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ORIGINAL ARTICLE

Predictors of malignant transformation in oral leukoplakia and proliferative verrucous leukoplakia: An observational prospective study including the DNA ploidy status

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Abstract

Background: This prospective observational study investigated the determinants of malignant transformation (MT) in localized oral leukoplakia (OL) and proliferative verrucous leukoplakia (PVL).

Methods: Demographic, clinical, histological, and DNA ploidy status data were collected at enrolment. Survival analysis was performed (MT being the event of interest).

Results: One-hundred and thirty-three patients with OL and 20 patients with PVL entered the study over 6 years (mean follow-up 7.8 years). The presence of OED, DNA ploidy, clinical presentation, and lesion site were associated with MT in patients with OL in a univariate analysis. In a multivariate model, OED was the strongest predictor of MT in patients with OL. Adding DNA ploidy increased the model's predictive power. None of the assessed predictors was associated with MT in patients with PVL.

Conclusions: DNA ploidy might identify a subset OL with low risk or minimal risk of MT, but it does not seem to be a reliable predictor in patients with PVL.

KEYWORDS

DNA ploidy, malignant transformation, mouth neoplasms, oral leukoplakia, proliferative verrucous leukoplakia

1 | INTRODUCTION

Oral potentially malignant disorders (OPMDs) are a heterogeneous group of disorders that have a non-negligible risk for malignant transformation (MT) ranging from 1.4% to 49.5%, depending on the specific disorder.[1] Localized oral leukoplakia (OL) is one of the most common OPMDs, characterized by the presence of "white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer."[2] On the other hand, proliferative verrucous leukoplakia (PVL) is a rare condition with multiple, multifocal, simultaneous leukoplakias that frequently cover wide areas of the oral mucosa.[3] PVL is considered a distinct form of multifocal

OL; these conditions have remarkably different MT rates (ranging from 49.5% to 53.2% and from 9.5% to 16.4%, respectively),[1,4–6] with PVL having the highest proportion of oral cavity cancer development compared with other OPMDs.[1,7] Patients with OL present either single flat or variable multifocal lesions,[8,9] and a correct interpretation of clinical features of multifocal oral leucoplakias is mandatory for the correct identification of PVL. PVL diagnosis is challenging and often achieved only retrospectively after careful clinical and pathologic correlation showing a history of persistent or recurrent and multifocal lesions.[10,11]

The presence of high-grade (described as moderate and/or severe) oral epithelial dysplasia (OED) is generally considered the best predictor for MT in cohorts of patients with OL. Nevertheless, a not negligible proportion of oral squamous cell carcinoma (OSCC) arises from OL histopathologically classified as nondysplastic.[12,13] This represents an important issue when we consider that in large cohorts more than 80% of OL do not harbor OED.[12] When faced with OL, the most prevalent OPMD, the presence of high-grade OED can identify no more than 5%–15% of high-risk patients,[12,14] so that the number of low-risk lesions far exceeds that of high-risk ones. Conversely, the diagnosis of PVL itself identifies patients with a high risk of MT. Therefore, a potential biomarker that could identify lesions with a high risk of malignant transformation would be of help to the largest proportion of patients with OL.

A recent meta-analysis, merging data from three retrospective cohort studies and two case–control studies not including PVL, concluded that "DNA aneuploidy is a useful marker of malignant transformation in OPMD, but a diploid result should be interpreted with caution" as "no malignant progression" is 82% more likely to occur in OPMD not harboring DNA aneuploidy.[15]

Current data on the predictive role of the DNA ploidy status come from retrospective studies,[16–19] often only focusing on lesions characterized by the presence of OED[20–23] and with little data collected from patients with PVL.[24–27] All previous studies harbor potential biases due to the retrospective study design and none of them compared the predictive value of a DNA aneuploid status in OL and PVL. Moreover, merging evidence together from the literature would suggest that the DNA ploidy status, as a single biomarker, seems to have limited value as a predictor of progression to cancer.[15,28]

The present prospective observational study aims to determine the MT rate of patients with OL and patients with PVL and to identify patient- and lesion-related features predictive for MT, including the DNA ploidy status assessed by high-resolution DNA flow cytometry (hr DNA-FCM). It also aims to consolidate our previously reported results evaluating the association of the DNA ploidy status with OED in different OPMDs.[29–35]

2 | METHODS

The present prospective observational cohort study was designed in accordance with the STROBE statement.[36] Patients with OL or PVL, excluding those with previous or present OSCCs, were recruited from November 2008 to December 2014 in the Oral Medicine and Oral Oncology Section of the University of Turin at the S. Luigi Gonzaga University Hospital (Orbassano, Turin).

Diagnosis was performed following the WHO criteria and therefore based on clinical and histopathological assessment.[3,7] Inclusion criteria selected cases with a clinical diagnosis of OL or PVL requiring either biopsy to complete the diagnostic workup (novel patients referred to the clinic) or histological assessment during the clinical follow-up (patients already receiving OL/PVL management). Exclusion criteria consisted of cases with prior history of OED or OSCC. Patients with carcinoma in the first biopsy or in a second biopsy within 2 weeks, or with inadequate DNA ploidy analysis data, exited the study and their data were not assessed further. The study was performed in accordance with the Helsinki Declaration of 1975 as revised in 1983. Patient written consent to enter the study and to have the DNA-ploidy assessment was obtained in every case during an interview according to the Institutional Ethic Committees (A.S.O.S. Luigi Gonzaga Prot. N. 11780). All clinical data were completely anonymous to safeguard the privacy rights of patients.

Anamnestic data were collected, including sex, age, and tobacco and alcohol habits. Smoking was considered a former habit if cessation occurred at least 6 months before the interview; exposure was considered heavy if consumption was ≥10 cigarettes/day. Alcohol exposure was considered high if consumption was ≥2 alcoholic units (AUs) per day. Consumption of 1 AU (equivalent to about 12 g of ethanol, or 1 glass of wine, or 0.33 L of beer, or 1 measure of liquor) or less than 2 AUs was considered light-to-moderate exposure. The registered clinical information consisted of clinical presentation (homogeneous or nonhomogeneous), lesion site (subsites: tongue, buccal mucosa, floor of the mouth (FOM), gingiva, lip, soft palate, hard palate), and presence/absence of multiple lesions. Clinical presentation was considered homogeneous in the presence of predominantly white, flat, thin or wrinkled lesions; and nonhomogeneous in the presence of mixed white-and-red (including speckled), nodular, granular, and verrucous lesions. The lesion site allowed us to determine if lesions were in an oral subsite characterized by high risk for MT (border/ventral tongue, FOM, soft palate), and to identify the histological type of oral mucosa: thin (border/ventral tongue, FOM, and soft palate), vestibular (buccal mucosa, lip), masticatory (dorsal tongue, gingiva, and hard palate).

