181 days	MDA 8-OHdG vitamin C vitamin E	Saliva serum	25	NA	Age range: 17–50	13/12	NA	NA	Compared with healthy controls, - MDA and 8-OHdG levels were increased in oral leukoplakia, submucosal fibrosis, and oral lichen planus cases, ($p < 0.05$); serum and salivary vitamin C and E levels were decreased ($p < 0.05$). Mean salivary and serum MDA, 8-OHdG, and vitamin C and E levels were significantly different in patients with oral leukoplakia, submucosal fibrosis, and oral lichen planus before the intake of curcumin and after treatment ($p < 0.05$).
Proliferation without exogenous stimulation									
Growth factors									
Beenken et al. (1994) USA Follow-up: 3 months	EGFR TGF-α HER-2/neu	Tissue (biopsy)	9	NA	NA	NA/NA	Dysplasia: 5 mild 1 moderate 3 severe	NA	Pretreatment expression scores for EGFR, TGF-a, and HER-2/neu in leukoplakia were increased when compared to their expression scores in normal-appearing mucosa. TGF-α expression ratios in leukoplakia were significantly reduced after treatment with 13-cis-retinoic acid.
Beenken et al. (1999) USA Follow-up: 1 month	TGF-α EGFR	Tissue (biopsy)	11	NA	NA	NA/NA	Dysplasia: 5 mild 3 moderate 3 severe	NA	TGF-α and EGFR scores were increased in dysplastic oral leukoplakia specimens when compared with adjacent normal-appearing mucosa. TGF-α expression scores in normal-appearing mucosa adjacent to dysplastic tissue were higher than those seen in healthy controls.

(Continues)

TABLE 2 (Continues)

Author Year Country Study Design Duration	Biomarker[a]	Specimen type	Leukoplakia (n)	Risk factors: leukoplakia No. of cases/smoking or alcohol use	Age, (year) Mean (±SD)[b] (Range)	Sex #M/#F	Histopathology No. of cases/type	Site of lesion: No. of cases/location	Outcomes/Results Biomarker–Malignancy link
Wan et al. (1999) USA Follow-up: 1 month	Neu proteins	Blood and oral mucosa cells	25	NA	NA	NA/NA	NA	NA	Changes in the protease activity in oral mucosal cells after therapy correlated with the changes in the neu protein levels in oral mucosal cells ($p < 0.001$) and serum ($p < 0.001$). In addition, the level of neu protein in oral mucosal cells correlated with the serum neu protein concentration in patients with oral leukoplakia before treatment ($p < 0.001$).
Uehara et al. (2010) Japan Follow-up: NA	Mean nuclear variation	Tissue (biopsy)	7	NA	NA	8/12	Leukoplakia samples: epithelial dysplasias	Leukoplakia samples: 4 tongue hard palate 1 soft palate 1 buccal mucosa	The mean nuclear areas: Before PDT: range: 54.1 to 158.8 μm², (median = 91.4 μm², SD = 23.0 μm²); After PDT: range: 24.6 to 106.8 μm² (median = 66.5 μm², SD = 21.7 μm², p = 0.0920). Significant differences in PCNA LI values before and after PDT were observed in: Recurrent group (p = 0.0496); non-recurrent group (p = 0.0188) with decreased PCNA LI values after PDT).
Cell signaling									
Saintigny et al. (2011) USA Follow-up: median of 7.5 years	Proteasome machinery, MYC ribosomal components	Tissue (biopsy)	35	Smokers: 22 current 35 former 29 never Alcohol use: 49 current 8 former 29 never	Median: 57.5 range: (23–90)	45/41	32 dysplasia 54 hyperplasia	NA	Gene sets associated with proteasome machinery, MYC upregulation, and ribosomal components were associated with a high risk to develop OSCC.
Saintigny et al. (2018) USA Follow-up: The median follow-up of the 51 patients who did not develop OSCC was 6.08 years	MET	Tissue (biopsy)	35 (pts who developed cancer)	Smokers: 42 current 45 former 33 never Alcohol use: 70 current 10 former 40 never	NA	61/59	44 dysplasia 6 hyperplasia	NA	No MET expression: Leukoplakia cases (26/120; 21.7%). MET expression: low (score 1), 34 (28.3%) mild (score 2): 39 (32.5%) high (score 3) 21 (17.5%).Time to cancer progression varied across 4 Met expression groups (score 0 through 3), p = 0.001). Oral cancer-free survival decreased in patients with high MET expression levels ($p < 0.001$). MET overexpression in leukoplakia: HR: 3.84 (95% CI: 1.59–9.27, $p < 0.003$) for malignant transformation.
Sakata et al. (2017) Japan Follow-up: NA	SMAD4	Tissue (biopsy)	127 (leukoplakias that did not transform)	NA	Median: 61.7 range: (19–87)	67/60	Cases: 13 no dysplasia 7 mild dysplasia 3 moderate/severe dysplasia Controls: 88 no dysplasia 0 mild dysplasia moderate/severe dysplasia	64 (42.7%) gingiva 52 (34.7%) tongue 21 (14.0%) buccal mucosa 13 (8.7%) other oral cavity sites	No. with malignant transformation: 23/150 (15.3%). Malignant transformation associated with: SMAD4 expression (p = 0.0017) -lymphocyte infiltration (p = 0.0054). Low SMAD4 expression: -significant prognostic factor in pts with leukoplakia (hazard ratio, 2.632; p = 0.043) -correlated with high lymphocyte infiltration (p = 0.00035). -significant correlation between low SMAD4 expression and high lymphocyte infiltration (p = 0.00027).
Insensitivity to antigrowth signals									
Cell cycle regulators									
Lee et al. (2000) USA Follow-up: 7 years (range: 0.2–10.6)	p53 RAR-β CP LOH micro-nuclei	Tissue (biopsy)	70	Smokers: 39 current 31 former/never Tobacco chewers: 10 yes/60 no Alcohol use: 49 current 21 former/ never Cancer history: 11 yes/59 no	NA	33/37	9 moderate/severe dysplasias 61 hyperplasia/mild dysplasia	27 tongue/FOM 43 others	31.4% of patients developed OSCC. High chromosomal polysomy (CP) expression, p53 protein accumulation in the parabasal layer, and LOH at chromosome 3p or 9p: associated with higher cancer risk (p = 0.0008).

