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PHOTODYNAMIC THERAPY AGAINST MULTIDRUG-RESISTANT ORAL STRAINS OF *CANDIDA SPP.*

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Photodynamic therapy against multidrug-resistant oral strains of *Candida spp.*

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ABSTRACT

Introduction. Candida species are a common cause of severe oral infections that have recently proven resistant to antifungal drugs. The emergence of multidrug-resistant (MDR) strains of *Candida spp*. stresses the need for the development of new antimycotics without collateral events for systemic health. Infection with *C. glabrata* and *C. albicans* is present in over 60% of prosthesis wearers, who, often being elderly patients, often have non-optimal health conditions. Therefore, there is a need to find an effective solution that does not further weaken the body. Photodynamic therapy (PDT) is an established treatment for various oral infections, and it involves the presence of three elements: (i) a photosensitizer that adheres specifically to microbial cells; (ii) light with a specific wavelength for each photosensitizer; and (iii) the presence of oxygen. The reaction between these three elements determines the formation of singlet oxygen and free radicals, which selectively kill the microbial cells.

Aim. The aim of this thesis is divided into three phases: 1) *in vitro* study to evaluate the effectiveness of 3 different wavelengths (660, 630, and 460 nm) in combination with 3 different commercial photosensitizers (Methylene Blue derivative, Toluidine Blue, and Curcumin+ Hydrogen Peroxide) on Candida colonies in collaboration with Oral Biotechnology Laboratory, University of Cagliari (*in vitro* study 1); 2) evaluation of new types of natural photosensitizers (olive oil, lactoferrin, saffron, pompia and strawberry juice) in PDT against *Candida spp*. through a series of in vitro studies. This step also comprised a model in human cell culture infected with *C. albicans* (*in vitro* study 2-3); 3) clinical evaluation of the effectiveness of photodynamic therapy protocols with commercial photosensitizers through a case series of treated patients with oral refractory candidiasis.

Methods *in vitro studies:* To evaluate the commercial photosensitizers (PS) and the natural elements, oral MDR isolates of *C. albicans, C. krusei, and C. glabrata* were assayed by the Kirby Bauer test, performed in accordance with EUCAST protocols. To evaluate commercial PS, an experiment with the microplate assay method has been performed. An *in vitro C. glabrata* subprosthetic candidiasis infection model to evaluate the efficacy of PDT with the curcumin-based photosensitizer has also been realized.

Specific protocols for the natural photosensitizer:

Olive Oil test. For each strain, four different combinations were evaluated: (i) 100 µl EVOO oil; (ii) 100 µl EVOO oil previously activated with 3% H_2O_2 (EVOO-H); (iii) 100 µl EVOO oil + 5 minutes of polarized light (380-3400 nm, 25 Watt), (iv) 100 µl of (EVOO-H) irradiated with the same light; (v) 100 µl of EVOO + 5 minutes of 660 nm diode laser irradiation ; (vi) 100 µl of (EVOO-H), + 5 minutes of 660 nm diode laser irradiation.

Lactoferrin test. 20 mg of bovine lactoferrin was dissolved in 1 mL of Sabouraud broth; this solution was used in four different combinations and tested against each strain: (i) solution as it is; (ii) solution activated with 3% H_2O_2; (iii) solution activated by light at 310-350 nm; (iv) solution activated both with 3% H_2O_2 and light at 310–350 nm (the maximum light absorption was previous evaluated by spectrophotometer).

Saffron test. A solution of 0,3% of Saffron dissolved in distilled water was used, the maximum light absorption evaluation was at 430 nm diode light. For each strain, two different combinations were evaluated: (i) saffron solution alone; (ii) saffron solution activated by a diode light at 460 nm, 7 watts of power for 1 minute. The Petri dishes in all *in vitro* tests described were then incubated at 37 °C for 48 h.

Cell culture and miRNA evaluation. Recent data underline the role of miRNAs as predictive markers for Candida infection. In this work, we infected HEK cells with a MDR

strain of *C. albicans*, and 1 and 2 minutes after PDT, we evaluated the expression level of miRNAs 146a and 155 in the culture RNA extract by real-time PCR (relative quantitation).

Methods in Clinical study: patients with a diagnosis of refractory Candidiasis have been treated with commercial PDT protocols, with follow up at 1 week and 1 month after. A healing evaluation through photo documentation was recorded.

Results: Commercial PS. The halos of inhibition with Toluidine Blue 1% and Methylene Blue 1% activated by lights at 630 and 660 nm were equal to 0, while the halo of inhibition relating to the curcumin-based photosensitizer was 46 mm. The latter, if activated by polarized light between 380-3400 nm was even more effective than activation with dedicated diode light at 460 nm. Prosthetic device infected with *C.glabrata* decreased the CFU values from 1500/cm^2 to 20 CFU/cm^2 after the PDT session.

Olive Oil Test. The clinical isolates of *Candida spp*. have shown different behaviour with the different assayed experimental groups. While *C. glabrata* resulted sensitive with all combinations, with a 50% increase of inhibition haloes by using polarized light. *C. krusei* was insensitive. *C. albicans* resulted inhibited only with (EVOO-H) light-activated.

Lactoferrin test. Group (iv): LF, H_2O_2, and light showed the best results, with inhibition haloes ranging from 30 to 40 mm for all analysed strains. The group (iii) (LF+ light) showed halo of inhibition of 30 mm against C. glabrata and 22 mm of *C. albicans*.

Saffron Test: Only the solution activated by the 460 nm light showed activity against *C. krusei*, with a halo of inhibition of 20 mm. No antimicrobial activity was shown against *C. albicans* and *C. glabrata.*

Cell culture and miRNA. The expression level of two miRNAs was significant in comparison with the negative control; in fact, infected cells represented an expression

level ranging from 2 to 3-fold higher than non-infected cultures. In addition, after PDT, a 50% decrease in miRNA 146a and 155 expression patterns was observed after 2 minutes of PDT treatment. This suggested a possible role of PDT in modulatory effects against inflammatory status due to Candida infection.

Clinical evaluation: 12 patients were treated; 7 cases of angular cheilitis (PDT effective with all the 3 commercial photosensitizers); 2 cases of chronic denture stomatitis (1 refractory to Toluidine Blue 1% protocol and without complete healing with the curcumin-based PS protocol), 1 case of chronic hyperplastic candidiasis, 1 case of oral leucoplakia infected with *Candida spp*. (successfully treated with PDT) and 1 case of Candida infection under orthodontic device (curcumin-based PS protocol). In 9 cases we have a clinically complete healing, in 3 cases we have partial results (denture candidiasis and orthodontic-associated candidiasis).

Conclusions: Among the commercial photosensitizers tested, only the curcumin-based PS performed well both *in vitro* and *in vivo*. Olive oil alone or activated with H_2O_2 could be considered a very performant photosensitizer against drug-resistant Candida spp. if illuminated by polarised light. Solutions based on H_2O_2 and lactoferrin can be considered a promising PS in PDT and in the eradication of *Candida spp*. in MDR. Saffron solution at 0.3% could be used against the *C. krusei* infection. Among those tested, the mixture of lactoferrin and hydrogen peroxide is the most effective natural photosensitizer. Further studies could combine EVOO, which has shown excellent results against *C. albicans* and *C. glabrata* but not against *C. krusei*, with saffron, which has shown good activity against *C. krusei.* In this way, a completely ingestible photosensitizer could be obtained, which can prove to be a very useful tool in PDT for paediatric and non-cooperative patients without the risks associated with accidental ingestion. The decrease in the microRNA expression pattern through the experiment on infected cell cultures after in vitro PDT demonstrates the effectiveness of the therapy, its safety, and lays the foundations for a new method of evaluating fungal activity *in*

vivo. Clinical evaluation showed better results than *in vitro* studies on the Methylene Blue PDT protocol, and sub-prosthetic candidiasis has been the most difficult type of fungal infection to eradicate.

Chapter 1 - General introduction

Following different sanitary reports in worldwide, every year 1.6 million people are killed by fungi infections [1], and the yeast-like microorganisms *Candida albicans* causes more than 150 million mucosal infections and over 200,000 fatalities annually as a result of invasive and widespread illness. For example, economically, the cost of treating Candida infections in the United States each year is around $2 billion [2], and the cost per person in the European Union is comparable. About 75% of all Candida infections are caused by *C. albicans*, which represents a significant burden on global health and is getting worse. Candida typically coexists with healthy oral mucosa and other parts of the body like the throat, gut, and vagina without posing any health risks. If it spreads unchecked or if it gets deep into the body, Candida can result in infections. It might, for instance, lead to serious oral infections or of internal organs tissues like the kidney, heart, or brain (invasive candidiasis).

In this context oral cavity is a unique ecological niche for microbial colonization, and Oral fungal infections are present in humans for millennia, e.g. Hippocrates (ca. 460–370 BCE) described two cases of oral lesions, similar to aphthosis, in debilitated patients that could be considered oral candidiasis forms [3].

The mouth is an entry portal for the human body through which air, solids, and liquids pass. The oral microbiota is the set of microorganisms that inhabit the mouth. Fungi are a minor component of the oral microbiota but can cause several types of damage

to the oral mucosa and the mouth [4]. Candida species are considered the most present type of fungi in the oral cavity, but other less common fungal infections can develop oral manifestations such as mucormycosis, aspergillosis, blastomycosis, histoplasmosis, cryptococcosis, and coccidioidomycosis [4]. Often, the synergistic action of several types of fungal microorganisms can create an oral mucosa infection [3]. Candida is a unicellular, dimorphic (blastospore and mycelium) eukaryote cell with sexual or asexual reproduction i.e., by bunding. Outside its cell membrane, there is an external cell wall. The plasma membrane contains large quantities of ergosterol. The most common species are *Candida albicans* (the most frequent), *C. glabrata*, *C. tropicalis*, *C. krusei*, C. *parapsilosis*, *C. guilliermondii*, and *C. dubliniensis*. In the oral cavity, the rate *Candida albicans*/non albicans species is 68/32%. *Figure 1*. Instead, in patients with candidemia, the association is similar, except for the *C. krusei*, *Figure 2*, [5,6].

In the oral cavity, we can find more than one type of Candida; the mixed colonization with *C. albicans* and *C. glabrata* has been found to be very frequent and is associated with a higher probability of tissue damage [5]. Some authors have demonstrated that *Candida spp*. could have a mutualistic relationship with other bacteria i.e. Streptococci, in particular with: *S. mitis, S. oralis, S. gordonii, and S. sanguis.* Other researchers have found a co-aggregation between *Candida spp., Porphyromonas gingivalis,* and *S. mutans,* which could favor the development of periodontic infections and tooth decay [3]. *Lactobacillus spp*., instead, can modulate the Candida infection, and for this reason, *L. helveticus and L. plantarum* have been proposed as probiotics against oral Candidiasis [3]. The most common site of adhesion in the oral mucosa is the tongue, but a very important role for the spread of this fungus is the saliva [4]. It has been reported that in 45% of newborns, 45–65% of healthy children, 30–45% of healthy adults, and up to 74% of older people, *Candida spp*. could colonize the oral cavity [4]. Saliva contains several ions, including sodium, potassium, cadmium, chloride, bicarbonate, and phosphate, responsible for the buffering properties against *Candida*

spp. that grows in an acid ph. All those conditions that involve a reduction in salivary flow or a qualitative alteration of saliva predispose to a greater probability of developing candidiasis, such as xerostomia from radiation therapy, Sjogren's syndrome, diabetes not compensated, oral prostheses, smoking, and bad oral hygiene [3-8]. Other conditions that can favor the mycotic spread are prolonged use of antibiotics, debilitating diseases such as HIV infection, organ transplantation, and cancer. The adhesion of *Candida spp*. is mediated by bacterial components and some mycotic proteins such as ALS2, ALS3, and others that are responsible for the transformation in the hyphae form. When the fungus is in this form, it can invade the oral mucosa barrier directly through ALS3p anchorage or receptor-induced endocytosis. The presence of candidalysine is the most important factor for epithelial tissue damage [8]. The integrity of the mucous tissue, the ability of the immune system to produce interleukins, in particular IL 17, the salivary buffering capacity, and bacterial eubiosis can very well counteract adhesion and invasion of the oral epithelium [8].