Tissue samples were obtained by incisional biopsy (formalin-fixed and routinely processed to complete the workup of OL/PVL and to determine the presence and grade of OED) and by microbiopsy (immediately frozen at 20°C and stored for later measurements by high-resolution DNA flow cytometry [hr DNA-FCM], as previously detailed).[29]

Histological diagnosis of OED required the consensus of two pathologists based on WHO criteria at the time of diagnosis. OED grading was directly equivalent to the WHO 2017 three-grade system.[2] A binary OED scale was also used for the analysis, that is, no-OED or OED. Where a patient with OL had only one single lesion this was identified as the "index lesion"; if multiple samplings were performed due to the lesion's size, the worst histological diagnosis was then considered. In the presence of multiple lesions (patients with OL or patients with PVL) requiring multiple samplings, the sample characterized by the worst histological diagnosis identified the index lesion. The above-cited data on clinical presentation and lesion site refer to the index lesion.

In the presence of a confirmed diagnosis of OL or PVL, frozen samples collected by microbiopsy were submitted to high-resolution flow cytometry DNA ploidy analysis at the IRCCS AOU-San Martino-IST, Genoa. Samples were prepared and analyzed as previously reported[29] to evaluate the DNA index (DI). Briefly, tissue samples were processed to obtain DAPI-stained nuclei suspensions following the method of Otto et al.[37] with modifications. High-resolution DNA flow cytometry (hr DNA-FCM) of these samples was used as previously described[29] to obtain DNA content histograms and to evaluate the DI. DNA diploid controls were represented by sex-specific human lymphocytes. The mean CV of the corresponding DNA diploid GO-G1 peaks was $1.2\% \pm 0.2\%$. The DNA diploid ($DI = 1$) and aneuploid sublines ($DI \neq 1$) were sorted using a Cyflow Space FCM equipped with a PPCS unit (Partec GmbH, Muenster, Germany) with purity of about 99%. The degree of DNA aneuploidy ($DI \neq 1$), expressing the amount of abnormal DNA content relative to normal, was calculated as the ratio of the mean channel number of the DNA aneuploid G0–G1 peak(s) to the mean channel number of the DNA diploid G0–G1 peak. The DI aneuploid values were subdivided into DNA near-diploid aneuploid ($DI \neq 1$ and $DI < 1.4$) and high aneuploid ($DI \geq 1.4$).

Patients were managed irrespective of the DNA ploidy status. Both patients with OL and patients with PVL were instructed to remove modifiable risk factors for SCC (i.e., exposure to tobacco and alcohol). In patients with OL, if a clear margin was achievable and if removal was feasible with acceptable postoperative function, then indication for complete removal was given in the case of (1) lesions harboring OED, (2) nonhomogeneous lesions, (3) lesions on a high-risk anatomical site. In patients with PVL, indication for complete removal was given for lesions with moderate-to-severe OED, while indication for removal of nondysplastic lesions was considered on a case-by-case basis. Indefinite follow-up was undertaken for both treated and untreated patients with OL/PVL every 3–6 months on a case-by-case basis.

2.1 | Malignant transformation endpoint

Data on MT and the vital status of patients were updated in June 2022 by merging information from the clinical charts of the Oral Medicine and Oral Oncology Section of the University of Turin (where all the enrolled patients received follow-up) and information from institutional databases for patients lost to clinical follow-up. Information on vital status of patients, causes of death, and hospitalizations or medical visits potentially related to MT was retrieved from the following databases: (1) the Registry Office of Turin-ATO (Anagrafe di Torino); (2) the Unitary Network of the Regional Administration-RUPAR (Rete Unitaria Pubblica Amministrazione Regionale); (3) the Regional Unitary Archive of Patients-AURA (Archivio Unico degli Assistiti); (4) the Hospital Discharge Register-SDO (Schede di Dimissione Ospedaliera). Failure to retrieve a patients' records from the above databases resulted in the exclusion of the patient from the final data analysis.

Data regarding follow-up visits from the clinical charts were integrated with data regarding new diagnoses from the Hospital Discharge Records-SDO of all other regional Italian hospitals and the Regional Unitary Archive of Patients-AURA. The vital status for each patient that did not develop MT or was not already registered as deceased was checked in the Unitary Network of the Regional Administration-RUPAR and the Registry Office of Turin-ATO.

All living subjects no longer attending the follow-up visits were contacted by telephone: a recall visit was offered and data on the current oral conditions and MT were collected.

2.2 | Statistical analysis

First, the mean and standard deviation of the continuous variables and frequencies for the category variables of interest were calculated and stratified by lesion type (OL vs. PVL). The association between the variables and MT was assessed using chi-square or t test, accordingly. Since it is known that OL and PVL have very different clinical and histopathological features, as well as risk of MT, all analyses presented in this study were stratified by lesion type.

In cancer epidemiology, studies exploring predictive factors for adverse pathological outcomes, such as MT, are better described by accounting for the timing of these events, as well as their probability; so we then used Kaplan–Meier curves and multivariate Cox proportional-hazards model to assess the risk of MT, which was our event of interest. Cases not exhibiting the event of interest were censored either at the date of last follow-up or the date of death. The follow-up time was calculated by subtracting the date of enrolment from the date of MT for those who had the event of interest; for the others, it was calculated subtracting the date of enrolment from the date of censoring or death. This analysis was possible only for cases with OL, due to the low number of cases with PVL ($n = 20$). At this point, we first performed univariate analysis using Kaplan–Meier curves and log-rank test in order to compare the time-to-event curves of several variables of interest potentially linked with MT. Cox proportional-hazards models were used to investigate multiple factors that increase the risk for MT in OL lesions (as seen in the univariate Kaplan–Meier analysis) in a joint multivariate model. The effect estimates were presented as hazard ratios (HRs) and 95% confidence intervals (CIs). In the first model, we included only DNA aneuploidy. In the second, third and fourth models we added consecutively, one by one, the following variables: OED, high-risk site, and heterogeneity (variables that showed the highest risk for MT in the univariate analysis).

