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Massimo Petruzzi
Alberta Lucchese
Gianna Maria Nardi
Dorina Lauritano
Gianfranco Favia
Rosario Serpico
Felice Roberto Grassi

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Massimo Petruzzi,[a,]* Alberta Lucchese,[b] Gianna Maria Nardi,[c] Dorina Lauritano,[d] Gianfranco Favia,[a] Rosario Serpico,[b] and Felice Roberto Grassi[a]
[a]University of Bari "Aldo Moro," Dipartimento Interdisciplinare di Medicina, Sezione Malattie Odontostomatologiche–Policlinico di Bari, Piazza Giulio Cesare, 11- 70124 Bari, Italy
[b]Second University of Naples, Multidisciplinary Department of Medical-Surgical and Odontostomatological Specialties; Via L. De Crecchio, 6 80138 Naples, Italy
[c]University "La Sapienza" of Rome, Department of Oral and Maxillo Facial Sciences, Via Caserta, Rome, Italy
[d]University of Milano Bicocca, Department of Neuroscience and Biomedical Technologies, Via Cadore, 48, Monza, Italy

Abstract. The objective was to compare toluidine blue (TB) and autofluorescence (AF) for the detection of oral dysplasia and squamous cell carcinoma (OSCC) in clinically suspicious lesions according to conventional examination. Fifty-six clinically suspicious lesions were subjected to AF and TB examination. Data were compared using two different scenarios: in the first, mild dysplasia was considered as positive, while in the second, it was considered as negative. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), accuracy, and concordance were calculated. AF sensitivity and specificity were 70.0 and 57.7%, respectively, while TB showed a sensitivity of 80% and a specificity of 61.5%. The sensitivity increased in the second scenario in both AF (76.5%) and TB (88.2%). The specificity decreased in AF and TB, showing the same value (51.3%). PPV was higher in TB than in AF (70.6 versus 65.6%) and similarly for NPV (72.7 versus 62.5%). In the second scenario, TB PPV was 44.1% and NPV was 90.9%; AF PPV was 40.6% and NPV was 83.3%. TB showed greater accuracy than AF in the first scenario (62.5 versus 58.9%). AF and TB are both sensitive but not specific in OSCC and dysplasia diagnosis. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: 10.1117/1.JBO.19.7.076003]

1 Introduction

Survival rates for oral squamous cell carcinoma (OSCC) have not shown significant improvement over the past 50 years: the 5-year and 10-year relative survival rates are 59 and 48%, respectively.[1] This is paradoxical if we consider that an effective screening for OSCC is a simple noninvasive procedure, which needs only a 5-min visual inspection of the oral mucosa with lighting, gauze, and gloves.[2] Adjunctive screening technologies have contributed in the last decades to the decrease of death rates in several malignant pathologies. Additional noninvasive techniques for the OSCC have been proposed to increase the visual inspection sensitivity and specificity.[3]

Toluidine blue (TB) is the most frequently used adjunctive complementary technique to assess oral mucosal neoplastic disorders. Introduced in 1964 by Niebel and Chomet, TB can be considered as the dean of the auxiliary techniques employed in the detection of OSCC. TB is a basic thiazine metachromic dye; its acidophilic properties are responsible for its affinity for nucleic acids, and therefore, TB binds nuclear material of tissues with a high DNA and RNA content.[4] Positive lesions are stained in royal blue, while the negative ones appear pale blue or do not capture the dye.[5]

Autofluorescence (AF) induced by the Visually Enhanced Lesion Scope system (VELscope®; LED Dental Inc., White Rock, B.C.) is a manual device that detects the loss of fluorescence of dysplastic and neoplastic tissues by applying direct fluorescence. The loss of fluorescence is the consequence of a series of histological and biochemical alterations like a nicotinamide adenine dinucleotide increase (and consequential flavin adenine dinucleotide decrease), changes in collagen biochemistry due to the breakdown of the extracellular matrix by dysplastic cells, and neoangiogenesis.[6] It consists of a source of light that emits a wavelength of 400 to 460 nm and a manual unit for direct visualization. Under this light, normal oral mucosa emits a green AF, whereas pathological areas absorb the fluorescent light and appear dark. The loss of fluorescence is an indicator of tissue derailment in the neoplastic direction.

The aim of this study was to compare TB and AF in the detection of oral dysplasia and OSCC in clinically suspicious lesions according to conventional light examination.

