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IDENTIFICATION OF THE GENETIC AND THE EPIGENETIC PROFILE OF ORAL LEUKOPLAKIA FOR DIAGNOSTIC AND PROGNOSTIC PURPOSES

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PROJECT 1

Early diagnosis of Oral Squamous Cell Carcinoma: direct healthcare costs and role of education for dental and medical practitioners

INTRODUCTION

Epidemiology:

Oral Squamous Cell Carcinoma (OSCC) is a malignant tumour originating from epithelial cells of the oral cavity. It is the most prevalent malignancy of the head and neck region and affects almost all sites of the oral cavity, including the lips, tongue, gums, and the floor of the mouth.

OSCC tend to invade locally, in particular to the lymph nodes of the head and neck region. However, metastatic spread through blood vessels may also occur, and lungs can be frequently involved.

Epidemiological data indicate that OSCC is far from being a rare pathology. In fact, in terms of incidence head and neck cancers (oral cavity and pharynx) rank as the sixth more common neoplasia in the world among all malignant tumours. More in detail, the rank of head and neck cancers differs between developed countries and developing countries (8^{th} vs 3^{rd} respectively) [1]. Of note, the majority of head and neck cancers are OSCC.

In 2022, almost 389,485 oral cavity cancers and 188,230 oral cancer deaths occurred worldwide [2].

Risk factors play a pivotal role. A significant percentage of OSCC cases have been identified in the Indian subcontinent due to the habit of chewing betel leaves and tobacco [3,4]. In Taiwan, OSCC is the leading cause of death among young males aged 25 to 44, likely due to the progressive increase of betel consumption in this area [5,6].

In Italy, the incidence of OSCC is 8.4 cases per 100,000 inhabitants per year reaching 12.1 cases per 100,000 inhabitants annually. Noteworthy, epidemiological trends shows an increasing occurrence of "juvenile" cases and a persistent lack of improvement in terms of prognosis and treatment outcomes [7]. Moreover, the male-to-female ratio for OSCC has shifted from 3:1 to 2:1 over the past two decades. A plausible explanation for this trend is the rising prevalence of cigarette smoking among the female population[1].

Despite OSCC typically manifests in the fifth to seventh decade of life, in recent years a notable increase in the incidence of cases diagnosed in individuals under the age of 45 (particularly in the United States and Europe) has been reported [8–10] .

Oral Squamous Cell Carcinoma is acknowledged for its unfavourable prognosis. The overall 5-year survival rate following diagnosis ranges from 45 to 55% [7,11–13].

In Italy, the annual mortality rate is approximately 3,000 individuals. This trend remained unchanged unlike other malignancies such as breast cancer, colon cancer, or melanoma, whose survival rates experienced in recent years a significant increase mainly due to the introduction of immunotherapy [7].

Risk Factors

Tobacco smoking and excessive alcohol consumption stand out as primary risk factors for the development of OSCC accounting for approximately 80% of cases [14,15].

However, literature also suggests the involvement of other factors in the mechanism of oral carcinogenesis or co-carcinogenesis: infectious agents, nutritional and dietary factors, conditions of poor oral hygiene, chronic oral trauma, ultraviolet radiation, states of immunosuppression and genetic factors.

Tobacco

The risk of developing OSCC is 5-9 times higher in smokers compared to non-smokers, and this risk exhibits a dose-dependent relationship [16]. The likelihood of OSCC doubles when more than 20 cigarettes are consumed per day. Furthermore, for patients who persist in smoking post-diagnosis, the risk of developing a second neoplasia after resective surgery is up to six times higher than that in patients who quit[17].

The carcinogenic impact of tobacco smoke primarily stems from mutagenic compounds present in combustion products, with over 70 identified carcinogenic compounds. These include polycyclic aromatic hydrocarbons (e.g., benzopyrene, anthracene), tobacco nitrosamines (e.g N-nitrosonornicotine, N-nitrosodimethylamine), aromatic amines (e.g., 2-toluidine), aldehydes (e.g., formaldehyde), metals, and organic compounds [18].

Direct thermal irritation of tobacco smoke on mucosae appears to function as a significant co-factor. For instance, the practice of reverse smoking (common in regions

such as Andhra Pradesh and the Philippines, where individuals smoke with the lit end inside the mouth) is correlated with an higher risk of malignant transformation [19].

Similarly, tobacco exposure beyond smoking is also associated with OSCC development. In specific regions such as India and Southeast Asia, the widespread use of betel leaves in combination with tobacco, areca nut, and other irritating compounds not only triggers the onset of submucous fibrosis (a pre-malignant lesion with a high risk of cancer) but also correlates with increased rates of OSCC incidence [20].

Alcohol

In the United States, about one-third of individuals affected by OSCC are heavy alcohol consumers [17]. Heavy drinkers (more than 100 grams of alcohol per day) have a 30 times higher risk of developing oral and oropharyngeal carcinoma [21]. Like cigarette smoking, the relative risk for alcohol also appears to be dose dependent. Epidemiological studies have demonstrated that smoking and alcohol are independent risk factors; however, when combined, they exhibit a non-additive but synergistic effect, resulting in an incidence increase of 6-15 times in consumers of both [22].

Experimental studies have shown that ethanol itself does not have mutagenic action; therefore, the carcinogenic action of alcoholic beverages would be due to indirect mechanisms. Alcohol could act as a solvent for other carcinogens, following an irritative and dehydrating action on the mucosa [23]. This mechanism would justify the synergistic oncogenic effect of alcohol and tobacco. However, the independent risk factor role of alcohol is not yet clear.

Fungal and Viral Infection

The potential involvement of yeasts and viruses in triggering or acting as co-factors in the malignant transformation of OSCC has been a subject of extensive investigation [24].

Specifically, Candida spp. has been identified as capable of producing potent mutagens, such as N-Nitroso bezilmetilamine, which appears to play a pivotal role in cancer development. Candida spp. is also linked to potentially malignant lesions, including hyperplastic chronic candidiasis, with a moderate to high risk of progressing into OSCC [25]. However, distinguishing between Candida-associated premalignant lesions and Candida super-infection of premalignant/malignant lesions, as well as

understanding the precise role of Candida spp. in oral carcinogenesis, remains an ongoing challenge [26].

Human Papilloma Virus (HPV)

Recent studies have associated Human Papillomavirus (HPV) infection with the development of Head and Neck Squamous Cell Carcinoma (HNSCC), particularly genotypes 16 and 18. HPV demonstrates a preference for the pharynx, where lymphoid tissue is abundant and invasion of the mucosal barrier is facilitated [26]. In the oral cavity, HPV-associated OSCC tends to manifest posteriorly, at the base of the tongue and in proximity to the palatine tonsils, while occurrences in the anterior part of the oral cavity are comparatively less frequent [27]. The oncogenetic effects of HPV are mediated by associated oncoproteins, such as E6 and E7, which interfere with crucial pathways, including TP53, inducing tumoral degeneration [28].

UV Radiations and Immunodepression

Prolonged exposure to UV radiation is a well-established risk factor for the development of OSCC of the lower lip. Increased awareness of risk factors among the general population in recent years has contributed to improved incidence rates and survival for lip cancer, characterized by a less aggressive behaviour compared to other forms of OSCC [29].

Immunodepression

Immunodepression significantly impacts tumorigenesis and, in the context of OSCC, individuals undergoing bone marrow transplants with associated immunosuppressant regimes face a 6-10 times higher risk of developing OSCC [30].

Similarly, HIV-positive patients with AIDS exhibit an elevated risk of OSCC compared to the general population. The compromised immune system in these patients makes it easier for tumors to evade immune surveillance, resulting in more pronounced clinical effects [31].

Diagnosis of Oral Squamous Cell Carcinoma:

Oral cavity lesions include a wide range of entities of different etiologies including neoplastic, premalignant, inflammatory and non-neoplastic origin. Oral lesion are

particularly prevalent in general population and can affect various sites including the tongue, lips, floor of the mouth, hard and soft palate, gingiva and buccal mucosa [32].

The accurate diagnosis of these lesions and the precise identification of the underlying pathology is of pivotal importance for both clinicians and pathologists [33]. Indeed, despite many classification schemes for oral lesions simply rely on the clinical appearance, a significant discrepancy may exist between clinical assessments and histopathological findings [33]. Consequently, pathological assessment should not be underestimated.

Indeed, oral mucosal lesions frequently presents as nonspecific signs of white-red lesions or erosive-ulcerative lesions [34].

Specifically, diagnosis and identification of oral cancer is of great importance[35], given its aggressive behavior, low survival rate and a significant higher risk of secondary oral neoplastic lesion. Unfortunately, the incidence of oral cancers in the population has exhibited a concerning rise over the years, especially among younger generations, potentially attributed to evolving habits and lifestyle changes [33].

In general, the diagnosis of OSCC is primarily based on a thorough clinical examination of the oral mucosa and subsequently confirmed through a biopsy for histological examination [36].

However, the diagnosis of OSCC is challenging as it is susceptible to misinterpretation.

Despite the oral cavity is easy accessible for direct clinical examination and despite a significant proportion of oral carcinomas (over 50%) arise from Oral Potentially Malignant Disorders (OPMDs) [37,38], OSCC is frequently diagnosed in advanced stage[33].

Indeed, it is estimated that an early diagnosis of OSCC is achieved in less than half of the cases.

When OSCC is diagnosed in its early stages the 5-year survival rate reach 80-90%, whereas it diminishes to 5-20% in cases of advanced-stage diagnosis [39]. This trend highlights the importance of early diagnosis on treatment and prognosis as diagnostic delays often entail the implementation of multi-modal invasive therapies, which, in turn, adversely impact the residual quality of life.

In 10-30% of cases, patients treated for OSCC may exhibit secondary tumor manifestations that exert a negative impact on prognosis, even when the initial diagnosis occurs early [40]. These secondary tumor manifestations can be categorized into Local Recurrences (LR), if stemming from cells of the primary tumor, and Second Primary Tumors (SPT), if they emerge as independent events from the primary tumor[41].

As a result, despite recent advancements in diagnostic and treatment modalities, the 5-year survival rate for this pathology consistently hovers around 50% [42].

Oral Potentially Malignant Disorders:

As aforementioned, numerous oral carcinomas (>50%) originate from sites that previously harbored premalignant lesions referred as Oral Potentially Malignant Disorders (OPMDs) [37,38].

Currently, is defined as OPMDs any oral mucosal abnormality that is associated with a statistically increased risk of developing lip or oral cancer over the course of the patient's lifetime [37].

Oral Leukoplakia (OL), Proliferative Verrucous Leukoplakia (PVL), Oral Erithroplakia, Oral Submucous Fibrosis (OSF), Oral Lichen Planus (OLP), Oral Lichenoid Lesion (OLL), Actinic Keratosis (AK), Oral Graft Versus Host Disease (oGVHD), Discoid Lupus Erythematosus and Dyskeratosis Congenita are the most common OPMDs [37].

All these lesions, primarily afflicting middle-aged or elderly individuals with a prevalence skewed towards males [43], display a varied spectrum of clinical features. These features include diverse colour variations, presenting as white, red, or a combination of both, and exhibit topographic changes encompassing plaque/plateau, smooth, corrugated, verrucous, granular, and atrophic presentations [43,44].

Moreover, OPMDs and are not confined to specific anatomical sites within the oral cavity, demonstrating the potential to affect any region, and may present as either uni- or multifocal entities [45].

The clinical trend of OPMDs, characterized by a tendency to remain static, progress, or regress, is unpredictable in majority of cases [43,46,47]. However, it's essential to note that only a minority of cases advance to a malignant state [37,38].

The interplay of ethnicity and the prevalence of specific cultural risk factors significantly influences the type and incidence of OPMDs in distinct populations [37]. In South Asian populations, the prevalent habit of betel quid and areca nut chewing significantly contributes to an increased prevalence of Oral Submucous Fibrosis (OSF)[48–50]. Conversely in Western populations OSF is uncommon and OL emerges as the most prevalent among OPMDs [37].

Oral Leukoplakia:

According to the WHO definition, Oral Leukoplakia (OL) is characterized as "A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" [37,51,52] and stands out as one of the most prevalent potentially malignant disorders, with a reported global prevalence of 2.6-4.1% [49,53,54].

Two recent population-based studies conducted in Italy, specifically in the metropolitan area of Turin and in the Sicilian population, showed a prevalence of 1.15% and 3.2%, respectively [55,56].

It is noteworthy that, despite leukoplakia is one of the most common OPMDs, it is still characterized by a diagnosis of exclusion without distinctive clinical or histological features.

The typical presentations of OL include two primary clinical phenotypes: homogeneous and non-homogeneous one [57]. Homogeneous OL is raised and whitish, while non-homogeneous lesions exhibit alternating red and white areas, as well as varying thickness. Non-homogeneous OL is also referred to as leuko-erythroplakia or erythroleukoplakia, depending on the predominant color [37,58,59].

Proliferative Verrucous Leukoplakia (PVL) represents a third and rare type of oral leukoplakia even if some authors tend to consider it a distinct clinical entity [57]. The name refers the warty and multifocal appearance of PVL [60]. PVL is associated with a high risk of malignant transformation and tends to recur after removal, progressively increasing in size over time [61–63].

Generally, the majority of OL cases are asymptomatic. Symptoms, when present, are usually associated with non-homogeneous forms and, depending on the site of occurrence, may include a simple sense of presence, discomfort, or a burning sensation, especially when consuming hot or spicy foods. In non-homogeneous forms, symptoms are often caused by colonization by Candida albicans: this super-infection, but not the leukoplakia itself, disappears after antifungal therapy [64].

The proportions of malignant transformation reported in studies exhibit significant variability, ranging from 0.13% to 34.0% [58,65] with an annual malignant transformation rate ranging around 1-1.5% [66,67].

The significant variability in the data is primarily attributed to the fact that the diagnosis of leukoplakia is not always objective and unanimous. In some instances, benign lesions may be mistakenly diagnosed as leukoplakia, or conversely, lesions that are already in an early stage of tumour development may be classified as leukoplakia[68].

Regrettably, there are currently no pathognomonic factors or specific data that allow for the precise prediction of which OL may undergo malignant transformation [58,69].

Several clinicopathological factors may play a role in the malignant transformation of OL, including sex, age, tobacco smoking, alcohol consumption and systemic diseases[58,70]. However, to date, only the presence of Oral Epithelial Dysplasia (OED) at histological level has been recognized as a significant risk factor associated with the malignant transformation of OL. The risk of malignant transformation gradually increases with the severity of OED [51,71–73].

Furthermore, scientific literature reported other clinical variables associated with an higher risk of malignant transformation: female gender, nonsmoker status, extended duration of leukoplakia, localization on the tongue and/or floor of the mouth, size exceeding 200 mm2, non-homogeneous type [62,71]. In particular, non-homogeneous leukoplakias carry a 20%–25% risk of cancer progression versus 0.6% – 5% in homogeneous cases [57].

From a molecular standpoint, the overexpression of Podoplanin, chromosomal loci abnormalities (such as loss of heterozygosity) and the presence of DNA aneuploidy

emerge as the most promising indicators for predicting the malignant transformation of OL lesions [62,65,71,74].

The diagnosis of leukoplakia is a diagnosis of exclusion based on clinical and histological confirmation. Histological assessment has the role to correctly differentiate other lesions with a similar clinical appearance and to stage the lesion in relation to the presence or absence of dysplasia. Histological findings can range from hyperkeratosis to severe dysplasia, including cases of carcinoma in situ [75].

Thus, for the correct diagnosis of OL, it is necessary to differentiate lesions or disorders that manifest within the oral mucosa as white and/or red lesions [76]. Misdiagnosis and improper classification of OL have led to confusion and underestimation of the rate of malignant transformation. [37].

Indeed, white lesions are not uncommon within the oral cavity and can stem from a range of causes. Majority of white oral lesions are benign [77]. Most of these benign white lesions are incidental findings, often attributed to mechanical friction, parafunctional habits, chemical or tobacco-related contact reactions [52,77].

Specifically, benign oral white lesion can be classified in four main groups: white lesion originated from genetic disorders, white lesion caused by local injury, white lesions caused by infections and immune-mediate keratotic lesions [64].

White lesions caused from genetic disorders are relatively rare and, among them, White Sponge Nevus is the most common. However, other conditions fall under this category, including hereditary benign intraepithelial dyskeratosis and various congenital genodermatoses. Each of these conditions exhibits specific and distinctive histopathologic features, allowing for diagnosis by a pathologist [64].

White lesions resulting from local injury encompass various conditions, including leukoedema, contact injuries and frictional keratoses. Leukoedema is a white or whitish-gray edematous lesion of mucosae, particularly the buccal and labial mucosa. It is harmless and represents a variation of the normal appearance rather than a disease [64].

Additionally, contact injuries may occur when mildly caustic substances are kept in contact with a particular site for prolonged periods. Examples include the use of smokeless tobacco or chewing gum, which can lead to localized white lesions in the

affected area [64]. Otherwise, literature refers to smoke-related reactive oral white lesion as “tobacco pouch keratosis” [77] and “nicotinic stomatitis” (also referred as “smokers’ palate”) [37].

Frictional keratoses, on the other hand, develop due to chronic irritation or trauma, leading to the thickening of the epithelium and the formation of white lesions. Various terms are utilized to describe these white patches: “frictional keratosis” when found on alveolar ridges is referred to as “alveolar ridge keratosis (ARK)”, while a white line along the occlusal plane is referred to as “linea alba buccalis” and “morsicatio buccarum” is a condition characterized by chronic irritation or injury to the commissures and/or to the buccal mucosa caused by repetitive chewing, biting, or nibbling [37].

Furthermore, certain manifestations of candidiasis, such as plaque and pseudomembranous forms, can lead to the development of white lesions in the oral cavity. Candidiasis is a fungal infection caused by Candida species, and these lesions may result from the overgrowth of the fungus on the oral mucosa [64].

On the other hand, "benign migratory glossitis" typically refers to migratory erythema or erythematous lesions rather than white lesions. It involves areas of erythema surrounded by white borders, creating a map-like appearance. While geographic tongue is considered a benign condition and not necessarily related to infections, it falls under the category of immune-mediated keratotic lesions due to its inflammatory nature [64].

Oral Lichen Planus:

Oral Lichen Planus (OLP) is an autoimmune pathology affecting the oral mucosa, characterized by a chronic inflammatory infiltrate primarily composed of T lymphocytes attacking the basal layer. This process is triggered by an antigenic change of unknown origin that occurs in the mucosa of predisposed individuals. The main targets of this local cell-mediated immune response are epithelial cells of the basal layer , which, following an unidentified insult, express an altered self-antigen [78,79].

This condition is more commonly diagnosed in females over the age of 50 (male-to-female ratio 1:4) and exhibits a global prevalence of 1.01% (with a higher prevalence

in Europe at 1.43%). It shows a slight predilection for females and an inverse association with common risk factors such as smoking and alcohol consumption [80].

The manifestations of OLP vary from patient to patient and can evolve during the natural course of the disease [81]. Common features include the presence of multiple lesions with a symmetrical distribution; a crucial aspect for clinical diagnosis is the ability to recognize papules or their confluence, giving rise to typical linear or reticular lesions known as Wickham's striae. The most typical sites involved are the buccal mucosa starting from the third posterior, the tongue, and the gums [78,81].

Another manifestation of OLP is the formation of white plaques, morphologically similar to leukoplakia. However, they can be differentiated by the simultaneous presence of reticulo-papular lesions at the periphery or by the preferential localization, which often affects the dorsal surface of the tongue. Papulo reticular and plaque clinical manifestations characterize the most frequent clinical presentation of oral lichen planus, often with a limited or absent symptomatic component [78].

Clinical forms in which atrophy, erosion, and, more rarely, blister formation define atrophic erosive manifestations of OLP and are associated with pain, burning sensation and functional impairment. In most cases, atrophic erosive lesions co-exist with typical white manifestations of lichen planus, helping in the provisional phase of clinical diagnosis. Gingival localization presents a clinical picture known as desquamative gingivitis, requiring a differential diagnosis with plaque-related gingivitis and other blistering diseases [81].