One of the study's objectives was to assess the potential of DNA ploidy to identify high- or low-risk lesions, so next we compared DNA ploidy with an OED, an already well-known predictor of MT. The presence of OED and DNA aneuploidy is often (but not always) intertwined, and so we first explored the association and correlation of the two variables in our study sample, independent of MT, using the chi-square test, which may be of use for comparison with previous and future studies. We then calculated the positive and negative predictive values (PPV and NPV, respectively) of DNA ploidy and OED. The PPV and NPV of combined OED and DNA ploidy was also calculated, as undertaken by Zaini et al. However, since PPV and NPV consider only the event of MT and not the time-to-MT, we calculated the Harrel's concordance index (C-index after each Cox regression model). The C-index is the most widely used metric for the global evaluation of prognostic models in time-to-event analysis and can be considered an analogue to the ROC curve. With a score that ranges from 0 to 1 (with a value of 0.5 representing random discrimination), a higher C-index indicates a better prognostic model. All analyses were performed using STATA (Stata Corp. 2021. Stata Statistical Software: Release 17).

3 | RESULTS

3.1 | Patients

Over the 6-year enrolment period, 269 patients were assessed for eligibility and 153 entered the study (Figure 1): one-hundred and thirty-three patients with OL and 20 patients with PVL, as described in Table 1. Patients with OL and patients with PVL differed in terms of the mean age at diagnosis, the percentage of females, the number of lesions, the clinical aspect of the lesion, and the percentage of patients exposed to tobacco. When considering patients with OL, the average age at enrolment was around 60 years old, while patients with PVL were 10 years older. The sex ratio within OL was relatively balanced, while there was a significant female predominance among patients with PVL. Although both OL and PVL presented primarily as plaques (superficial, solid, elevated lesions: 64% vs. 75%, respectively), the second most common clinical aspect of OL was the presence of patches (flat area of color change: 26%), while verrucous lesions were the second most common clinical aspect of PVL (20%). Irrespective of the index lesion site, high-risk sites for MT (border/ventral tongue, floor of the mouth, and soft palate) were variably affected in 61 subjects (39.9%). By contrast, the remaining 92 patients (60.1%) had lesions on sites not at high-risk (vestibular/buccal mucosa, gingiva/alveolar ridge, hard palate, lip). Patients with PVL were significantly less exposed to tobacco or alcohol when compared to patients with OL. Interestingly, OL and PVL index lesions showed differences in DNA ploidy but not in OED. The difference in prognosis between the two lesion types is also evident in our study population: in a mean follow-up of 7.8 (range 0.5–12.8; SD 3.0; 95% CI 7.3–8.3) years, 50% of patients with PVL showed MT, while this was true only for 4.5% of the OL ones.

3.2 | Cases with malignant transformation

Malignant transformation occurred in 6 out of 133 (4.5%) patients with OL and in 10 out of 20 (50%) patients with PVL. None of the progressing patients were lost to clinical follow-up before MT, so that clinical data on MT was successfully obtained at the enrolment center itself. As detailed in Table 2, carcinoma arose in a different/distant subsite from the index lesion in 1 out of 6 (17%) OL-progressing patients and 3 out of 10 (30%) PVL-progressing patients. In two out of the three PVL-progressing patients the subsite of MT had no lesions at enrolment. There was a female predominance in cases that developed MT in both OL and PVL lesions (5/6 in OL and 10/10 in PVL).

All OL-progressing patients had a histological diagnosis of classic SCC involving the tongue (two cases), the

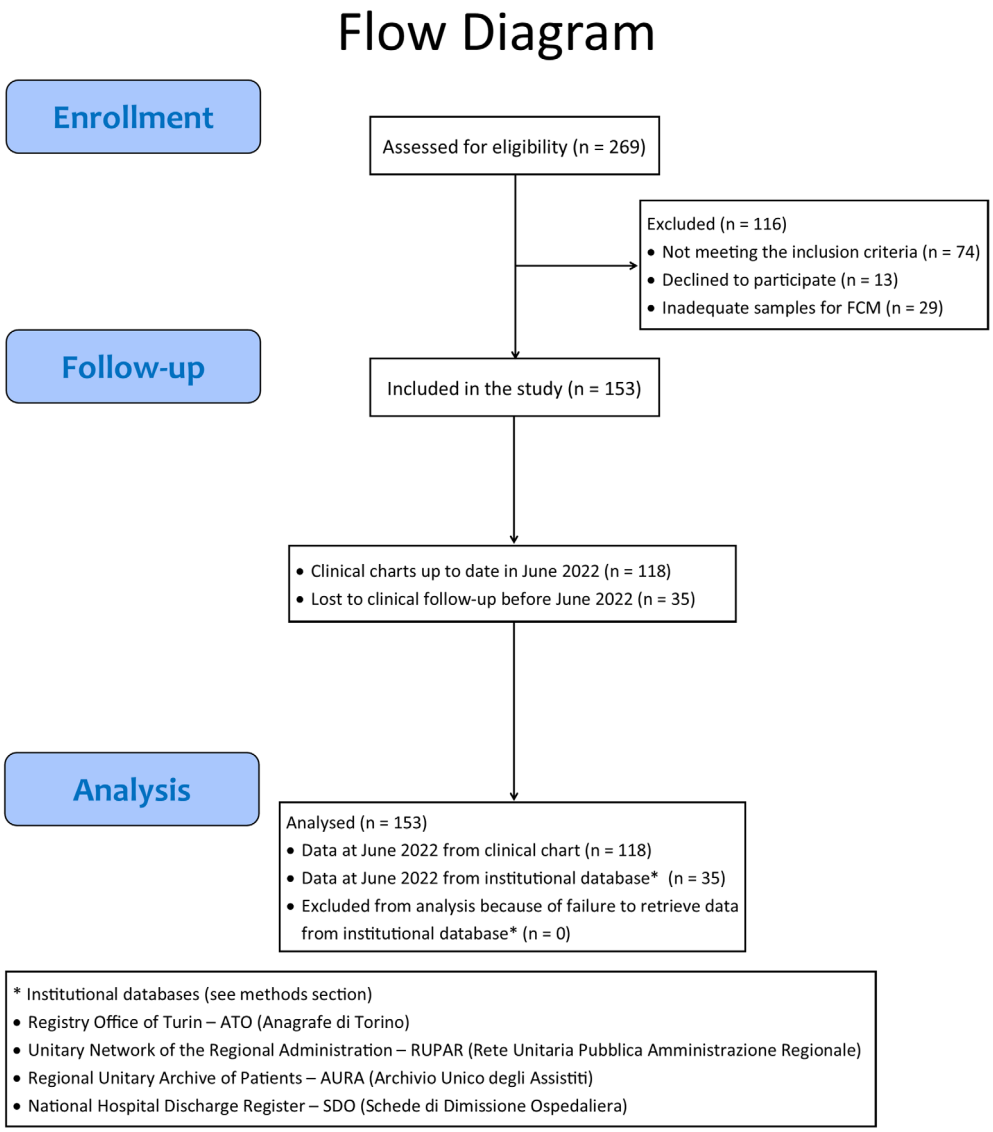

FIGURE 1 Flow diagram of the study [Color figure can be viewed at wileyonlinelibrary.com]