2 Materials and Methods

2.1 Study Design

This is a double center, cross-sectional study, in which patients with suspicious premalignant and/or malignant oral mucosal lesions were observed in oral pathology and medicine outpatient settings. The clinic centers included in this study were from oral

*Address all correspondence to: Massimo Petruzzi, E-mail: massimo.petruzzi@uniba.it

medicine communities with heterogeneity in social and economic circumstances and for which local investigators had committed to providing good-quality data during the study follow-up.

2.2 Patients Recruitments

Patients were identified in clinics at two study sites: the Dental Clinic of the University "Aldo Moro" of Bari, and the Dental Clinic of the Second University of Naples and were enrolled after completing institutionally approved informed consent forms. Patients aged 18 years or over, male and nonpregnant, nonlactating women of all races able to provide written and informed consent were eligible for this study. Patients who had a history of oral lesions or were at high risk for an oral lesion were identified and asked to participate. After written consent was obtained, they were enrolled in the study. Patients who did not have a lesion identified during the conventional visual exam were excluded as well as patients with advanced and clinically obvious OSCC. The procedures followed were approved by the local ethical committee (n.4375) and in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2002.

2.3 Mucosal Examination

1. Conventional light examination: Suspicious lesions were first identified with a conventional visual examination under incandescent projected light. Suspicious lesions were considered to be leukoplakia, erythroplakia, leukoerythroplakia, and ulcers not related to trauma or autoimmune diseases. Lesions were photographed and data regarding the conventional visual examination were recorded.

2. AF examination: The VELscope® device was purchased from the Italian distributor (Mectron s.p.a. – Carasco, Genova) for LED, Vancouver, Canada. For evaluation of the suspicious lesions, the room was shaded and the hand piece covered with a lens cover. The excitation blue light was projected on the oral mucosa by a dichroic mirror. Through the back of the hand piece, tissue AF >480 nm was identified as green light. According to the literature, the loss of the normal tissue fluorescence was judged as a malignant or dysplastic alteration.[5] Red or orange fluorescence was not considered as malignant according to the literature.[7] VELscope pictures were rated as positive or negative by four experienced experts in oral medicine previously calibrated. The four experts involved in this study have routinely used the VELscope since 2008 and analyzed ~2000 patients prior to this study.

3. Toluidine blue staining: the TB staining test was performed using the TBlue® oral lesion marking system manufactured by Zila Inc. (Phoenix, Arizona). The staining procedure was carried out according to Mashberg recommendations.[8,9] The oral rinsing protocol was: 20 s prerinse with 30 ml of 1% acetic acid; 20 s water rinse; 20 s rinse/gargle with 10 ml of TB solution; 20 s postrinse with 30 ml of 1% acetic acid

(twice); a final water rinse. Only blue royal stained lesions were considered positive according to Gandolfo et al.[10] TB lesions were rated as positive or negative by four experienced experts in oral medicine previously calibrated in pairs.

2.4 Biopsy

A surgical biopsy was performed for histopathological assessment. All clinically identified lesions underwent biopsies irrespective of the findings with TB and the results of AF. Multiple biopsies were performed in multifocal lesions based on clinical findings; large lesions were excised *in toto*. All specimens were placed in 10% buffered formalin for fixation and then submitted for histopathological evaluation by a senior oral pathologist blinded to the clinical findings. We interpreted the histopathological outcomes in two different scenarios. In the first, positive lesions were considered: dysplasia (mild, moderate, and severe) and OSCC (*in situ*, microinvasive, invasive). In the second scenario, lesions that showed mild dysplasia were considered as negative.

2.5 Photo Documentation

Photo documentation was carried out with a Nikon D70s, equipped with a Nikkor 105-mm Macro Lens and Macro Ring Lite (Sigma EM-140 DG, Nikon Corp., Tokyo, Japan). For all white-light pictures, the same parameters for ISO, exposure time, and aperture were used (ISO 800, 1/60 s, f 22). The automatic white balance set used for the AF pictures was the same as that for the white-light pictures. For correct visualization and documentation of AF patterns, the examination room was darkened. A dedicated coupler (PhotoMed International, Van Nuys, California) was attached to the macro lens and the VELscope eyepiece was then fitted to the coupler. For all AF-light pictures, the same parameters for ISO, exposure time, and aperture were used (ISO 1600, 1/60 s, f 08). The magnification factor as well as the angle of documentation were adjusted to the clinical picture so as to allow correct identification of altered autofluorescence patterns.