The disease's course can be chronic, with periods of exacerbation of clinical manifestations and phases of apparent remission [78,81].

Secondary Tumor Manifestations:

Another distinctive characteristic of OSCC is the high risk of developing secondary tumour manifestations (17-30%), even following complete surgical excision of the primary tumour [35]. This risk encompasses both synchronous tumours, which occur within six months of the initial neoplasia, and metachronous tumours, which develop subsequently [82]. Secondary tumours typically occur within a year from the diagnosis of index tumour, with 20% of cases presenting between the 2nd and 5th year [35,83].

Second neoplastic events can be classified into second primary tumors (SPTs) which are independent from the index tumor at the molecular level, and local recurrences or metastases which, by contrasts, are genetically related to the primary tumor.

Two main theories have been proposed to explain the emergence of secondary tumours: "premalignant cell migration" and "field cancerization". These theories may coexist, giving rise to both LR and SPT.

The "premalignant cell migration" theory elucidates how certain tumour cells from a carcinoma can disseminate from the main mass, utilizing biological fluids such as saliva or blood, as well as biological structures like lymphatic vessels or even through epithelial migration. This enables the development of a second neoplasia remotely from the original mass, genetically identical to the primary one [35,84].

The "field cancerization" theory was initially proposed by Slaughter et al. in 1953 [85]. Following observations from extensive tissue samples the authors described the characteristics of multiple oral tumours: (1) oral and oropharyngeal cancer develops in multifocal areas of altered cells, interpreted as arising from independent events; (2) the tumour is surrounded by abnormal tissue; (3) both oral and oropharyngeal carcinomas often consist of multiple independent masses that merge into a single tumour mass; (4) the presence of abnormal tissue adjacent to the neoplasia explains the occurrence of SPTs and LRs.

From a practical standpoint, at any given moment, a basal layer cell may undergo genetic modification due to a mutagenic stimulus, giving it a proliferative advantage over other cells. Its replication, facilitated by genetic damage, leads to the formation of a patch of daughter cells, all bearing the same mutation. Each of these daughter cells can undergo further genetic damage, accumulating over time, and as they replicate more rapidly than non-mutated cells, spatially amplifying the phenomenon, a field may emerge, extending throughout the oral cavity, as well as contiguous structures such as the oropharynx or larynx. Over time, additional mutagenic stimuli on this field may lead to the development of carcinoma. Surgical removal, even if radical, is insufficient to eliminate the entire field of genetically damaged (but clinically or histologically healthy) cells. If these cells acquire new DNA damage, a new tumour independent of the previous one may arise. Consequently, field cancerization theory, can explain why

the number of SPTs is high in former OSCC patients. For the same reason, operated patients should undergo close follow-ups and are considered at high risk for additional tumor development.

Nonetheless, both LR or SPT have a negative impact on prognosis [86].

Prognostic markers in surgically treated OSCC patients: a review

As mentioned earlier, the annual mortality rate for Oral Squamous Cell Carcinoma (OSCC) in Italy is approximately 3,000 individuals [7].

Despite improvements in patient survival due to multimodal treatment and enhancements in quality of life through free tissue transfer, OSCC continues to pose a significant threat to mortality [87].

To date, the clinical assessment of OSCC relies on the TNM staging system, but additional tumour features, such as histologic grade and tumour depth, serve as well-established indicators of prognosis [12,88].

However, the 5-year survival rate for OSCC is significantly influenced by the high number of patients (up to 30%) experiencing recurrences post-initial treatment, leading to an unfavourable prognosis [35]. Typically, symptoms of secondary tumours manifest within a year after the initial occurrence, with 20% of cases emerging between the 2nd and 5th year after the initial adverse event [35,83].

Thus, despite recurrence of OSCC significantly affects its prognosis, our understanding of the recurrence patterns and influencing factors remains limited [89,90]. This limitation can be attributed to the diverse nature of previous studies, variations in treatment strategies, and the distinct nature of squamous cell carcinomas originating from different subsites of the oral cavity. The lack of specific biomarkers for predicting each patient's disease burden not only hampers the development of effective treatment plans but also results in deficiencies in monitoring for recurrence [91].

Over the years, several histological parameters, such as the size of surgical margins, extent of depth of invasion, the pattern of invasion and perineural spread have been identified to assess the pre-operative risk of developing new secondary tumour manifestations [88].

In the context of surgical treatment for head and neck cancer, the status of the surgical margin emerges as the most crucial prognostic factor [92]. Traditionally, margin status is assessed intraoperatively using frozen sections [93]. A positive surgical margin is associated with a 90% increase in local recurrence and has been demonstrated to elevate the risk of all-cause mortality at 5 years by 90% in oral cavity cancer [94,95]. However, consensus on the definition of a "clear" or "negative" surgical margin is lacking, leading to considerable variability in how margins are evaluated both intra- and post-operatively[96].

The literature presents a range of distances considered as safe margins for OSCC: 1.6 mm [97], 2.2 mm [98], 2.5 mm and 3.5 mm [99], up to 5 mm [100,101]. These diverse responses underscore the complexity of margin sampling, with varying interpretations among both surgeons and pathologists [93].

Therefore, to mitigate subjectivity and enhance accuracy, various other histological markers have been explored.

For instance, perineural invasion (PNI) has emerged as a factor associated with unfavourable outcomes [102,103]. Recent evidence indicates that OSCC is a neurotropic tumour, and neoplastic cells can spread through nerve fibers into surrounding tissues, escaping local disease control and increasing the risk of secondary tumour manifestations[102]. Numerous studies have demonstrated the association of PNI with advanced Tumor (T) and node (N) stages, extranodal extension, poor tumour differentiation, lymphovascular invasion and increased depth of invasion [104], making it one of the most significant negative prognostic factors in OSCC [105–108].

However, recently depth of invasion (DOI) and worst pattern of invasion (WPOI) have been proposed as the most pathological predictors for OSCC recurrence [109].

The inclusion of DOI in the new AJCC staging system for oral cavity malignancies emphasizes its significance in determining the prognosis of these tumours [88]. Depth of invasion is defined as the measurement from the basement membrane zone to the deepest point of cancer cell invasion [110]. Recently, several reports have demonstrated a lower likelihood of disease-free survival as the DOI increases [111–113].

The WPOI refers to the infiltration of tissue by cancer cells at the tumour/host interface and is considered a critical factor in histological grading systems, particularly for OSCC. Detection of WPOI typically occurs histologically after surgical tumour removal [114]. In 2005, Brandwein-Gensler et al. identified and validated five WPOI categories for OSCC[90]. The low-invasiveness types include type 1, characterized by broad pushing borders and cohesiveness; type 2, featuring broad pushing finger-like growths or separate large tumour islands; and type 3, consisting of invasive tumour islands with more than 15 cells per island. The high invasiveness types comprise type 4, with invasive tumour islands containing fewer than 15 cells per island that are separated from the main tumour mass, and type 5, involving tumour satellites of any size located 1 mm or further distant from the main tumour or the next closest satellite with intervening normal tissue. Subgroups of these types were defined based on invasive properties and patient survival rates [115,116]. The American Joint Committee on Cancer staging system recently recognized the presence of a WPOI of 5 at the advancing tumour edge as a prognostic key in oral cancer [117].

Over time, there has been an increasing emphasis on carefully stratifying the risk of secondary tumoral manifestations by analysing molecular markers located in "clear" surgical margins of resection. This emphasis is rooted in the "field cancerization theory", which suggests that the persistence of abnormal tissue after surgery serves as an explanation for the high rate of second neoplastic manifestations [85].

For instance, Brennan et al. in their study identified 25 patients with primary squamous-cell carcinoma of the head and neck, all of whom had a p53 mutation and had undergone what appeared to be complete tumour resection based on negative histopathological assessment. Molecular analysis was conducted on 13 out of these 25 patients, revealing a positive presence of a p53 mutation in at least one tumour margin. Among these patients, 38 percent (5 out of 13) experienced local recurrence of carcinoma. In contrast, none of the 12 patients with negative margins showed local recurrence ($P < 0.02$ by the log-rank test) suggesting a significant correlation between the presence of a p53 mutation in tumour margins and the likelihood of local recurrence in patients who initially appeared to have undergone complete tumour resection [118].

Graveland et al. retrospectively examined 35 patients with HNSCC among whom 16 developed LR, while 19 remained disease-free for at least 4 years. Various molecular analyses, including Loss of Heterozygosity (LOH) at chromosomes 3p, 9p, and 17p, p53 immunostaining, Ki-67 immunostaining, and histopathological grading of paraffin-embedded surgical margins, were conducted and correlated with LR. Despite tumour-free histopathological margins with a minimum 5 mm distance from the tumour, the persistence of a field post-excision was identified as a significant LR risk factor. LOH at 9p and p53 immunostaining emerged as the most predictive factors (hazard ratios 3.17 and 3.46, p-values 0.027 and 0.017, respectively). Moreover, combining LOH at 9p and/or a large p53-positive field yielded the highest predictability (hazard ratio 7.06, $p < 0.01$) [119].

Moreover, De Carvalho et al. retrospectively examined tissue specimens from 55 patients with HNSCC who underwent tumour resection. The study aimed to assess the utility of evaluating the expression of PTHLH, EPCAM, MMP9, LGALS1, and MET in histologically negative surgical margins and to establish the correlation of these tumour-related alterations with clinical and prognostic parameters. Quantitative RT–PCR analyses were employed to determine the differential gene expression in normal mucosa, HNSCC, and negative margin samples. The results indicated that 38% of the histologically negative surgical margins analysed exhibited overexpression of at least one of the evaluated genes. Specifically, overexpression of MMP9 and PTHLH in the surgical margins was found to be associated with the development of SPT ($p = 0.002$). Additionally, MMP9 overexpression was linked to lower rates of local control, as evidenced by the log-rank test ($p = 0.022$; Hazard Ratio = 4.186; $p = 0.035$) [120].

Similarly, Govindaraj et al. aimed to evaluate the expression of Ki-67, Cornulin, and ISG15 in non-involved mucosal surgical margins and assess the association of clinicopathological prognostic factors with local relapse in OSCC. The researchers immunohistochemically stained surgical margins from the study group (relapse, n = 23), control group (non-relapse, n = 32), and normal oral mucosa (n = 5) using Ki-67, Cornulin, and ISG15 antibodies. Statistical analysis was conducted to explore the association between marker expression and clinicopathological prognosticators with local relapse in OSCC. The findings revealed a significant decrease in Cornulin expression in the surgical margins of the study group ($p = 0.032$). Low Cornulin

expression was notably associated with local relapse ($p = 0.004$) and non-tongue primary tumours ($p = 0.013$). Regression analysis further identified low Cornulin expression ($p = 0.018$) and increased patient age ($p = 0.008$) as predictors of local relapse in OSCC, with a 34-fold and 18-fold increased risk, respectively. Although Ki-67 expression in non-involved mucosal surgical margins was higher in patients with OSCC relapse compared to those without relapse, this difference did not reach statistical significance [121].

Experimental part

Early diagnosis of Oral Squamous Cell Carcinoma (OSCC) is of great importance, as it remains the most crucial factor for improving the survival and remaining quality of life of patients. Furthermore, early diagnosis of oncological disorders can also be economically beneficial for the National Health Service.

Although this may seem self-evident, there is a surprising lack of literature on the economic “value" of early OSCC diagnosis in Italy. The existing studies are sparse, with only one other study conducted by otolaryngologists, which includes but does not specifically address the oral cavity [122].

Additionally, it is important to acknowledge that effective and timely treatment of OSCC requires a series of coordinated steps by multiple healthcare providers (general dentists, oral pathologists, and maxillofacial surgeons). The initial and essential step in this process is the identification of a suspicious lesion by the general dentist, even if only to refer the patient to a specialist. General dentists are often the first to examine patients, but do they possess the diagnostic skills necessary for early detection? Then, how can their diagnostic performance be improved?

Thus, in this first research project we aimed to analyse two key aspects: the economic aspects of early diagnosis and the diagnostic capabilities of general dentists.

A) Direct health-care costs of oral cancer

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According to Globocan, the estimated number of cases and deaths attributable to oral cancer in Italy in 2020 were 4,037 and 1,583, respectively [123]. Despite its relatively low incidence, oral cancer poses a significant public health concern.

Studies analysing the costs of illness in Oral Squamous Cell Carcinoma (OSCC) have gained increasing importance. While the patient's clinical benefit remains the primary aim for treatment planning, evaluating the economic burden of the disease is crucial for healthcare policy decision-making, resource allocation and research priorities.

The economic burden of OSCC can be categorized into:

- *Direct medical costs*: These include the costs of diagnosis, treatment, and follow-up care. Treatment modalities may involve surgery, chemotherapy, radiation therapy, or a combination of these.
- *Indirect and intangible costs*: These encompass expenses related to productivity and income reduction due to illness, disability, or premature death. Additionally emotional and psychological burdens, pain and suffering experienced by the patient and their loved ones, and financial toxicity fall into this category [124].

Recent studies have evaluated the direct costs of OSCC care worldwide [122,125–129]. However, only a few investigations have exclusively examined patients diagnosed with carcinoma of the oral cavity and lip.

Moreover, the source of data may vary: some studies have analysed the economic burden of head and neck carcinoma using institutional or administrative databases, while others rely on reviews of clinical records. Databases provide data from larger cohorts of patients but are less likely to offer specific clinico-pathological variables influencing healthcare costs.

Geographic location is another factor influencing the economic burden of OSCC, as healthcare costs and productivity losses can differ by country or region. Most studies

originate from the USA, while only a few have been conducted in Europe. Specifically, only one study has analysed the costs of patients affected by head and neck carcinoma (including oral cavity) in Italy [122], assessing direct healthcare costs from diagnosis to treatment in 879 patients through an institutional database.

Against this backdrop, the present project aims to retrospectively evaluate the direct costs of oral cancer treatment at a tertiary Italian hospital in Bologna, Italy. The focus is on assessing the relationship between disease stage, direct costs, and post-surgical complications, apart from specific disease survival.

Methods:

Study population: This retrospective observational study was conducted at Sant'Orsola-Malpighi Polyclinic Hospital in Bologna, Italy. The study included consecutive patients diagnosed with OSCC who underwent surgical treatment at the oral and maxillofacial surgery unit between January 2018 and January 2020. All participants provided informed consent.

The inclusion criteria were as follows:

- Histological diagnosis of OSCC involving various sites within the oral cavity, such as the tongue, lip, floor of the mouth, buccal mucosa, gingival tissues, hard and soft palate, retromolar area, and tuber maxillae.
- First presentation of OSCC within the specified study period.
- Surgical resection of OSCC with curative intent, with or without adjuvant chemotherapy or radiotherapy.
- A total follow-up period of 3 years.

Patients with a history of oral cavity tumours and head & neck carcinomas of the nasopharynx, oropharynx, and hypopharynx were excluded from the study. Ultimately, 63 cases met the inclusion criteria and were included in the study population.

Treatment modalities: All patients underwent evaluation by a multidisciplinary team for head and neck cancer, comprising maxillofacial surgeons, otolaryngologists, plastic and reconstructive surgeons, radiation and medical oncologists, pathologists, radiologists, and other allied healthcare personnel. Following the diagnostic workup

and multidisciplinary discussion, all 63 patients underwent surgical resection of OSCC in adherence to standard treatment practices [12].

The surgical approach was tailored based on the clinical stage and involved composite resections, including excision of the primary tumour with ipsilateral or bilateral neck dissection, depending on the N-status. For early stages, both primary closure and local flap placement were performed. Microvascular reconstruction was undertaken in patients with locally advanced disease.

Post-operative adjuvant therapies were administered in accordance with current guidelines for head and neck cancer [130]. Post-operative radiotherapy typically consisted of a 30-session cycle and needed to be initiated within 7 weeks after surgical treatment, following a planning CT or PET-CT scan based on the T and N status of each patient. The intensity-modulated radiotherapy (IMRT) technique was employed, with a dose ranging from 50 to 63 Gy, depending on the risk of disease recurrence and anatomical site. Concomitant chemotherapy involved 3-6 cycles of cisplatin for all patients.

Clinical follow-up occurred every two months during the first year after treatment, every three months during the second year after surgery, and subsequently every six months. A CT scan or MRI of the head and neck region was requested every six months during the initial three years after surgery and then annually, following internal guidelines.

Data collection: As a preliminary step, we created a de-identified, confidential, and password-protected database containing clinicopathological details for each patient from the time of diagnosis to 3 years post-diagnosis.

Clinicopathological information encompassed age, gender, smoking habits, ASA status, tumour location, diagnosis date, date of surgical treatment for oral cancer excision, TNM stage classification following AJCC criteria (8th edition) [88], histological grade (defined according to Kademani et al.[12]), the necessity and type of maxillofacial surgical reconstruction, adjuvant radiotherapy and chemotherapy, duration of surgical treatment (in minutes), duration of intensive and ordinary recovery (in days), and the need for adjuvant radiotherapy and/or chemotherapy.

All postoperative visits, postoperative CT or MRI examinations, management of treatment complications, investigation for recurrence, and treatment of recurrence were documented. The study population was categorized into four disease stages in accordance with the AJCC criteria (8th edition) [88].

Cost analysis and calculation: Billing records from the Emilia Romagna healthcare system were utilized to determine costs, excluding operating theatre costs (TABLE 1).

TABLE 1. Cost calculation related to clinical category.

Treatment	Characteristics	Mean Cost	Reference
Surgery	Operating room cost per minute. Including medical staff, nursing staff and equipment.	€30 per min	Institutional cost
Intensive therapy	Intensive room cost per day. Including equipment, nursing and medical staff, drugs, nutrition, and accommodation costs.	€1,383 per day	Emilia Romagna regional health care system
Hospitalization	Hospital stay cost per day. Including equipment, nursing and medical staff, treatment, catering, and accommodation costs.	€615 per day	Emilia Romagna regional health care system
Chemotherapy	Average cost between 3 and 6 cycles of Cisplatin.	€383.5 (from 3 to 6 cycles)	Emilia Romagna regional health care system
Radiotherapy	Cost of 30 sessions of radiotherapy.	€5,670 (30 cycles)	Emilia Romagna regional health care system
Follow-up visits	Cost of a follow up visit at the Maxillofacial Unit where the standard protocol is: - 1st year: 1 every 2 months. - 2nd year: 1 every 3 months. - 3rd, 4th, and 5th year: 1 every 4 months.	€18 for visit	Emilia Romagna regional health care system
CT scan	Cost of CT scan. The standard protocol for this instrumental exam is: - 1st, 2nd, and 3rd year: 1 every 6 months. - 4th and 5th year: 1 every year.	€142.05 for CT scan	Emilia Romagna regional health care system

The hospital charges in the regional healthcare system do not accurately represent the true healthcare costs for operating theatre procedures. Due to state subsidies to hospitals, charges are significantly lower to align with the Italian healthcare system's policy of universal hospital coverage. An institutional cost of 30 € per minute of theatre time was considered, as previously described [131].

Outpatient visits for psychology and social work were not included in this study. Since outpatient dental care is provided by external institutions, costing data for this analysis were not available.

For each patient, we calculated costs associated with surgical procedures, hospitalization, adjuvant radiotherapy and chemotherapy, follow-up visits, and the occurrence of complications.

Statistical analysis: Demographic and clinical parameters were summarized using classic descriptive statistics. Non-parametric tests, such as Chi-squared and Fisher's exact tests, were employed to assess any between-group differences in demographic, surgical, and post-surgical characteristics across different disease stages (stage I vs. stage II vs. stage III vs. stage IV).

One-way ANOVA analysis with multiple range tests and Chi-square analysis were used to examine significant differences in costs between different disease stages (stage I vs. stage II, stage III, stage IV).