FOM (two cases), and the buccal mucosa (two cases). By comparison, 5 out of 10 PVL-progressing patients had a diagnosis of verrucous carcinoma (VC) mainly involving the masticatory mucosa (two cases on the gingiva, two in the hard palate, and one on the buccal mucosa); the remaining SCC lesions were located on the border/ventral tongue (two cases), gingiva, buccal mucosa, and FOM. The onset of VC and the involvement of masticatory mucosa were significantly more frequent in patients with PVL ($p = 0.037$ and $p = 0.037$, respectively).

3.3 | Time-to-event analysis

In a mean follow-up of 7.8 (range 0.5–12.8; SD 3.0; 95% CI 7.3–8.3) years, MT occurred in 16 subjects, as detailed in Data S1, Supporting Information. The MT rate was 4.9 per 1000 person-years (95% CI 2.23–11.05) in patients with OL and 73.1 per 1000 person-years (95% CI 39.32–135.82) in patients with PVL (Figure 2).

The univariate Kaplan–Meier analyses reported in Data S1 showed that OL patients with DNA aneuploidy, OED, high-risk site location, nonhomogeneous clinical aspect, and presence of erosive/ulcerative features have an increased risk of MT. Smoking and alcohol use does not seem to be associated with MT. The results of this analysis in patients with PVL lesions were inconclusive and/or unreliable due to the small number of cases with PVL ($n = 20$) and can only be considered suggestive.

The multivariate Cox proportional-hazard models were applied only to the OL group due to the larger number of cases and also because we believed that identifying prognostic factors for MT would be more clinically relevant for patients with OL (Table 3). Other than DNA aneuploidy, which is of specific interest in the present study, we decided to include OED, clinical aspect, and

TABLE 1 Characteristics of the 153 cases of oral leukoplakia (OL) and oral proliferative verrucous leukoplakia (PVL)

	OL (133 cases) *n* (%) or mean (SD)	PVL (20 cases) *n* (%) or mean (SD)	χ^2 or Student's *t* test
Age	60.08 (13.98)	70.8 (12.26)	0.001
Sex			0.003
Female	66 (49.6%)	17 (85.0%)	
Male	67 (50.4%)	3 (15.0%)	
Clinical presentation[a]			0.209
Homogeneous	109 (82.0%)	14 (70.0%)	
Nonhomogeneous	24 (18.0%)	6 (30.0%)	
High-risk site (overall)			0.615
No	81 (60.9%)	11 (55.0%)	
Yes	52 (39.1%)	9 (45.0%)	
Multiple lesions			<0.001
No	60 (45.1%)	0 (0%)	
Yes	73 (54.9%)	20 (100%)	
Clinical aspect[a]			0.011
Plaque	85 (63.9%)	15 (75.0%)	
Erosion/ulcer	8 (6.0%)	0 (0%)	
Patch	34 (25.6%)	1 (5.0%)	
Verrucous	6 (4.5%)	4 (20.0%)	
Lesion site[a]			0.940
Tongue	28 (21.1%)	3 (15.0%)	
Buccal mucosa	46 (34.6%)	9 (45.0%)	
Floor of the mouth	13 (9.8%)	2 (10.0%)	
Gingiva	25 (18.8%)	4 (20.0%)	
Lip	2 (1.5%)	0 (0.0%)	
Soft palate	5 (3.8%)	1 (5.0%)	
Hard palate	14 (10.5%)	1 (5.0%)	
Oral epithelial dysplasia[a]			0.499[b]
No OED	112 (84.2%)	18 (90.0%)	
Mild OED	18 (13.5%)	0 (0.0%)	
Moderate OED	1 (0.8%)	2 (10.0%)	
Severe OED	2 (1.5%)	0 (0.0%)	
DNA ploidy[a]			0.840
DNA diploid	96 (72.2%)	14 (70.0%)	
DNA aneuploid	37 (27.8%)	6 (30.0%)	
Ever smoker			<0.001
No	41 (30.8%)	18 (90.0%)	
Yes	92 (69.2%)	2 (10.0%)	
Pack-year	18.75 (23.58)	2.0 (8.9)	0.014
Alcohol use			0.036
Nonregular	81 (60.9%)	17 (85.0%)	
Regular	52 (39.1%)	3 (15.0%)	

[a]Index lesion.
[b]Overall presence versus absence of OED.

TABLE 2 Details of the 16 patients with malignant transformation

Patient	Sex	Disorder	Histology	DNA index	Lesion site[a]	MT site	Time to progression (years)	Multiple lesions	Clinical presentation[a]	Mucosa[a]	Treatment[a]	Smoke	Pack-years	Alcohol use
1	F	OL	Mild OED	1.00	Buccal mucosa	Buccal mucosa	0.8	Yes	Nonhomogeneous	Vestibular	Ablation	Non-smoker	0	Nonregular
2	F	OL	Moderate OED	1.10	FOM	FOM	3.3	No	Homogeneous	Thin	Excision	Former smoker	20	Regular
3	F	OL	Severe OED	1.00	Ventral tongue	Ventral tongue	1.8	Yes	Nonhomogeneous	Thin	Excision	Active smoker	20	Nonregular
4	F	OL	No OED	1.40	Ventral tongue	Ventral tongue	1.7	Yes	Nonhomogeneous	Thin	Ablation	Former smoker	5	Nonregular
5	M	OL	Mild OED	1.76	FOM	FOM	5.2	No	Nonhomogeneous	Thin	Excision	Heavy smoker	55	Nonregular
6	F	OL	Mild OED	1.12	Ventral tongue	Buccal mucosa	7.3	Yes	Homogeneous	Thin	Excision	Non-smoker	0	Nonregular
7	F	PVL	Moderate OED	1.00	Ventral tongue	Ventral tongue	0.6	Yes	Homogeneous	Thin	Excision	Non-smoker	0	Nonregular
8	F	PVL	No OED	1.00	Ventral tongue	Ventral tongue	11.2	Yes	Homogeneous	Thin	Observation	Non-smoker	0	Nonregular
9	F	PVL	No OED	1.00	Gingiva/alveolar ridge	Gingiva/alveolar ridge	3.0	Yes	Homogeneous	Masticatory	Excision	Non-smoker	0	Nonregular
10	F	PVL	No OED	1.00	Buccal mucosa	Gingiva/alveolar ridge	11.2	Yes	Homogeneous	Vestibular	Observation	Non-smoker	0	Nonregular
11	F	PVL	No OED	1.00	FOM	Gingiva/alveolar ridge	3.0	Yes	Homogeneous	Thin	Observation	Active smoker	1	Nonregular
12	F	PVL	Moderate OED	1.00	Buccal mucosa	Buccal mucosa	0.9	Yes	Homogeneous	Vestibular	Ablation	Non-smoker	0	Nonregular
13	F	PVL	No OED	1.09	Buccal mucosa	Buccal mucosa	2.4	Yes	Nonhomogeneous	Vestibular	Excision	Non-smoker	0	Nonregular
14	F	PVL	No OED	1.00	FOM	FOM	5.1	Yes	Homogeneous	Thin	Excision	Non-smoker	0	Nonregular
15	F	PVL	No OED	1.11	Buccal mucosa	Hard palate	4.1	Yes	Nonhomogeneous	Vestibular	Excision	Non-smoker	0	Nonregular
16	F	PVL	No OED	1.00	Hard palate	Hard palate	1.0	Yes	Nonhomogeneous	Masticatory	Observation	Non-smoker	0	Nonregular