One of the most important local factors promoting Candida colonization is the presence of dentures. On the adults, a percentage between 60% and 100% of denture wearers have Candida in their mouth, which is interesting data overall if compared with non-wearers patients [9]. Protheses determine an alteration in the flow of oxygen and saliva to the underlying tissues, creating an acidic, anaerobic environment that is conducive to Candida growth. Mechanical features of the acrylic denture, such as porosity and hydrophobicity, must be taken into consideration as factors that promote the Candida infection [10]. Since prosthesis wearers are mainly elderly subjects, they are certainly also the age group of the population most frequently affected. Non-albicans Candida species have been found overall in 80-year-old patients compared to younger patients [11], Link notes 1,2.

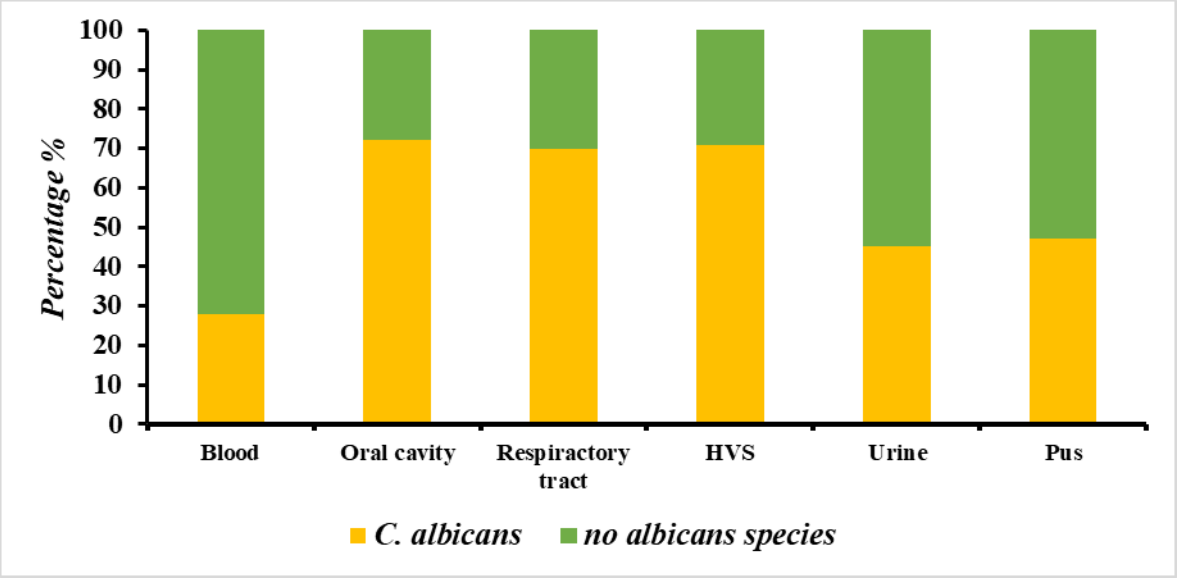

Fig.1. Distribution % of *Candida albicans* and non-albicans isolated from clinical samples analyzed in USA from 2005 to 2013 [5].

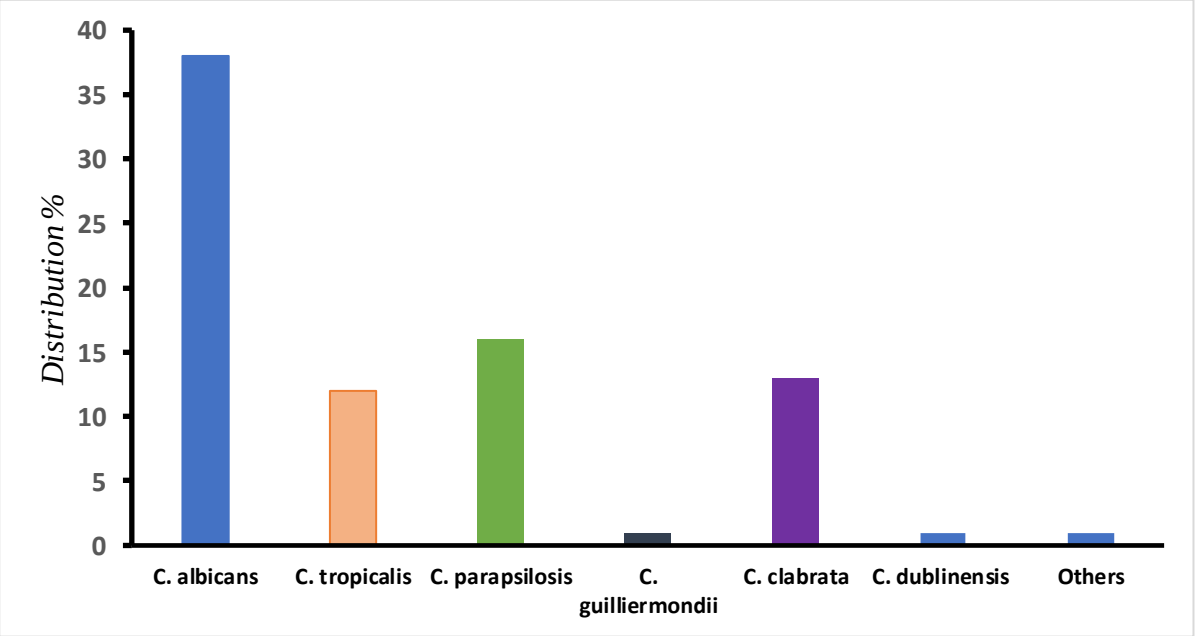

Fig.2. Distribution % of *Candida* species. responsible for candidiasis in humans [6].

Chapter 2 - *Candida spp.*: biology ecology and pathogenesis in the host

As described before, this genus can exist in yeast or multicellular (hyphae, pseudohyphae) forms. This microbe's ability to flip between many phenotypes makes it unusual. In fact, multiple occurrences of the spontaneous transition between the two phenotypes are possible and are due to environmental changes or biological host stimuli, i.e., oral microbiota profile changes or the host immune motif. Studies now provide strong evidence that changes in the regulation of gene expression are the cause of these phenotypic transitions, which include a very complex biochemical pathway. The pathological species, e.g., *C. albicans*, have a phenotype that includes white, smooth colonies, but the alternative phenotypic variant has flat, grey colonies. The two types express antigens differently and have different tissue affinities. *Candida spp.* is extremely adaptable to environmental changes thanks to this evolutionary benefit. In the same way, these phenotypic-genotypic variations are included in the dramatic shift from commensal to pathogenic [12]. A pathogenic sign in this fungus, is characterized by the initial expression of adhesins, when yeast cells attach to the surfaces of host cells. The yeast-to-hypha transition and directed growth via thigmotropism are triggered by contact with host cells. Through increased endocytosis, invasin expression mediates the host cell's uptake of the fungus. It has been suggested that adhesion, physical pressures, and the production of fungal hydrolases can help the second type of invasion, or fungal-driven active penetration into host cells, by dismantling barriers. The creation of biofilms with yeast cells in the lower half and hyphal cells in the upper part can result from the adhesion of yeast cells to abiotic (like dentures) or biotic (host cells) surfaces. It has been reported that *C. albicans* phenotypic flexibility (switching) affects the antigenicity and biofilm

development in the host. Along with these virulence features, a number of fitness parameters also affect fungal pathogenicity. In particular we can observe:(i) a strong stress response mediated by heat shock proteins (Hsps); (2) auto-induction of hyphal formation through amino acid uptake; (3) excretion of ammonia (NH_3) and concurrent extracellular alkalinization; (4) metabolic flexibility and uptake of various compounds as carbon (C) and nitrogen (N) sources; and (5) uptake of essential trace metals like iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn). *Figure 3.*

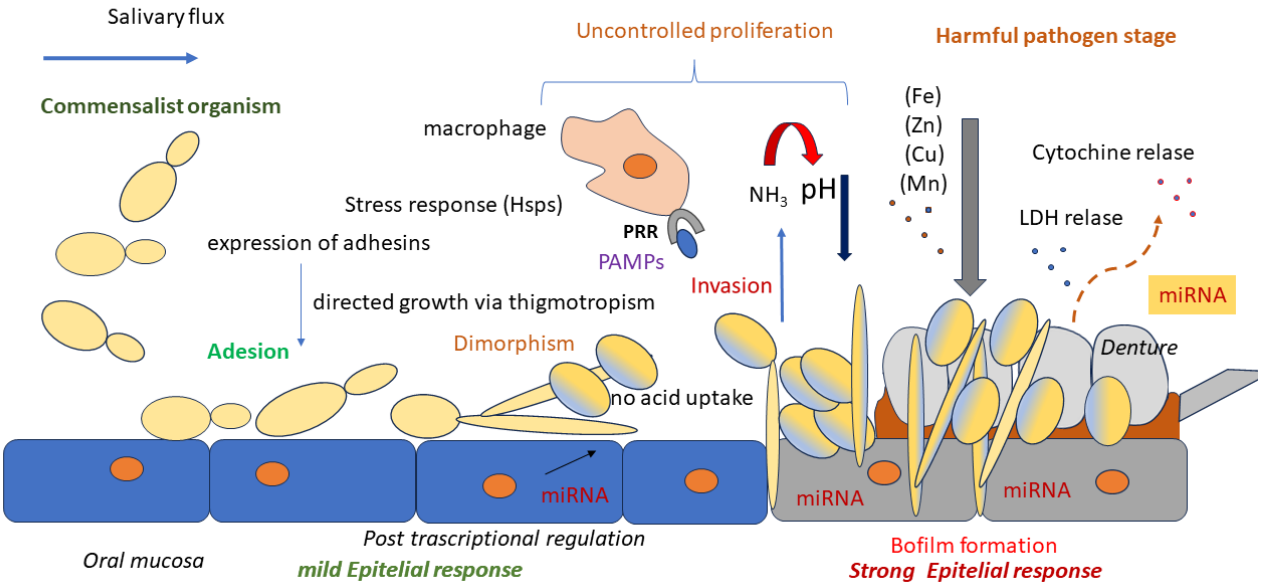

Fig.3 An overview of the main *C.albicans* pathogenicity mechanisms**.**

Antifungal resistance in *Candida spp*.

Antifungal resistance in *C. albicans* is less common, but it has been documented in cases of prolonged antifungal therapy and recurring infections, including chronic mucocutaneous candidiasis and recurrent oropharyngeal candidiasis in individuals with uncontrolled human immunodeficiency virus infection. While some non-albicans Candida species, like *Candida krusei*, have inherent resistance to some kinds of

antifungals or are less sensitive to them, other non-albicans Candida species, like *Candida glabrata*, have developed resistance after being exposed to antifungal agents. Multidrug resistance (resistance to more than one drug class) is becoming more frequently reported, as in the case of some oral clinical isolates of *Candida albicans* [13].

Some amazing ability to withstand antimycotics has made it harder to treat some clinical isolates of *Candida species* strains. Candida spp. has a high level of innate and acquired resistance mechanisms, which it uses to evade most drugs. Additionally, a recently identified mechanism of *C. albicans* adaptive antibiotic resistance, which comprises biofilm-mediated resistance and the raising of multidrug-tolerant persistent cells, is in charge of infection recurrence in the oral cavity.

For example, the standard clinical protocols recommend local application of Fluconazole or Ketoconazole as an alternative to Nystatin or Miconazole as part of the current therapeutic strategy for infection control in patients with oral candidiasis, particularly when extension into the oesophagus is suspected. In several clinical studies, it was discovered that fluconazole was superior to a Nystatin suspension for treating oral thrush in HIV-positive patients or otherwise healthy newborns [13].

Recently, oral candidiasis has been treated with second-generation triazoles such Voriconazole. This azole specifically has broad-spectrum efficacy against yeasts, molds, and species of Aspergillus. The azole susceptibility pattern for clinical isolates of *Candida albicans* in the oral cavity from non-immunocompromised individuals has only been briefly described in a few articles.