A multiple linear regression with stepwise selection was conducted for the entire study population to assess the relationship between costs and various variables, including age (<65/>65), gender (male/female), smoking status (no/yes), ASA status (ASA 1-2 vs. ASA 3-4), tumour location (tongue and floor of mouth/cheek/gingiva and hard palate), grading (G1/G2/G3), T stage (T1-2/T3-4), N stage (N0 vs. N+), disease stage (stage I vs. other stages), radiotherapy (yes vs. no), chemotherapy (yes vs. no), surgical reconstruction (no flaps vs. local flaps vs. microvascular flaps), and surgical complications (yes/no).

Additionally, a Cox proportional hazard model was fitted to confirm the association between disease-specific survival and the disease stage. A significance level of $P<0.05$ was considered for all analyses.

Results:

Study population: The study included a total of 63 patients, with 33 males and 30 females, and a median age of 69.6 ± 11.9 years (range 39-91). Twenty patients were smokers, while forty-three were non-smokers. Tumor locations varied, with the tongue being the most common (33.3%), followed by the lower gingiva (23.9%), cheek (19%), and others. The TNM classification revealed a distribution across different stages, with 17 patients classified as stage I, 14 as stage II, 8 as stage III, and 24 as stage IV. Surgery involved various procedures, including primary closure, local flaps, and microvascular reconstruction, with a median surgical and reconstruction time of 370.9 minutes.

Post-surgical hospitalization, including intensive care and ordinary units, had a median duration of 13.5 days, and the majority (79.4%) required intensive care. Treatment modalities included surgery alone for 54% of patients, adjuvant radiotherapy for 46%, and concomitant chemo-radiotherapy for 14.3%. Post-surgical complications occurred in 18 patients, with 16 being loco-regional and 2 reconstruction-related.

During follow-up, 28.6% of patients experienced a second loco-regional neoplastic manifestation, including local and regional relapses and lung metastasis. Twelve of the 17 patients with relapses died of the disease.

Cost and price analysis: The estimated mean total direct cost for OSCC treatment and post-surgery surveillance was €26,338.5, with a range of €1,386.3 to €70,473 per patient (TABLE 2). One-way ANOVA analysis indicated a significant difference between stages of the disease ($F=22.79$; $p<.001$). Post hoc analysis revealed that stage I had a significantly lower mean patient cost compared to other stages.

One-way ANOVA analysis also demonstrated significant differences between stages for the cost of surgical treatment, cost of intensive and ordinary therapy, and cost of adjuvant radiotherapy and chemotherapy.

Multilevel mixed logistic regression analysis identified advanced diagnosis (stage III and IV), complex surgical procedures involving microvascular flaps, and loco-regional recurrences as variables significantly related to a higher cost of OSCC treatment and

post-surgical surveillance. This comprehensive analysis sheds light on the economic implications associated with different disease stages and treatment modalities.

TABLE 2. Mean, minimum and maximum cost (in euros) for patient according to clinical disease stage. Entries in boldface indicate statistically significant p-values.

	Stage I	Stage II	Stage III	Stage IV	*p value*
Cost of surgical treatment	3,771.18 (300-10,200)	8,809 (540-15,000)	13,800 (750-24,600)	16,801 (6,900-30,000)	**<.0.01**
Cost of intensive recovery	650.8 (0-2,766)	1,185.4 (0-1,383)	1,383 (0-2,766)	1,613 (1,383-2,766)	**<.0.01**
Cost of ordinary recovery	3,472 (0-13,530)	4,963.9 (0-11,685)	7,303 (1,230-13,530)	12,505 (4,305-29,520)	**<.0.01**
Cost of adjuvant therapy (radiotherapy and chemotherapy)	356.1 (0-5,670)	432.4 (0-5,670)	4,348.4 (0-5,670)	5,025.2 (0-5,670)	**<.0.01**
Cost of instrumental examinations during follow-up period	726.9 (142,05-852,3)	740.7 (0-852,3)	710.3 (142,05-852,3)	526.8 (0-852,3)	.07
Cost of follow-up visit	206.5 (54-234)	208.3 (18-234)	202.3 (72-234)	158.3 (0-234)	.31
Complications and secondary tumours treatments	8,775 (0-11,505)	11,560 (0-24,370)	6,971 (0-19,068)	7,259.3 (0-37,518)	.9
TOTAL	10,733 (1,386-21,894.3)	19,642.9 (1,626-44,461.5)	30,361.4 (21,262-46,136.7)	39,957.1 (18,294-70,473)	**<.0.01**

Discussion:

In this retrospective study, we calculated the direct costs associated with the multimodal treatment and follow-up of patients diagnosed with OSCC in a cohort from a tertiary Italian hospital. Managing oral cancer poses significant economic challenges due to complex treatment regimens and associated healthcare costs. The calculated average cost per patient amounted to €26,338.5, representing a substantial financial burden, equivalent to 77% of the annual salary of an average Italian individual.

Stratifying costs by disease stage revealed noteworthy variations, ranging from €10,733 for stage I patients to €39,957.1 for those with advanced disease (stage IV). This underscores the escalating economic burden with disease progression. Comparisons

with Polesel et al. indicate significant agreement despite differences in study design and patient populations, emphasizing consistent trends in average costs across various disease stages [122].

On the other hand, a Greek study reported lower direct costs per patient (€7,450) for squamous cell carcinoma limited to the oral cavity [126]. Interestingly, the Greek investigation observed significant cost variations based on clinical stage, ranging from €4,088.9 for stage I to €12,803.7 for stage IV similarly to Italian studies [122,126].

Despite finding similar average direct costs per patient in two European studies analysing patients treated for head and neck cancer, divergent results have been reported in studies conducted in non-European countries. For instance, Van Agthoven et al. estimated an average cost of €25,543 in a study conducted in two major hospitals in the Netherlands [128], while in the UK, the estimated cost amounted to approximately €23,500 in the first two years of treatment [132]. In contrast, in India and Iran, the median cost per patient was 170,343 INR (€1,904.74) [127] and $9,022 (€8,307.10) [133], respectively. Not surprisingly, direct costs appear to be higher in countries with elevated living costs. Pollaers et al. reported a mean cost of $92,958 AUD (€57,137.66) in Australia [125], whereas Lairson et al. (2017) estimated $139,749 (€128,548.12) for the management of oropharyngeal cancer patients in the USA [134].

Our data, in agreement with previous reports, confirm the economic burden of oral cancer treatment on the healthcare system. Similarly, the progressive increase in costs during disease progression emphasizes the importance of early diagnosis and intervention, not only for patients' survival and quality of life but also for decision-making in healthcare policies, resource allocation, and research priorities.

In the present study, only 17 cases were diagnosed with stage I (26.9%), 14 with stage II (22.2%), 8 with stage III (12.7%), and 24 at stage IV (38.1%). Notably, data from this study suggest that a simple transition from stage I to stage II implies a significant increase in costs (average cost stage I €10,733 vs. average cost stage II €19,642.9). This underscores the need for cost reduction policies to adopt strategies to improve early diagnosis in oral cancer management.

In many cases, oral cancer at early stages is asymptomatic and can mimic benign conditions. Despite the ongoing debate about the cost-effectiveness of oral cancer screening programs, there are potential benefits. High-risk patients could be identified and treated during the premalignant phases of the disease (e.g., OPMDs) or early-stage cancers, potentially reducing mortality. Current methods for identifying and monitoring high-risk patients, such as conventional oral examination and incisional biopsy with histological assessment, have limitations related to the clinical experience of practitioners and the invasive nature of incisional biopsy. To enhance early detection rates for OSCC, various non-invasive diagnostic aids, including technologies using dyes, autofluorescence, toluidine blue, and non-invasive sampling procedures based on saliva or brushing cell collection for molecular marker analysis, have been proposed. Studies evaluating the economic implications of oral cancer screening programs, especially those incorporating non-invasive diagnostic aids, are essential to bridge the knowledge gap regarding their clinical advantages and cost-effectiveness in OSCC patient management [135].

Moreover, data from the present study highlight a direct correlation between treatment costs and certain variables: the appearance of neck nodal metastases and complex reconstructive surgical procedures. This finding aligns with a recent paper by Porta-Vasquez et al., where the costs for diagnostic and therapeutic procedures were determined by the ASA grade, tumour size, lymph node infiltration, and the presence of metastases [129]. Pollaers et al. and Polesel et al. also identified the development of local recurrences as a significant cost predictor [122,125]. Nodal metastasis is a well-recognized independent predictor of survival [136] and is usually closely related, both spatially and chronologically, to the associated squamous cell carcinoma [137].

Therefore, there is an urgent need to identify novel techniques and technologies that can reduce loco-regional recurrence rates in oral cancer patients, not only to improve patient survival but also to decrease the economic burden of the illness. For instance, several authors have proposed preoperative molecular markers to identify aggressive OSCCs and assist surgeons in planning the most appropriate treatment option [138–140]. This information can be valuable in deciding on neck management for patients with cT1N0 OSCC to identify those at high risk of occult nodal metastases. Recently, there has been a trend toward "personalized medicine," where specific patient information is

used to calculate predictive nomograms and optimize patient care. These models are likely to become increasingly important not only for patient outcomes but also to reduce the direct costs of treatment in the future. Finally, this study underscores that long and complex multimodal treatments, often associated with advanced cancers, are linked to higher costs. While costs should not compromise decisions related to oral cancer treatment and patient survival, new technologies must be economically sustainable, recognizing that healthcare systems have finite resources.

This study has several limitations that warrant consideration. Firstly, the data were derived from the records of a relatively small cohort of patients from a single institution, potentially limiting the generalizability of our findings to other regions in Italy. However, it is noteworthy that our study population aligns with existing literature in terms of staging at diagnosis and loco-regional recurrence rates, and the calculated cost of treatment is consistent with that reported in the existing literature. Secondly, our study focuses solely on the direct costs of oral cancer treatment.

As highlighted by various authors [122,125,126,134], oral cancer treatment is associated with numerous indirect costs, including reduced productivity, loss of income, expenses related to comorbidities, a potential reduction in life years, and a decrease in overall quality of life. These indirect costs are significant and should be considered to provide a more comprehensive understanding of the true economic impact of the disease.

Conclusions:

The findings from this study underscore the significance of early diagnosis and a personalized preoperative treatment approach for OSCC in not only reducing the risk of loco-regional recurrence but also in contributing to the overall reduction of healthcare costs and enhancement of patients' quality of life. Strategies aimed at minimizing costs should prioritize the development of diagnostic and prognostic tools that accurately identify individuals at a high risk of OSCC development. This approach enables the determination of the most suitable treatment options based on the patient's risk profile before the primary tumour resection, ultimately leading to more effective and economical healthcare interventions.

B) The role of academic and continuing education in early diagnosis of Oral Squamous Cell Carcinoma

(data not *published*)

Introduction:

OSCC mortality is closely related with the stage at diagnosis: the 5-year OSCC survival rate reaches 80-90% when the carcinoma is diagnosed at early stages, whereas it diminishes to 5-20% in cases of advanced-stage diagnosis [39]. Diagnostic delays necessitate the implementation of highly invasive therapies, which, in turn, adversely impact the residual quality of life with significant effects on swallowing, speech, and physical appearance [141]. Actually, an early diagnosis of OSCC is achieved in less than half of the cases [39].

Since OSCC can be suspected and/or detected just by visual and tactile examination, dentists are one of the most likely groups of health-care practitioners that can detect oral cancer at an early stage and refer the patient to a specialist (specialist in oral medicine or maxilla-facial surgeon).

So, dentists can well play a key role in early detection and prompt diagnosis of oral cancer and are professionally responsible for providing a comprehensive oral cancer examination for their patients.

However, many General Dental Practitioners (GDPs) fail to suspect or diagnose promptly OSCC. The obstacles in undertaking a routine oral examination by GDPs have been recognized and include practitioners' lack of knowledge and experience [142,143], other than absence of familiarity with oral mucosal lesions which may lead to diagnostic delay [144].

In this scenario, the need for different strategies of education for GDPs in the field of oral medicine is desirable [143].

The aim of the present study was to evaluate the attitude of dental students and practitioners with different levels of experience in correctly detecting oral carcinoma by evaluating clinical images representative of all oral mucosa sites and clinical appearance.

Materials and methods:

An anonymous survey based on 40 clinical images of lesions was distributed using the software Qualtrics^{XM}. All photographic images were obtained with a Nikon D7500 camera equipped with a Nikon AF-S Micro Nikkor 85mm lens and two Nikon Wireless Remote Speedlight SB-R200 flashes. One investigator (D.M.) and one reviewer with long-term oral medicine experience (D.B.G.) selected 40 clinical images, anonymized for all personal data that could lead to patient identification. Specifically, 20 images were representative of oral cancers at different stages (10 early-stage oral cancers and 10 advanced-stage oral cancers) and the remaining 20 images were representative of benign oral lesions with different diagnoses (inflammatory, reactive, OPMDs).

The images were also homogeneously distributed according to the following clinical variables: the presence or absence of a white component within the lesion, site (tongue-floor, cheek, palate-gingiva) and clinical appearance of the lesion (white lesion, ulcer, neoformation) (TABLE 3).

TABLE 3. Clinical images included in the questionnaire classified by clinical aspect and site of onset.

Lesion		**OSCC**	**Benign lesions**	**TOTAL**
White lesion	*Yes*	9	12	40
	No	11	8	
Site	*Tongue/floor of mouth*	10	7	40
	Cheek	2	8	
	Gum	7	4	
	Hard palate	1	1	

The online survey was distributed to three different groups of participants:

Group 1: 25 dental students, who had recently attended the undergraduate Oral Medicine module of the dental program of University of Bologna (DS).

Group 2: 30 junior general dental practitioners (JDP), (<3 years of clinical experience).

Group 3: 44 senior general dental practitioners (SDP), (> 10 years of clinical experience).

Each participant who consented to receive the questionnaire viewed all 40 clinical images. For each image, participants were asked to assess the suspicion of oral carcinoma by choosing one of the following options: Yes, Positive Uncertainty, Negative Uncertainty, No. Each clinical image could be viewed for a maximum of 1 minute. Only participants who answered to all 40 questions were included in the study population.

Statistical Analysis:

Quantitative variables are presented as means ± standard deviations, while categorical variables are presented as frequencies and percentages. One-way ANOVA and multiple range tests with Bonferroni correction were used to evaluate any between-group differences (Group 1 vs. GROUP 2 vs. GROUP 3) in terms diagnostic accuracy considering a 2-score model (sum of positive answers "yes+uncertain/positive" in case of OSCC images and negative answers "no+uncertain/negative" in presence of benign lesion for each group) and in terms of uncertainty (sum of "positive uncertainty and negative uncertainty for each group).

One-way ANOVA was also used within each group to identify any differences concerning the following variables: diagnosis (carcinoma vs. non-carcinoma), carcinoma stage (early diagnosis vs. advanced one), clinical appearance of OSCC (exophytic vs. ulcerated vs. verrucous), clinical appearance of non-carcinoma (white-red lesion vs. ulcerated lesion vs. new growth), and presence of a white component (yes vs. no). In all analyses conducted, p-values < 0.05 were considered significant.

Considering 2-scores model obtained we calculated the sensitivity, specificity and diagnostic accuracy with the associated 95% confidence intervals (CIs).

Intra-group inter-operator agreement was assessed by calculating Fleiss' kappa, which provides a chance-corrected measure of agreement among three or more raters. The following kappa scores were used: $\kappa < 0.4$ for poor agreement, $0.4 \leq \kappa < 0.6$ for moderate agreement, $0.6 \leq \kappa < 0.8$ for substantial agreement, and $\kappa \geq 0.8$ for good agreement. In this analysis variability parameters were assessed considering all the 4 scores "positive", "uncertain/positive", "uncertain/negative" and "negative" (4-score model), considering 3 scores obtained grouping the two "uncertain" scores (3-score model) and considering 2 scores obtained grouping the overall positive and negative scores (2-score model).

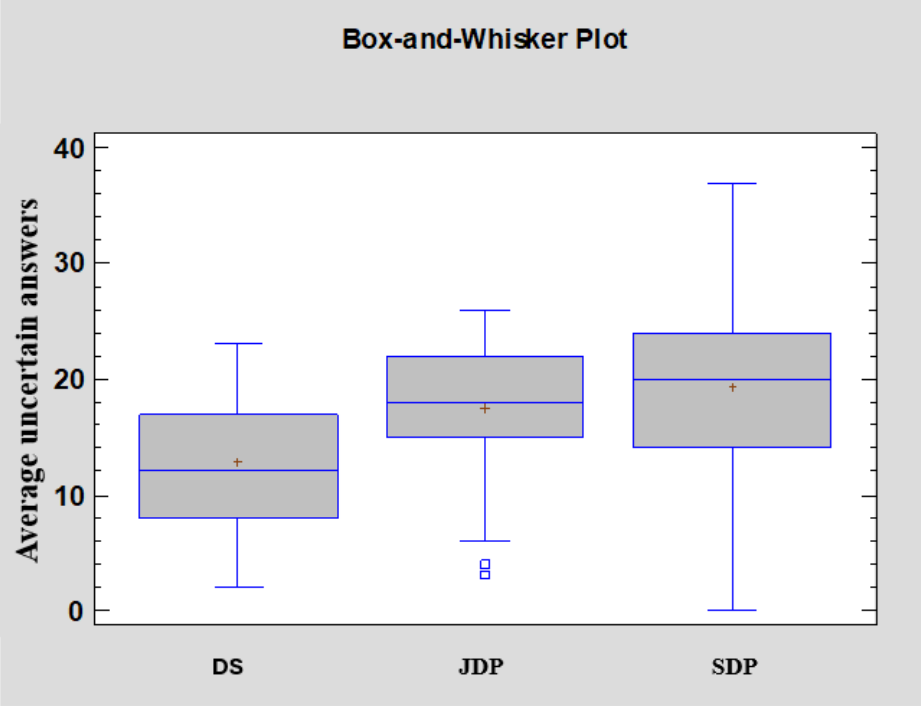
Results:

Group 1 consisted of 25 third-year students, Group 2 comprised 30 JDPs, while Group 3 included 44 SDPs (more than 10 years since graduation). Group 1 completed the test in an average of 724.77 ± 233.22 seconds, Group 2 in 718.83 ± 178.8 seconds, and Group 3 in 1010 ± 711.93 seconds (p=ns).

Between-groups comparison:

One-way ANOVA showed a significant between-group difference (F = 14.34; p<.001) in terms of diagnostic accuracy on the basis of 2-scores model (FIGURE 1).

FIGURE 1: Boxplot showing the mean score obtained in the clinical image-based survey among the three groups (DS, JDP, SDP)



Specifically, DS group obtained a mean score of 32.24 ± 2.9 (min. 26 – max. 38), JDP group obtained a mean score of 27.9 ± 2.9 (min. score 20 – max. score 33) and SDP group 3 showed a mean score of 28.7 ± 2.9 (min. score 22 – max. score 35).

One-way ANOVA, also revealed a significant between-group difference in terms of uncertainty (F 7.49; p<.05) considering the sum of "uncertain/positive" and "uncertain/negative" answers (FIGURE 2): DS group showed uncertainty in the 32% of answers, JDP group in the 43.75% of answers and SDP group in 48.5% of answers.

Multiple range test showed that DS group has a significant higher mean score related to diagnostic accuracy and a significant lower level of uncertainty respect to JDP and SDP group.

No significant differences have been found between JDP and SDP group.