TABLE 2 (Continues)

Author Year Country Study Design Duration	Biomarker[a]	Specimen type	Leukoplakia (n)	Risk factors: leukoplakia No. of cases/smoking or alcohol use	Age, (year) Mean (±SD)[b] (Range)	Sex #M/#F	Histopathology No. of cases/type	Site of lesion: No. of cases/location	Outcomes/Results Biomarker~Malignancy link
Nagao et al. (2017) Japan Follow-up: NA	p53 ki67	Tissue (biopsy)	4	Smokers: 1 former 3 never Alcohol use: 2 regular 2 never	Median: 66	2/2	Responders: dysplasia (1/4) no dysplasia (3/4) Non-responders: dysplasia (4/12) no dysplasia (5/12)	Responders: tongue/FOM (4/4); Non-responders: (7/12), other (5/12)	p53 expression: greater in basal layers vs. parabasal layers. Mean parabasal labeling index (LI) of p53 was higher in non-responders (26.0) than in responders (11.2) (p = 0.028). Ki67 LIs were similar between groups.
Tanic et al. (2009) Serbia Follow-up: 4 years	p53	Cases: tissue (biopsy) Controls: blood	32	All smokers	NA	NA/NA	30 mild/no dysplasia 32 moderate/severe dysplasia	NA	p53 gene was mutated in 13 cases (40.6%). The majority of mutations were localized on exon 6. Four patients with moderate instability and mutated p53 underwent malignant transformation.
Visioli et al. (2012) Brazil Follow-up: 3–6 years	p53 p21WAF1	Tissue (biopsy)	36	NA	Mean: 50.1 SD (±14.5)	19/17	NA	11 gingiva 10 tongue 8 buccal mucosa 6 lip 1 palate	Overexpression of p53 and p21WAF1: observed in dysplastic and non-dysplastic leukoplakias.
Loss of heterozygosity									
Mao et al. (1996) USA Follow-up: median time to OSCC progression: 63 months (range: 5-92)	Markers located at chromosomes 9p21 & 3p14	Tissue (biopsy)	37	22 smokers	57.5	20/17	32 dysplasia 52 hyperplasia and/ or hyperkeratosis	NA	8/37(22%) developed OSCC. LOH: (19/37) (51%). - Cases (37%) with LOH at either 9p21 or 3p14 (or both) developed cancer—One case (6%) without LOH underwent malignant transformation. Time to cancer development was shorter in LOH (p = 0.039).
Ion channels									
Fernandez-Valle, Rodrigo, Garcia-Pedrero, et al. (2016) Spain Follow-up: median time to OSCC progression: 25.5 months (range: 7-308).	Kv3.4	Tissue (biopsy)	62	28 smokers (45%) of whom 14 (22.6%) also consumed alcohol	61.1 SD: ±12.41 Range: (30-85)	35/27	43 hyperplasia mild dysplasia 7 moderate dysplasia 4 severe dysplasia/ carcinoma in situ	Cannot discriminate between leukoplakia and OSCC	Detection of Kv3.4 + immunostaining noted in: -hyperplastic leukoplakias 7/43 (16%) -leukoplakic lesions with dysplasia 8/16 (50%) (p = 0.008). No correlation observed between Kv3.4 expression in oral leukoplakia compared to OSCC samples (p = 0.86). Leukoplakias with Kv3.4 neg expression: 8/13 (61.5%) showed strong Kv3.4 immunostaining in after subsequent OSCC development
Fernandez-Valle, Rodrigo, Rodriguez-Santamarta, et al. (2016) Spain Follow-up: median time to OSCC progression: 39 months (range: 7 -226)	HERG1 protein expression	Tissue (biopsy)	62	28 smokers (45%) of whom 14 (22.6%) also consumed alcohol	Mean 61.1 SD ± 12.4 Range: (30-85)	27/35	43 hyperplasia 8 mild dysplasia 7 moderate dysplasia 4 severe dysplasia/ carcinoma in situ	NA	HERG1 status of lesions on OSCC development: -HERG1-(+): 8/22 (36%); HERG1(-neg): 18/39 (46%)