In non-immunocompromised patients, Orrù et al.2008 in Sardinia (Italy) reported a relatively high prevalence of resistant strains isolated in the oral cavity. In fact, in a total of 81 patients, 21% harboured fluconazole-resistant strains, and among these isolates, they were also cross-resistant to Voriconazole (83,3%) and 66.6% to KTC. The homozygous point mutation G464S in the ERG11 gene was responsible for fluconazole resistance [13].

Another profile for drug-resistant *C. albicans* isolates was described by Asaad et al., 2023, in comparison with the previous work, on contrary Voriconazole resistance was observed in many of the clinical isolates [14].

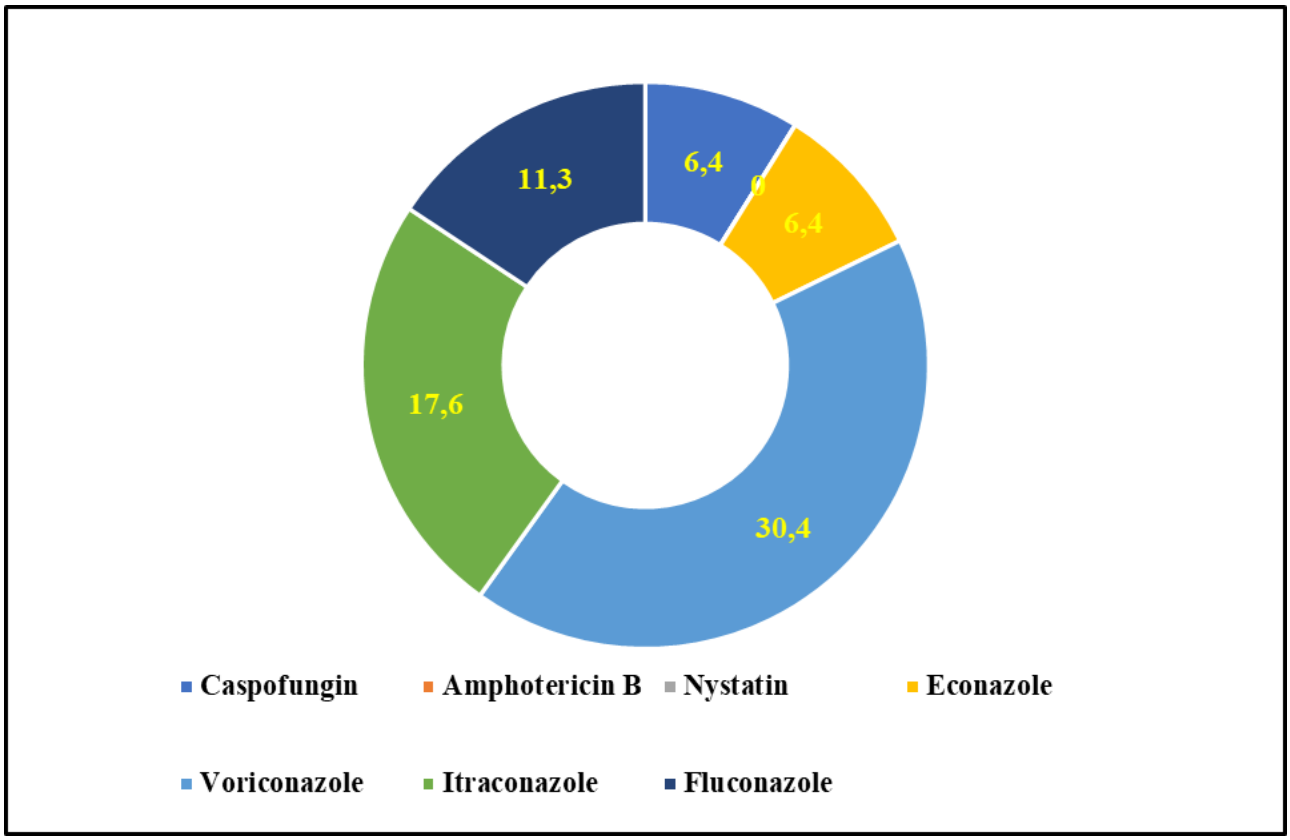

Fig.4 Frequency of drug resistance in the *C. albicans* clinical isolates in Egypt **[14].**

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The presence of MDR strains of Candida clinical isolates has led to a rising need for and interest in the research and development of alternative therapeutic approaches that offer fresh approaches against Candida infections. For this reason, the development of new drugs is urgently needed.

The development of novel antifungal medicines and diagnostics depends critically on our understanding of the pathogenicity mechanisms that Candida albicans employ during infection. Antifungal medications were traditionally made to have fungicidal

effects, or to kill the pathogenic microorganism. But recently, a novel and exciting antifungal approach has been put forth that particularly targets virulence factors. Numerous virulence factors, including dimorphism, the release of proteases, and the expression of adhesins and invasins, have been proposed as desirable targets, and recent studies have expanded our knowledge of the *C. albicans* factors and activities that contribute to virulence. A prospective therapeutic target has been identified as the heat shock response, which includes the large and small heat shock proteins.

In this context, novel and promising therapeutic approaches are developed by increasing the oxidative stress level inside *Candida spp* components.

In this context, in this dissertation novel, and promising therapeutic approaches are evaluated and described, to treat oral Candida infections. These protocols are based by increasing the oxidative stress level inside fungal cells. This by using a combinate therapy e.g., through light-sensitive molecules and oxygen derivates: the Photodynamic therapy.

Chapter 3 – Clinical patterns of Oral Candidiasis

We can distinguish Oral Candidiasis based on the colour of the lesions. White lesions include acute pseudomembranous candidiasis and chronic hyperplastic candidiasis; red lesions include acute and chronic erythematous candidiasis, angular cheilitis, median rhomboid glossitis, and linear gingival erythema. Other rare oral types not included in these categories are cheilocandidiasis, chronic mucocutaneous candidiasis, and chronic multifocal candidiasis.

Acute Pseudomembranous Candidiasis

This type is considered the most typical clinical form of oral Candidiasis, regarding about one third of cases. Newborns, immunodepressed and old patients are most affected. Clinical features are presence of white patches that with the help of a gauze, can be removed, leaving an erythematous mucosa area. These patches with desquamated epithelial cells and fungal hyphae form the pseudomembranes. The lesions are usually asymptomatic and affect the tongue, lips, buccal mucosa, gingival tissues, palate, and oropharynx. If symptomatic, a burning sensation was reported by the patients in the mouth, oral bleeding, and changes in taste perception [14,15].

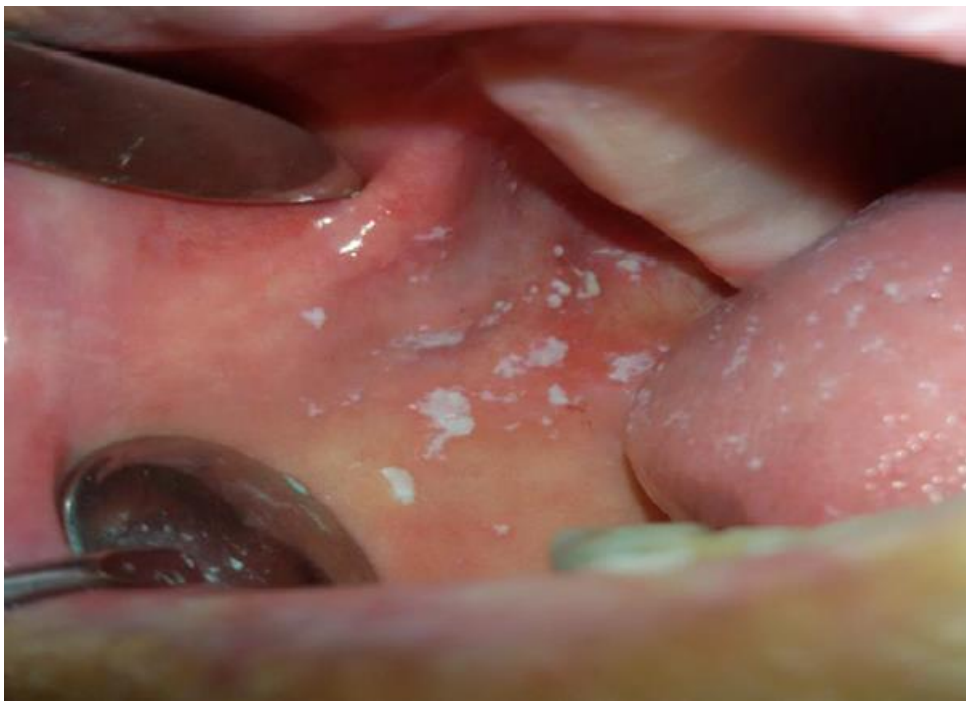

Fig 5. Pseudomembranous candidiasis in a highly debilitated cancer patient. Note the location of the pseudomembranes especially in the buccal mucosa, the site most affected by this type of candidiasis.

Acute atrophic candidiasis

This type of candidiasis is characterized by the presence of atrophy of the mucous membranes, in the absence of whitish pseudomembranes. It manifests itself above all on the hard palate and sometimes on the dorsum of the tongue which appear strongly reddened. Patients often experience discomfort and burning. In addition to the infection caused by the fungus, we have other comorbidities, such as nutritional deficiencies (such as iron and vitamin B12), use of corticosteroids, diseases such as HIV and uncompensated diabetes mellitus [8,9].

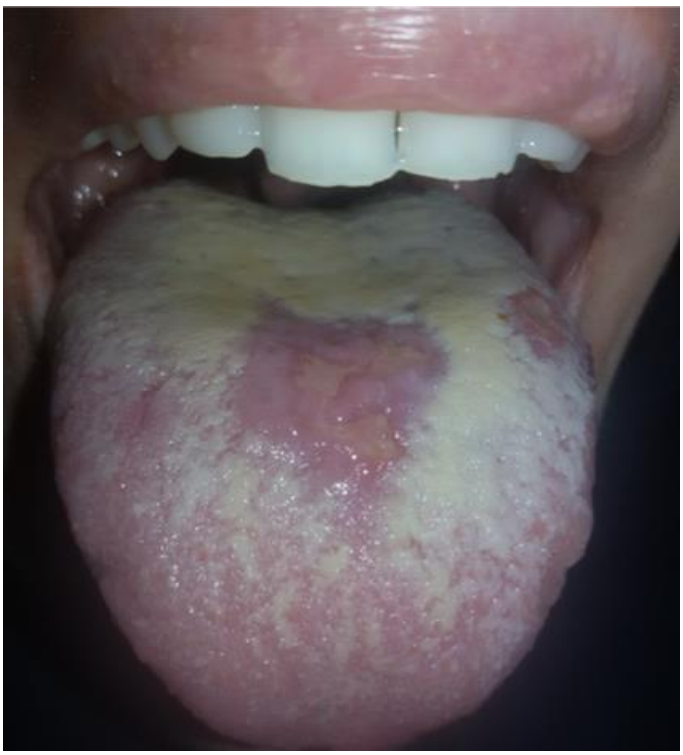

Fig 6. Atrophic candidiasis of the tongue in a patient with aphthosis under cortisone therapy. See the atrophy of the central lingual part, which made it difficult to clean the lingual back, with consequent stagnation of food debris.

Chronic atrophic Candidiasis

It is a condition of chronic atrophy of the oral mucous membranes which occurs above all in the palate of patients with prostheses or orthodontic appliances. It affects 65% of prosthesis wearers, and some factors can help its onset: the incongruity of the prosthesis, wearing the prosthesis during the night, incorrect cleansing of the prosthesis. The affected area appears as a large red spot whose perimeter coincides with the ends of the prosthesis. Often in these cases there is also the presence of another form of candidiasis, angular cheilitis. It is classified into 3 types:
type 1 the extension of the erythema perfectly coincides with the prosthetic adhesion area; type 2 the erythematous area develops only in the central part of the prosthetic adhesion; type 3 hyperplasia of the subprosthetic tissues is present [14,15].