FIGURE 2: Boxplot showing the average uncertain answers in the clinical image-based survey among the three groups (students, JDPs and experienced SDPs)

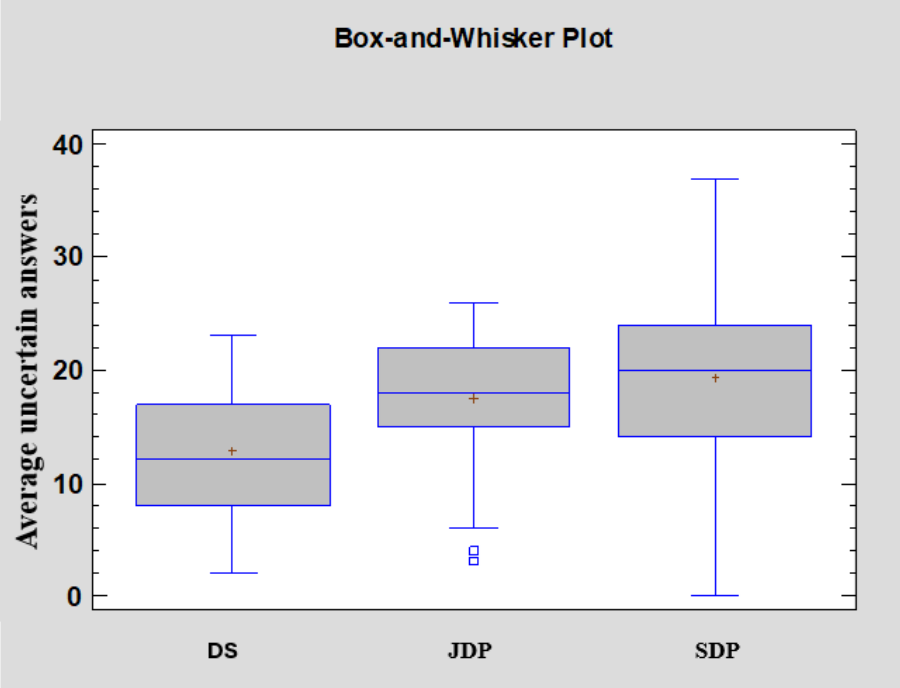


TABLE 4 described clinical variables that showed a significant difference for each group in terms of diagnostic accuracy (2-scores model).

We found that DS group showed a sensitivity of 88% (84%-91.9%), respect to 71.3% of JDP group and 70.9% of SDP group.

Considering only early-stage carcinoma DS group showed a sensitivity of 79.6% (74.2%-84.9%), significantly higher respect to 56.7% of JDP group and 55.3% of SDP group. TABLE 5 resumed values of sensitivity and specificity of three different groups of volunteers.

TABLE 4: Percentage of correct diagnoses for each group according to analysed variable.

		Group 1 (25 participants)	p. value	Group 2 (30 participants)	p. value	Group 3 (44 participants)	p. value
Actual diagnosis	OSCC	21.9/25	**0.04***	21.4/30	0.68	31.2/44	0.8
	Non-OSCC	18.3/25		20.4/30		32/44	
OSCC	Early diagnosis	19.8/25	**0.01***	17/30	**<0.001***	25.6/44	**0.002***
	Late diagnosis	24.6/25		26/30		38/44	
Clinical presentation of OSCC	Exophytic	23.5/25	**<0.001***	21.7/30	**0.02***	31.5/44	**0.04***
	Ulcerated	22.5/25		24.7/30		35.7/44	
	Verrucous	11/25		9.5/30		16/44	
Clinical presentation of non-OSCC lesion	White lesion	23/25	**<0.01***	24.3/30	**0.02***	38.6/44	**0.001***
	Ulceration or bullous lesions	13.5/25		11.5/30		16/44	
	Neoformation	16.7/25		21.4/30		34/44	
Locations	Tongue/floor	18.7/25	0.3	18.1/30	0.07	27.1/44	**0.04***
	Cheek	22/25		24.6/30		36.9/44	
	Gingiva/palate	22.5/25		21.8/30		33.4/44	
Whitish lesion	Yes	19.9/25	0.8	21.2/30	0.8	31.6/44	0.99
	No	20.3/25		20.6/30		31.6/44	

TABLE 5. Sensitivity and specificity estimates based on dichotomous division of responses ("yes + positive uncertainty" vs. "negative uncertainty + no")

	Sensitivity (95% CI)	**Specificity (95% CI)**	**Sensitivity (cT1-cT2)**	**Sensitivity (cT3-cT4)**
Group 1	88% (84%-91.9%)	73.2% (68.7%-77.5%)	79.6% (74.2%-84.9%)	96.4% (92.4-100%)
Group 2	71.3% (67.7%-74.9%)	68% (64.1%-71.8%)	56.7% (51.8%-61.6%)	86% (82.6%-89.4%)
Group 3	70.9% (67.9%-73.9%)	72.8% (69.5%-75.9%)	55.3% (51.2%-59.4%)	84.7% (81.9%-87.6%)

Intra-group inter-operator agreement calculated by Fleiss' kappa showed a moderate agreement for DS group and SDP group in 2-scores model and only in DS group in 3-scores model. A poor agreement has been calculated in JDP group has been calculated, independently to the score model grouping (TABLE 6).

TABLE 6. Intra-group inter-operator variability. Group 1 is composed of 25 third-year dentistry students, Group 2 by 30 JDP and Group 3 by 44 SDP.

		Two categories	Three categories	Four categories
Group 1	Overall agreement	78.2%	60.99%	54.36%%
	Free-marginal kappa	0.56 95% CI [0.44, 0.69]	0.41 95% CI [0.32, 0.51]	0.39 95% CI [0.29, 0.49]
		Moderate agreement	Moderate agreement	Poor agreement
Group 2	Overall agreement	68.59%	49.78%	40.30%
	Free-marginal kappa	0.37 95% CI [0.26, 0.48]	0.25 95% CI [0.18, 0.31]	0.20 95% CI [0.13, 0.28]
		Poor agreement	Poor agreement	Poor agreement
Group 3	Overall agreement	70.31%	52.67%	41.28%
	Free-marginal kappa	0.41 95% CI [0.31, 0.50]	0.29 95% CI [0.24, 0.34]	0.22 95% CI [0.16, 0.28]
		Moderate agreement	Poor agreement	Poor agreement

Discussion:

OSCC survival largely depends on the stage of disease and extent of spread at the time of diagnosis. Therefore, early diagnosis is crucial for improving survival rates [39].

A dental practitioner's attitude and cancer specific-knowledge are the key factors that contributed to promptly identifying suspicious lesions for OSCC, especially in the early stages of development [142–144]. In this regard, the present study aimed to evaluate the

diagnostic attitude of dental students and general practitioners in correctly detecting suspicious lesions for oral carcinoma.

The data obtained from the survey showed that the group of dental students correctly diagnosed a significant higher number of clinical images and that their levels of uncertainty in responses resulted significantly lower compared to the two groups of general dental practitioners. As consequence, dental students showed higher levels of sensitivity, especially for early-stage carcinoma (DS group 79%; JDP 55.3%; SDP 56.7%). Dental students also showed an intra-group moderate agreement whereas JDP and SDP showed poor agreement, independently to the score-model. It is worth noting that dental students completed the questionnaire after finishing the course in "Oral Pathology and Medicine", which includes lectures and internships in the Oral Medicine ward during the third year of dental program of university of Bologna. It is one of the first clinical internships for the students during the dental program, as the first two years are typically focused more on basic science courses. The results therefore suggest that an intensive training program in oral medicine improves diagnostic abilities, even in students without clinical experience.

Fewer studies have investigated the acknowledgment of students after attending academic courses in oral medicine and pathology. Kujan et al. identified the limited number of internships in Oral Medicine wards during dental education as a significant requirement on diagnostic skill development [145]. Hassona et al. in their study have attempted to analyse diagnostic capabilities of students using clinical images. Participants in this study were exclusively students, divided into groups based on their year of study and authors demonstrated that students in later years achieved higher diagnostic accuracy [146]. Our findings reveal higher values in students attending not in the final years but in the third year and may appear discordant compared to the Jordan-based study of Hassona et al. However, the discrepancy may be related to differences of dental program among different universities in different country. University of Bologna schedules the intensive internship and training in oral medicine during the first half of third year of dental program.

Data obtained from the survey underscored low level of diagnostic accuracy and higher level of uncertainty among general dental practitioners without specialty in oral medicine, both junior and senior one. A possible interpretation of these data may be that evaluation and management of oral mucosal lesions occur sporadically for GDPs, who frequently handle clinical issues related to other branches of dentistry such as prosthetics, surgery, endodontics, and others.

These findings are consistent with the scientific literature. Several previous studies on groups of dentists have achieved similar results highlighting the importance of continuous training [147–150]. Pentenero et al., Sardella et al. reported knowledge acquired during graduation is seen to significantly weaken in the absence of continuing education.

Brocklehurst et al., reported uncertainty in diagnostic steps and treatment decisions for oral lesions, opting to refer patients to specialists instead [151]. While this may initially appear positive as GDPs acknowledge their limitations in Oral Medicine and Pathology and seek specialist input, it leads to a burden on specialists, including managing cases that could potentially be handled more straightforwardly, thereby contributing to lengthy waiting lists for specialist consultations [151].

One proposed solution to enhance general dentists' ability to identify oral mucosal lesions is the development of programs of continuous education or the introduction of diagnostic aids easy-to-perform and reliable measure of oral cancer risk to alert primary care providers, general dentists and other frontline screeners.

The present study may present limitations: diagnostic accuracy was assessed through an anonymous questionnaire consisting of clinical photographs of various oral lesions. Certainly, the use of a questionnaire based on digital images instead of physical patients presenting oral lesions may represent a limitation. However, the use of photography for educational purposes in Oral Medicine and Pathology has been confirmed as a reliable method for documenting the presence of oral lesions [152]. In addition, the viewing of clinical photographs has been identified as an effective method for training practitioners' diagnostic abilities, as those who regularly observe oral lesions exhibit higher diagnostic accuracy [113,153].

Conclusions:

This study highlights how an academic education can improve the diagnostic performances of undergraduate dental students without clinical experience.

Conversely, dentists, regardless of their level of experience in other branches of dentistry, demonstrate unsatisfactory diagnostic performance. This underscores the importance of postgraduate education to improve diagnostic skills among practitioners with the aim of reducing diagnostic delays for OSCC.

Notably, continuous and repeated training is necessary as the study reveals that the diagnostic abilities of dental practitioners tend to decline over time if not practiced.

PROJECT 2

Predictive role of a minimally invasive procedure based on 13-gene DNA methylation analysis from oral brushing in high-risk patients

INTRODUCTION

Epigenetic landscape in oral carcinogenesis

The term epigenetics refers to the analysis of various types of modifications affecting DNA or the surrounding regions, ultimately influencing gene expression without altering the DNA sequence. This means that these modifications change the phenotype without affecting the genotype. These changes prevent transcription factors from reaching their usual binding sites on DNA, resulting in a direct alteration of gene activation status. Epigenetic modifications include processes such as acetylation, ubiquitination, phosphorylation, but the one that has garnered the most interest in the field of oncology to date is methylation [154].

Based on current scientific literature, methylation seem to be capable of modifying gene expression on par with deletions and chromosomal alterations [154,155].

A characteristic of methylation is the binding of a methyl group to cytosines within a gene, facilitated by the enzyme methyltransferase. However, methylation does not occur at all cytosines; this is particularly true for those within the promoter sequences of the gene of interest, organized in so-called "CpG islands" (5′-cytosine-phosphate-guanine-3′ islands). These islands are easily identifiable as they consist of series of adjacent cytosines and guanines that tend to repeat and, importantly, remain unmethylated. This sets them apart from other cytosines scattered throughout the gene[156].

The methylation status can be altered in both positive (hypermethylation) and negative (hypomethylation) directions, indicating an increase or decrease in the number of methyl groups on the promoter sequences. Specifically, if the CpG island within the promoter sequence of a tumour suppressor gene is methylated, it can trigger a process with consequences similar to mutations or deletions of DNA bases, solely through histone deacetylation or chromatin compaction. This results in the silencing of the affected gene[156]. On the other hand, global hypomethylation event taking place in the genome would lead to genomic instability and carcinogenesis [157].

Considering that hypo and hypermethylation can be detected by various detection methods such as methylation-specific PCR, bisulfite sequencing and methylation assay and that the methylation status can be relatively easily identified in the DNA of cells present in biological fluids such as serum, saliva, and urine, it can be used as a diagnostic

tool in patients with malignant and potentially malignant disorders of the oral cavity [157,158].

Lastly, methylation is a reversible mechanism, and thus, in the future, it could become a potential therapeutic target to reactivate silenced genes using methylation inhibitors or histone deacetylation [156].

For this reason, numerous studies have analysed the methylation status of genes known to be involved in carcinogenesis, whether they are tumour suppressors or oncogenes. It has been observed that these genes are expressed differently in patients with cancer compared to healthy individuals [156].

Hypermethylation appears capable of inactivating genes involved in the tumour cells' response to chemotherapy, as well as genes crucial for cell repair and protection against external insults, known as "caretaker genes". The silencing of a tumour suppressor gene is central to the carcinogenic process [156].

Promoter hypermethylation has been observed in various types of tumours affecting an increasing number of genes associated with cancer development. Examples include tumour suppressor genes (e.g., p16INK4a, p14ARF, p15INK4b, p73, Rb), DNA repair genes (e.g., O6-methylguanine-DNA-methyltransferase (MGMT), hMLH1, BRCA-1), carcinogen detoxifiers (GSTP), and inhibitors of metastasis and angiogenesis (e.g., E-cadherin, TSP-1, DAPK). These epigenetic changes are frequently linked to the downregulation of gene expression and seem to play a crucial role in the occurrence of the multiple genetic events necessary for driving tumour progression [159].

In particular, each tumour seems to exhibit specific patterns of methylation, as suggested by the BRCA1 gene, which is altered exclusively in ovarian and breast carcinoma [160].

Regarding OSCC, altered methylation patterns have been observed compared to normal tissue [158,159,161], and this is true even for mucosa distant from the neoplastic lesion (due to "field cancerization") [162].

This suggests that DNA methylation alterations are actually an early phase of the carcinogenic process, preceding even genetic mutations and influencing the malignant evolution of the cell. Methylation is currently better understood and more promising, at

least in oncology, compared to other epigenetic control mechanisms such as histone acetylation.

Predictive role of methylation in oral pre-malignancy: a review of literature

The rationale for utilizing epigenetics as predictive indicators of premalignancy is grounded in the belief in a higher hierarchy of the epigenome over the transcriptome and proteome [69].

It is widely acknowledged that epigenetic DNA reprogramming plays a role in all stages of cancer evolution [163–165] and that the patterning of the DNA methylome precedes the initiation of cancer-like stem/progenitor cells [163].

Therefore, comprehending and identifying aberrant epigenetic alterations, including DNA methylation, is crucial for revealing early cancer biomarkers [166,167].

Aberrant promoter methylation is recognized to accumulate in various organs, particularly in high-risk tissues such as gastric mucosae with Helicobacter pylori infection, in liver tissue at the precancerous stage, in colonic mucosae with ulcerative colitis, and in oesophageal mucosae [168–171].

These previous reports substantiate the hypothesis that the accrual of aberrant methylation in OPMDs generates epigenetic field defects that pave the way for malignant transformation [167].

Indeed, the accumulation of aberrant methylation in non-cancerous lesions, such as gastric mucosae with Helicobacter pylori infection, produces epigenetic field defects leading to malignant transformation [168,169] similar to the mechanism of transformation from OPMD to OSCC.

However, in the realm of oral malignancy, while numerous reports elaborate on methylation silencing in OSCC [161,172–174], there is a paucity of studies concentrating on methylation in OPMDs, particularly OPMDs with a high risk of malignant transformation .

The literature, especially in the past, has reported conflicting and heterogeneous data on OPMDs. The definitions of various OPMDs, particularly leukoplakia, have been updated in recent decades, making different studies not entirely comparable.

Shidrar et al. in their review highlighted that the most commonly reported hyper-methylated loci in OL were p16, p14, MGMT and DAPK [176].

Infact, in a study by Asokan et al., the methylation levels of p16, p15, hMLH, MGMT, and E-cadherin were assessed in tissues derived from incisional biopsies of a control group (5 normal healthy individuals), 10 OL patients, and 10 OSCC patients. No methylation was observed among the five genes in the control group, while OL patients exhibited 60% methylation in p16 and E-cadherin genes and 30% methylation in the case of MGMT [177].

Liu et al. also investigated p16, DAPK, MGMT and GSTP1 genes methylation level from 111 biopsies from OL of which 34 with dysplasia at the time of biopsy. Unfortunately, an interpretable methylation pattern for p16, DAPK, MGMT, and GSTP1 was obtained in 82, 87, 106, and 110 biopsies only. The results showed that the p16 tumour suppressor gene exhibited promoter hypermethylation in 21 of 82 (25.6%) cases, the DAPK gene in 28 of 87 (32.2%), and the MGMT gene in 32 of 106 (30.2%) lesions. No aberrant methylation was found for the GSTP1 gene in 110 leukoplakia lesions analysed. The authors concluded that epigenetic mechanisms of inactivation, such as aberrant methylation of p16, DAPK, and MGMT genes, occur early in head and neck tumorigenesis and might play a role in the progression of OL. [178]

Similarly, Takeshima et al. identified a high frequency of hypermethylation involving p14, p15, and p16 in 64 patients with OL, of which 44 and 20 were histopathologically diagnosed with mild and severe dysplasia, respectively. Importantly, no hypermethylation was observed in normal epithelium [179].

Ghosh et al. evaluated the methylation levels of the genes SH3GL2, p16, p14, and p15 in tissue samples from 40 dysplastic leukoplakia cases with normal oral mucosa adjacent to the lesion. SH3GL2 exhibited the highest level of methylation, with 42% (17/40) of cases showing methylation. This was followed by p15 with 27% (11/40), p14 with 20% (8/40), and p16 with 17% (7/40) methylation [180].

In a study by Bathia et al., the methylation status of promoter region of MGMT and p16 was analysed in blood and tissue samples from 54 patients affected by OPMDs, 11 OL without dysplasia and 22 with dysplasia. The study also included 16 healthy donors as a

negative control and 76 OSCC cases as a positive control. Interestingly, MGMT and p16 genes exhibited a similar methylation pattern in both tissue and blood DNA samples from patients with premalignant oral lesions (OL with and without dysplasia, oral submucous fibrosis, and oral lichen planus) and OSCC. This pattern significantly differed from that observed in healthy donors' samples [181].

Using oral rinse samples, Lopez et al. demonstrated abnormal hypermethylation of the genes p16, p14, and MGMT in patients affected by OL. Aberrant promoter methylation of these genes was detected in 82% (28/34) oral rinses. Specifically, the tumour suppressor gene p16 was hypermethylated in 44% (15/34) of the patients, p14 promoter hypermethylation was observed in 4 cases (12%), while 19 patients (56%) showed MGMT promoter hypermethylation [159].

However, various biomarkers beyond p16, p14, MGMT and DAPK have been investigated in this context.

For instance, in a study conducted by Abe et al., 24 OL tissues were investigated, with 13 exhibiting dysplasia and 11 without dysplasia. The study examined the methylation status of eight genes (TSPYL5, EGFLAM, CLDN11, NKX2-3, RBP4, CMTM3, TRPC4, and MAP6), which were previously found to be methylated in their promoter regions in OSCC tissues. The results indicated that seven out of the eight genes, excluding EGFLAM, were methylated in their promoter regions in OL tissues as well. Additionally, OLs with dysplasia showed a significantly higher number of methylated genes compared to those without dysplasia ($p < 0.0001$). However, no association was observed between the grade of dysplasia and the methylation status of the identified genes [175].

Meanwhile, Gao et al. in a small population counting 4 OL with dysplasia described two showing DBCCR1 hypermethylation [182].