2.6 Data Analysis and Statistics

Histopathological results were considered as the gold standard and TB and AF outcomes were compared to them. The χ^2 test (significance set at $p < 0.05$) was used for additional analysis for categorical variables and relationships in the contingency tables. In detail, the χ^2 test was used to test AF and TB properties (positive or negative) versus the gender, localization of the lesions, and histopathologic results (absence or presence of dysplasia/OSCC).

Global validation of the test results was established by calculating the sensitivity, specificity, and accuracy and both the positive and negative predictive values from contingency tables. The Cohen's kappa coefficient was used to evaluate the inter-rater agreement between (1) AF and TB, (2) histopathologic results and AF (in the first scenario), (3) histopathologic results and TB (in the first scenario), (4) histopathologic results and AF (in the second scenario), and (5) histopathologic results and TB (in the second scenario). Concordance was evaluated according to Landis and Koch who defined values <0 as indicating no agreement, 0 to 0.20 as slight, 0.21 to 0.40 as fair, 0.41 to
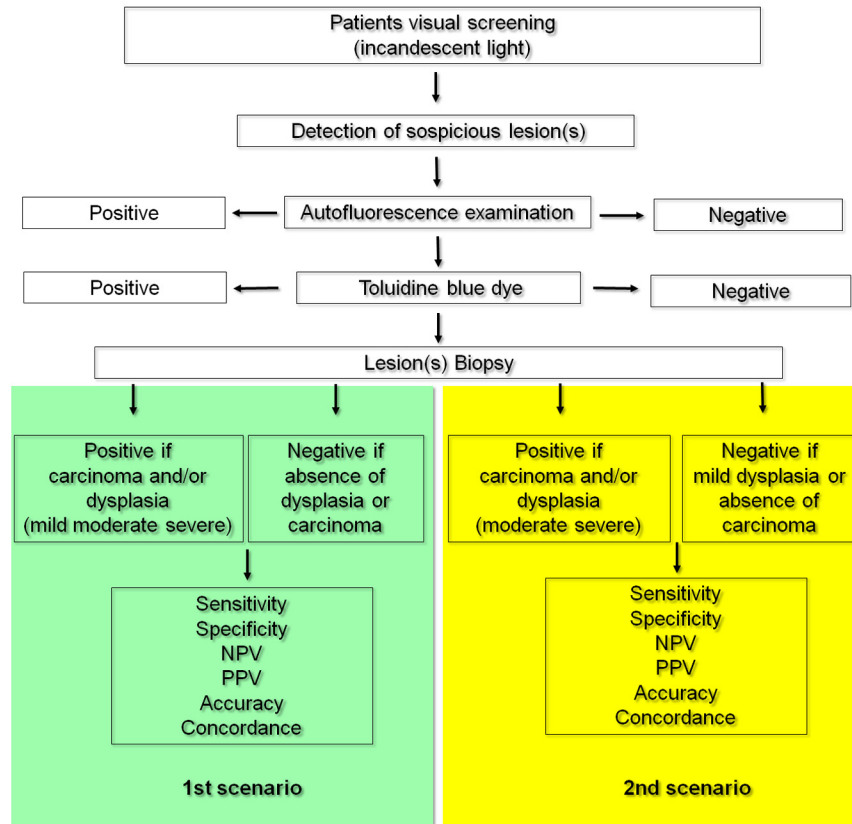
Fig. 1 The flow chart that resumed the study protocol.

0.60 as moderate, 0.61 to 0.80 as substantial, and 0.81 to 1 as perfect agreement.[11] Statistical data were analyzed both considering mild dysplasia as positive and then considering mild dysplasia as negative.

In Fig. 1, the study flow chart is represented.

3 Results

Between September 2012 and September 2013, 49 patients (22 female and 27 male) with an average age of 56.7 years were included in our study. Biopsies were performed in 56 lesions examined after the AF and TB examination. The histopathologic evaluation revealed the absence of dysplasia and/or OSCC in 26 lesions (46.4%). In the remaining 30 lesions, 13 (23.2%) were mild dysplasia, 2 (3.6%) were moderate dysplasia, 4 (7.1%) were severe dysplasia, and 11 (19.7%) were OSCC.