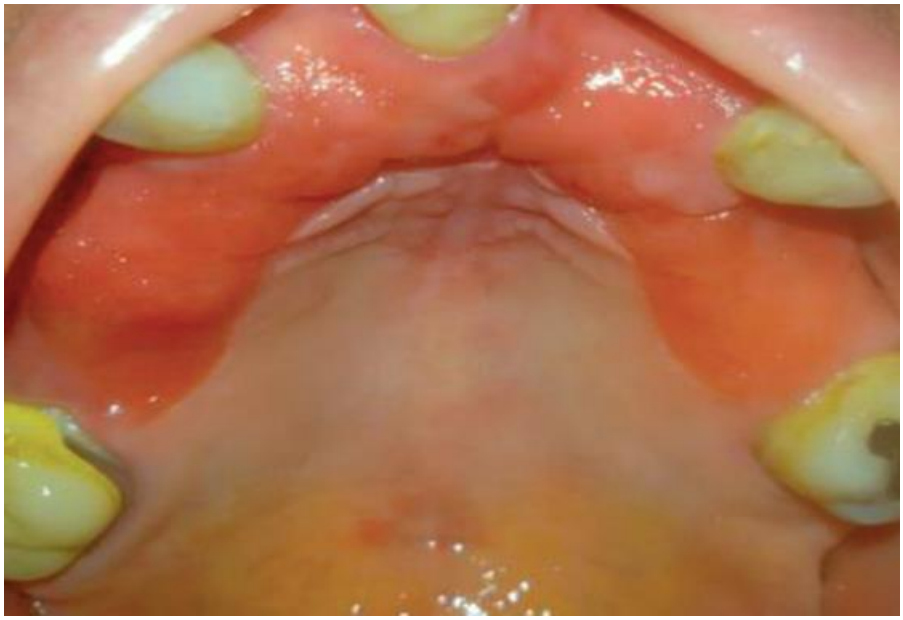

Fig 7. Chronic atrophic Candidiasis type 1.

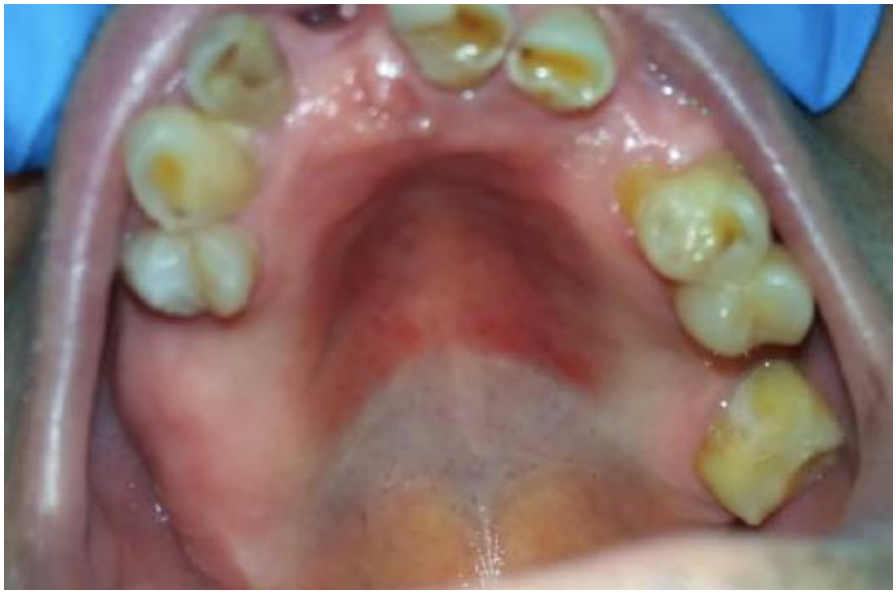

Fig 8. Chronic atrophic Candidiasis type 2.

Median rhomboid glossitis

It is a condition of absence of lingual filiform papillae in a central area of the lingual dorsum, often rhomboidal in shape, anterior to the circumvallate papillae. In reality it

is an alteration of the lingual tissue that occurs in about 1% of the population. When it is infected by the fungus, as a *locus minoris resistentiae*, it produces a mold-like lesion on the palate. In that case it must be treated pharmacologically. It is often asymptomatic. Its appearance could be a first sign of HIV infection [14,15].

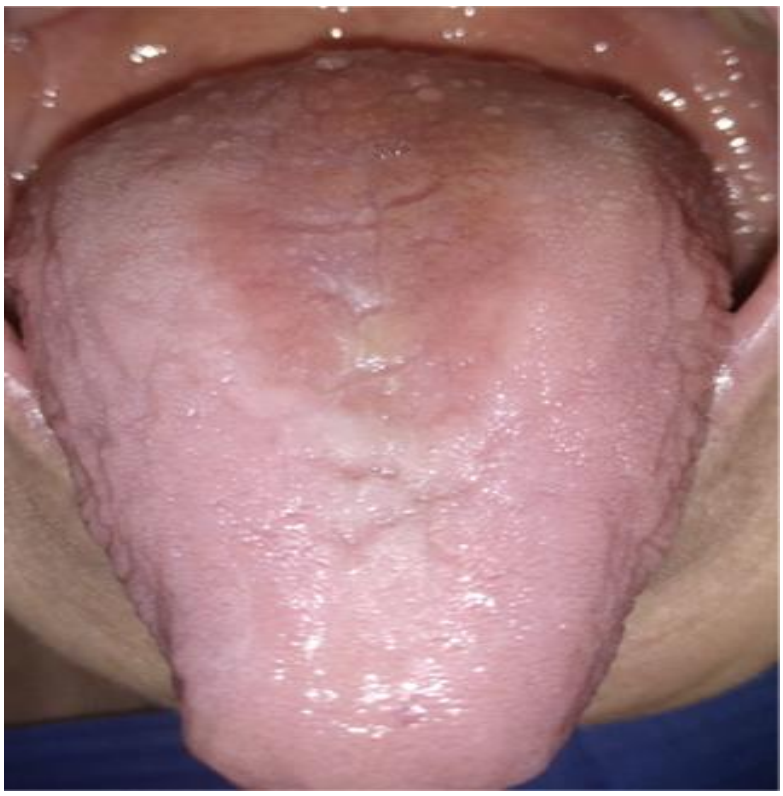

Fig 9. Median rhomboid glossitis. In this case the patient presented this condition from an early age without mold lesions on the palate.

Angular cheilitis

It is a condition that often affects both sides of the labial commissures. It presents with erythematous areas, desquamation and often fissuring of the lips. Sometimes it's quite sore. The infection by the fungus is often due to a mechanical factor which determines an introversion of the lips with consequent salivary stagnation (reduction of the vertical height of the middle third of the face) with the formation of a humid

environment which not only facilitates the growth of *Candida* but also of bacterial species such as *Staphylococcus aureus*. Sometimes it can be associated with nutritional deficiencies such as iron, folate, vitamins B [14,15].

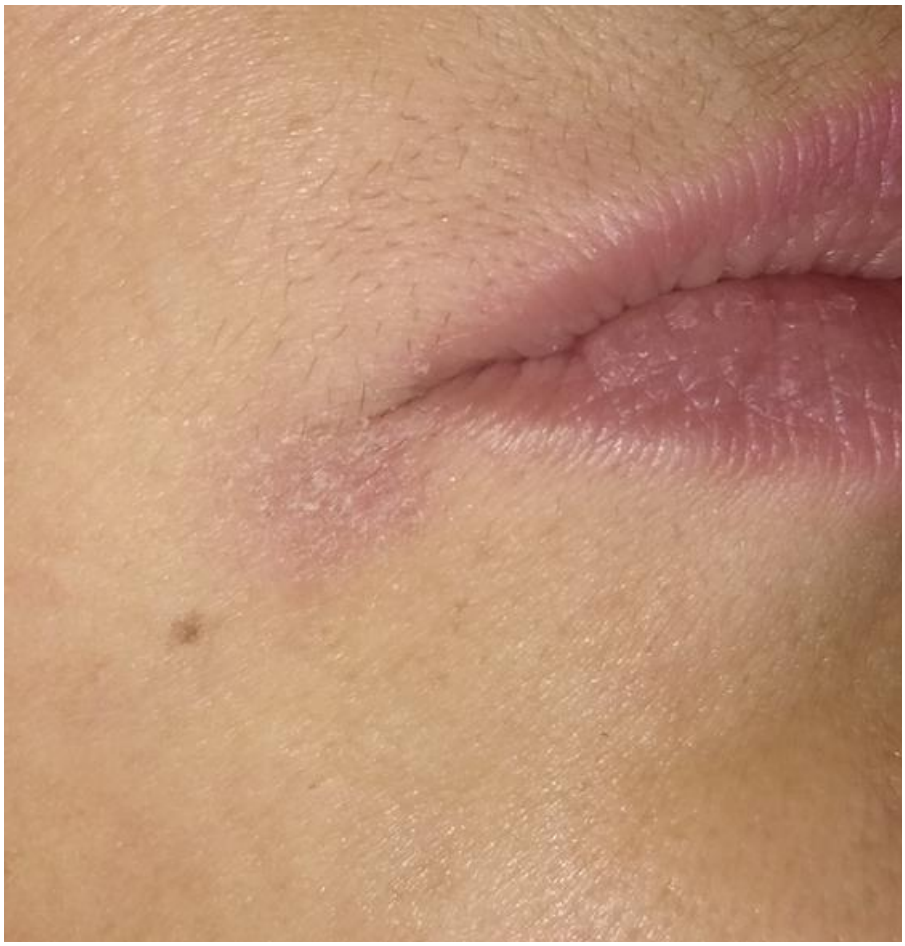

Fig 10. Angular cheilitis in a patient with correct vertical height and Vitamin B12 deficiency.

Linear gingival erythema

In this case the fungal infection appears between two adjacent interdental papillae as a diffuse erythema on the keratinized gingival mucosa. We often have a co-infection with other bacterial microorganisms. Curiously, this condition is often sensitive to

antiseptics such as 1% chlorhexidine or topical antibiotic therapies, just as if it were actually a bacterial infection.

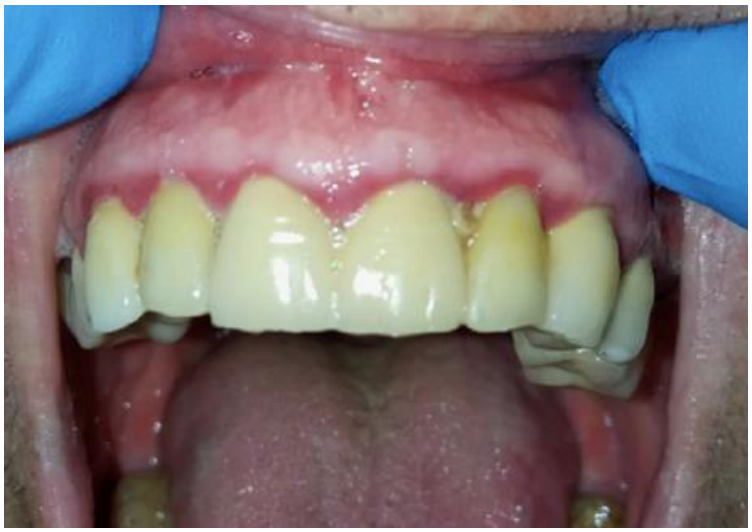

Fig 11. Linear gingival erythema in a patient with poor oral hygiene.