Presence of hypermethylation of the hMLH1 and hMSH2 promoters was highlighted in OL tissue sample by Sengupta et al. as well. In particular, 4 out of 27 (15%) leukoplakia samples exhibited hypermethylation exclusively of the hMLH1 promoter, while 5 out of 27 (18%) leukoplakia samples exhibited hypermethylation exclusively of the hMSH2 promoter. Simultaneous promoter hypermethylation of both genes was observed in 8 of

27 OL samples (30%) while 10 of 27 OL samples (37%) did not show hypermethylation in the promoter of either gene [183].

Similarly, Youssef et al. evaluated RAR-β2 methylation pattern in 124 tissue samples from OL and 66 (53%) of them showed methylation of RAR- β2 [184].

Moreover, Cheng et al. collected oral scrapings from 5 normal oral mucosa subjects, 107 OL patients (26 exhibiting no dysplasia, 50 with mild dysplasia, and 31 with moderate/severe dysplasia), and 95 oral squamous cell carcinoma patients. They evaluated the methylation levels of ZNF582, PAX1, SOX1, NKX6.1 and PTPRR genes. It was observed that both ZNF582 and PAX1 methylation rates gradually increased as well as the grade of dysplasia of the lesion. [185]

Juan et al. evaluated PAX1 and ZNF582 methylation pattern from tissue samples as well. In their follow-up study they paired 60 healthy donors and 111 OL (81/111 with dysplasia) and observed that methylation levels of the two genes was higher in patients with mild dysplasia and moderate dysplasia or worse than for patients with a normal histopathology. Also, the study validated that the ZNF582 and PAX1 methylation has an higher incidence and hazard ratio of malignant progression in in OPMDs patients [186].

Majority of researchers have focused on investigating the methylation pattern from tissue samples. Interestingly, Pattani et al. took a different approach by analysing the methylation status of the KIF1a and EDNRB genes from saliva samples. They conducted their study in the context of lesions with both low and high confirmed risk, including 43 dysplastic leukoplakia/erythroplakia cases, as determined by clinical and histological examinations. This study revealed that anomalous methylation of EDNRB could serve as an independent factor for assessing the risk of OPMDs. Interestingly, EDNRB was found to be hypermethylated in both dysplastic lesions and microinvasive carcinomas. Furthermore, this observation was not influenced by age, gender, or race.[187].

Despite the fact that most studies investigating methylation levels in OL have utilized biopsy-confirmed tissue samples and employed standard, validated, and reproducible methods for methylation analysis, there is notable heterogeneity among these studies. This heterogeneity is evident in terms of sample size, control sampling methods (paired vs. different healthy samples), methylation analysis (PCR, NGS, etc.) and the

consideration of socio-economic and lifestyle factors (e.g., tobacco and alcohol use). Regrettably, there is often a lack of emphasis on reporting data from control groups in these studies [176].

Nevertheless, also the molecular mechanism orchestrating the malignant transformation of OL remains elusive, specific molecular and epigenetic markers able to identify individuals at higher risk of developing OSCC have not yet been isolated [167].

Infact, as concluded by Villa and Celentano et al. in a recent systematic review 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 [57].

Validation of potential biomarkers should be prioritised in future studies to ensure the specificity and sensitivity of biomarkers in diagnosing OSCC and OL [167].

EXPERIMENTAL PART

13-gene DNA methylation analysis from oral brushing: background

As aforementioned, several authors have proposed molecular markers and/or non-invasive procedures to assist clinicians in diagnosing and identifying lesions at risk of malignant transformation. However, to date, none of these methods are routinely used in clinical practice.

Since 2012, the research group working at the Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna for the early diagnosis and treatment of patients with OSCC has developed a molecular test based on a non-invasive sampling method – oral brushing – and the epigenetic analysis of a broad panel of genes using Next Generation Sequencing (NGS).

The test specifically analyses the methylation status of a panel of 13 genes (*GP1BB, ZAP70, KIF1A, LRRTM3, TERT, PARP, FLI1, NTM, LINC0059, EPHX3, ITGA, mir139, and miR296*) implicated in oral carcinogenesis. The obtained data are combined using a calculation algorithm that provides a dichotomous result for easy interpretation: positive in the presence of epigenetic alterations characteristic of OSCC and negative in the absence of such alterations.

Originally designed for the early detection of OSCC, the method is currently under patent at the University of Bologna (patent filed on November 4, 2016, under No. 102016000111174 for: METHOD FOR DETERMINING A HEAD AND NECK SQUAMOUS CELL CARCINOMA).

The method was initially validated in a 2017 study, demonstrating high sensitivity and specificity. Specifically, 28 out of 29 brushing samples from OSCC-affected mucosa (96.6%) tested positive, while all 65 samples from the control group tested negative (100%) [161].

Notably, the sensitivity and specificity data were substantially confirmed in a larger multicentre study involving 10 other Italian oral medicine centres [188].

In 2020, a preliminary study was published testing the method on Oral Potentially Malignant Disorders (OPMDs), yielding promising results. In this study, we identified a significant relationship between high-grade dysplasia and positive brushing specimen values collected in patients affected by OL. Specifically, 22/31 OL showed positive

values due to aberrant methylation pattern of the 13 genes composing the panel. In OL patients, dysplasia was the only variable significantly related to positive values: 10/10 OLs with high-grade dysplasia were positive with respect to 12/21 OLs without dysplasia [158].

Finally, we managed to publish a paper where a positive result in an oral brushing specimen collected from regenerating mucosa after resecting OSCC was the most powerful variable related to the appearance of a secondary tumour [162].

In conclusion, the promising diagnostic results achieved through our developed methodology for oral cancer have established a robust foundation for the future of our research.

Currently, our research group is primarily dedicated to prospectively and prognostically assessing outcomes in individuals at risk of OSCC. This research trajectory seeks to offer a comprehensive view of the effectiveness of our methodology, not only in diagnosing but also in predicting the progression of the disease in susceptible individuals, including those affected by OPMDs or those who have undergone surgical treatment for OSCC.

This advancement is pivotal for enhancing the clinical management of high-risk patients and further solidifying the efficacy of our approach in combating oral cancer.

13-gene DNA methylation analysis from oral brushing: method description

An appealing characteristic of epigenetic alterations is their perceived stability and easy detectability in bodily fluids [173]. In recent years, several researchers have investigated the methylation status of a group of genes using saliva or samples obtained through oral brushing [187,189–192].

Given the potential for identifying specific markers that can distinguish neoplastic or high-risk lesions through non-invasive methods like saliva collection or exfoliative cytology, our research group has developed a non-invasive procedure to provide an effective first-level test for identifying high-risk patients.

This method involves a minimally invasive oral-brushing sampling technique and the subsequent DNA methylation analysis of a preselected panel of 13 genes in the oral mucosa, as previously described by Morandi et al. [161].

In brief, DNA from exfoliated cells was purified using the MasterPure Complete DNA Purification Kit™ (MC85200; Lucigen, Middleton, WI, USA) and treated with sodium bisulfite using the EZ DNA Methylation-Lightning Kit™ (D5031; ZymoResearch, Irvine, CA, USA), following the manufacturer's instructions. Subsequently, quantitative DNA methylation analysis of specific genes (*ZAP70, ITGA4, KIF1A, PARP15, EPHX3, NTM, LRRTM1, FLI1, MIR193, LINC00599, MIR296, TERT, and GP1BB*) was conducted through next-generation sequencing (NGS). The libraries for sequencing were prepared using the Nextera™ Index Kit following a two steps approach with a locus-specific bisulfite amplicon approach [161]. Each NGS experiment was designed to allocate at least 1000 reads/amplicon, with the aim to reach a depth of coverage of 1000×. These libraries were loaded onto MiSEQ (15027617; Illumina, San Diego, CA, USA). The FASTQ output files underwent quality control (>Q30) and were converted into FASTA format within the Galaxy Project environment [193].

The methylation ratio of each CpG site was computed using various tools concurrently: BSPAT (http://cbc.case.edu/BSPAT/index.jsp accessed on 29 December 2020) [194], BWAmeth in a Galaxy Project environment (Europe) followed by the MethylDackel tool (https://github. com/dpryan79/MethylDackel accessed on 29 December 2020), EPIC-TABSAT [195] and Kismeth [196].

In a previous study, ROC analysis identified the most discriminative CpG sites, which were then used to develop a selection algorithm based on multiclass linear discriminant analysis [161].

This approach enabled accurate identification of OSCC at a threshold of 1.0615547, demonstrating optimal sensitivity and specificity values (area under the curve = 0.981). Values surpassing the threshold of 1.0615547 were deemed positive [161].

A) Oral brushing and DNA methylation analysis for prognostic assessment in patients at risk of developing oral cancer

Screening populations for the early detection of asymptomatic oral carcinoma or precursor lesions is an attractive strategy to improve the survival and quality of life of OSCC patients. Our research group has developed and validated in a recent multicentre study a method based on quantitative DNA methylation analysis of a panel of 13 genes [161,188]. The high sensitivity and specificity of the method stimulated us to evaluate the predictive role of the procedure in patients at high risk of developing OSCC: patients with oral potentially malignant disorders (OPMDs) and patients surgically treated for OSCC.

In this vein, a case report was initially published, describing how our methodology successfully predicted the malignant transformation of an apparently low-risk OL, characterized by homogeneity and absence of dysplasia, and, concurrently, the occurrence of a second carcinoma in the same patient.

A 13-Gene DNA Methylation Analysis Using Oral Brushing Specimens as an Indicator of Oral Cancer Risk: A Descriptive Case Report

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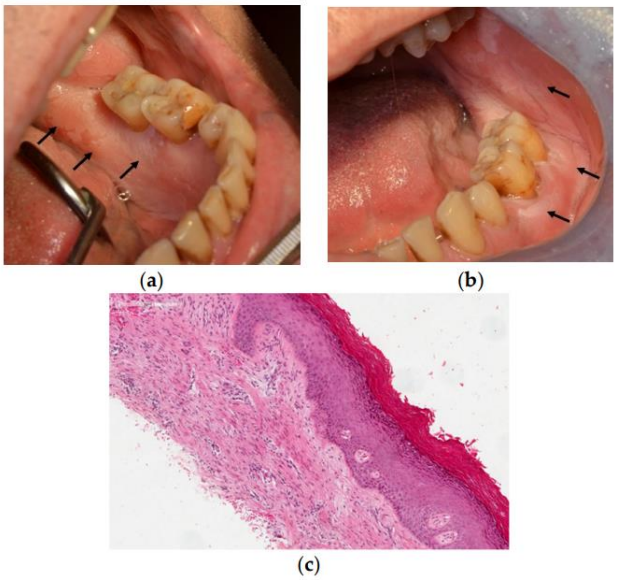
Case Presentation

Patient History:

In December 2016, a 68-year-old non-smoking male was referred to the Department of Biomedical and Neuromotor Sciences, Section of Oral Sciences. The patient presented with an asymptomatic white lesion in the oral mucosa involving the lingual and vestibular gingiva near dental element #37, the left cheek, and a portion of the soft palate (Figure 3a,b). His medical history included a previous diagnosis of diabetes and

hypercholesterolemia, for which he was receiving treatment with metformin, atorvastatin, and cardioaspirin.

FIGURE 3. (a-c). Clinically homogeneous oral leukoplakia (OL): black arrows point out sites of OL extension involving the lingual and vestibular gingiva near dental element #37, the left cheek, and a portion of the soft palate **(a, b)**. Haematoxylin and eosin staining (HE) of a white lesion showing compact hyperkeratosis and hypergranulosis without dysplasia (HE 10x) **(c)**.

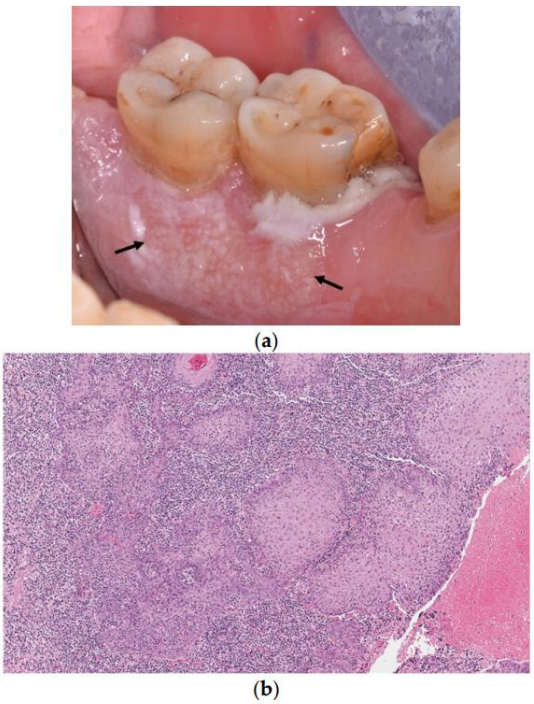


During clinical examination, the white lesion was observed to be non-removable, homogenous, and exhibited well-defined borders. No pain or evidence of ulceration was noted during the initial examination. The patient had a non-contributory medical history. Following the clinical evaluation and after excluding potential etiological causes, a provisional clinical diagnosis of OL was established. Subsequently, an incisional biopsy was performed to attain a definitive clinical-pathological diagnosis. Histological assessment revealed acanthosis, hypergranulosis, hyperkeratosis, and non-specific inflammatory cells, without dysplastic characteristics (Figure 3c).

Consequently, the lesion was definitively classified as Oral Leukoplakia (OL) based on the criteria described by Warnakulasuriya et al. [197]. The patient underwent clinical follow-up every 6 months. During a routine follow-up visit in March 2019, a proliferative and dyshomogeneous area was noted in the lesion located in the lingual gingiva near dental element #37 (Figure 4a). The patient reported discomfort and pain corresponding to element #37. An incisional biopsy and histological assessment revealed the presence

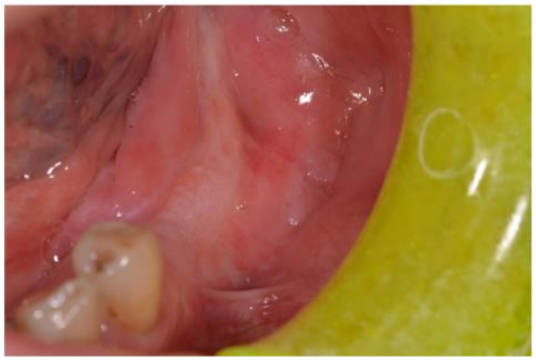
of a well-differentiated, micro-invasive Oral Squamous Cell Carcinoma (OSCC) (Figure 4b).

FIGURE 4. (a-b) Black arrows indicate non-homogeneous and proliferative lesion involving the lingual gingiva near element #37 **(a)**. The histological assessment revealed the presence of a well-differentiated, verrucous-type, and keratinizing OSCC with micro-invasive foci (HE 5x) **(b)**.



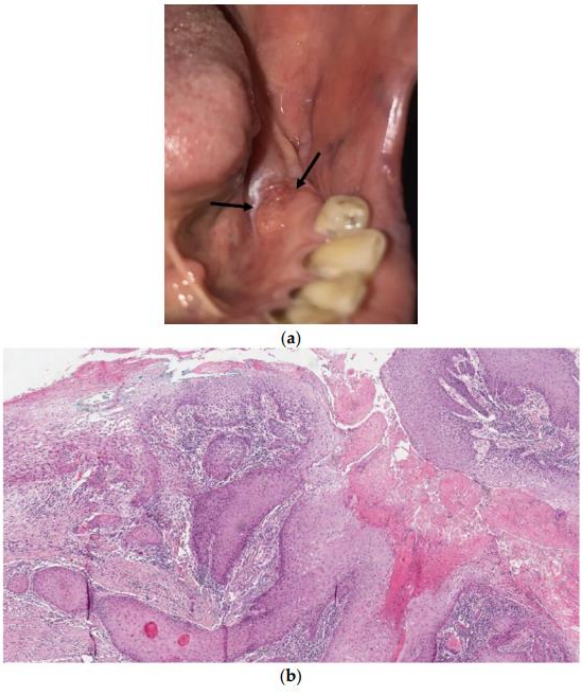
Complete surgical resection of the OSCC, along with concomitant extraction of element #37, was performed following standard treatment practices [12]. The final pathological classification revealed a pT1N0 OSCC with a clear margin of resection and a low pattern of invasion (P1 based on the classification by Chang et al. [198]). The depth of invasion was <4 mm, and there was an absence of perineural and vascular invasion. After the surgery, the patient was included in a regular oncological follow-up program, undergoing clinical, instrumental, and radiological examinations in accordance with international National Comprehensive Cancer Network guidelines (Figure 5).

FIGURE 5. Apparently clinically healthy mucosa 6 months after resecting the OSCC



During the routine follow-up one year later, a clinically (Figure 6a) and histologically (Figure 6b) confirmed secondary Oral Squamous Cell Carcinoma (OSCC) tumour developed in the anterior area of the gingiva related to the index tumour, and the secondary OSCC was surgically resected. The patient is currently free from disease but continues to undergo routine oncological follow-up.

FIGURE 6. (a, b) Black arrows showed the presence of a proliferative lesion in the area previously surgically treated for OSCC **(a).** The histological analysis revealed the presence of a secondary tumor (HE 5x) **(b).**



During the routine follow-up one year later, a clinically (Figure 6a) and histologically (Figure 6b) confirmed secondary Oral Squamous Cell Carcinoma (OSCC) tumour developed in the anterior area of the gingiva related to the index tumour. The secondary

OSCC was surgically resected. As of now, the patient is free from disease but continues to undergo routine oncological follow-up.

13-Gene DNA Methylation Analysis:

We employed our recently developed non-invasive procedure, previously described at page 46.

Brushing samples were collected at five different time points, as outlined in Table 7: at the time of OL diagnosis, obtained from the surface of the white lesion (December 2016); concurrently with the biopsy that led to the diagnosis of the index Oral Squamous Cell Carcinoma (OSCC) in the proliferative area with homogeneous dye (April 2019); 6 months after surgical removal of the primary OSCC in the regenerative area following the initial OSCC resection (October 2019); at the time the secondary tumour appeared, collected from the tumour mass (May 2020); and 6 months after surgical removal of the secondary tumour in the regenerative area following the second OSCC resection (December 2020).

TABLE 7. Quantitative methylation levels of the most informative CpGs of each gene and methylation scores derived from the algorithm for all the five brushing samples to which the patient was subjected

Genes	Oral Leukoplakia	Index OSCC	Regenerative Mucosa Six Months after Primary OSCC Resection	Secondary Tumor	Regenerative Mucosa Six Months after Secondary Tumor Resection
Date of brushing sampling collection	December 2016	March 2019	September 2019	March 2020	October 2020
KIF1A	0.17647	0.58333	0.0349	0.3942	0.0638
ZAP70	0.82342	0.90934	0.998	0.9291	0.7821
GP1BB	0.37096	0.79415	0.751	0	0.7586
LRRTM3	0.34285	0.33333	0.6538	0	0.1447
TERT	0.04323	0.10909	0.0006	0.1897	0
PARP	0.03448	0.55445	0.1594	0	0.0054
FLI1	0	0	0	0	0
NTM	0.15948	0.72847	0.7643	0	0
LINC0059	0.03473	0.18089	0.1102	0.1479	0.0015
EPHX3	0	0.86813	0	0.6223	0
ITGA4	0.59198	0.37885	0.1186	0.0006	0.0405
MIR193	0.31818	0.69214	0.5033	0.0699	0.1632
MIR296	0.05555	0.04123	0	0.94	0.0005
Methylation score (threshold: 1.061554)	1.61	5.21	1.85	8.14	0.47

The 13-gene DNA methylation analysis yielded positive results in specimens obtained from primary (score 5.21) and secondary (8.14) OSCC. Positive scores were also determined for the OL lesion diagnosed 28 months prior to the primary oral cancer's onset (score 1.61) and in the brushing sample collected from the regenerated clinically

healthy area 6 months after resecting the primary tumour and 8 months before the appearance of the secondary tumour (score 1.85). Lastly, the brushing sample collected from regenerative oral mucosa 6 months after resecting the secondary OSCC yielded a negative result (0.47) (Table 7).