(Continues)

TABLE 2 (Continues)

Author Year Country Study Design Duration	Biomarker[a]	Specimen type	Leukoplakia (n)	Risk factors: leukoplakia No. of cases/smoking or alcohol use	Age, (year) Mean (±SD)[b] (Range)	Sex #M/#F	Histopathology No. of cases/type	Site of lesion: No. of cases/location	Outcomes/Results Biomarker~Malignancy link
Sustained angiogenesis									
Growth factors									
Nayak et al. (2015) India Follow-up: median time to OSCC progression: 29.41 months	FGF-2 FGFR-2 FGFR-3	Tissue (biopsy)	43	NA	NA	NA/NA	NA	NA	Expression of FGF-2 and FGFR-2: associated with HR 28.2 (95% CI: 3.29–241.72; $p < 0.002$) and HR 11.5 (95% CI: 2.20–59.91; $p < 0.004$) of cancer progression, respectively.
Yang et al. (2014) NA country Follow-up: up to 5 yrs	GDF15	Serum	24	NA	NA	NA/NA	NA	NA	Serum GDF15 level: increased leukoplakia and OSCC cases compared with healthy controls ($p < 0.001$). OSCC cases with GDF15 < 346.9 ng/L had improved 3-year disease-free survival rate compared to cases with GDF15 ≥ 346.9 ng/L. (64.3% vs. 56.5%)
Podoplanin									
Kawaguchi et al. (2008) USA Follow-up: 7.5 years	Podoplanin	Tissue (biopsy)	163	Smokers: 52 current 60 former Alcohol use: 85 current 18 former	NA	NA/NA	101 hyperplasias 49 dysplasias	NA	Podoplanin (+) lesions: more common in older patients ($p = 0.016$), females ($p = 0.02$), and those with dysplastic leukoplakias ($p = 0.04$). 23% of the patients progressed to OSCC. Podoplanin was an independent risk factor for malignant transformation (HR = 3.087; 95% CI, 1.530–6.231; $p = 0.002$).
Saintigny et al. (2009) USA Follow-up: median of 7.5 years	ΔNp63 podoplanin	Tissue (biopsy)	162	Smokers: 56 current 5 former 1 never Alcohol use: 93 current 19 former 50 never	NA	85/77	NA	NA	Oral cancer was associated with: -ΔNp63 and podoplanin status ($p < 0.0001$), -ΔNp63 status and intraepithelial inflammatory cell clusters (EIC) ($p = 0.0003$), -between podoplanin status and presence of EIC ($p = 0.0012$). Presence of all three biomarkers was associated with dysplastic histology ($p = 0.016$) and older age ($p = 0.004$). Cumulative incidence for developing oral cancer at 3 years of follow-up: 53% for cases with all three biomarkers compared to 11% of other patients. Patients with podoplanin-positive leukoplakia and those with ΔNp63-positive leukoplakia had a higher incidence of OSCC ($p < 0.0001$).
Glucose-regulated proteins									
Lin et al. (2010) Taiwan Follow-up: median of 5.4 years (range: 0.02–6.8)	GRP78	Tissue (biopsy)	28	NA	NA	NA/NA	NA	NA	Overexpression of GRP78 was associated with increasing malignant potential in 14% of leukoplakia patients. Patients with GRP78 overexpression had a poorer 6-year same-site premalignancy-free survival rate than those without (44.0% vs. 89.6%, respectively; $p > 0.002$).