Chronic hyperplastic Candidiasis

Chronic hyperplastic candidiasis is a type of candidiasis in which there is the formation of real whitish plaques, which cannot be removed. It has a prevalence of 1.61% in the world population and is important to recognize as it has the potential for malignant transformation. It mainly affects the buccal mucosa bordering the internal part of the labial commissures (58,9%) and the lingual dorsum (15,7%). Its differential diagnosis occurs with leucoplakia and lichen planus, other potentially malignant pathologies. A recent review of the literature has shown that about 12% of chronic hyperplastic candidiasis can evolve towards carcinogenesis [16]. It is a common condition among smokers and tobacco chewers, but also conditions of prosthetic decubitus, hypovitaminosis, lowered immune system, can contribute to the development of this condition. Treatment is based on topical antifungal therapies, retinoic acid, bleomycin or surgical removal with a cold blade, Laser, or cryotherapy, especially for the purpose of histological evaluation. Dysplasia is present in approximately 15% of chronic

hyperplastic candidiasis. The presence of candida can assist in the carcinogenic process through the production of oncogenic proteins such as N-nitroso-benzyl-methylamine and triggering chronic inflammatory processes producing proinflammatory cytokines (IL-1, IL-1, IL-6, IL-8, IL-18, TNF, IFN), leading to a disbalance in immune surveillance mechanisms and changes in the tissue environment.

However, the WHO has not included chronic hyperplastic candidiasis among the potentially malignant lesions of the oral cavity [16]. Furthermore, Candidalysin is a cytolytic toxin produced by *Candida spp*. that had shown to induce NF-kB and MAPK pathways. According to recent studies, the potential for malignant transformation of oral leucoplakia is between 9.5 and 9.8%, therefore a lower rate than that of chronic hyperplastic candidiasis.

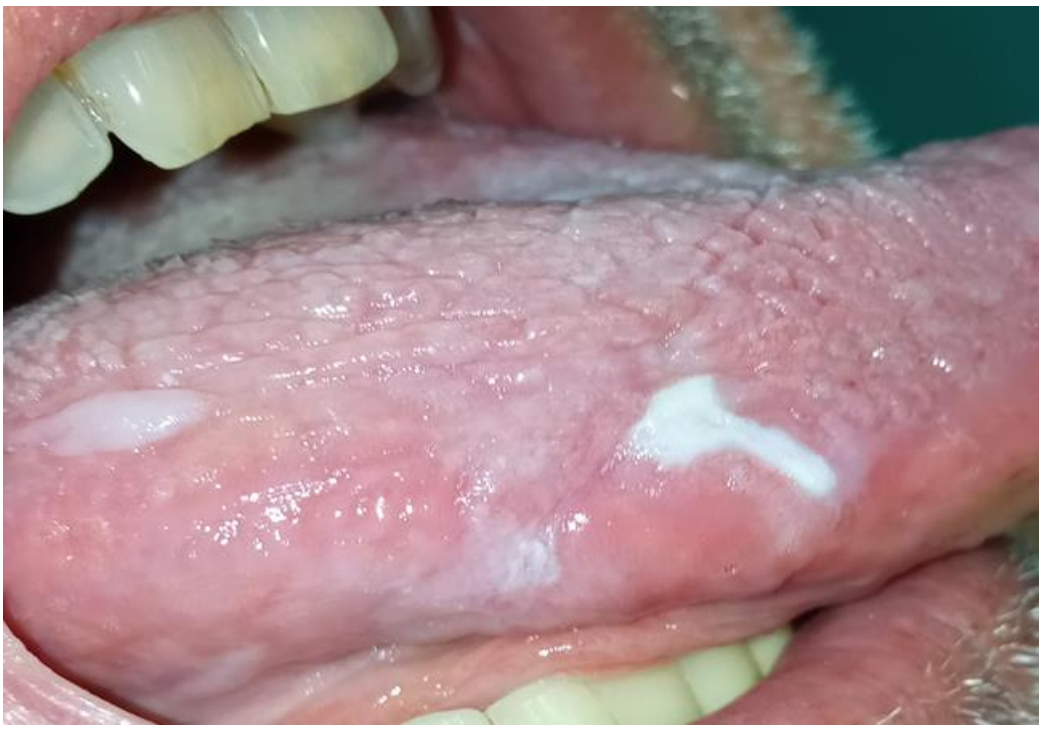

Fig 12. Oral leucoplakia on the tongue. It could be a differential diagnosis with Chronic hyperplastic Candidiasis.

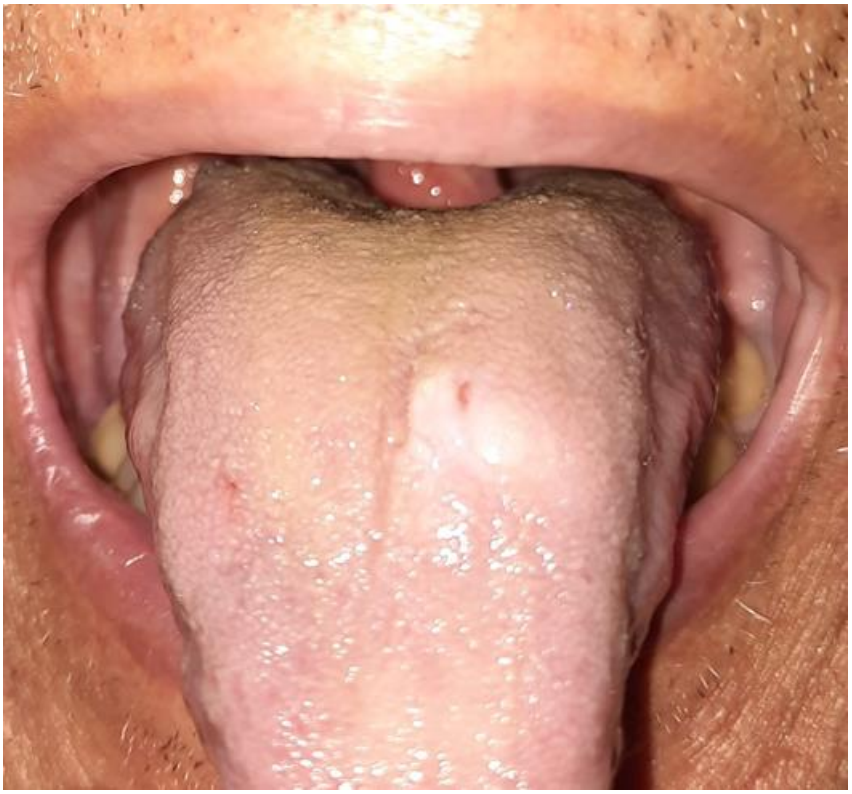

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Fig 13. Oral lichen planus lesion on the central part of the dorsal tongue. It could be taken into consideration for differential diagnosis of Chronic hyperplastic Candidiasis.

Chapter 4 – Clinical diagnosis for oral candidiasis

The diagnosis of candidiasis is mainly clinical but is also supported by a laboratory diagnosis to identify the strain of *Candida*, the fungal load, and the resistance profile to antifungals. In the case of chronic hyperplastic candidiasis, an adjunctive incisional biopsy is necessary to evaluate by histological examination the presence of neoplastic cells. In this case, some authors recommend, after topical or systemic therapy of this particular variant of mycotic lesion, to re-perform an incisional biopsy to evaluate any degree of dysplasia and the presence of fungal hyphae [13, 16]. In addition to culture tests, molecular biology evaluations may also be useful, *Figure 14*. In the case of sub-prosthetic candidiasis, it is important to take samples with sterile brushes, even on the surface of the prosthesis, which adheres to the palate [16].

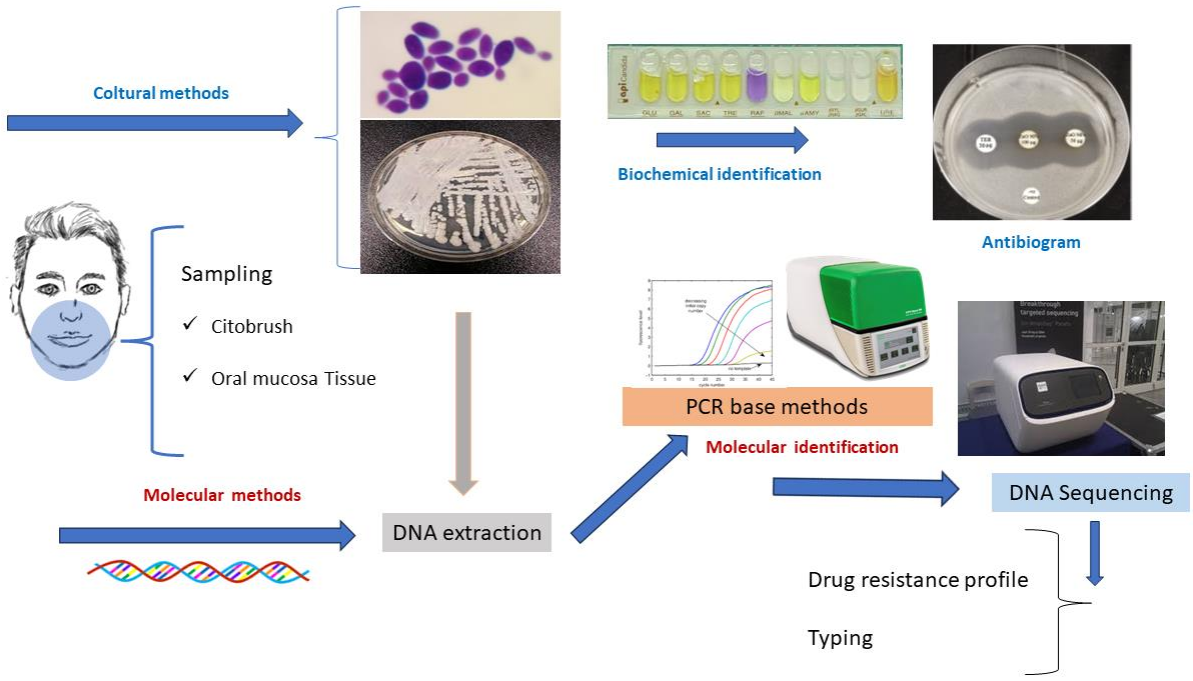


Fig.14. Operational flow for laboratory diagnosis for candidiasis.

Clinical approaches for oral candidiasis therapy and treatment.

In most cases the treatment for candidiasis is topical pharmacological, in some refractory cases it is proceeded with systemic therapy. The most common used topical drugs are nystatin 100,000 IU, clotrimazole, miconazole and ketoconazole, Figure 4. Nystatin is recommended in oral suspension for 4 daily applications; however, it presents major problems as it is extremely rich in sugars and therefore not indicated in decompensated diabetic patients or in subjects with a high risk of caries. It can also be associated with vomiting [13]. In more advanced or refractory cases, the gold standard is fluconazole 200 mg or 100 mg for 7-14 days. The use of fluconazole in single administration at a dosage of 150 mg can be useful in the initial forms of candidiasis in oncological patients. It is a kidney excreted drug which therefore cannot be administered in patients with renal insufficiency [13].

Given the limits and interactions of antifungal drugs, research has moved towards other therapeutic approaches, especially on a natural basis. An Indian study evaluated the use of garlic mousse as an antifungal, compared with clotrimazole [17]. In another study the use of an aqueous solution of garlic was proposed in subjects with oral candidiasis, compared to nystatin, giving overlapping results [18]. The use of supplementation with lactoferrin has been successfully proposed in the literature [19], also some plants extract, such as Melaleuca Alternifolia [20], Cinnamon [21], Origanum vulgare [22]. The latter has shown to have a certain inhibition capacity of fungal phospholipases at aqueous concentrations of 1-5-10%. However, these studies are mainly carried out *in vitro* or on animals. Among the proposals suggested for the non-pharmacological treatment of oral candidiasis we also have probiotics. There are only 8 clinical trials performed on patients in this regard, with extremely variable dosages and timing, so it is not currently possible to establish a gold standard for the

treatment of oral candidiasis with probiotics. We know that the most studied microorganism is *Lactobacillus rhamnosus* which has been shown to alter the morphology of the fungus, the ability to adhere and to have access to nourishment [23]. Another Lactobacillus evaluated by prevuois work *L. crispatus*, is able to alter b-defensin and cytokine production and toll-like receptor expression of *C. albicans* [23].
Among the modern electromedical devices, good results have been achieved by the use of ozonated water in patients with a high intraoral fungal load. In a clinical trial, the researchers compared the CFU of patients before and after the following treatments: 10 ml of ozonated water per day; 2 daily applications of clotrimazole. The reduction in CFU was 60% in the group treated with ozonated water and 32% in the group treated with clotrimazole [24]. Another recent in vitro study confirmed these interesting results about ozonated water against oral Candidiasis [25].
Among other electromedical devices, the results obtained by decontamination with photodynamic therapy are very encouraging. These are mainly in vitro studies and case reports, but recently clinical trials have also demonstrated efficacy through the use of natural photosensitizers [26]. The discussion of this subject, extremely important as an object of experimentation, will be resumed later.