Discussion:

Individuals diagnosed with OPMD and/or treated for oral carcinoma are regarded as having a high risk of developing OSCC. Implementing a lifelong follow-up program, which includes visual and tactile assessments, represents the optimal strategy for early detection of malignant occurrences.

This case report highlights the limitations of current diagnostic procedures in identifying patients undergoing malignant transformation and underscores the potential clinical application of a minimally invasive procedure based on the methylation level of a panel of 13 genes in oral brushing specimens. The patient in question initially received a diagnosis of OL and subsequently developed two metachronous oral malignant manifestations during the follow-up period. The brushing cell collection, 13-gene DNA methylation analyses, and score calculations were conducted at five different times during the patient's course of care.

The brushing specimens collected in December 2016, before the incisional biopsy confirming the OL diagnosis, and in April 2019, before the incisional biopsy confirming the malignant transformation of OL into OSCC, both showed altered methylation patterns (scores exceeding the threshold value of 1.0615547). Similarly, brushing samples collected in October 2019 from the clinically healthy mucosa 6 months after surgical resection of OSCC, in May 2020 from the tumour mass of the second cancer, and in December 2020 from the clinically healthy mucosa 6 months after surgical resection of the second cancer also exhibited altered methylation patterns.

Four out of five brushing specimens displayed methylation scores above the threshold, indicating an aberrant methylation profile. Interestingly, the diagnostic procedure detected high scores in brushing samples collected from the primary and secondary tumours, confirming its diagnostic value.

Additionally, a positive score (1.61) was identified in a brushing specimen collected at the diagnosis of OL, two years prior to the neoplastic transformation. Notably, despite the clinical and histological features of OL, characterized by a homogeneous lesion without histological dysplasia, not indicating a substantial risk of malignant transformation, the positive score suggests the presence of genetic and epigenetic alterations associated with the subsequent development of a secondary neoplastic manifestation.

Furthermore, a positive score (1.81) was determined in the brushing sample collected from apparently healthy mucosa eight months before the onset of the secondary cancer. These findings align with previous studies, affirming the presence of genetic and epigenetic alterations associated with the emergence of a secondary neoplastic manifestation in tissue adjacent to the tumour or in clinically and histologically normal mucosa [162,199–202]. However, these observations contrast with the clinical and histological characteristics of primary OSCC, which do not suggest a high risk of relapse (keratinizing-type squamous cell carcinoma of the verrucous type, T1N0M0 with clear margin of resections, absence of perineural infiltration and vascular infiltration, and a depth of invasion <4 mm).

The case report suggests that 13-gene DNA methylation analysis of oral brushing specimens has diagnostic and predictive potential for screening and longitudinally monitoring patients at risk of OSCC transformation. The study advocates for the collection of brushing cells at different intervals during follow-up, such as every 6 months, to enhance the understanding of oral cancer risk for individual patients. However, further studies with extended follow-up periods are needed to validate this hypothesis.

The application of a non-invasive or minimally invasive procedure based on oral brushing, mouth rinsing, or saliva for analysing epigenetic markers has been proposed as a diagnostic aid for identifying patients at risk of developing oral cancer. While several studies have investigated the diagnostic value of biomarkers in oral cancer [189,203–207], none have been widely implemented in diagnostic work-ups and to the best of our knowledge, there are only two multicentre studies performed with the aim to validate a non-invasive or minimally epigenetic procedure [188,204].

In this report, we were able to compare the methylation levels of single informative CpGs from the panel of 13 genes in brushing specimens collected at five different times. As gene methylation is a reversible process, this analysis during long-term follow-up provides valuable insights into the oral carcinogenesis process.

The 13-gene panel comprises two miRNAs (MIR296 and MIR193a) previously associated with various cancers, a long non-coding RNA (Linc00599), and several protein-coding genes (GP1BB, ZAP70, KIF1A, PARP15, FLI1, NTM, TERT, EPHX3, LRRTM1, ITGA4). Notably, ZAP70 exhibited hypermethylation in all four positive brushing specimens, indicating that altered methylation of this gene may represent an early and stable event in oral carcinogenesis. In contrast, KIF1A, TERT, and EPHX3 displayed aberrant methylation patterns only in brushing samples related to the primary and secondary OSCC. These findings align with previous research, such as the hypermethylation of EPHX3 specifically in OSCC samples [208].

Despite the clinical criteria indicating a local recurrence based on the timing of appearance [209], the epigenetic data revealed distinct pathways between the primary and secondary tumours. Five out of thirteen genes (LRRTM3, PARP, NTM, ITGA4, and MIR193) exhibited hypermethylation only in primary OSCC, GP1BB showed hypomethylation only in primary OSCC, while MIR296 displayed hypomethylation only in secondary OSCC. This underscores the importance of further investigations to assess the role of epigenetic changes as biomarkers for identifying clonal relationships among multiple oral cancers.

Conclusion:

In this study, we present the clinical application of 13-gene DNA methylation analysis in oral brushing specimens for the management of a patient who developed a premalignant lesion followed by two subsequent neoplastic lesions in the oral cavity over a 4-year period. The use of a non-invasive or minimally invasive procedure based on molecular markers holds promise as a valuable diagnostic tool for clinicians in identifying and monitoring patients and lesions at risk of malignant transformation.

However, drawing definitive conclusions from the examination of a single patient within a relatively short follow-up period is not feasible. An ongoing trial, involving brushing cells collected at various intervals during the follow-up period from a substantial number of high-risk OSCC patients (including those with OL and those surgically treated for OSCC), is crucial to validate the potential of our procedure.

Two prospective studies with larger populations have been conducted. Specifically, 13-gene DNA methylation analysis from oral brushing has been applied on patients surgically treated for OSCC and on patients affected by OPMDs, respectively.

B) 13-gene DNA methylation analysis of oral brushing sample as a potential surveillance tool for periodic monitoring of treated patients with oral cancer

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Aims:

Oral squamous cell carcinoma (OSCC) stands out with one of the highest mortality rates among all cancers [123], largely attributed to a notable incidence of loco-regional recurrence. A subsequent cancer, encompassing local recurrences, lymph-node metastases, and second primary tumors, emerges in 20–50% of OSCC patients following multimodal therapy, contributing significantly to cancer-related deaths [35].

Managing recurrent OSCC remains a formidable challenge. Many authors consider surgical salvage as the primary option for recurrent OSCC due to the limited efficacy of chemo/radiotherapy for achieving loco-regional control [210,211], coupled with the need to balance these treatments against high toxicity [212]. Therefore, a timely diagnosis of loco-regional recurrence is crucial for achieving curative surgical excision and ensuring a favorable prognosis [213].

Standard methods for evaluating loco-regional control typically involve clinical assessment, incisional biopsy with subsequent histological examination, and imaging. However, repeated incisional biopsies are invasive and may not be suitable for the follow-up of previously treated OSCC patients. Additionally, imaging, while valuable, is characterized by its high cost and exhibits limitations in terms of specificity and positive predictive value [214].

The repeated analysis of OSCC biomarkers at adequate follow-up intervals using a minimally invasive sampling procedure may be an attractive strategy to evaluate loco-regional recurrence.

Normal mucosa with regular DNA methylation levels at the surgical site might undergo aberrant methylation before the recurrence of OSCC, as epigenetic alterations tend to manifest early in the process of carcinogenesis [155].

In a recent study, we devised a method for the early detection of OSCC, relying on the DNA methylation patterns of 13 genes derived from non-invasive oral brushing samples. These 13 genes exhibited abnormal methylation patterns in patients with OSCC or high-grade dysplasia, as demonstrated in prior studies [161,208,215–217].

In this project, the analysis of DNA methylation in the 13-gene panel was conducted on oral brushing samples collected at various intervals throughout the oncologic follow-up of patients who had undergone surgical treatment for oral squamous cell carcinoma (OSCC). The primary objective was to assess the correlation between changes in methylation levels and the occurrence of secondary OSCC. The study aimed to explore the predictive utility of the 13-gene DNA methylation analysis for identifying secondary oral carcinoma in individuals previously treated for OSCC.

Methods:

Study population: The study comprised 61 consecutive patients diagnosed with OSCC at the Department of Biomedical and Neuromotor Sciences, Section of Oral Sciences, University of Bologna. These patients underwent intent-to-cure surgical resection at the Maxillofacial Surgery Unit, Sant'Orsola Hospital, between 2014 and 2019. The research adhered to the principles outlined in the Declaration of Helsinki, and ethical approval was obtained from the local Ethics Committee (study number 14092, protocol number 899/CE). Informed consent was obtained from each participant.

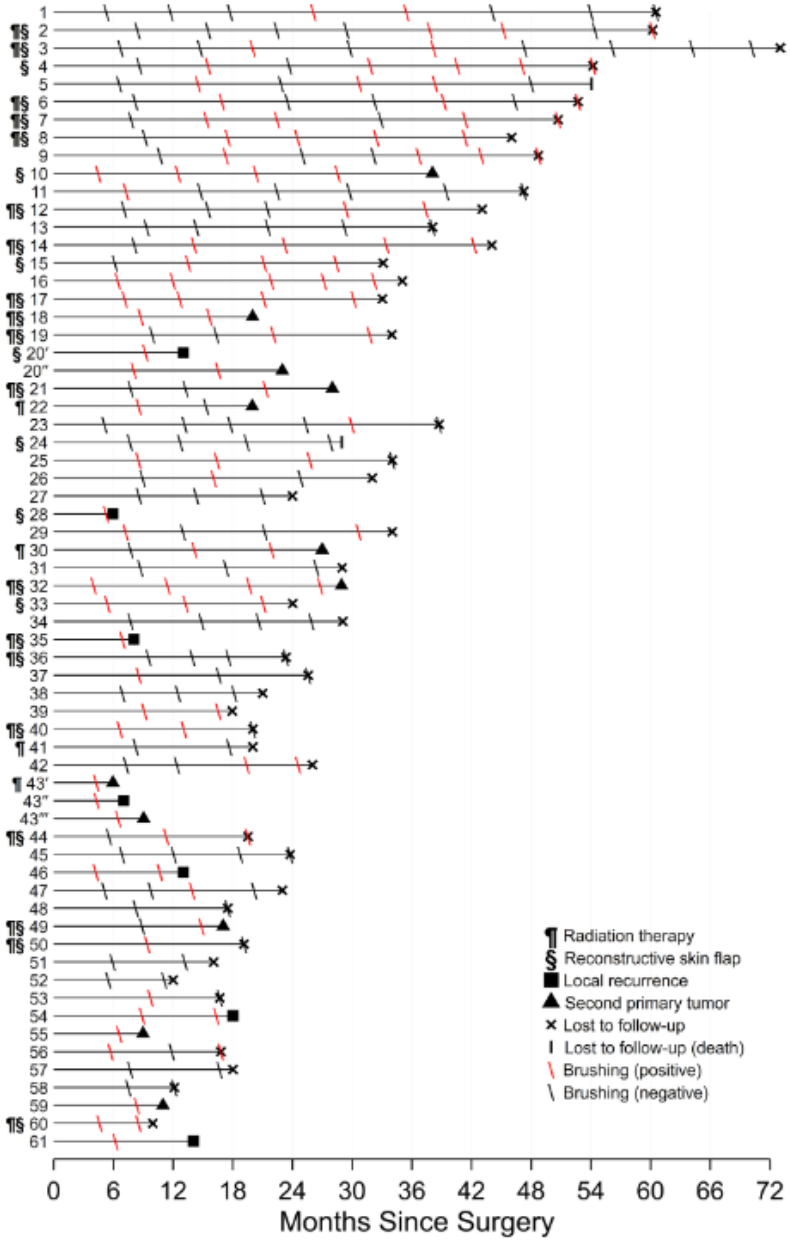
Standard surgical practices were followed for the surgical resection of OSCC as outlined by Kademani et al [12]. Histological analyses of preoperative biopsies and surgical specimens were conducted at the Sections of Anatomic Pathology of the University of Bologna and Sant'Orsola Hospital. The study specifically included OSCC patients who underwent complete surgical resection without margin involvement. All participants showed no clinical or radiographic evidence of relapse within 4 months post-initial treatment.

Subsequently, patients underwent routine follow-up, including clinical, instrumental, and radiological examinations, in accordance with the International National Comprehensive Cancer Network guidelines [130]. A multidisciplinary team, comprising ear-nose-throat specialists (ENT), maxillofacial surgeons, radiation and medical oncologists, and radiologists, formed an outcome review panel. During the follow-up period, patients received regular clinical and endoscopic evaluations. For patients with an advanced stage of disease and a high risk of relapse, head and neck computed tomography and magnetic resonance imaging were performed every 6 months during the initial 3 years. In contrast, patients with early-stage disease underwent clinical assessment, with imaging reserved for those exhibiting local symptoms or suspected local relapse. It's worth noting that some patients in this study were also part of previous studies [158,162].

Oral brushing sample collection: Prior to any cancer treatment, oral brushing sample collection and DNA methylation analysis were conducted to assess the presence of an altered methylation pattern in the tumor mass, following the protocol described by Morandi et al. [137,161]. During the follow-up period, oral brushing samples were collected from a broad regenerative area after the surgical resection of the index tumor, extending beyond the margins of surgical resection, irrespective of the type of surgery employed (with or without reconstructive tissue transfer for surgical repair after resection). In cases involving free-flap reconstruction of the surgical defect, gentle brushing was performed over a wide area, encompassing both the reconstructive tissue used for surgical repair and the adjacent oral mucosa. Sample collection, as per a previously established protocol [158,161,162], was repeated in each patient. Baseline brushing samples were obtained 4–10 months after the surgical resection of primary OSCC or after radiation therapy in the case of multimodal therapy. Subsequent samples were collected every 4–10 months unless relapse or censoring occurred (Figure 7). The mean time between samples was 7.3 ± 1.5 months, with a median of 7.4 months and an interquartile range of 6.2–8.5. Brushing specimens were collected during follow-up visits at the Department of Biomedical and Neuromotor Sciences of the University of Bologna, the Section of Oral Sciences, and the Maxillofacial Surgery Unit of Sant'Orsola Hospital between 2014 and 2019. Preoperative clinical information, including age, sex, smoking status, and tumor location, as well as pathological information and staging results for surgical specimens (primary

tumor type, regional lymph node involvement, tumor grade, depth of invasion (DOI), perineural invasion, resection margins, and tumor stage), were recorded in accordance with the 8th American Joint Committee on Cancer criteria [88]. Variables related to index OSCC treatment, such as postoperative radiotherapy and free-flap reconstruction of the surgical defect, were also evaluated.

FIGURE 7. Time-to-relapse chart depicting the individual follow-up periods observed in the study. Patients No. 20 and No. 43 developed two and three tumors during the study period, respectively, and for this reason were included multiple times in the study design.



Disease-free survival (DFS), defined as the interval between primary OSCC resection and the occurrence of new loco-regional neoplastic manifestations (including tumor

progression such as local recurrences [LR], lymph node metastases [LNM], or distant metastases) or as second primary tumor, as well as death, were assessed at the final follow-up visit in December 2019. LR and SPT were differentiated using the criteria of Hong et al. [209], representing a modification of the definition provided by Warren and Gates [41]. LR was defined as a second neoplastic lesion with the same histological features, appearing within 2 cm and occurring less than 3 years after the index tumor. SPT was defined as a second neoplastic lesion located at a distance greater than 2 cm from the index tumor or a second lesion occurring more than 3 years after the index tumor. Any histopathologic differences between the second and primary neoplastic lesions or the presence of Epithelial Precursor Lesions (EPL) associated with the second tumor supported the hypothesis of an SPT.

Thirteen-gene DNA methylation analysis: A 13-gene DNA methylation analysis was performed as described at page 46.

Statistical analysis: Quantitative variables are presented as means ± standard deviations, while categorical variables are expressed as frequencies and percentages. Disease-free survival (DFS) was estimated using the Kaplan–Meier method, with the date of surgery as the starting point.

The association between exposure (oral risk score) and outcome (relapse) was evaluated through a nested case–control design, akin to a case–control study within a cohort study [218]. Cases comprised patients who experienced relapse during follow-up, and for each case, four time-matched controls were randomly chosen from those in the cohort who had not experienced relapse by the time of disease occurrence in the case. The "risk-set sampling" or "incidence density sampling" technique was employed to ensure a comparable time window for measuring methylation scores between cases and controls [219]. Cases and controls were categorized into three mutually exclusive groups: persistently negative (scores persistently < 1.0615547), persistently positive (scores persistently > 1.0615547), and mixed (variable scores). Matched controls were excluded if no samples were available between the dates of surgery and matching. The associations between score groups and relapse were estimated using a logistic regression model employing Firth's method, which is similar to the penalization of the log-likelihood by the Jeffreys prior, addressing the issues of separation or quasi-separation [220].

Unconditional Firth-type regression analysis was conducted by controlling for the matching factor used in risk-set sampling (i.e., time of case occurrence), and the matched follow-up period was included as a covariate in the model [221]. In a secondary analysis, adjustment for confounders was enhanced by incorporating propensity scores based on baseline patient characteristics as additional covariates, including age over 70 years, smoking, and hard palate tumor location. These variables were considered potential confounders due to their significant association with the outcome ($p = 0.10$) in simple (crude) regression analysis. The results are presented as odds ratios (ORs). The regression analysis was replicated separately on local recurrences (LRs) and second primary tumors (SPTs), treating competing outcomes as censoring events. P-values were computed using the penalized profile likelihood method [220]. Finally, the regression analysis was repeated to confirm the impact of each methylation beta value included in the last available score before the matching date.

Data were analyzed using Stata (version 17.0; StataCorp., College Station, TX, USA) and R (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria) software [222]. The significance level was set at $p < 0.05$ (two-sided).

Results:

This study enrolled 61 patients, comprising 35 (57%) females and 26 (43%) males, with a median age at the initial presentation of oral squamous cell carcinoma (OSCC) of 66.8 ± 13.0 (range: 36–91) years. The distribution of patients based on tumour stage and lymph node involvement was as follows: 22 patients classified as T1N0M0, 12 as T2N0M0, 4 as T3N0M0, and 11 as T4N0M0. Among those with lymph node involvement, 2 had T2N2M0, 2 had T2N3M0, 1 had T3N3M0, 3 had T4N1M0, 1 had T4N2M0, and 3 had T4N3M0. Adjuvant postoperative radiation therapy was administered to 23 (38%) of the sixty-one patients.

Baseline prognostic variables: Figure 7 displays a time-to-relapse chart illustrating all individual follow-up periods included in the study. Over a median follow-up of 28.9 months (interquartile range: 18–36.5), 19 secondary tumours were diagnosed, with two patients (No. 20 and No. 43) developing multiple tumours (three and two, respectively). Consequently, there were 64 observations in total. The secondary tumours were categorized according to the criteria established by Hong et al. [209]: seven patients had

local recurrences (5/7 limited to the oral cavity, and 2/7 with oral cavity involvement and lymph node metastases), and 12 had second primary tumours developed in the oral cavity. Additionally, seven patients died due to disease progression, while two deaths were unrelated to the disease.

TABLE 8. Baseline characteristics at index OSCC manifestation (n = 61).

TABLE 1 Baseline characteristics at index OSCC manifestation ($n = 61$).