Patients' gender and lesion localization were not significantly related to AF and TB outcomes ($p > 0.05$). In the first scenario (mild dysplasia considered as positive), a statistically significant relationship existed between AF and histopathologic outcomes ($p < 0.05$), and TB and histopathologic outcomes ($p < 0.05$). In the second scenario (mild dysplasia considered as negative), only TB appeared significantly related to histopathologic features ($p < 0.05$), while AF examination displayed a border-line value ($p = 0.05$) when compared to histopathologic data. In Table 1 the above-mentioned data are summarized.

Table 2 shows the results concerning sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPP), and accuracy. Considering mild dysplasia as positive, AF examination showed a sensitivity and specificity of 70.0

and 57.7%, respectively, while TB showed a sensitivity of 80% and a specificity of 61.5%. The percentage of sensitivity increased in the second scenario (mild dysplasia as negative) in both AF (76.5%) and TB (88.2%). On the contrary, the specificity decreased in AF and TB, showing the same value (51.3%). PPV was higher in TB than in AF (70.6 versus 65.6%) as was the NPV (72.7 versus 62.5). If considered in the second scenario, PPV of TB was 44.1% and that of NPV was 90.9%; AF PPV was 40.6% and that of NPV was 83.3%. TB showed greater accuracy than AF in both the first (71.4 versus 64.3%) and second scenarios (62.5 versus 58.9%).

The strength of agreement between AF and TB as well as the concordance AF/histopathologic features and TB/histopathologic features are reported in Table 3. AF and TB showed a diagnostic agreement of 33.7%, which is considered to be fair. Similarly, the concordance AF/histopathologic features (in both the scenarios) and TB/histopathologic features in the second scenario revealed a low percentage of agreement (27.8, 22.2, and 30.8%, respectively). A moderate agreement was recorded between TB and histopathologic features in the first scenario with a concordance rate of 42.0%.

Examples of lesions evaluated with AF and TB are showed in Figs. 2 and 3.

4 Discussion

This is the first study, to our knowledge, that has compared TB and AF screening results with histopathologic findings in lesions deemed to be clinically suspicious according to conventional light examination. Of the 56 observed lesions, only 30 (56%) were characterized by dysplastic or carcinomatous

Table 1 Comparison between data obtained considering mild dysplasia as positive or negative.

N. lesions in gender	Total n (%)	AF + (%)	AF − (%)	p value	TB + (%)	TB − (%)	p value
Male	28 (50.0)	16 (57.1)	12 (42.9)	>0.05	15 (53.6)	13 (46.4)	>0.05
Female	28 (50.0)	16 (57.1)	12 (42.9)		18 (64.3)	10 (35.7)	
Localization							
Tongue	25 (44.7)	18 (72.0)	7 (28.0)	>0.05	17 (68.0)	8 (32.0)	>0.05
Buccal mucosa	18 (32.1)	11 (61.1)	7 (38.9)		8 (44.4)	10 (55.6)	
Gingival	6 (10.7)	3 (50.0)	3 (50.0)		4 (50.0)	2 (50.0)	
Palate	3 (5.4)	1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)	
Vestibule	4 (7.1)	2 (50.0)	2 (50.0)		3 (75.0)	1 (25.0)	
Dysplasia[a] or carcinoma[b] (I scenario)							
Yes	30 (53.6)	21 (70.0)	9 (30.0)	**<0.05**	24 (80.0)	6 (20.0)	**<0.05**
No	26 (46.4)	11 (42.3)	15 (57.7)		10 (38.5)	16 (61.5)	
Moderate, severe dysplasia or carcinoma (II scenario)							
Yes	17 (30.4)	13 (76.5)	4 (23.5)	0.05	15 (88.2)	2 (11.8)	**<0.05**
No	39 (69.6)	19 (48.7)	20 (51.3)		19 (48.7)	20 (51.3)	

Note: AF, autofluorescence; TB, toluidine blue. Bold values are statistically significant.
[a]Mild 13, moderate 2, severe 4.
[b]11 oral squamous cell carcinoma.

aspects: AF and TB also showed good sensitivity rates when mild dysplasia was considered as negative, but did not show sufficient specificity. The main cause for the limited specificity was the rate of false positive results. The accuracy of the two tested methods decreased in the second scenario, indicating that TB and AF were also able to detect the initial changes related to dysplastic progression. Data about the low percentage of concordance between AF and TB suggest their complementary employment. AF and TB can be used in sequence on the same lesion, summing the properties of both the techniques, because AF does not invalidate the vital dye.