Prevention strategies

It is necessary to instruct the patients on cleansing manoeuvres of the prosthesis. In some cases, it is necessary not to wear the prosthesis during the night, to keep it dry in the air.
Remind patients under treatment that the use of chlorhexidine inhibits nystatin, and therefore cannot be used to clean the prosthesis [13].
Motivate patients to stop smoking and interrupt any antibiotic therapies responsible for the onset of oral candidiasis. In particularly predisposed subjects it is important to follow a diet rich in fibres and low in sugars to reduce the risk of developing oral

candidiasis. In children who develop forms of thrush during breastfeeding, the use of topical antifungal creams to be applied to the mother's nipple can be a good therapeutic strategy [13]. HIV-positive and diabetic patients must be carefully monitored with frequent visits to the oral pathologist and possible microbiological examinations due to the high risk, compared to the normal population, of developing candidiasis.

Chapter 5 – Other oral clinical condition associated with Candida infections.

Oral candidiasis can involve the pharyngeal region, causing dysphagia and therefore difficulties in nutrition and hydration.

In the most serious cases, very rarely, it can invade the bloodstream causing septicaemia with the need for immediate intervention with intravenously administered antifungal drugs [13].

Among the oral pathologies where a high fungal burden co-responsible for the pathology has been found, we have osteonecrosis of the maxillary bones due to bone resorption drugs (MRONJ) and hairy lingua nigra.

MRONJ

MRONJ stands for Medicate-Related Osteonecrosis of The Jaw. It is a multifactorial pathology in which patients subjected to pharmacological treatment with bone resorption drugs (especially bisphosphonates and monoclonal antibodies) spontaneously or following traumatic and/or surgical manoeuvres can develop bone tissue necrosis [27]. This phenomenon clinically leads to bone exposure with

surrounding erythematous mucosa, which does not regress with common antibiotic therapies, which can be localized in the maxillary or mandibular bone but more frequently along the mylohyoid line. Diagnosis is clinical and requires the patient to have three conditions: - have taken anti-resorption drugs in the last few years or months; - not be under radiation therapy; - have exposed bone without healing for at least 6-8 weeks [27]. Instrumental investigations such as CT and MRI are essential to see the real extent of the lesion in the bone. Bisphosphonates and monoclonal antibodies (e.g. Denosumab) are given to treat osteoporosis, to prevent or stop bone metastases, to treat multiple myeloma, and to cure Paget's disease. The treatment of this complex pathology goes beyond the scope of this work, but what is extremely important is that the activity of the aforementioned drugs, which inhibit the bone remodelling processes, through inhibition of the osteoclasts, is only one of the aspects that lead to the genesis of this pathology. In fact, the infection by Actinomyceti and Candida spp. plays a fundamental role in the onset and response to therapies of ONJ [28].

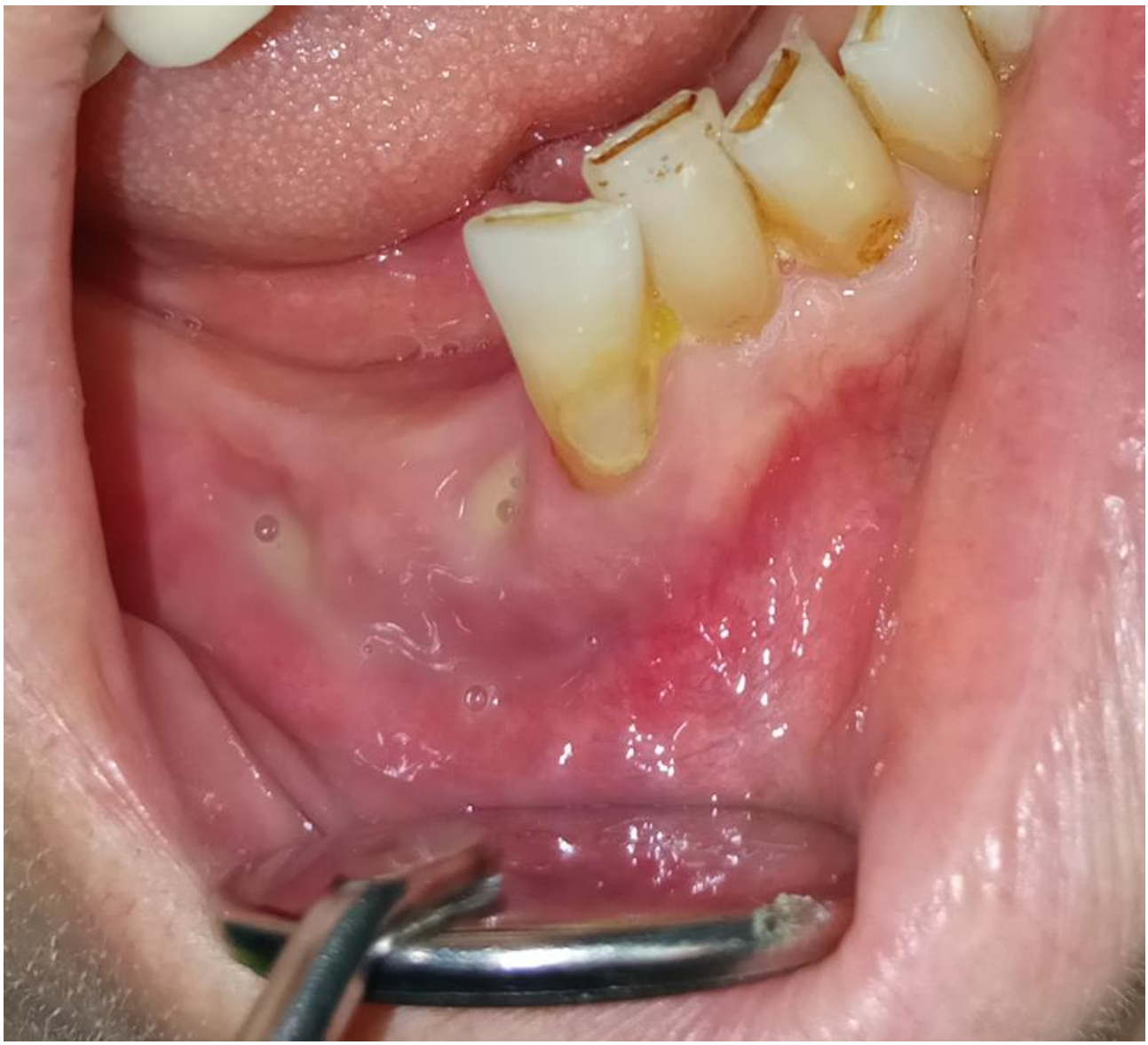


Fig. 15. Patient with MRONJ, with high fungal load in the mouth.

Black Hairy Tongue

Villous lingua nigra is a condition of dark discoloration of the dorsal mucosa of the tongue, with prolongation of the lingual filiform papillae, due to irritation. This condition can be linked to the consumption of smoke, alcohol, oxidizing mouthwashes, antibiotic drugs or immunosuppressants for prolonged use or particular physical

debilitation. The dark colour is linked to the presence of chromogenic bacteria and can vary from white to green, yellow, brown and black. Affects the central part of the lingual dorsum, does not include the lingual tip and margins. Often among the associated microorganisms it is possible to find the presence of *Candida spp*. [29].

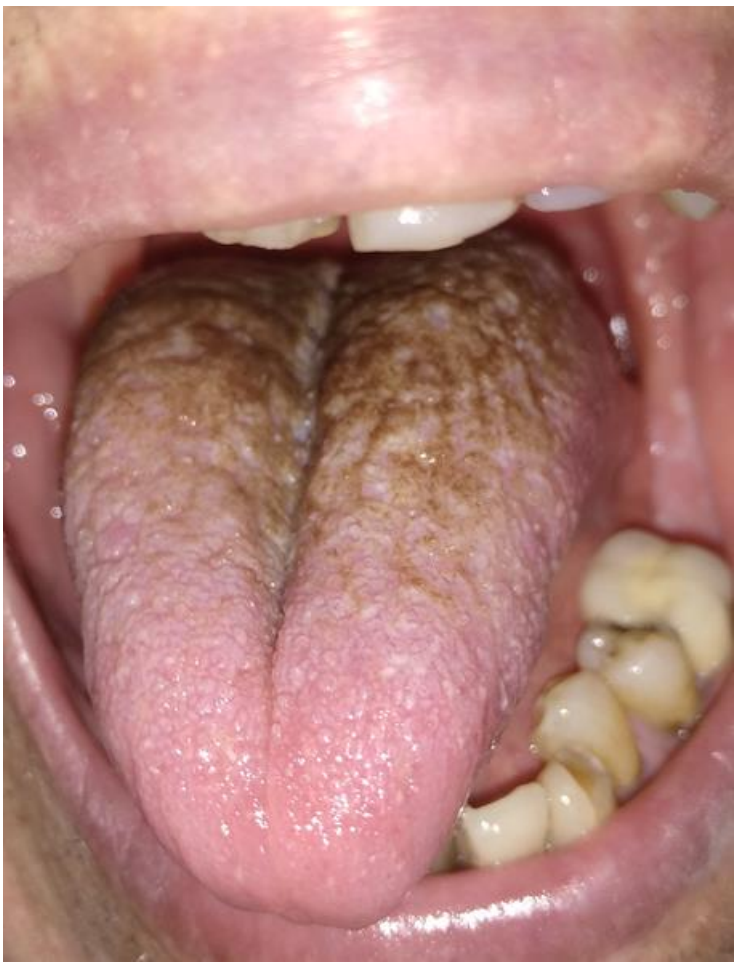


Fig 16. Black Hairy Tongue in an old patient treated with systemic antibiotics and corticosteroids.