Characteristic	*n* (%)
Female	35 (57%)
Age >70 years	27 (44%)
Smoker	13 (21%)
OSCC location	
Gum	23 (38%)
Tongue	16 (26%)
Labial gingival mucosa	11 (18%)
Hard palate	8 (13%)
Soft palate	2 (3%)
Floor of mouth	1 (2%)
Grading	
1	27 (44%)
2	25 (41%)
3	9 (15%)
T3/T4 size and extent	23 (38%)
Lymph node involvement	12 (20%)
Radiation therapy	23 (38%)
Reconstructive skin flap	26 (43%)
Perineural invasion	9 (15%)
Vascular invasion	4 (7%)
Depth of invasion ≥4 mm	27 (44%)
Surgical margin	
Clear	57 (93%)
Close	3 (5%)
Dysplasia at surgical margin	1 (2%)

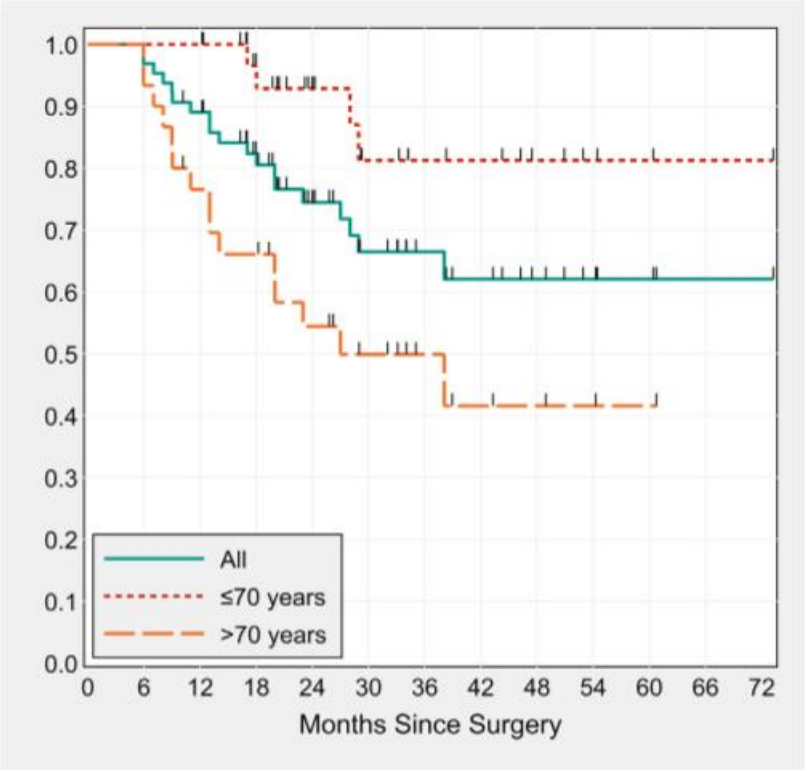
Abbreviation: OSCC, oral squamous cell carcinoma.

Thirteen-gene DNA methylation analysis: In the study, a pre-operative positive score was detected in all 61 OSCC patients in the population (mean value 3.77 ± 1.37). Figure 7 illustrates the analysis of 221 oral brushing specimens collected during the follow-up, with DNA amounts ranging between 100-500 ng. None of the clinical variables, including radiation therapy, were significantly related to a lower DNA amount. Nine

patients (14%) had a single oral brushing sample collected, all of whom experienced disease relapse. The remaining patients had multiple sample collections during follow-up.

Figure 8 presents the Kaplan–Meier Disease-Free Survival (DFS) curve. The log-rank test revealed that age > 70 years was associated with decreased DFS (p = 0.001). The relapse rate was 2.05 per 100 person-months among patients aged > 70 years compared to 0.39 per 100 person-months among those aged ≤ 70 years. A tumour located in the hard palate showed a significant negative association with DFS (p = 0.002), with an incidence rate of 3.78 per 100 person-months in this location compared to 0.86 per 100 person-months in other tumour locations.

FIGURE 8. Kaplan-Meier survival estimates of time to relapse after surgical resection of Oral Squamous Cell Carcinoma (OSCC), overall and by age group. The spikes indicate censoring times.



Cases were classified into persistently negative (n = 14, 22%), persistently positive (n = 20, 31%), and mixed (n = 30, 47%) score groups. Among the 30 patients with mixed profiles, 21 tested negatives after the first oral brushing sample collection performed at 4–10 months after OSCC surgical resection, while 9 tested positive. In these two groups, 3/21 (14%) and 1/9 (11%) second primary tumours (SPTs) were observed, respectively.

A pre-operative mean score of 3.14 ± 0.3 was calculated in the group of patients with persistently positive scores during the follow-up period, 2.8 ± 0.2 in the group of patients with mixed scores, and a mean score of 2.3 ± 0.4 was detected in the group of patients with persistently negative scores during the follow-up period. No correlations were found between pre-operative and post-operative scores during the follow-up period.

Table 9 presents the scores for the cases and matched controls. Controls were matched to cases at a ratio of 4:1 based on the follow-up duration. Among the 19 relapsed cases, 15 (79%) had persistently positive results before recurrence, whereas 4 (21%) had mixed results and none had persistently negative results.

TABLE 9. Distribution of brushing score results in cases and matched controls obtained via risk-set sampling; values are count (percentage) or mean ± standard deviation [range]

Brushing score	Relapse cases ($n = 19$)	Matched controls ($n = 66$)[a]
Results over follow-up period		
Negative	0 (0%)	28 (42%)
Positive	15 (79%)	23 (35%)
Mixed (both neg. and pos.)	4 (21%)	15 (23%)
Last result before matching date		
Negative	1 (5%)	32 (48%)
Positive	18 (95%)	34 (52%)

[a]Sample size is 66 instead of $19 \times 4 = 76$ because $76 - 66 = 10$ controls had no available cytological samples between the date of surgery and the matching date, and were thus discarded.

The regression analysis showed that compared to persistently negative patients, persistently positive patients had an almost 42-fold higher relapse likelihood (OR = 42.15, $p < 0.001$), and patients with mixed results had a 32-fold higher likelihood (OR = 31.96, $p = 0.006$). No significant differences were observed between the persistently positive and mixed groups, even after adjustment for baseline risk factors. Persistently positive patients had a 58-fold higher local recurrence (LR) likelihood and a 20-fold higher SPT likelihood compared to persistently negative patients.

Nearly all single methylation beta values included in the last available score before the matching date were significantly associated with increased relapse likelihood: ZAP70-16 (OR = 4.06, p = 0.000), GP1BB-1 (OR = 2.41, p = 0.010), MiR193-12 (OR = 2.02, p = 0.010), NTM-14 (OR = 2.00, p = 0.000), LRRTM1-3 (OR = 2.00, p = 0.000), KIF1A-22 (OR = 1.92, p = 0.000), PARP15-2 (OR = 1.60, p = 0.050), EPHX3-1 (OR = 1.60, p = 0.030), and LINC00599-1 (OR = 1.57, p = 0.040). These results indicate an increase in the odds of relapse of one standard deviation with an increase in the methylation beta values.

Discussion:

The study aimed to assess the utility of repeating a 13-gene DNA methylation analysis from oral brushing samples to determine the time-related risk of OSCC development and to identify local recurrences and second primary tumours. This approach represents a novel and minimally invasive tool based on DNA methylation analysis performed at different times to evaluate the risk of relapse during the follow-up of patients treated for primary OSCC.

The 13-gene DNA methylation analysis of repeat oral brushing specimens enabled the categorization of patients into three groups: persistently negative (n = 14; score < 1.06457), persistently positive (n = 20; score > 1.06457), and mixed (n = 30). This categorization was determined based on the methylation scores obtained from oral brushing samples collected at various intervals during the follow-up period.

The study's results revealed that patients with persistently positive (OR = 42) or mixed (OR = 32) scores had a significantly higher risk of OSCC relapse compared to those with persistently negative scores. Notably, none of the 14 patients with persistently negative scores developed a secondary tumour. In contrast, 15 of the 19 secondary carcinomas (7 local recurrences and 8 second primary tumours) had persistently positive scores during follow-up.

Among the surgically treated OSCC patients with mixed scores for the brushing samples collected every 6 months during follow-up, 21 (70%) of the 30 mixed results showed a negative score after the first oral brushing sample collection performed 4–10 months after OSCC surgical resection. However, the four patients with mixed results

who developed a SPT exhibited characteristic epigenetic alterations. Three of them initially had a negative result 6 months after OSCC treatment, but subsequent samples showed a positive result. This highlights a potential risk of underestimating the risk of a secondary tumour when relying on a single oral brushing sample, as evidenced by the case (No. 22) that developed a secondary event 5 months after a single negative test.

TABLE 10. Distribution of the scoring results during oncological follow-up in patients who developed multiple oral squamous cell carcinoma during oncological follow-up.

Cases	Index tumor date	Time distribution of oral brushing sampling collection, score calculation and secondary neoplastic manifestations					
Case 10	December 2015	April 2016: POS (2.19)*	December 2016: POS (1.45)*	August 2017: POS (1.35)*	April 2018: POS (1.26)*	February 2019: SPT‡	
Case 18	August 2016	April 2017: POS (2.76)*	November 2017: POS (1.18)*	April 2018: SPT‡			
Case 20	August 2016	May 2017: POS (3.39)*	September 2017: LR†	May 2018: POS (1.91)*	January 2019: POS (3.12)*	August 2019: SPT‡	
Case 21	September 2016	April 2017: NEG (−1.37)	October 2017: NEG (−0.56)	June 2018: POS (2.35)*	January 2019: SPT‡		
Case 22	September 2016	May 2017: POS (3.35)*	December 2017: NEG (0.49)	May 2018: SPT ‡			
Case 28	November 2016	April 2017: POS (8.88) *	May 2017: LR†				
Case 30	December 2016	July 2017: NEG (0.68)	February 2018: POS (2.08)*	September 2018: POS (4.06)*	March 2019: SPT‡		
Case 32	March 2017	June 2017: POS (1.27)*	January 2018: POS (2.54)*	September 2018: POS (3.04)*	April 2019: POS (1.79)*	July 2019: SPT‡	
Case 35	May 2017	November 2017: POS (2.34)*	January 2018: LR†				
Case 43	November 2017	March 2018: POS (1.15)*	May 2018: LR†	September 2018: POS (1.67)*	December 2018: SPT‡	June 2019: POS (1.64)*	September 2019: SPT‡
Case 46	December 2017	April 2018: POS (3.99)*	October 2018: POS (3.86)*	January 2019: LR†			
Case 49	March 2018	November 2018: NEG (0.96)	May 2019: POS (2.66)*	August 2019: SPT‡			
Case 54	June 2018	February 2019: POS (2.22)*	October 2019: POS (2.8)*	December 2019: LR†			
Case 55	June 2018	December 2018: POS (3.59)*	March 2019: SPT‡				
Case 59	August 2018	April 2019: POS (1.15)*	July 2019: SPT‡				
Case 61	March 2019	September 2019: POS (1.86)*	May 2020: LR†				

Abbreviations: LR†, local recurrence; NEG, negative test; POS*, positive test; SPT‡, second primary tumor.

The study discussed possible explanations for the changes in scores (positive and negative) in the mixed group. One hypothesis is that insufficient adult cancer stem cells or cancer cells might be present to repopulate the area of surgical intervention, leading to aberrant methylation patterns. Alternatively, tumour heterogeneity could contribute to

an insufficient number of altered epialleles crossing the threshold value, resulting in fluctuating methylation patterns over time.

TABLE 11. Odds ration estimates (p-values) for OSCC relapse obtained with unconditional Firth-type logistic regression; the full set of pairwise comparisons between the three exposure groups is presented.

	Reference: Low		Reference: High		Reference: Mixed	
Brushing score	**Unadjusted**	**Adjusted[a]**	**Unadjusted**	**Adjusted[a]**	**Unadjusted**	**Adjusted[a]**
Negative	1.00	1.00	0.02[b]	0.04[b]	0.03[b]	0.05[b]
	(·)	(·)	(<0.001)	(<0.001)	(0.006)	(0.020)
Positive	42.15[b]	28.12[b]	1.00	1.00	1.32	1.42
	(<0.001)	(<0.001)	(·)	(·)	(0.712)	(0.639)
Mixed (neg./pos.)	31.96[b]	19.75[b]	0.76	0.70	1.00	1.00
	(0.006)	(0.020)	(0.712)	(0.639)	(·)	(·)

[a]Controlled for age >70 years, smoking, and hard palate OSCC location via propensity-score covariate adjustment.
[b]Significant at the 5% level (*p*-value ≤0.05).

The study's results confirmed the predictive value of the 13-gene methylation analysis, with 18 out of 19 loco-regional relapses developing after a positive score on oral brushing sample collection. The intriguing finding of a negative score obtained before the development of a SPT in case No. 22 highlights the need for further studies to understand the implications of such cases. The study suggests that implementing a strict brushing sample collection interval, such as every 3–4 months, may enhance the ability of the 13-gene-based methylation analysis to identify patients at risk of secondary tumours.

Regarding the specific genes analysed, nine out of the 13 genes (ZAP70-16, GP1BB-1, MiR193-12, NTM-14, LRRTM1-3, KIF1A-22, PARP15-2, EPHX3-1, and LINC00599-1) showed a significantly altered methylation level in samples collected before the development of a secondary tumour compared to the remaining samples. This underscores the potential of DNA methylation as a molecular biomarker for detecting microscopic and histological cellular alterations after OSCC treatment.

The study also addressed two proposed mechanisms to explain the high rate of second neoplastic manifestations in the oral cavity. The concept of "field cancerization," introduced by Slaughter et al.[85], suggests the persistence of abnormal tissue after surgery, contributing to the high rate of second neoplastic manifestations. Additionally, the possibility of incomplete surgical resection of the primary tumour has been considered as a factor contributing to secondary oral cancer development [35].

The study's findings contribute valuable insights into the identification of epigenetic modifications associated with residual tumour cells or field effects responsible for LR or the development of a SPT after OSCC resection. The study suggests that the oral brushing cell collection procedure in a wide regenerative area is a minimally invasive approach capable of detecting these epigenetic changes, even when they may not be apparent in histological analyses of surgical margins.

Previous research has highlighted the significance of molecular alterations and dysplasia at negative surgical margins as risk factors for secondary neoplastic events.

The altered expression of genetic markers, including p53 [118,223–225] and hLy6D [226], as well as epigenetic markers, has been linked to the presence of minimal residual disease and local recurrence in the context of oral cancer.

Conversely, observations of dysplasia at the surgical margin of resection [105,227], loss-of-heterozygosity (LOH) [228], changes in the expression of Ki-67 [121,228], MMP9 and PTHLH [120] were noted in tumour-adjacent normal tissue, indicating associations with preneoplastic altered fields.

Notably, recent studies have elucidated a correlation between the presence of dysplasia at surgical margins of resection and the emergence of SPT [105,227]. Additionally, De Carvalho et al. have reported that altered expression of MMP9 and PTHLH in the analysis of negative surgical margins of resection is linked to the occurrence of SPT [120]. These findings underscore the significance of molecular markers in delineating the molecular landscape associated with oral cancer and its recurrence, providing valuable insights for clinical understanding and management.

The study proposes that oral brushing, despite its potential limitations in precisely identifying the extension of a preneoplastic field responsible for multiple tumours, offers a minimally invasive means to capture epigenetic modifications related to residual tumour cells or field effects.

The study's approach aligns with findings from other research demonstrating hypermethylation of specific genes in saliva samples collected post-diagnosis and treatment of primary OSCC [229,230]. This underscores the feasibility and value of oral

brushing samples as a non-invasive surrogate for tissue biopsies in profiling the epigenome of oral cancer.

However, the study acknowledges potential limitations, including the small study population and the relatively high proportion of elderly patients, considering the impact of aging on global genome methylation. Further investigation is warranted, especially in younger patients (<45 years old) with OSCC, to assess the reliability of the 13-gene DNA methylation analysis in this specific age group.

Conclusions:

The present study presents a promising application of a minimally invasive procedure involving the 13-gene DNA methylation analysis of oral brushing samples for the follow-up of patients surgically treated for oral cancer. The study indicates that a positive score on the methylation analysis exhibits high diagnostic accuracy, exceeding 90%, for the detection of emerging secondary neoplastic events. This suggests the potential utility of the 13-gene DNA methylation analysis as a valuable tool for monitoring patients post-surgery.

The observation of epigenetic instability in patients with oral squamous cell carcinoma (OSCC) further emphasizes the dynamic nature of epigenetic changes in the post-treatment period. This finding underscores the importance of understanding the temporal aspects of epigenetic modifications for effective monitoring and early detection of secondary neoplastic events.

Additionally, the study recognizes that biological factors potentially related to OSCC may influence the methylation status of surgically treated patients. Understanding these factors can contribute to a more comprehensive interpretation of the methylation analysis results and may facilitate personalized approaches to post-surgical monitoring.

In conclusion, the study provides valuable insights into the potential of the 13-gene DNA methylation analysis in the context of oral cancer follow-up. Future research with extended follow-up periods and refined surveillance intervals will be crucial for validating and optimizing the proposed methodology for clinical use.

C) Identification of the epigenetic profile of Oral Potentially Malignant Disorders for diagnostic and prognostic purposes via oral brushing and DNA-methylation analysis

(data not *published*)

Introduction:

The potential for OSCC screening exists, as there are recognized premalignant phases of the disease (oral potentially malignant disorders, OPMDs) during which high-risk individuals could be identified [231]. Currently, OPMDs are defined as any oral mucosal abnormality associated with a statistically increased risk of developing lip or oral cancer over the patient's lifetime [37].

OPMDs transform into oral cancers through various histopathological stages, progressing from hyperkeratosis and hyperplasia to various degrees of dysplasia (categorized as mild, moderate, or severe based on the presence and severity of cellular atypia and other structural changes in the epithelium), and ultimately to carcinoma in situ and invasive cancer. Histopathological evaluation of the grade of epithelial dysplasia is, still today, the most reliable method used to quantify the malignant potential of individuals with oral potentially malignant disorders [232].

Within the oral cavity, oral leukoplakia (OL), oral erythroplakia (OE), and oral lichen planus (OLP) are the most common OPMDs in Europe and North America. These lesions typically appear as white and/or red patches on the oral mucosa [233].

According to the WHO definition, OL is characterized as “A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer” [37,51,52].

As previously reported, the oral mucosa becomes white for and excess production of keratin as a response to injury (eg, frictional hyperkeratosis), bite trauma, excess production of keratin intrinsically from benign keratotic diseases (eg, genodermatoses as white sponge nevus or hereditary benign intraepithelial dyskeratosis), immune-mediated disease (eg, lichen planus) and oncogenic mutations (leukoplakia with dysplasia).

So not all white keratotic lesions on the oral mucosa are leukoplakias and the final diagnosis of OL involves a process of eliminating numerous lesions and disorders that can manifest within the oral mucosa [76].

“True” leukoplakia has been postulated to be the clinical expression of genetic and epigenetic alterations within the oral mucosa epithelium whose accumulation can facilitate the progression into OSCC. However, Currently, there's a lack of molecular techniques capable of distinguishing OPMDs, in particular OLs, from benign lesions and capable to identify the OPMD at risk of developing OSCC.

Recently, our research group has developed a non-invasive method to detect early-stage oral carcinomas through quantitative DNA methylation analysis of a 13-gene panel [161]. A specific algorithm has been developed and a score that exceeded a threshold value (1.0615547) was indicative of epigenetic alterations related to oral cancer.

In a previous paper, we observed epigenetic alterations in 100% of OL with presence of dysplasia, 57.1% of OL without presence of dysplasia and 16,7% OLP cases [158]. Moreover, 13-gene DNA methylation analysis successfully predicted the progression of one case of OL without dysplasia to malignant transformation [234].

The aim of this study was to establish an epigenetic profile that can differentiate OL from benign reactive oral lesions and to identify aberrant methylation patterns related to the progression of OPMDs to malignancy.

Materials and methods:

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the local Ethics Committee (study number 14092, protocol number 899/CE). All information regarding the human material used in this study was managed using anonymous numerical codes. Each participant gave informed consent.