Although conventional visual examination and tactile assessment of the whole oral cavity remains the gold standard for the identification of oral mucosal lesions, ancillary methods are useful to enhance the inspective capacity of the oral cavity in order to identify potential precancerous lesions as early as possible or to identify early OSCC. Both TB and AF are ancillary

Table 2 Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy percentage for autofluorescence and toluidine blue.

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
Considering mild dysplasia as positive					
AF	70.00	57.69	65.62	62.50	64.29
TB	80.00	61.54	70.59	72.73	71.43
Considering mild dysplasia as negative					
AF	76.47	51.28	40.62	83.33	58.93
TB	88.24	51.28	44.12	90.91	62.50

Note: AF, autofluorescence; TB, toluidine blue.

Table 3 Concordance percentage for autofluorescence and toluidine blue.

	Concordance[a]	Strength of agreement
AF/TB	0.337 (0.127) 0.088 to 0.586	Fair
Considering mild dysplasia as positive		
AF/Histopathologic	0.278 (0.129) 0.026 to 0.530	Fair
TB/Histopathologic	0.420 (0.121) 0.183 to 0.657	Moderate
Considering mild dysplasia as negative		
AF/Histopathologic	0.222 (0.110) 0.007 to 0.437	Fair
TB/Histopathologic	0.308 (0.100) 0.111 to 0.505	Fair

Note: AF, autofluorescence; TB, toluidine blue.
[a](Cohen's kappa coefficient) Mean (SE)$_{95\%}$ CI (range 0 to 1).
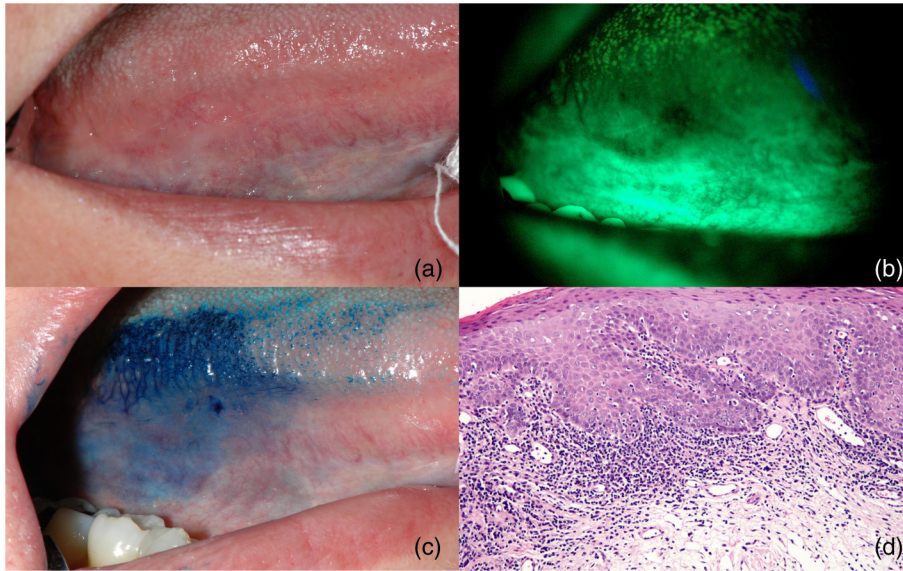
Fig. 2 (a) Clinical aspect of a lesion on the right border of the tongue. (b) The same lesion shows loss of fluorescence in a limited mucosal area. (c) Blue retention in the same area identified by the auto-fluorescence test. (d) The histopathologic exam of the lesion evidences mild dysplasia.

methods employed for this purpose. Different studies have separately evaluated the efficacy of AF or TB, but their direct comparison on the same lesions in the same patients had not yet been carried out.