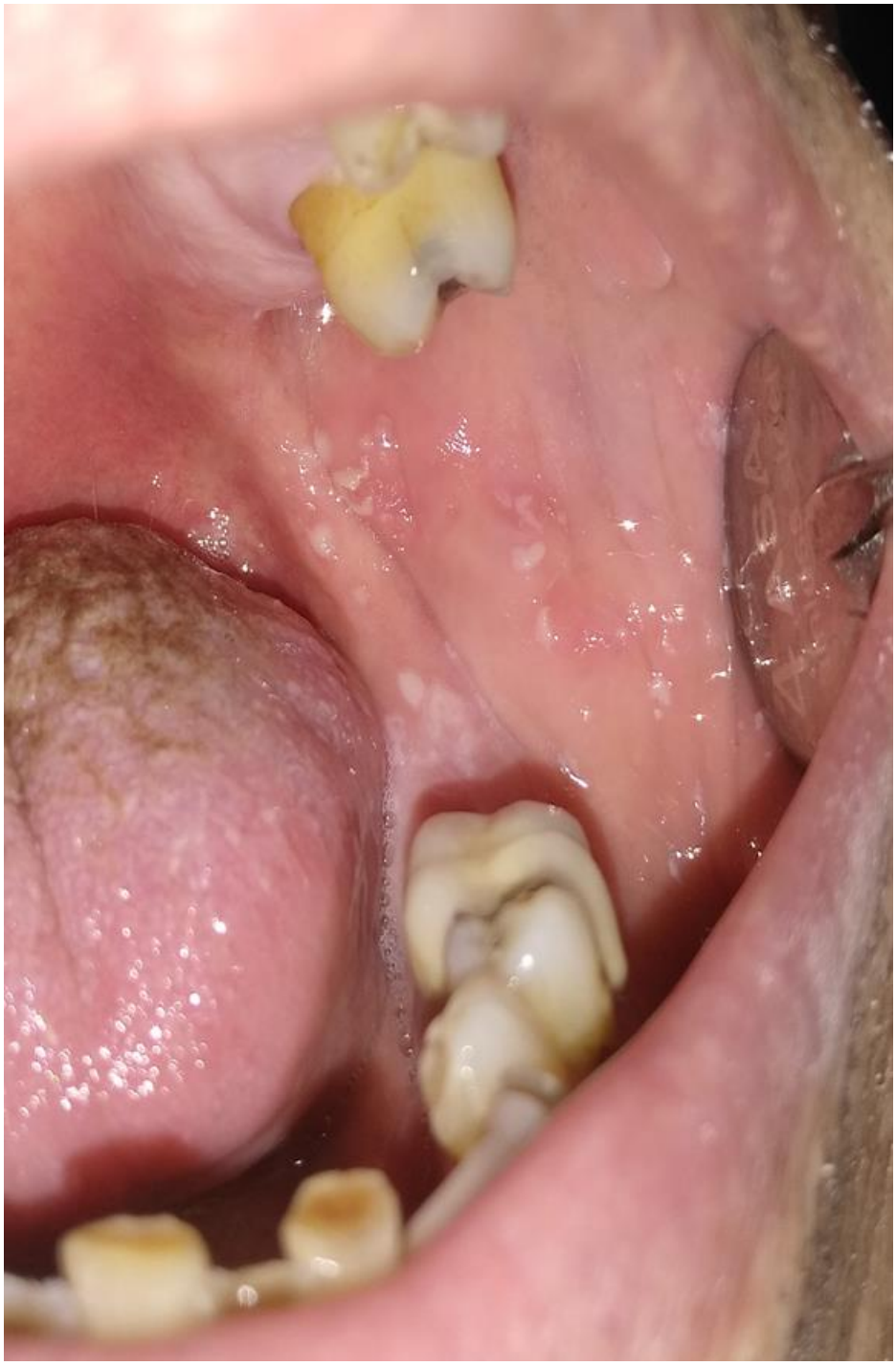
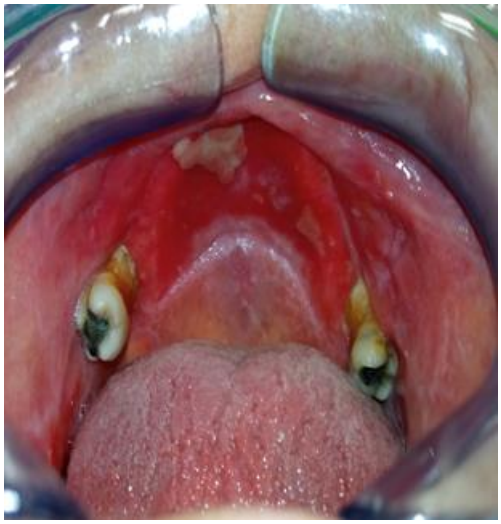


Fig 17. Same patient with clinical presentation of pseudomembranous candidiasis on the right buccal mucosa.

Other conditions linked to the development of Candidiasis

Some autoimmune oral diseases are treated with topical or systemic corticosteroid therapies. Prolonged use of these drugs can lead to the onset of fungal superinfections such as oral candidiasis. Among these autoimmune diseases, the most frequent is oral Lichen Planus. It is a condition of chronic inflammation of the oral epithelia which can sometimes affect the skin and genital tissue, especially in women (20% of cases). The appearance can be very varied, even if the basic lesion is the papule, a white lesion less than 3 mm in diameter. Often it is possible to see the organization of the papules in reticules, Wickam's striae, or in plaques, which generally have a symmetrical and bilateral distribution. Lichen can also be erosive, atrophic or bullous in nature and in this case is associated with a greater tendency to malignant transformation. The use of topical antifungals or oral decontaminants is important when using corticosteroids to treat oral lichen planus lesions to avoid fungal



superinfections that slow the healing process [30].

Fig 18. Palate erosions of Lichen Planus with Candida superinfection, in a patient treated with prolonged topical cortisone therapy and prosthesis wearer.

Chapter 6-Photodynamic Therapy

Photodynamic therapy can take place in the presence of three elements: a light with an appropriate wavelength, a photosensitizer, the presence of oxygen. When a photosensitizer is activated by light it can undergo two phenomena: losing energy or forming an oxygen triplet [31]. This second type of Photodynamic therapy is linked to the amount of oxygen, while the first one PDT can work in an anaerobiosis condition. The interaction that the photosensitizer has with oxygen can determine the formation of hydrogen peroxide (H_2O_2); hydroxyl radicals (-OH); superoxide radical oxygen (O_2); singlet oxygen (-O). All these products determine the killing of microorganisms due to damage to the cell membrane and their metabolic activity [31]. In type 2 photodynamic therapy we have two possibilities: 1) direct interaction with lipids, proteins, DNA and therefore their destruction; 2) interaction of the radicals produced with other oxygen molecules, which by oxidizing proteins, lipids, sugars, etc. create alterations in metabolic processes, such as that of calcium, inducing cell death [31].
The maximum absorption of light by the photosensitizer occurs with a wavelength between 600 and 800 nm, in fact a higher wavelength would not have enough energy to stimulate oxygen to produce radicals [31]. The penetration of the wavelength comprised between 700-1100 nm is wider than comprised between 400- 700 nm [32], *Figure 19*.

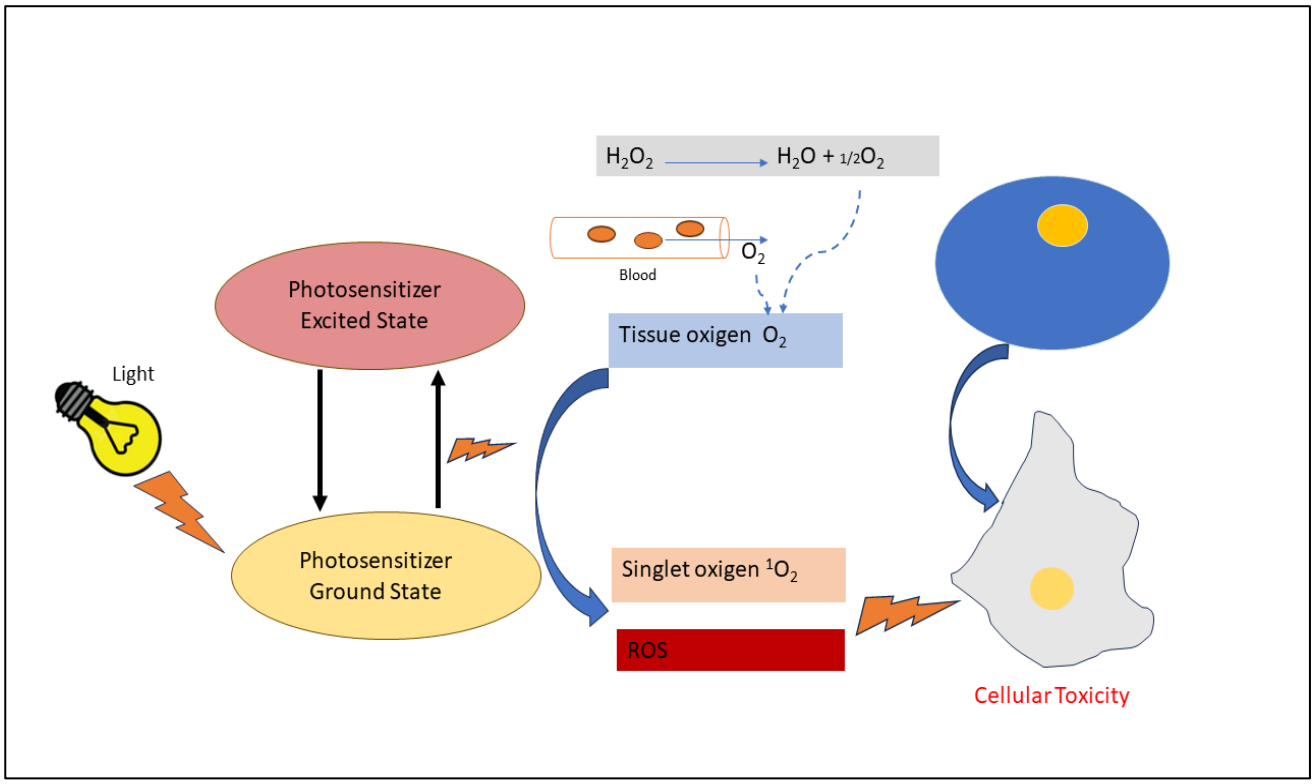


Fig.19. Schematic representation of a Photodynamic reaction.

Photosensitizer used in Photodynamic Therapy

In spectroscopy language, a compound that has a stable electronic configuration and has a ground singlet state containing a photo trigger electron, is called a photosensitizer. A photosensitizer (PS), a light-activated drug is also called a chromophore, which has unsaturated conjugated bonds to absorb a specific wavelength of light to become excited [33].

Photosensitizers have a very important role in the antimicrobial PDT (aPDT) process, because they are the main actors of the process, as absorbers of light energy. Several factors are taken into consideration when choosing photosensitizers. Photosensitizers must have some important features: 1) chemically pure; 2) produced under good production conditions with quality control and low production costs; 3) lead to better storage stability; 4) Non-toxic effect for normal healthy cell.

Different types of photosensitizers have been proposed in scientific literature, some

of natural origin, others of synthetic type.

Natural photosensitizers

From natural photosensitizer we have Furanocumarins, in which we have the presence of secondary metabolites of phenolic compounds called coumarins. They could be subdivided in:

(1) The linear (psoralens) group, which includes psoralen, bergapten, and xanthotoxin;
(2) The angular (angelicins) group, which includes angelicin, pimpinellin and sphondin.

Psoralen is present in numerous plant families in high concentration, including Moraceae, Leguminosae, Apiaceae and Rutaceae. If this compound is exposed to ultraviolet light, it can become cytotoxic. The use of Psoralen activated by UVA (PUVA) in the dermatological medium has been very successful, both for the treatment of autoimmune pathologies and for the treatment of tumor pathologies [33]. Alkaloids are derived from metabolites of substances containing the nitrogen group and are extracted from plants, especially flowering plants. Alkaloids have showed various effective properties, such as analgesic, local anaesthetic and pain relief, neuropharmacological, antifungal, antimicrobial, anti-inflammatory, anticancer, and numerous other activities. They are also beneficial as supplements, diet ingredients, pharmaceuticals such as camptothecin and vinblastine, which are used as chemo drugs. Barberine and Coraline have a high affinity for cell mitochondria easily inducing cell apoptosis even in cancer cells [33].
Poly-acetylenes and thiophenes derivatives are other types of plant-derived photosensitizers.
The first ones consist of conjugated double and triple bonds and are bio-synthetically cyclized into thiophene compounds.

They could be classified in 3 types:

(1) Straight-chain aliphatic acetylenes;
(2) Partly cyclized;
(3) Thiophene derivatives (addition of sulphur into polyacetylene).

These compounds could be activated by wavelength comprised between 314-360 nm. They have been found in many plant families such as Asteraceae, Campanulaceae, Araliaceae, Apiaceae, and Basidiomycete fungi groups. Polyacetylenes and thiophenes derivatives have been reported to have various pharmacological actions such as antimicrobial activities, antitumor, anti-inflammatory, anticancer, and analgesic [33].
Curcumin is a substance obtained from the roots of the *Curcuma Longa* plant. Curcumin extracted from *Curcuma longa* is widely used in food and cosmetic industries and as a spice and colouring agent. It is one of the most studied plants in the medical medium. It has characteristics such as anti-inflammatory, antitumoral, antioxidant, antibacterial, and chemotherapeutic effects, which make it valuable for photobiological application [31]. In photo dynamics it is used by activating it with blue light 405-460 nm. It has been documented that activation with UVB can determine an apoptotic effect against cancer cells and skin infections [33].
Flavonoids are a group of polyphenolic compounds consists primarily of a benzopyrone structure. They are commonly found in vegetables, cereals, herbs, flowers, fruits, seeds, and nuts and are responsible for many pharmacological activities. Previous works has shown that these plant-derived photosensitizers are used to treat diseases and have properties such as anti-inflammatory, anticancer, antiviral, antioxidant, and hepatoprotective.
Based on the degree of saturation, oxidation of the carbon ring and chemical structure in general, we can have seven subgroups: flavanones, flavones, flavonols, isoflavones,

anthoxanthins (flavanone and flavanol), anthocyanins, and chalchones [33]. One of the most famous of this group is quercetin, that is activated by λ =557 nm wavelength light.