Training Dataset:

We collected brushing specimens from 30 consecutive patients showing white and/or red oral lesions which underwent incisional biopsy and obtained a clinical and histological diagnosis of OPMD showing dysplasia. Lesions were considered dysplastic only in the presence of moderate/severe dysplasia following the criteria described in the

WHO/IARC Classification of Tumors, 2022 [235] . Additionally, we collected brushing samples from 50 consecutive patients presenting with benign reactive oral lesions (BRL). This cohort included 20 patients with oral white lesions due to frictional keratoses, 4 with oral fibroma, 10 with severe gingival inflammation caused by periodontitis, and single cases of Candida infection, bacterial infection, CMV infection, pyogenic granuloma, hairy leukoplakia, non-specific inflammation, hyperkeratosis related to smoking (3 patients), papilloma, pemphigus, pemphigoid, acute trauma (2 patients), non-specific ulceration, and a white sponge nevus lesion.

All The first two groups were composed by patients occurring to the Oral Medicine ward of the Dental Clinic of the University of Bologna for the first time after their general dentist advice to assess the nature of an oral white and/or red lesion spotted during routinary controls. All these lesions underwent oral brushing sample at presentation before definitive diagnosis was obtained. All samples of the present study were collected from October 1st, 2021 to October 31th, 2024 at the department of Biomedical and Neuromotor Sciences, section of oral sciences, University of Bologna. Histological examination was performed at the Department of Biomedical and Neuromotor Sciences, M. Malpighi Section of Anatomic Pathology at Bellaria Hospital, University of Bologna, Italy. All cases were examined by the same pathologist (M.P.F.)

As positive control group, 227 brushing specimens of OSCC patients were included into the training dataset, along with 245 healthy donors' samples serving as the negative control group. Positive and negative control group included samples analysed in previous studies [158,161,188].

Validation Dataset:

We validated our results using an independent and retrospective cohort comprising 60 cases, including clinically and histologically confirmed OPMDs referred to the Department of Biomedical and Neuromotor Sciences, Section of Oral Sciences, University of Bologna, Italy. Patients were enrolled from October 1st, 2015, to October 31st, 2023, with a mean follow-up period of 39.9 (10-96) months.

They consisted of 30 patients with a definitive clinicopathological diagnosis of OL and 30 patients with a clinical and histological diagnosis of OLP. The definition of OL

proposed by the WHO in 2017 [236] was used: 'A predominantly white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer'. All patients with a provisional OL diagnosis underwent histological analysis and a definitive diagnosis of OL was made when any etiological cause other than tobacco had been excluded and histopathology had not confirmed any other specific disorder as described by van der Waal et al. [237]. OLP histological diagnosis was based on the presence of irregular acanthosis, degeneration of the basal cell layer of the epithelium, and an inflammatory infiltrate in the upper chorion composed almost exclusively of mature lymphocytes. Brushing cell collection was always performed before incisional biopsy for histological assessment. All 60 OPMDs didn't show dysplastic alterations in histological analysis.

Brushing cell collection and 13 gene DNA methylation analysis:

A cytobrush was used to collect exfoliated cells from oral mucosa and DNA methylation was performed as previously described at page 56.

Quantitative DNA methylation analysis of the following genes was performed by next-generation sequencing (NGS): *ZAP70, ITGA4, KIF1A, PARP15, EPHX3, NTM, LRRTM1, FLI1, MIR193, LINC0059, MIR296, TERT and GP1BB*.

Statistical analysis

Each sample was analyzed as either a numeric or a dichotomous variable (positive/negative), according to the score generated from the algorithm and the cutoff value calculated previously (SG-OCRA^{TM}). In the training dataset Kruskal-Wallis analysis and Fisher's exact test were used to determine significant differences between group differences (OSCC, OPMD with dysplasia, BRL and healthy volunteers). For each CpG island, Kruskal-Wallis analysis and multiple range test with Bonferroni correction were performed to identify CpG islands with no significant differences in methylation levels of OPMD with dysplasia group and OSCC positive control group and with a significantly higher mean methylation level respect to BRL group and healthy volunteers. Receiver operating characteristic (ROC) curve analysis and a multiclass linear discriminant analysis have been calculated for CpG islands identified. In the validation dataset Kruskal-Wallis analysis and Fisher's exact test were used to determine

significant differences between group of OPMD without dysplasia and group of BRL using SG-OCRA ™ or the association SG-OCRA™-newly developed algorithm. Survival analysis has been also performed to identify predictors of malignant transformation in the group of OPMD without dysplasia. Survival rate was estimated using the Kaplan–Meier method. Statistical significance was evaluated using the log-rank test. Time was defined as the period between oral brushing cell collection and malignant transformation or the last follow up visit. SG-OCRA algorithm and the association between SG-OCRA and newly developed algorithm, other than other clinical and histological variables were analyzed for their relationship with the outcome of interest.

Only values of $p \leq 0.05$ were considered statistically significant in all analyses.

Results:

1.1. Training Dataset:

The training dataset included 30 patients with OPMD with dysplasia, 50 patients with benign oral lesions (BRLs), 227 patients with OSCC, and 245 healthy donors. Baseline demographic, clinical, and histological characteristics of the patients are summarized in Table 12a,b.

TABLE 12a. All demographic and site variables for training dataset are summarized.

Variables	Categories	OSCC (227 patients)	OPMD with dysplasia (30 patients)	BRL (50 patients)	Healthy donors (245 patients)
Age	<60	53	8	23	106
	>60	174	22	27	139
Gender	Male	111	16	25	120
	Female	116	14	25	125
Smoking habits	Yes	99	12	20	64
	No	128	18	30	181
Site	Tongue	103	14	12	0
	Cheek	42	4	15	245
	Gum	47	7	18	0
	Palate	35	5	5	0

Table12b. Clinical features for training dataset are here summarized

Diagnosis	Clinical features	Patients
OSCC (227 patients)	Exophytic	71
	Ulcerate	129
	Leukoplasic	27
OPMD with dysplasia (30 patients)	Homogeneous	14
	Non-homogeneous	9
	Verrucous	7

SG-OCRA^{TM} algorithm

207 of the 227 (91%) OSCC cases, 27 out of 30 (90%) dysplastic OPMDs, 15 out of 50 (30%) benign lesions and 19 out of 245 (8%) healthy donors were calculated as positive.

1.2. Development of a new algorithm:

To develop an algorithm useful to increase the specificity of our procedure we analysed a panel of 16 genes (*ZAP70, ITGA4, KIF1A, PARP, EPHX3, NTM, LRRTM1, FLI1, MIR193, LINC0059, MIR296, TERT, LINC0059, PAX1, miR137 and H19*), encompassing a total of 285 CpG islands. Specifically, Kruskal-Wallis analysis and Multiple Range Test with Bonferroni correction was performed to identify CpG islands that exhibited:

a) similar methylation levels between the OPMD with dysplasia group and OSCC;

b) distinct methylation profiles between the OPMD with dysplasia group and BRL group and the healthy volunteers;

c) not significantly different methylation level between BRL and healthy donors (an example of methylation profile can be seen in Figure 9);

Out of the 16 genes analysed, only 3 showed the previously described methylation profile CpG islands: LINC0059 (1 out of 20 islands: island number 9), miR-193 (1 out of 26 islands: island number 6) and GP1BB (12 out of 18 islands: island numbers 1-8, 10-13). ROC analysis was conducted on each of the 12 eligible CpG islands of GP1BB to assess differences between dysplastic OPMDs and BRLs. Island number 10 from GP1BB was chosen as the most discriminative (AUC: 0,759). The areas under the curve (AUCs) for the eligible CpG islands are summarized in Table 13.

As consequence, CpG island number 9 from LINC0059 and CpG island number 6 from miR-193 were selected for algorithm development.

FIGURE 9. Visualization of the eligible methylation profile of CpG island n.6 for the gene MIR193.

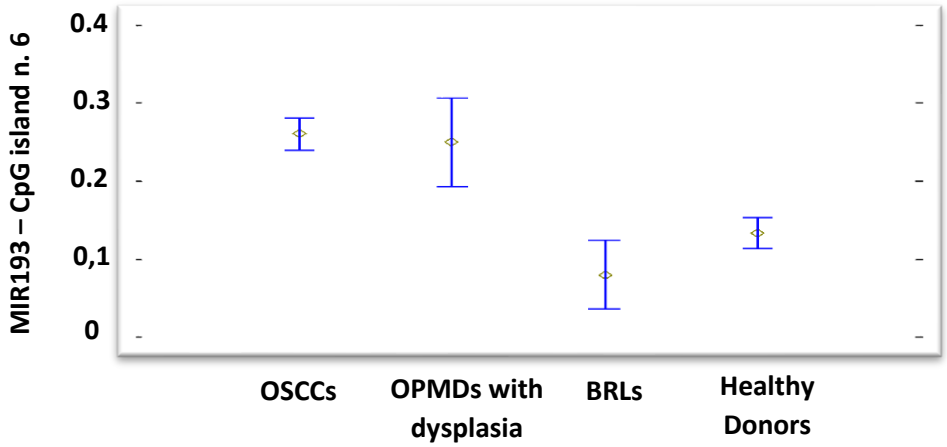


TABLE 13. Areas under the curves (AUCs) for eligible CpG islands. Selected CpG islands are highlighted in bold.

Gene	CpG Island	AUC
LINC0059	**9**	**0,694**
MIR-193	**6**	**0,655**
GP1BB	1	0,732
	2	0,741
	3	0,743
	4	0,757
	5	0,747
	6	0,748
	7	0,708
	8	0,729
	10	**0,759**
	11	0,707
	12	0,741
	13	0,743

A multiclass linear discriminant analysis (LDA) that weighted the contribution of each CpG island was employed to calculate the discrimination score. ROC curve analysis of these scores, differentiating dysplastic-OPMDs from benign oral lesion, yielded an area

under the curve (AUC) of 0.88. This analysis identified a threshold value of 0.6266667 as optimal for sensitivity and specificity, with rates of 57% and 94%, respectively.

Using this threshold, 17 out of 30 (57%) OPMD with dysplasia exceeded the threshold value, whereas only 3 out of 50 (6%) BRLs showed a positive score with the newly developed 3-gene algorithm.

2.1 Validation Dataset:

The validation dataset included 60 patients with OPMDs showing no dysplasia, with a mean follow-up of 39.9 months (range: 10-96 months). Specifically, 30 patients were diagnosed with OL and 30 with OLP.

During the follow-up period, 5 out of 60 (8,3%) of these lesions progressed: 1 developed high-grade dysplasia and 4 developed OSCC. Baseline demographic and clinical characteristics of the patients composing validation dataset are summarized in Table 14.

TABLE 14. Baseline demographic and clinical characteristics for validation dataset summarized.

Groups	Clinicopathological variables	Categories	Patients
OPMD showing no dysplasia (60 patients)	Age	<60	27
		>60	33
	Sex	Female	35
		Male	25
	Smoking habits	Yes	20
		No	40
	Clinical features	OL	30
		OLP	30
	Site	Tongue	14
		Cheek	35
		Gum	7
		Palate	4
	Clinical features	Homogeneous	22
		Non-homogeneous	32
		Verrucous	6

SG-OCRA™ algorithm identified a positive score in 23 out of 60 (38%) cases, while the newly developed 3-gene algorithm showed a positive score in 16 out of 60 (27%) cases. 13 out of 60 cases (22%) were positive with both the 13-gene and 3-gene algorithms.

Chi square analysis showed no significant difference between the group of BRLs groups of the training dataset and OPMD without dysplasia of the validation dataset when comparing the results of SG-OCRA™. Indeed, 23/60 were calculated as positive in the OPMD without dysplasia respect to 15/50 of BRL group (p=0.15).

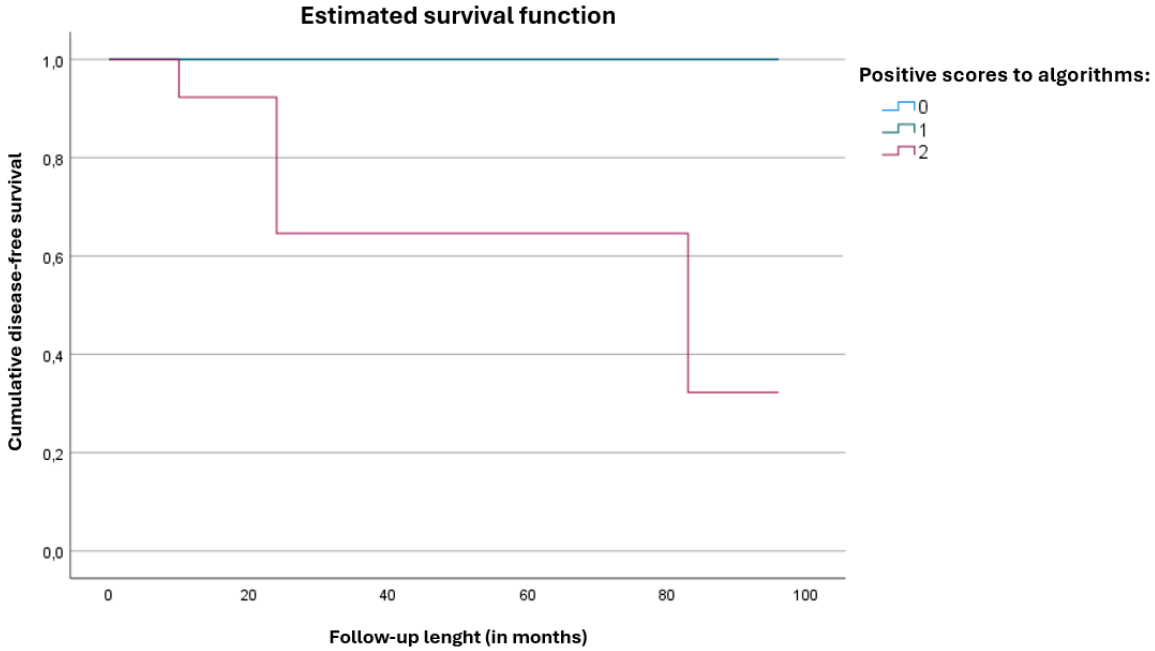
Conversely, Chi square analysis revealed a significant difference between the group of BRLs groups of the training dataset and OPMD without dysplasia of the validation dataset when comparing the results of SG-OCRA™ associated to newly developed 3-gene algorithm. Indeed, 13/60 OPMDs without dysplasia resulted positive with both algorithms respect to 3/50 of BRLs lesions (p<.02) (Table 15).

TABLE 15. Chi-Square Analysis Comparing BRLs from the training dataset and OPMDs without dysplasia from the validation dataset using the SG-OCRA and newly developed 3-gene algorithm

Algorithms	**OPMDs without dysplasia**	**BRLs**	**p-value**
SG-OCRA™	23/60 positive score	15/50 positive score	p=0.36
SG-OCRA™ associated to 3-gene algorithm	13/60 double positive score	3/50 double positive score	**p=0.02***

Finally, Kaplan-Meier analysis showed that a positive score of both algorithms was related to progression into OSCC or high-grade dysplasia. Indeed, all 5 lesions that progressed to high-grade dysplasia or OSCC showed a positive score with both algorithms, achieving a significant difference (Chi-Square = 22.3, P-value = 0.000009) compared to other groups showing one or no positive scores with the two algorithms (Figure 10).

FIGURE 10. Kaplan-Meyer curve displaying cumulative disease-free survival (Y axis) and follow-up length (X axis). Validation dataset population is stratified for the number of cumulative positive score to the two algorithms (0, 1 or 2). Log-rank analysis revealed significant differences among the three populations (chi-square 19.1, p. < 0.001).



Discussion:

Our research group previously developed a non-invasive method based on the analysis of 13-gene DNA methylation from oral brushing samples to correctly detect OSCC. In previous studies, this method successfully distinguished healthy oral mucosa from mucosa affected by OSCC and high-grade dysplasia [161,188].

The diagnostic value of 13-gene DNA methylation analysis in oral brushing samples was assessed in a multicentre Italian clinical trial, providing a sensitivity of 93.6%, a specificity of 84.9%, a Positive Predicting Value (PPV) of 86.6%, a Negative Predicting Value (NPV) of 92.8% and accuracy of 89.4%.

High sensitivity, also in presence of cT1-2 OSCC and lesions with high-grade dysplasia, suggests its clinical usefulness as a first-line screening tool, prior to invasive incisional biopsy, for oral cancer detection in the general population.

However, OSCC is often preceded by OPMDs, clinically presented as white lesions. Most white lesions are benign reactive lesions (e.g. frictional keratoses) or keratoses from inflammatory conditions (e.g., lichen planus) and the diagnosis is usually evident

from histopathology. At the opposite, leukoplakia is the term used for a white lesion that is precancerous and is defined by the World Health Organization (WHO) as ‘‘a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer.’’ To correctly predict oral cancer transformation, it is essential to recognize white lesions with a risk of malignant transformation compared to benign reactive lesions that carry no increased risk. For example, there is consensus on management or ‘‘best practice’’ guidelines for the management and treatment of white lesions whit presence of dysplasia, much less for leukoplakias that at histological level showed “hyperkeratosis with no evidence of dysplasia”. Therefore, there is a consensus that future research should prioritize the validation of potential biomarkers to ensure their specificity and sensitivity in diagnosing OPMDs [167].

In view of the high sensitivity and specificity of our procedure for OSCC, we attempted to identify an epigenetic profile capable in differentiating OL from benign reactive oral lesions and to identify aberrant methylation patterns related to the progression of OPMDs to malignancy.

In this study, the method was first tested on a training dataset comprising 30 “true” OPMDs exhibiting high-grade dysplasia and 50 benign reactive white and/or red oral lesions. In this scenario, 13-gene DNA methylation analysis from oral brushing (reported as SG-OCRA score) detected as positive 27 out of 30 (90%) dysplastic OPMDs and 15 out of 50 (30%) reactive benign oral lesions.

We identified 30% of false positive cases (15/50 BRLs detected as positive) and with the aim to increase the specificity of our procedure, we identified three CpG islands related to genes LINC0059, miR-193, and GP1BB that could discriminate dysplastic OPMDs from benign reactive lesions. Therefore, linear discriminant analysis was employed to generate a score that weighs these CpG islands, leading to the development of a new three-gene-based algorithm. When tested on the training dataset, this new algorithm demonstrated excellent specificity (94%) with a positive score only in 3 of 50 BRL cases.

Subsequently, we evaluated the role of SG-OCRA together with the score calculated from the newly developed 3-gene algorithm on a validation dataset comprising 60 low-risk OPMDs with no or low-grade dysplasia as these lesions, as previously reported, present significant diagnostic and prognostic challenges [238,239].

Notably, The SG-OCRA algorithm alone was unable to differentiate between OPMDs without dysplasia and reactive lesions (see Table 15). In contrast, a positive score of both algorithms, significantly differentiated two groups of specimens; indeed, 13 of 60 OPMDs without dysplasia compared to 3 of 50 BRLs.

Interestingly, a positive score for both algorithms resulted in a significant variable related to the malignant transformation of OPMDs without dysplasia. Indeed, all five OPMDs without dysplasia transformed during follow up (four evolved into OSCC and one into high grade dysplasia) presented double positive scores. This suggests that, with limitations related to the small sample size and relatively short follow-up period, that DNA methylation analysis from oral brushing may represent a potentially valuable tool for identifying OPMDs with a higher likelihood of transformation. Specifically, the combined approach of two algorithms enhances specificity and allows for a more focused identification of high-risk cases.

Further future studies are necessary to confirm these preliminary findings.

Conclusions:

The present study evaluated our non-invasive method based on oral brushing samples followed by DNA methylation analysis in populations comprising high-risk OPMDs, low-risk OPMDs, and benign reactive oral lesions. The 13-gene algorithm demonstrated a 30% false-positive rate when assessing reactive benign oral lesions. To increase the specificity of the procedure, a new algorithm was developed with the aim of correctly differentiating white lesions with the potential for malignant transformation from benign reactive oral lesions. The combination of the 13-gene DNA methylation score and the new algorithm correctly differentiated OPMDs without dysplasia from BRL lesion and identified OPMDs that evolved into OSCC during follow-up. If future studies confirm these preliminary data, our procedure may be proposed as a surveillance tool for the periodic monitoring of OPMDs.

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