Few studies have compared the different noninvasive diagnostic methods employed in the ancillary diagnosis of OSCC. Balevi, using a probabilistic statistical model, analyzed the PPV and false positive rate of AF, OralCDx, and TB in three clinical scenarios: total population, adults (≥40 years), and adults affected by visually obvious oral lesions. In this last population (not similar to our study population), the Balevi's results indicated a false positive rate of 98.68 (TB), 91.48 (AF), and 91.89 (OralCDx), while the PPVs were 1.32 (TB), 8.52 (AF), and 8.11 (OralCDx). The author concluded that TB staining, AF, and

OralCDx are not specific enough to distinguish noncancerous lesions from true cancerous lesions in the general population, but they may be beneficial in opportunistic screening programs or in cancer referral clinics when the pretest probability of oral cancer is likely to be >10%.[12] Patton et al., in their systematic literature review, found that the sensitivity of TB as a diagnostic adjunct varied from 38 to 98% (median, 85%) and the specificity varied from 9 to 93% (median, 67%). PPV ranged from 33 to 93% (median, 85%) and NPV from 22 to 92% (median, 83%).[13] Patton's review considered only two studies eligible for AF[6,14] in which the reported sensitivities of AF as an adjunct to visual examination were 98 and 100%; specificities were 100 and 78%; PPVs were 100 and 66%, and NPVs were 86 and 100%, respectively.
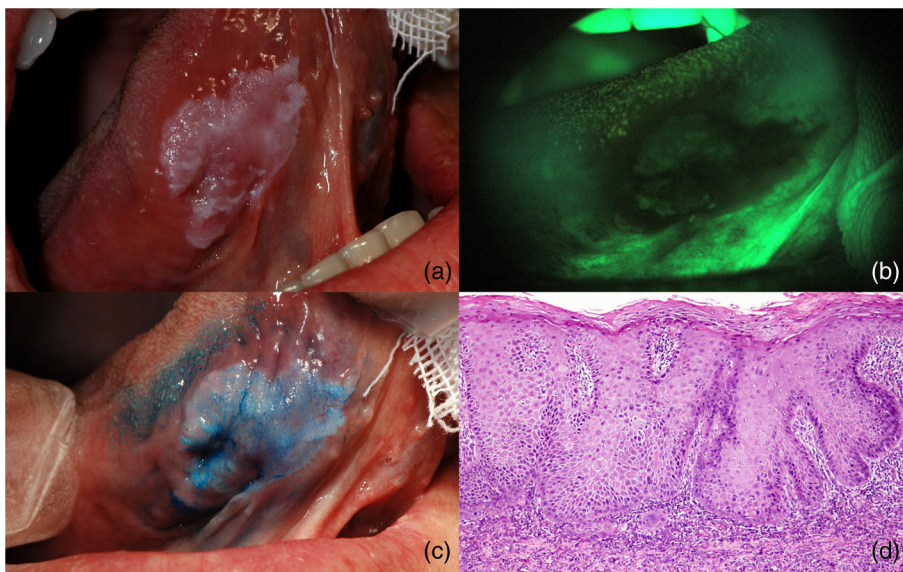

Fig. 3 (a) Leukoplakia of the tongue ventral surface. (b) The lesion shows a marked loss of fluorescence. (c) Blue royal staining in limited areas of leukoplakia. (d) The histopathologic exam of the lesion evidences mild dysplasia.

A direct comparison between AF and chemiluminescence plus TB was performed in a cross-sectional study in which the authors recorded a sensitivity of 0% and a specificity of 75.5% for the chemiluminescence, while AF showed a sensitivity of 50% and a specificity of 38.9%.[15] Patients submitted to the chemiluminescent examination were different from the AF ones while in the present study, the comparison was performed on the same lesions of the same patients. Also, when compared to TB, the chemiluminescent exam resulted in improving the brightness and/or sharpness of margin in 61.8% of identified suspicious lesions. Biopsied lesions with TB stain retention reduced a false positive rate of 55.26% while maintaining 100% for NPV.[16] A more recent cross-sectional study between TB and chemiluminescence suggests that TB retention test may be better suited than chemiluminescence to detect high-risk oral precancerous lesions.[17]

Güneri et al. designed a comparative study between TB and brush cytology in order to evaluate the diagnostic efficacy of the two tested methods.[18] Mild and moderate dysplasia were considered as negative. They found a concordance (TB/brush cytology) of 30% for benign lesions and 61% for malignant lesions and concluded that adjuncts identified 92% of carcinoma in situ and SCC, in contrast to clinical findings alone in which 62% of these lesions were identified.