(Continues)

TABLE 2 (Continues)

Author Year Country Study Design Duration	Biomarker[a]	Specimen type	Leukoplakia (n)	Risk factors: leukoplakia No. of cases/smoking or alcohol use	Age, (year) Mean (±SD)[b] (Range)	Sex #M/#F	Histopathology No. of cases/type	Site of lesion: No. of cases/location	Outcomes/Results Biomarker–Malignancy link
Gene transcription modification									
miRNA expression									
Xiao et al. (2012) China Follow-up: median of 32 months (range: 20–47)	miRNA	Tissue (biopsy)	7 (progressing leukoplakias)	Smokers: 3 current 4 never/former Alcohol use: 4 current 3 never/former	Mean: 55.4 SD ± 16.3	3/4	Non-transformed leukoplakias: 11 no/mild dysplasia 5 moderate dysplasia 4 severe dysplasia	NA	Leukoplakia cases who OSCC had -upregulation of: miR-31, miR-142-5p, miR-33a, miR-1259, miR-146b-5p, miR- 886-3p, miR-886-5p, miR-519d, and miR-301a -downregulation of: miR-572, miR-611, miR-602, miR-675, miR- 585, miR-623, miR-637, and miR-1184. FISH analysis confirmed high miR-31 expression in leukoplakia that underwent malignant transformation.
Yang et al. (2013) China Follow-up: median time of 35 months (range: 26–46)	miRNA	Saliva	15	Smokers: 7 current 3 former 5 never/former	Mean 57.6	3/4	All 15 samples were LGD (mild to moderate dysplasia). Among those that progressed 5 were severe dysplasia (3/5 with SCC micro-foci), 2 CIS, 1 moderate to severe dysplasia SCC micro-foci	5 lateral tongue 2 lateral palate 6 buccal mucosa 1 palate 1 lower lip	There was upregulation of miR-708, miR-10b, miR-19a, miR-30e, miR-26a, miR-660 and downregulation of miR-99, miR-15a, miR-197, miR-145, and miR-150. miRNAs were differentially expressed in non-progressing low-grade dysplasias with different progression potentials. miR-181c and miR-181b were underexpressed in progressing low-grade dysplasias and overexpressed in non-progressive low-grade dysplasias.
DNA methylation									
Foy et al. (2015) USA Follow-up: For the 12 patients who did not develop OSCC (median follow-up, 7.64 years) and the patients who did develop OSCC (group B, median follow-up, 2.15 years)	DNA methylation	tissue (biopsy)	24	NA	NA	NA/NA	Reported in the Supporting Information Appendix S1	NA	86 candidate genes were identified. Methylation of *PENK, FOXI2,* and *AGTR1* promoter regions was associated with the development of OSCC.

#: number; CIS: carcinoma in situ; F: females; FOM: floor or the mouth; leukoplakia (*n*): leukoplakia group sample size; LOH: loss of heterozygosity; M: males; NA: not available; OSCC: oral squamous cell carcinoma; PDT: photodynamic therapy; pt(s): patient(s); SCC: squamous cell carcinoma y: year(s).

[a]Please see Table 4 for explanation of biomarkers; [b]Some studies reported the median and not the mean.

ion channels (Fernandez-Valle, Rodrigo, Garcia-Pedrero, et al., 2016; Fernandez-Valle, Rodrigo, Rodriguez-Santamarta, et al., 2016), sustained angiogenesis factors (Kawaguchi et al., 2008; Lin et al., 2010; Nayak et al., 2015; Saintigny et al., 2009; Yang et al., 2014), and epigenetic biomarkers (Foy et al., 2015; Xiao et al., 2012; Yang et al., 2013). Studies tended to include small sample sizes, under-reported or variably reported histopathological data, did not address potential confounding, reported limited/variable follow-up data, or lacked a control group. Notably, 14 of the 25 studies were components of chemoprevention trials (Beenken et al., 1994; Foy et al., 2015; Kawaguchi et al., 2008; Lee et al., 2000; Mao et al., 1996; Massarelli et al., 2005; Nagao et al., 2017; Rai et al., 2010; Saintigny et al., 2009, 2011, 2018; Uehara et al., 2010; Wan et al., 1999; Yang et al., 2013). Inclusion of subsets from chemoprevention trials may have introduced bias regarding reported malignant transformation rates and accuracy of prognostic biomarkers.