[a]Index lesion.

risk site in the final model since they were the variables most strongly associated with MT in the univariate analysis. Although the clinical aspect of the lesion, in particular the presence of an ulcerative lesion, also seemed to be associated with MT, the small number of patients with ulcerative lesion and MT did not allow us to use this variable in the final model.

DNA ploidy in a univariate model was predictive of MT. However, after adding the OED and clinical aspect to the model, the HR for DNA ploidy decreased and the confidence intervals widened. The multivariate Cox analysis showed that OED is the variable most strongly associated with MT, even when it was mutually adjusted for other factors, which is in agreement with what was previously known. The C-index when using only DNA ploidy was 0.69; when adding OED, heterogeneity, and risk site to the model, the estimate of the C-index increased up to 0.93, indicating a good prognostic model. Nonetheless, when comparing the C-value resulting from the already recognized predictive factors (OED, high-risk site, heterogeneity) with or without the data of DNA ploidy status no significant variations were observed.

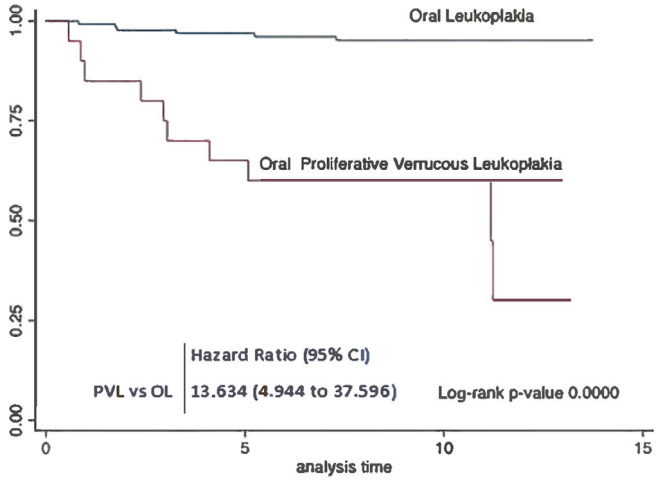

FIGURE 2 Kaplan–Meier survival curves comparing oral leukoplakia (OL) versus oral proliferative verrucous leukoplakia (PVL) and hazard ratio with 95% confidence interval (95% CI) from the Cox regression model [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Positive and negative predictive values

Analyses were performed in order to assess if a DNA aneuploid status and/or the presence of OED were

TABLE 3 Cox regression model restricted to OL lesions

	Model 1		Model 2		Model 3		Model 4	
Characteristics	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
DNA aneuploidy	5.29	0.97–28.87	1.67	0.275–10.156	1.24	0.19–8.01	1.72	0.25–11.67
Dysplasia			23.81	2.432–233.133	17.50	1.682–182.140	10.72	0.89–129.46
High-risk site					2.89	0.256–29.93	1.99	0.15–25.65
Heterogeneity							3.81	0.60–24.17
Harrel's concordance index	0.69		0.89		0.91		0.93	

Note: Number of patients with oral leukoplakia 133, number of malignant transformations 6, time at risk 1209.016 years.

TABLE 4 DNA ploidy status related to the presence of oral epithelial dysplasia

	OL			PVL		
	No OED ***n* (%)**	**OED** ***n* (%)**	**χ^2 test**	**No OED** ***n* (%)**	**OED** ***n* (%)**	**χ^2 test**
DNA ploidy			0.001			0.329
DNA diploid	87 (77.7%)	9 (42.9%)		12 (66.7%)	2 (100%)	
DNA aneuploid	25 (22.3%)	12 (57.1%)		6 (33.3%)	0 (0%)	
High DNA aneuploidy			0.044			0.732
No	108 (96.4%)	18 (85.7%)		17 (94.4%)	2 (100%)	
Yes	4 (3.6%)	3 (14.3%)		1 (5.6%)	0 (0%)	

positive predictors of MT, and if a DNA diploid status and/or the absence of OED were negative predictors of MT. When considering only patients with OL, a DNA diploid status resulted in an NPV of 97.9% (95% CI 93.8–99.3) and a DNA aneuploid status in a PPV of 10.8% (95% CI 6–18.6). The presence of dysplasia resulted in a PPV of 23.8% (95% CI 14.9–35.9). When considering the DNA ploidy status and the absence of OED together, the NPV approached 100%, as no MT occurred in patients with OL whose index lesion showed both absence of OED and a DNA diploid status. In the presence of both OED and a DNA aneuploid status, the PPV rose to 25% (95% CI 10.7–48).

Due to the low number of cases such analyses were not performed in the PVL group.

3.5 | DNA ploidy status and OED

A DNA aneuploid status was positively associated with the presence of OED (Table 3): overall, DNA aneuploidy was observed in 52.2% of dysplastic lesions and in 23.8% of nondysplastic ones (chi-square test $p = 0.005$). The distribution of the DNA ploidy status related to the presence of OED is presented in Table 4.

When comparing progressing cases from OL and PVL groups (Table 5), patients with OL who underwent MT more frequently already had OED at the index lesion; the difference observed in age at enrolment tended to decrease slightly when considering the age at MT. Significant associations between lesion/patient-related features and MT were observed only in patients with OL, as detailed in Table 6.