We considered two different scenarios in this study: in the first, mild, moderate, and severe dysplasia, carcinoma in situ, or invasive carcinoma were considered as positive, while in the second, mild dysplasia was considered as negative. The rationale of this division was justified by two different meta-analysis data sets that provided evidence that the risk of developing invasive OSCC in mild, moderate, and severe dysplasia was ∼8, 13, and 24%, respectively, in 4.3 years.[19,20] An interesting debate about this topic arose from an article by Epstein et al., who analyzed oral lesion biopsies identified and evaluated by visual examination, chemiluminescence, and TB.[16,21]

One of the potential limits of our study is that it was conducted by clinical experts in oral medicine and oral pathology. As such, the results may not be generalizable to the general population of practitioners who are not familiar with assessing suspicious lesions on a regular basis. AF and TB are both sensitive but not specific to diagnoses of OSCC and oral dysplasia. However, in a tertiary care setting, they may be useful as an adjunct to conventional visual examination. One of the most important limitations in AF use is that the threshold between fluorescence loss and diminished fluorescence is arbitrary and related to the experience of the user. Similarly, in the TB use, the border line between royal blue and pale blue is very subjective. A computer-aided colorimetric analysis may eliminate the interobserver bias, as proposed by Maeda et al., who applied this method in unstained lesions surrounding OSCCs and oral potentially malignant disorders using iodine.[22] Our study design does not permit a full evaluation of the primary prevention performance of these tests but rather considers them in the tertiary care setting as a diagnostic aid in secondary and tertiary prevention of OSCC. Moreover, we designed a cross-sectional study in which the clinical expert first observed the lesion using AF and then observed using TB. So in this instance, all the AF information was available and could affect the TB determination.

A recent Cochrane review stated that "no robust evidence was identified to support the use of other adjunctive technologies like TB, brush biopsy, or fluorescence imaging within a primary care environment. Further randomised controlled trials are recommended . . . ."[23] Our study can be considered useful for the evaluation of the most suitable technique in the lesions inspection that appear suspicious at the conventional visual examination. Further studies with larger cohort of patients are needed to better understand the potentiality of the different ancillary methods employed in the OSCC prevention and early detection.

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Massimo Petruzzi is an assistant professor at the University of Bari "Aldo Moro," Italy. He is a postgraduate in oral pathology and medicine, and a specialist in oral surgery. He works at the Hospital Policlinico di Bari, Italy, in the section of oral pathology and medicine. His fields of interest are preneoplastic and neoplastic oral lesions (diagnosis, treatment, and prognosis) and oral autoimmune diseases (diagnosis, treatment, and prognosis). He is an active member of SIPMO (Italian Society of Oral Pathology and Medicine).

Alberta Lucchese is an assistant professor at the Second University of Naples (SUN), Italy. She is a postgraduate in oral pathology and medicine. She works at Policlinico di Napoli, Italy, in the section of oral pathology and medicine. Her fields of interest are proteomic applied to oral sciences and early diagnosis of oral cancer. She is an active member of SIPMO.

Gianna Maria Nardi is an assistant professor at the Università La Sapienza of Rome, Italy. She works at the Policlinico Umberto I of Rome, Italy, in the section of oral hygiene. Her field of interest is the new technologies applied to oral sciences.

Dorina Lauritano is an assistant professor at the University of Milano Bicocca, Italy. She works at the Ospedale San Gerardo di Monza, Italy, in the section of oral pathology and medicine. She is a member of SIPMO. She is the scientific coordinator of the Post Graduate in Oral Medicine and Laser Therapy at University of Milano Bicocca.

Gianfranco Favia is a full professor of oral pathology and medicine at the University of Bari "Aldo Moro." He is a specialist in surgery and anatomical pathology. He is the director of the dental clinic of the Policlinico di Bari. His fields of interest are head and neck cancers (clinical and pathological aspects), bone osteonecrosis (BRONJ) and head and neck vascular malformation. He is an active member of SIPMO.

Rosario Serpico is a full professor of oral pathology and medicine at SUN, Italy. He works at the Policlinico di Napoli, Italy, as the director of the section of oral pathology and medicine. His fields of interest are oral immunopathology, oral cancer screening, and epidemiology. He is an active member of SIPMO.

Felice Roberto Grassi is a full professor of periodontology and implantology at the University of Bari "Aldo Moro," Italy. He works at Hospital Policlinico di Bari, Italy. His fields of interest are the new technologies applied to oral sciences and oral surgery.