3.2 | Quality assessment of the studies

Low risk of bias was observed in "statistical reporting," "study participation," "study attrition," and "outcome measurement" (81%, 70%, 52%, and 52% of included studies, respectively), while moderate risk of bias in "study confounding" was found in 70% of studies and in "outcome measurement," "prognostic factor measurement," and "study attrition" in 48% of the studies. Percentage of studies with high risk of bias was relatively low and varied between 0% and 11% across the different domains (Figure 2; Table 3).

4 | DISCUSSION

This systematic review was directed to identification of biomarkers that could predict the likelihood of malignant progression over time in patients affected by oral leukoplakia (Table 4). Therefore, only studies applying longitudinal designs were included. Overall, the 25 included studies documented a wide range of biomarkers derived from three different anatomic sources: serum, tissue, and saliva.
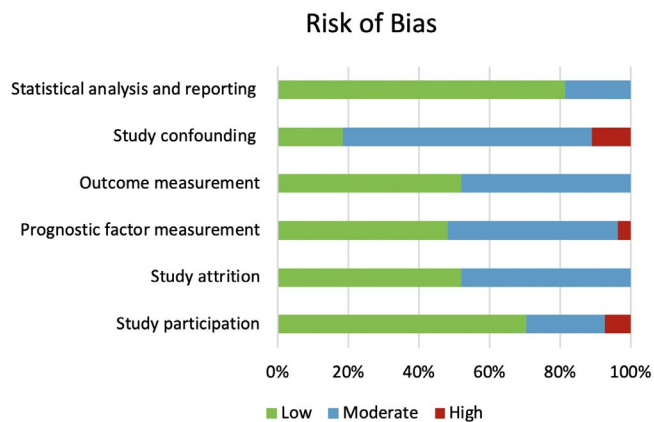
Risk of Bias

FIGURE 2 Risk of bias in the 25 included studies according to the Quality in Prognosis Studies (QUIPS) criteria (Hayden et al., 2013)

Heterogeneity of biomarkers investigated across these studies precluded direct interstudy comparison in virtually all cases.

Whereas selected biomarkers that were examined longitudinally seem promising, major limitations delineated in the current studies prevent definitive clinical application at this time. Most of the studies: (a) included small sample sizes with the largest comprising only 162 patients (Kawaguchi et al., 2008); (b) suffered from a paucity of histopathological data for leukoplakia and a lack of reporting of demographics and potentially confounding factors such as age, tobacco smoking, and alcohol consumption (Table 2); (c) reported on limited and variable follow-up data and/or did not include a control group. As such, no negative or positive predictive value was reported for the biomarkers. Each study included in the review evaluated distinct biomarkers, with the exception of podoplanin that was evaluated by two research groups (Kawaguchi et al., 2008; Saintigny et al., 2009), but continues to require further investigation. Thus, potential validity of biomarkers examined to date remains to be demonstrated in other studies. Several included papers reported on biomarkers examined in the context of chemoprevention trials (14/25 studies) where intervention may have potentially impacted the malignant transformation rates and accuracy of prognostic biomarkers relative to risk detection for malignant progression of oral leukoplakia (Beenken et al., 1994; Foy et al., 2015; Kawaguchi et al., 2008; Lee et al., 2000; Mao et al., 1996; Massarelli et al., 2005; Nagao et al., 2017; Rai et al., 2010; Saintigny et al., 2009, 2011, 2018; Uehara et al., 2010; Wan et al., 1999; Yang et al., 2013). Finally, most studies exhibited a moderate risk of bias in association with missing data, no consideration of potential confounding, and availability of limited follow-up data (Table 3). Future studies should include a longer follow-up (the longest one reported in the studies included for this systematic review was 7.5 years; by Saintigny et al., 2009) to capture all cases of oral leukoplakia that undergo malignant transformation. This requires a larger effort with multiple centers and resources involved. Notably, biomarker assessment was not conducted in a blinded manner in any of the included studies, further introducing additional potential for bias.