TABLE 5 Characteristics of cases with malignant transformation (MT) stratified by disorder (oral leukoplakia [OL] and oral proliferative verrucous leukoplakia [PVL])

	OL *n* (%) or mean (SD)	PVL *n* (%) or mean (SD)	χ^2 test or Student's *t* test
Age at enrolment	66.3 (15.45)	72.1 (8.32)	0.047
Age at MT	70.0 (14.59)	76.4 (9.69)	0.076
Time to progression (months)	39.67 (29.72)	50.40 (47.37)	0.355
Sex			0.375
Female	5 (83.3%)	10 (100.0%)	
Male	1 (16.7%)	0 (0.0%)	
OED			0.035
No	1 (16.7%)	8 (80.0%)	
Yes	5 (83.3%)	2 (20.0%)	
DNA ploidy			0.118
DNA diploid	2 (33.3%)	8 (80.0%)	
DNA aneuploid	4 (66.7%)	2 (20.0%)	
SCC histology			0.093
Verrucous	0 (0.0%)	5 (50.0%)	
Squamous	6 (100.0%)	5 (50.0%)	
MT at masticatory mucosa			0.302
No	2 (33.3%)	7 (70.0%)	
Yes	4 (66.7%)	3 (30.0%)	
MT at index lesion			0.511
No	1 (16.7%)	3 (30.0%)	
Yes	5 (83.3%)	7 (70.0%)	
Ever smoker			0.036
No	2 (33.3%)	9 (90.0%)	
Yes	4 (66.7%)	1 (10.0%)	
Alcohol use			0.375
Nonregular	5 (83.3%)	10 (100.0%)	
Regular	1 (16.7%)	0 (0.0%)	

TABLE 6 Association between lesion/patient-related features and malignant transformation (MT) stratified by disorder (oral leukoplakia [OL] and oral proliferative verrucous leukoplakia [PVL])

	OL			PVL		
	No MT *n* (%) or mean (SD)	MT *n* (%) or mean (SD)	χ^2 or Student's *t* test	No MT *n* (%) or mean (SD)	MT *n* (%) or mean (SD)	χ^2 or Student's *t* test
Age at enrolment	59.8 (13.9)	66.3 (15.4)	0.263	69.5 (15.6)	72.1 (8.3)	0.648
Sex			0.091			0.060
Female	61 (48.0%)	5 (83.3%)		7 (70%)	10 (100%)	
Male	66 (52.0%)	1 (16.7%)		3 (30%)	0 (0%)	
OED (any grade)			<0.001			0.136
No	111 (87.4%)	1 (16.7%)		10 (100%)	8 (80%)	
Yes	16 (12.6%)	5 (83.3%)		0 (0%)	2 (20%)	
OED (moderate/severe)			<0.001			0.136
No	126 (99.2%)	4 (66.7%)		10 (100%)	8 (80%)	
Yes	1 (0.8%)	2 (33.3%)		0 (0%)	2 (20%)	
DNA ploidy			0.030			0.329
DNA diploid	94 (74.0%)	2 (33.3%)		6 (60%)	8 (80%)	
DNA aneuploid	33 (26.0%)	4 (66.7%)		4 (40%)	2 (20%)	
High DNA aneuploidy			0.002			0.305
No	122 (96.1%)	4 (66.7%)		9 (90%)	10 (100%)	
Yes	5 (3.9%)	2 (33.3%)		1 (10%)	0 (0%)	
Clinical presentation			0.010			1.000
Homogeneous	107 (84.3%)	2 (33.3%)		7 (70%)	7 (70%)	
Nonhomogeneous	20 (15.8%)	4 (66.7%)		3 (30%)	3 (30%)	
High-risk site overall			0.023			0.178
No	80 (63.0%)	1 (16.7%)		7 (70%)	4 (40%)	
Yes	47 (37.0%)	5 (83.3%)		3 (30%)	6 (60%)	
Multiple lesions			0.071			
No	58 (45.7%)	5 (83.3%)		0 (0%)	0 (0%)	
Yes	69 (54.3%)	1 (16.7%)		10 (100%)	10 (100%)	
Ever smoker			0.925			1.000
No	40 (31.5%)	2 (33.3%)		9 (90%)	9 (90%)	
Yes	87 (68.5%)	4 (66.7%)		1 (10%)	1 (10%)	
Alcohol use			0.249			0.060
Nonregular	76 (59.8%)	5 (83.3%)		7 (70%)	10 (100%)	
Regular	51 (40.2%)	1 (16.7%)		3 (30%)	0 (0%)	

4 | DISCUSSION

4.1 | MT rate

The overall and annual MT rates observed in the present study for OL are lower when compared to the meta-analysis by Iocca et al. (MT rate 8.6%, annual transformation rate 1.56%) and to recent data from the Netherlands (annual transformation rate 4.9%)[1,38]; this could be related to the relatively lower prevalence of OED in the present cohort (15%). Data from the PVL group are consistent with the previously cited meta-analysis, reporting an overall MT rate of 49.5% and an annual transformation rate of 9.3%.

The meta-analysis by Mehanna et al.[39] reported a mean time-to-MT for OED of 4.3 years. In the literature, the risk of MT is most often considered the highest within the first 5 years of diagnosis,[9] although a couple of studies identified the first 2 years after OED diagnosis as the period with the highest risk,[40,41] and one study described a consistent annual transformation rate.[38] In the present cohort, a large proportion of patients with OL (52/133; 39%) had follow-ups ranging from 9 to almost 13 years, and all MTs occurred within 7.3 years from enrolment. All patients with a lesion harboring moderate-to-severe OED (three patients with OL and two patients with PVL) were submitted to surgical excision; within the frame of an observational prospective study, they were treated irrespectively of the DNA ploidy status. Among them, one patient with OL did not undergo MT, the remaining four patients had MT at the same site in a mean period of 19.5 months. Irrespective of treatment, MT following a diagnosis of OED occurred in a mean period of 34.1 months, even though dysplastic lesions were surgically removed in five out of six patients. The shorter time-to-progression related to the presence of OED observed in the present study did not reach statistical significance but is consistent with previously reported data.[18]

In OL cases where MT occurred after 62 and 87 months, respectively, a field effect may well be considered responsible for progression. In the PVL group, a couple of patients had MT more than 11 years after enrolment. The present data show that, compared to OL, PVL seems to have a slower path toward cancer but a longer-lasting risk of MT.

MT occurring in a subsite different/distant from the site of the index lesion was observed in both patients with OL and patients with PVL. Such evidence is in keeping with, and supports, the field-cancerization model in oral carcinogenesis, and further validates the current terminology of Oral Potentially Malignant *Disorder* as a nonsite-specific indicator of risk of likely future malignancies. This also corroborates data recently reported by Bagan et al. in a group of patients with OL that underwent surgical/laser treatment, and where 9 out of 26 MT were observed in the absence of recurrence after OL treatment.[42]

4.2 | Clinical predictors

The present results on lesion- and patient-related features frequently investigated as predictors for MT (sex, age, lesion site, and tobacco/alcohol exposure) support the differences already identified between OL and PVL.[10] Although the male-to-female ratio among patients with OL was rather balanced, there was a striking female predominance among progressing patients. However, our univariate Kaplan–Meier analysis, although indicative, did not show strong evidence for differences between sex, probably due to the small sample size. Similar scenarios with more frequent MTs in females not reaching statistically significant association have already been reported[38] and could also be related to suboptimal sample size.

Data from the OL group also support evidence from the literature on both the significantly high risk for MT in nonhomogeneous lesions and the different amounts of risk depending on the lesion location.[9] Consistent with numerous studies conducted in Western countries and China, no association between smoking habits and MT[9,43] was found. Considering the relatively high prevalence of smokers among those patients with OL (68.4% were classified as ever-smokers) and the lack of association between smoking and MT in the same group, it could be that smoking plays a major role in the occurrence but not in the progression of OL to cancer. The prospective design of the present study allowed the complete collection of data on alcohol consumption; retrospective studies are frequently characterized by a large amount of missing data in this respect. In any case, even alcohol exposure was not significantly associated with MT.

PVL was more commonly found in older women, and patients with PVL were significantly less exposed to classical risk habits for SCC (tobacco smoking and alcohol intake) compared to patients with OL. Features of the present PVL cohort support the recent proposal of changing terminology to proliferative leukoplakia since many but not all lesions are verrucous.[11] Patients with PVL were more likely to have MT in oral subsites characterized by masticatory mucosa (gingiva and hard palate) and very often developed VC. Finally, the present results underline the long-lasting high risk of MT, with progression occurring in 2 out of 10 cases more than 11 years after enrolment and the frequent development of second primaries, which occurred in 5 out of 10 progressing patients (data not shown).

4.3 | Oral epithelial dysplasia

The onset of OED usually follows the appearance of OL/PVL. At diagnosis, most OL and the vast majority of PVL do not yet harbor OED; currently, OED presence is recognized as the main predictor in identifying OL patients with a high risk of progression.[1,7,41] Within the literature, the prevalence of OED varies in OL, ranging from 57% to 85%.[8,12,28,42,44] However, in almost all the reported cohorts, the number of low-risk lesions largely surpasses that of high-risk lesions, and many reports of MT of keratotic, nondysplastic lesions can also be

found.[12,13] Data from the present study (at the upper end of the range) are based on the assessment of presence and grade of OED according to the WHO 2017 criteria.[2] In the 5th Edition of the WHO Classification of Head and Neck Tumours, architectural and cytological features of OED have been expanded, and early lesions of PVL demonstrate many of the newly added architectural features, including premature keratinization, sharp lateral margins, skip keratoses, and increased keratin.[45,46] The presence of corrugated hyperortho- or para-keratotic lesions with a verruco-papillary architecture, with or without minimal cytologic atypia (typical in early PVL), should be considered as mild dysplasia.[45] In recent years, the incorporation of the histologic features of differentiated dysplasia has been proposed, in addition to the ones observed in classic dysplasia; the aim is to improve the predicting value of the histological assessment for MT, and promising results have been reported.[47,48] Similarly, a recent consensus paper on histopathology of PVL added other important architectural criteria for dysplasia, such as a thickness of keratin greater than half the thickness of the underlying epithelium, and hyperkeratosis with atrophy in the absence of cytologic atypia.[49] Such features were not considered at the time of diagnosis in the present cohort.

The Cox analysis based on the present data found OED among the strongest predictors of MT in OL: 23.8% of patients harboring OED at enrolment progressed to MT; 0.9% of patients not harboring OED at enrolment progressed to MIT. The application of the newly proposed criteria for OED[45,46,49]—potentially able to upgrade the no-dysplasia lesions from "no-dysplasia" to "dysplasia"—would have been of little help in predicting MT in the present OL cohort. However, the newly proposed criteria could increase the proportion of early PVL lesions showing OED, thus improving OED's predictive value in the PVL group.

When considering models of oral carcinogenesis based on the sequential accumulation of genetic events, nondysplastic OL may already harbor early genetic changes. Recent models suggest that the appearance of OED and subsequent MT could be characterized by a sequential accumulation of genetic events (according to a neutral model) and by the occurrence of specific genetic flaws acting as actual driver-events (according to a punctuated model), respectively.[50] Neutral and punctuated evolution may explain the highly heterogenous genetic landscape of OPMDs (including OL) and are consistent with field cancerization leading to MT in different sites of the oral mucosa. In this context, further investigation of the molecular fingerprint of lesions characterized by the newly proposed criteria for OED could be of great interest.

4.4 | DNA ploidy status in OL

The presence of OED already identifies a subgroup of patients with a high risk of progression to cancer; but finding a biomarker able to detect patients earlier with chromosome instability (CIN)—and for this reason more prone to develop OED itself—would be of utmost value and would allow an even earlier stratification of patients. Currently, no single reliable biomarker is available; clinicians usually consider together several patient- and lesion-related features aimed at stratifying the risk of progression in a single patient; and biomarkers' assessment has still not entered the routine clinical-practice of Oral Medicine Practitioners (OMPs).[51] Most studies assessing the predictive value of the DNA ploidy status concluded that it has limited value in predicting progression to cancer as a single biomarker[16,17,20,21]; the combined use of OED and DNA ploidy has been shown to be a better predictor for identifying low-risk lesions.[18,22] This accords with our own findings, with the C-index indicating a better model when using both DNA ploidy and the presence of OED. Although the present study has a relatively large sample size compared to many previous prospective studies investigating OL, the small number of events of interest (MT) in some subcategories of patients may lead to imprecise estimates with wide confidence intervals.