Ten studies aimed to identify objectively measured molecular biomarkers for potential utility in identifying patients with oral leukoplakia at higher risk of malignant transformation. The remaining 14 studies that incorporated biomarker assessment as part of chemoprevention trials had other objectives. We defined the potential role of each of the biomarkers reported in these studies based on their relational juxtaposition to essential hallmarks of cancer (Hanahan & Weinberg, 2011).

Based on evidence available to date, candidate biomarkers for cancer progression in patients affected by leukoplakia examined in this systematic review lacked substantive evidence as harbingers of risk for malignant transformation of oral leukoplakia. However, we only included those studies that specifically described "leukoplakia"; studies that described dysplasia only were excluded. Consequently, some biomarkers with prognostic value for oral dysplastic lesions (and possibly some leukoplakia cases) may have been missed. In addition, some of these biomarkers were captured in the Y3, Y4, or Y5

TABLE 3 Quality assessment of the studies according to the Quality in Prognosis Studies (QUIPS) criteria (Hayden et al., 2013), illustrated in Figure 2

Author, Year	Study participation	Study attrition	Prognostic factor measurement	Outcome measurement	Study confounding	Statistical analysis and reporting
Beenken et al., 1994	High	Moderate	Moderate	Low	Moderate	Moderate
Beenken et al., 1999	High	Moderate	Moderate	Low	Moderate	Moderate
Chang et al., 2013	Moderate	Moderate	High	Moderate	Moderate	Low
Fernandez-Valle, Rodrigo, Garcia-Pedrero, et al. (2016)	Low	Low	Low	Low	Low	Low
Fernandez-Valle, Rodrigo, Rodriguez-Santamarta, et al. (2016)	Moderate	Low	Low	Moderate	Moderate	Low
Foy et al., 2015	Low	Low	Low	Low	Moderate	Low
Kawaguchi et al., 2008	Low	Low	Low	Moderate	Moderate	Low
Lee et al., 2000	Low	Low	Low	Low	Moderate	Low
Lin et al., 2010	Low	Moderate	Moderate	Moderate	High	Low
Mao et al., 1996	Low	Low	Low	Moderate	Low	Low
Massarelli et al., 2005	Low	Low	Moderate	Low	Low	Low
Nagao et al., 2017	Moderate	Low	Moderate	Moderate	Moderate	Moderate
Nayak et al., 2015	Low	Moderate	Moderate	Moderate	Moderate	Low
Rai et al., 2010	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Saintigny et al., 2009	Low	Low	Low	Low	Moderate	Low
Saintigny et al., 2011	Low	Low	Low	Low	Low	Low
Saintigny et al., 2018	Low	Low	Low	Low	Moderate	Low
Sakata et al., 2017	Low	Low	Low	Low	Low	Low
Tanic et al., 2009	Low	Low	Low	Low	Moderate	Low
Uehara et al., 2010	Low	Moderate	Moderate	Moderate	Moderate	Low
Visioli et al., 2012	Low	Low	Moderate	Moderate	Moderate	Low
Wan et al., 1999	Low	Moderate	Moderate	Moderate	Moderate	Moderate
Xiao et al., 2012	Low	Moderate	Low	Moderate	Moderate	Low
Yang et al., 2014	Moderate	Moderate	Low	Moderate	Moderate	Low
Yang et al., 2013	Moderate	Moderate	Moderate	Low	Moderate	Low

TABLE 4 Biomarkers used with leukoplakia specimens in the 25 studies displayed in Table 2

Biomarker acronym	Biomarker name and function
CP	Chromosomal polysomy
CRP	C-reactive protein; acute inflammatory marker
E-cadherin	Molecule that makes cells adhere to each other (cadherin = "calcium-dependent adhesion")
eGFR, EGFR	Epidermal growth factor receptor
E-selectin	Cell adhesion molecule only expressed on endothelial cells activated by cytokines; inflammatory marker
FGF	Basic fibroblast growth factor and signaling protein
FGFR	Fibroblast growth factor receptor
GDF	Growth differentiation factor
GRP	Glucose-regulated protein
HER	Human epidermal growth factor receptor
HERG	Human ether-à-go-go related gene
ICAM	Intercellular adhesion molecule; inflammatory marker
IL	Interleukin, cytokines (secreted proteins, signal molecules)
Ki-67	Antigen, nuclear protein
Kv3.4	Potassium voltage-gated channel gene
M-CSF	Macrophage colony-stimulating factor; inflammatory marker
MET	Mesenchymal–epithelial transition
MDA	Malondialdehyde; oxidative marker
miRNA	Micro-**ribo**nucleic **a**cid
MMP	Matrix metalloproteinase
MYC	Family of regulator genes and proto-oncogenes that code for transcription factors (MyC: **My**elo**c**ytomatosis)
ΔNp63	Tumor protein p63
8-OHdG	8-hydroxydeoxyguanosine
p21WAF1	Tumor protein, cell cycle protein regulators
p27	Protein that regulates the cell cycle
p53	Tumor protein, cell cycle protein regulators
Podoplanin	Mucin-type protein, specific lymphatic vessel marker
RAR-β	Retinoic acid receptor-Beta
SAA	Serum amyloid A; inflammatory marker
SMAD4	Mothers against decapentaplegic homolog 4 gene
TGF	Transforming growth factor; inflammatory marker

categories and will be reviewed separately in future publications by our group. One example of such a biomarker comes from the study by Zhang and colleagues who examined 296 oral premalignant lesions and showed that LOH at 3p and/or 9p was present in 20% of cases that underwent malignant transformation (Zhang et al., 2012), further supporting the role of LOH in malignant transformation of oral leukoplakia. Although LOH may indeed be a predictive biomarker for malignant transformation, this systematic review highlights that both clinical and histopathological descriptors of lesions should be included in future studies on the topic to ensure robustness of data, but also to allow subsequent meta-analyses should more than one study investigate the same marker or set of markers.

This systematic review disclosed that insufficient longitudinal evidence is currently available to support identification of biomarkers that could improve current methods for detection of leukoplakia and any subsequent malignant disease progression. Therefore, the strength of the current evidence precludes advancement of any candidate biomarkers examined to date into clinical practice. While a variety of technologies and laboratory techniques were employed to measure biomarker expression (including IHC, PCR, ELISA, and next-generation sequencing), outcomes of these studies based on the current body of evidence do not achieve the standards required to identify and validate candidate biomarkers investigated to date. While recent advancements in biomarker development using microarray technology, mass spectrometry, next-generation sequencing, and proteomic technologies have facilitated accomplishment of relatively rapid screening in real time, one of the major challenges that remain unaddressed is the validation in large longitudinal trials to assess the true effectiveness of these biomarkers. Reliable, sensitive, and specific biomarkers are needed to predict malignant transformation in patients affected by leukoplakia and inform physicians on correct treatment decisions. Thus, their relative value

remains equivocal pending validation in future appropriately designed studies, with no advances currently observed. Well-designed, randomized controlled studies with proper follow-up are required to propose and validate biomarkers for the purpose of establishing a new basis for their clinical and diagnostic utility. Consequently, clinicians continue to rely on tissue biopsy and histopathological assessment of oral dysplasia as the gold standard to determine the risk of malignant transformation and management strategies for patients with oral leukoplakia.

Limitations in tissue availability of leukoplakia samples (often small, formalin-fixed paraffin-embedded) and the preinvasive nature of leukoplakia may limit its systemic detectable expression in saliva and blood. Two promising biomarkers identified in this systematic review included podoplanin and cell cycle regulators. Podoplanin was identified as a possible independent predictor for cancer progression (hazard ratio = 3.1; 95% CI: 1.5–6.2) (Kawaguchi et al., 2008), and p27 loss was shown to be an independent factor for malignant transformation ($p = 0.02$) (Massarelli et al., 2005). However, despite the potential for greatly improving current clinical tools and collective recent advances, there is still insufficient longitudinal evidence to support the identification of these and other biomarkers that could improve the current methods for detection of leukoplakia and any subsequent malignant disease progression based on the 25 studies that met eligibility for inclusion in this systematic review. Thus, oral epithelial dysplasia on histopathological assessment of a biopsy specimen currently remains the best predictor for transformation to invasive squamous cell carcinoma.