Despite extensive data from the literature, and although it is a more objective technique than OED evaluation, DNA ploidy status, evaluated either by Image or Flow Cytometry (ICM or FCM), is currently routinely assessed in practice by just under 10% of OMPs in Europe and Australia.[51] This could be related to a number of issues: the cost for each test (not greater than €50) is not prohibitive, but both ICM and FCM require facilities unavailable in most laboratories; and, primarily, the prospective validation of a strong predictive role of DNA ploidy is still lacking. Cox regression models based on our results show that DNA aneuploidy might not be as strong predictor as the other variables. It remains to be clarified if DNA aneuploidy acts as an effect modifier to other predictor variables.

Validation of biomarkers for OL/PVL progression is quite challenging due to the low prevalence of OL/PVL, the relatively long progression-time, and their low rate of transformation, particularly for OL cases not already harboring OED.

Over the last 20 years, the predictive value of DNA aneuploidy for MT in OPMDs has been investigated in retrospective small-case series, often by association with OED grade; a fairly recently published meta-analysis highlighted the limited amount of scientific evidence from the five retrospective studies selected, even if aneuploidy was found to be associated with a 3-fold overall

increased risk of progress to cancer.[15] A later prospective observational study, including 181 OL with a variable presence of OED, found the DNA ploidy status to be a reliable marker for progression only in a univariate logistic regression assessment (OR 6.5 with 95% CI 1.9–22.8); this accords with our own univariate analysis. However, the authors did not mention any related assessment for the presence of OED.[52] This should not be omitted, given the recognized association between OED and a DNA aneuploid status[22,53–55] already reported in our previous studies.[29,56] The highest predictive values for MT have been obtained by combinations of DNA aneuploidy and severe OED.[18]

It has been reported that DNA aneuploidy could indicate a long-term risk, potentially extending to more than 12 years.[57] In the present cohort, the two OL patients with MT occurring more than 5 years after enrolment had DNA aneuploidy associated with mild OED at the index lesion, and one of them had MT in an oral subsite different from that of the index lesion. This could support the ability of DNA aneuploidy to identify patients with CIN, favoring field cancerization. Conversely, two patients with PVL who progressed more than 11 years after enrolment had neither OED nor DNA aneuploidy at the index lesion.

In recent years, researchers have focused on the negative predictive value (NPV) resulting from a DNA diploid status.[23,57] Even if the low prevalence of OED and the small number of events of interest (MT) could limit the strength of the present results, a remarkably high NPV was observed in the OL group, supporting the reliability of a DNA diploid status associated with the absence of OED to identify patients with minimal or no risk of MT. It should be taken into consideration, however, that NPV and PPV are dependent on the prevalence of MT inside the study population and, therefore, might be study specific. It is also reasonable to assume that prognostic biomarkers might lose strength over time,[58] which means that measures such as PPV and NPV depend on the length of follow-up and the time to progression. Nevertheless, our findings regarding NPV and PPV are comparable to those from previous studies, confirming the predictive value of OED and the benefits of using DNA ploidy to distinguish low-risk lesions.

4.5 | DNA ploidy status in PVL

As suggested by molecular evidence, MT of PVL seems not to follow the same pathway as that in the presence of OED; this is consistent with the observed significantly high MT rate, even in the absence of OED or DNA aneuploidy. We note that OED was observed in 2 PVL index lesions and neither of them harbored DNA aneuploidy, irrespective of the recognized association between OED and DNA aneuploidy. In the present cohort, after excluding patients already harboring OED, the higher risk of MT observed in PVL was not associated with a significantly higher prevalence of DNA aneuploidy. Therefore, the higher risk of MT observed in PVL when compared to OL cannot be associated with CIN detectable by a DNA ploidy analysis. Different oncogenic pathways are supposed to characterize OL and PVL,[59] and current data offer weak evidence of possible correlations between DNA aneuploidy and MT in PVL. Future research could address other features of genome instability potentially involved in MT. Three papers retrospectively investigated the DNA ploidy status using, respectively, ICM,[26] Fairfield image-based analysis,[25] and FCM.[24] These studies considered DNA aneuploidy to be a reliable aid for the early recognition of PVL, the prediction of MT, and the selection of patients for aggressive treatment. A recent meta-analysis confirmed the potential value of DNA aneuploidy in predicting MT in PVL, but the DNA ploidy status was not considered separately from the presence of OED.[27] When faced only with data from 14 patients progressing to MT at least 6 months after the first biopsy (Table 7), DNA aneuploidy could predict MT

TABLE 7 DNA ploidy status in PVL samples from the literature

	Kahn et al.[24]		Klanrit et al.[25]		Gouvea et al.[26]	
	DNA diploid *n* (%)	DNA aneuploid *n* (%)	DNA diploid *n* (%)	DNA aneuploid *n* (%)	DNA diploid *n* (%)	DNA aneuploid *n* (%)
Histological diagnosis						
No OED	0 (0%)	3 (100%)	1 (50%)	1 (50%)	4 (33%)	8 (67%)
Verrucous hyperplasia	4 (40%)	6 (60%)				
Mild OED	0 (0%)	1 (100%)	4 (100%)	0 (0%)	3 (14%)	18 (86%)
Moderate–severe OED	0 (0%)	3 (100%)	3 (43%)	4 (57%)	0 (0%)	15 (100%)
SCC-VC	0 (0%)	7 (100%)	1 (100%)	0 (0%)	0 (0%)	15 (100%)

in the absence of OED in just three subjects (21%), which is similar to what was observed in the present study. Nevertheless, here we found that the DNA ploidy status had no significant predictive value for MT in PVL.

5 | CONCLUSIONS

This study confirms the baseline differences between OL and PVL, such as age, sex, clinical presentation, and site. We report a female predominance in patients with OL and patients with PVL that progressed to cancer. Several considerations on oral carcinogenesis could suggest that a single biomarker is unlikely to be a good predictor of MT. The present analyses indicate that OED remains the strongest predictor for MT, but they also suggest that DNA ploidy might have a role in identifying an OL with low-risk or minimal-risk of MT eligible for cost–benefit tailored management. This potential role should be confirmed in larger sample-size studies; it would represent an important aid, since most OL do not harbor OED. The present evidence is not able to shed light on PVL. The high and long-lasting risk for MT does not seem to be associated with any patient- or lesion-related predictor, nor does the reported evidence suggest potential correlations with DNA aneuploidy.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The participants of this study did not give written consent for their data to be shared publicly. Therefore, due to the sensitive nature of the research, supporting data are not available.

ETHICS STATEMENT

Patient written consent was obtained in every case during an interview according to the Institutional Ethic Committees (A.S.O.S. Luigi Gonzaga Prot. N. 11780). Patients signed informed consent to enter the study and to have the DNA-ploidy assessment.

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SUPPORTING INFORMATION

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

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