4.1 | Future directions

The past decade has seen an increase in availability and access to high-throughput profiling and sequencing technologies for genomic analysis and increased bioinformatic approaches to support rapid and comprehensive investigations of multi-omics data in the precancer and cancer research domain. The growing capacity for integration and systems analysis of multi-omics data needs to be pursued to help elucidate interactions between genetic and epigenetic alterations being recognized in leukoplakia and promote identification of a prognostic biomarker or panel of biomarkers with improved potential for cancer prediction and simultaneously definition of pathways involved in malignant transformation including candidate molecules which could be targeted by therapeutic interventions to impede progression of oral leukoplakia to invasive cancer. Notably, the era of precision medicine has seen achievement of this goal in the context of some cancers, including breast cancer. Achievement of this goal is further linked to definition of specific genetic alterations associated with leukoplakia and processes that drive malignant transformation. Increased understanding of these processes will also promote further identification of candidate biomarkers that may represent detectable molecular signals arising as a consequence of transformational processes. In the future, additional well-designed research that defines triggers of leukoplakia progression through integrated, ad hoc computational analyses is needed to leverage genomic and other meta-data in defining appropriate candidate biomarkers and tailored molecular targets with translational potential to mitigate risk of malignant transformation associated with leukoplakia.

As more high-throughput technologies such as next-generation sequencing are utilized in the discovery of diagnostic, prognostic, and predictive biomarkers, it is incumbent on researchers to better understand biomarker types and to better plan study designs to suit the biomarker type but also the question at hand. In the case of oral leukoplakia malignant transformation, assessing DNA tissue biomarkers through genome-wide association studies or even exome sequencing in affected individuals may provide insight into genetic mutations or aberrations of susceptibility in affected individuals, and, if followed up over time, may lead to discovery of a susceptibility profile for malignant transformation, as it is known that not all leukoplakias progress to malignancy. These biomarkers can be tested retrospectively on biobanked samples, and would hasten biomarker discovery as patient recruitment for biomarker study design would not necessarily need to proceed prospectively. DNA tumor biomarkers of oral leukoplakia samples themselves may offer equally interesting insights, but would require a prospective study design. These biomarkers would be amenable for testing therapy response and pharmacodynamics, but may suffer from lack of reproducibility as different somatic mutations may be present in different parts of the lesion, thus highlighting the importance of biopsy site selection and multiple sampling of oral leukoplakia. Nonetheless, having this insight would dramatically increase our ability to discuss treatment options with patients, and would be invaluable prognostic predictors for therapy. Finally, RNA, protein, or metabolite biomarkers can be assayed as DNA tumor biomarkers, but have the advantage of being tested in a variety of biosamples including blood, saliva, gingival crevicular fluid, tissue, or cell scrapes, would equally be amenable to therapy testing and monitoring of response, would provide reproducibility at any one point in time, and could be used in prospective study designs, but additionally would be suitable as surrogate markers of disease. Furthermore, their discovery can elucidate molecular pathways involved in malignant transformation and provide more comprehensive insights into this process.

Ideally, collaborative research, at the international level, strategically designed to systematically collect and analyze data is required in order to (a) achieve a robust, adequately powered study population for follow-up of patients with leukoplakia and (b) observe progression in a larger subset of patients than is normally possible in single-center studies due to the relatively low rate of malignant transformation. Patients with newly diagnosed leukoplakia should be included with subsequent longitudinal follow-up, and collection of structured data at specifically defined time points. Future studies should also focus on identifying biomarkers by integrating -omics data with environmental and lifestyle variables with the final goal of identifying more informative predictors of cancer progression that supersede dependence on histopathological diagnosis alone. Collection of these data into a centralized repository in the context of good histopathology data would further create a resource amenable to application of bioinformatic approaches and predictive

modeling to identify the most informative variables for screening across time.

5 | CONCLUSIONS

This review identified insufficient longitudinal evidence to support validated prognostic biomarkers for oral leukoplakia. Further studies are needed to identify molecular targets with the potential to mitigate risk of malignant transformation of oral leukoplakia to advance precision medicine approaches to management of patients with these lesions.

CONFLICT OF INTEREST

The authors have no conflict of interest. The authors would like to thank Dr. Jim Berryman, Liaison Librarian, Brownless Biomedical Library, University Library, The University of Melbourne, for his guidance developing the literature search strategy.

AUTHOR CONTRIBUTION

Alessandro Villa conceptualization, data collection, paper writing. Antonio Celentano conceptualization, data collection, critical review of the paper. Ingrid Glurich conceptualization, critical review of the paper. Wenche S. Borgnakke conceptualization, critical review of the paper. Siri Beier Jensen critical review of the paper. Douglas E. Peterson critical review of the paper. Konstantina Dellicritical review of the paper. David Ojedacritical review of the paper. Arjan Vissink critical review of the paper. Camile S. Farah conceptualization, critical review of the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.