Chrysanthemin, also known as cyanidin-3-glucoside, is a kind of anthocyanin that has an absorption wavelength of 550 nm, that have photoactivable characteristics. It was used in antimicrobial PDT against the periodontopathic bacterium *Porphyromonas gingivalis* using a green light laser in an in vitro biofilm model. It was observed that this plant-derived compound efficiently reduced *Porphyromonas gingivalis* in biofilm.

Another flavonoid very tested is Riboflavin, that is also been used in antimicrobial PDT, activated with light at λ= 360-440 nm of wavelength.

Anthraquinone (AQ) is the most common type of natural quinone found in higher plants. The shikimic acid/mevalonate and acetate/malonate routes yield anthraquinones, which are generally categorized as monomeric and dimeric anthraquinones. They are activated with light at λ = 380-460 nm of wavelength [33].

Synthetic photosensitizers

Phenothiazinium is a subgroup of synthetic dyes. The most common used phenothiazinium dyes are methylene blue (Mb) and toluidine blue. Methylene blue is one of the most used photosensitizers in photodynamic therapy. It was initially born as an antimalarial drug, against methemoglobinemia, hepato-pulmonary syndrome and some authors have proposed it for the treatment of Alzheimer's disease. Being a derivative of phenothiazine, it absorbs light with a wavelength of 630-680 nm [34].

The photosensitizing therapy carried out with Methylene Blue has demonstrated efficacy against various microorganisms such as *S. aureus, P. aeruginosa*, including oral microbes such as *S. mutans, P. gingivalis* and *Aggregatibacter spp*., proving to be effective in the prevention of caries and peri-implantitis [34]. A certain affinity of methylene blue with melanin is documented in scientific literature and therefore

photodynamic therapy with this compound has been proposed for the treatment of melanoma. There have also been excellent results in the treatment of osteosarcoma [34]. Toluidine blue, also a derivative of phenothiazine, has been introduced as a substance able to help the clinician to recognize cancer cells. It has demonstrated a certain antimicrobial activity against various microorganisms, especially against *Candida albicans*. These are mostly *in vitro* studies, still very few *in vivo* studies, and the most performing wavelength for the activation of this molecule is 630 nm [35]. Other synthetic dyes are Eosin Y, Erythrosine (ERY) and Rose Bengal (RB) which belong to anionic xanthene dyes derived from Fluorescein. All these dyes have an absorption peak in the green wavelength range (480-550 nm) [36].

Tetra-pyrroles

They are one of the largest and firstly type of photosensitizers. Tetra-pyrrole structures are called "pigment of life" because of their abundancy in nature (e.g. in haemoglobin or chlorophyll). The most frequently used of this group are porphyrins and phthalocyanines. Peak absorption of phthalocyanines is in the red region at 670 nm. We have diverse phthalocyanines (Pc). Among these agents, Zinc phthalocyanine (ZnPc) is the most studied Pc for aPDT [36]. The wavelength to activate the porphyrins is instead between 405 and 550 nm. Some bacteria tend to accumulate large number of porphyrins making them susceptible to killing when irradiated with blue light or UV, such as *Propionibacterium acnes spp.*, *Prevotella spp.*, *Porphyromonas gengivalis*, *Aggregatibacter actinomycetemcomitans* [36].

New Photosensitizers

Indocyanine green (ICG) is a chemical compound that entered the medical field about 60 years ago. In 1959 the American FDA approved its use for liver diseases. In 1963 it

was also used in the cardiac medium and for the evaluation of cerebral perfusion [37]. ICG is a closed-chain, cyanine dye that is soluble in two moieties of benzoyl indole. The molecular weight of ICG is 774.96. To increase solubility Sodium iodide is added to ICG that determine the dark brown colour of ICG. When diluited with water, the ICG solution becomes green [37]. The luminous excitation of the molecule occurs between 600 and 900 nm, with a peak around $\lambda = 875$ nm. The half-life is between 120 and 240 seconds. It has recently been proposed in photodynamic therapy in the medical medium, with success also in the treatment of some tumors as well as in an antimicrobial sense. Its use has been proposed in periodontal therapy [38].

In recent times, the need to enhance the natural and synthetic photosensitizers already on the market has tried to amplify their activity adding nanoparticles. According to a recent review on the subject, the most frequently added nanoparticles are metallic ones (gold and silver), in second place are silica-derivatives, followed by metal oxides. Among the most studied photosensitizers in conjunction enriched with inorganic nanoparticles we have Methylene Blue, *Curcuma Longa*, Toluidine Blue, Rose Bengal [39].

Type of Light Source

Laser

The laser as a light source in PDT has a unique feature: the ability to generate a monochromatic, coherent beam of light with a very narrow bandwidth. A laser essentially consists of an active medium, which can be solid, gaseous or liquid; a resonance cavity, inside which the active medium is located; an energy source that stimulates the active medium through electrical noise or light. The latter is frequently made up of solid material such as glass, crystal, fibre, powder, or gaseous such as the excimer laser. The simplest resonance cavity consists of a series of mirrors, which amplify the emission of fluorescence, derived from the excitation of the active

medium. The most common used types of lasers in PDT are diode lasers, either in pulsed or continuous mode [40]. Dye lasers are lasers with a liquid active medium which circulates constantly within a circuit to avoid overheating. They produce light with a wavelength between 600 and 650 nm. They need a high-power energy source, over 10 watts, so they are awkward to manage. Diode lasers are lasers whose active medium consists of semiconductors, activated by a source of electrical energy. They are generally much lighter and easier to transport than dye lasers, but produce a wider beam, with angular dispersion. They generally have a wavelength between 415 and 670 nm [40].

LED light

These are light sources with semiconductors that emit light spontaneously without being activated by an energy source. Not having a resonance cavity, the light produced is not coherent and sometimes results in a lower absorption of the same by the photosensitizer. They are practical, inexpensive, and do not require large sources of electricity. In medicine they are also used for the thermal effect produced following the conversion of low-level electricity into optical energy [40].

LAMPS.

Lamps were the first light sources used in PDT. These include fluorescent lamps, incandescent lamps, xenon lamps, sodium lamps. They have a very broad wavelength spectrum with oscillations ranging from 300 to 1200 nm. A filter is therefore necessary in order not to expose biological tissues to wavelengths in the ultraviolet range. Very easy to use, cheap, are still used in some cases in PDT [40].

Parameters in Laser and Non-laser PDT

The Power Density is the Power used in Watt spread over the Application Area (expressed in square centimetres). The Fluency or Energy Density determines the Applied Energy expressed in Watts per Application Time with respect to the Radiated Area. The areas irradiated by this beam of photons are different and so the power density and the effects inside the cells produced will be very different. As an example, 1 W delivered through a 400 µm diameter optical fiber will produce a power density of 796 W/cm^2 while the same 1 W delivered through an 8-mm diameter therapy handpiece will produce a power density of only 2 W/cm^2. Energy density is frequently reported in research literature but the spot area at the tissue is often omitted. Irradiation is strongly dependent on the distance between the light and the irradiated tissue. This increases or decreases as you feel or move the light source away from the tissue. Often in PDT the light source is kept about 0.5-1 cm from the injured tissue [41]. In physics, the wavelength of a periodic wave is the distance between two crests or troughs of its waveform, and is commonly denoted by the Greek letter lambda. Wavelengths between 600 and 750 nm go less deeply, from 750-900 nm there is greater tissue penetration depth [41].

Variability of parameters in PDT

In the scientific literature there are very different protocols for PDT of the same pathology. In fact, the variables that come into play are many: the type, concentration and quantity of photosensitizer; the type of light source, the wavelength, the power density and the fluence; the pre-irradiation time, i.e. how long the photosensitizer takes to bind to the target cells; how many minutes of irradiation and how many total sessions are necessary to reduce that phenomenon. The evaluation of the quantity of oxygen available in that injured tissue, the quantity of mitochondria and the cellular

metabolism of different microorganisms, target of PDT are just some of the factors that can influence the success of the photodynamic process [40].

PDT in Oral infection diseases

Among the infections that afflict the oral cavity, we have endodontic infections, supported by microorganisms that colonize the inside of the tooth and which can also give rise to infections in the periapical bone tissue. One of the most important microorganisms in this infectious process is *Enterococcus faecalis*, which is also the most studied in *ex vivo* and *in vitro* models. The difficulty of eradicating this microorganism responsible for endodontic re-infections and refractory infections has prompted researchers to make use of PDT in eradicating this microorganism. Researchers have found that the photosensitizer curcumin activated by LED (450 nm, 67 mW/cm^2, and 20.1 J/cm^2), had shown very interesting results in reducing CFU of *E. faecalis* [41]. Other authors have tested Ce6 methyl ester (Zn (II)e6Me), a chlorophyll-derived photosensitizer, activated by red light (627 nm, 75 mW, 3150 J/cm^2) for 90 s, with success against *E. faecalis*. Furthermore, an *in vitro* study found that aPDT using 500 g/mL of Chlorella plus 660 nm diode laser at an energy density of 23.43 J/cm^2 is effective against *E. faecalis* biofilms [41].

The most frequent infectious disease at the gingival level is periodontitis. It is an infectious process mediated by various microorganisms including *P. gingivalis, Aggregatibacter* and which leads to inflammation and destruction of bone and gingival tissue, with loss of stability of the dental elements. Among the natural photosensitizers that have shown the most effectiveness we have *Curcuma Longa* activated by blue LED lights (450–470 nm, output power density 1.2 W/cm^2). Ce6, was also successfully tested as photosensitizer against *Eikenella corrodens*, *Actinomyces odontolyticus*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Slackia exigua* , *Atopobium rimae* , *A. actinomycetemcomitans* , and *P. gingivalis* in planktonic

phase and within subgingival oral biofilms communities [41]. During orthodontic treatment, which aims to recreate an occlusal balance using fixed or mobile devices worn in the oral cavity, one of the most important problems is often the prevention of caries, linked to the accumulation of plaque and tartar that the orthodontic therapy itself entails. Riboflavin or vitamin B12 has been shown to be very effective when activated by blue lights in lowering the CFU of *Streptococcus mutans* and *S.sanguis* [41]. Rose Bengal with a light source for illumination (375 nm, 3 mW/cm^2) could be very useful to reduce the bacterial load of S. Mutans, the most important etiological agent of dental cavity.

Among the most frequent viral infectious diseases in the oral cavity, we have herpes simplex type 1 infection. It is a condition that involves the formation of vesicles filled with infectious liquid which, when they explode, give rise to erosions and the formation of crusts if they affect the vermilion border of the lips. Although the preferred site of the microorganism is the lips, it can occur in other sites of keratinized mucosa such as the hard palate and the gingiva. The first contact with the virus, especially in children and adolescents, can lead to the invasion of the entire oral mucosa in 10% of cases. The treatment considered the gold standard for this condition is represented by topical or systemic antivirals in particularly extensive cases. The reduction of sensitivity to pharmacological treatments has prompted several researchers to find different solutions to eradicate the microorganism, including PDT [42]. The most used photosensitizer for the treatment of herpes simplex infection is Methylene Blue, at concentrations ranging between 0.1 and 1%, activated by lights with a wavelength of 660 nm. In an in vitro study indocyanine green also showed good results when activated by wavelengths of 810 nm [43-44].

Among the very frequent viral pathologies in the oral cavity, we also have HPV lesions. These are often single exophytic lesions (squamous papilloma, verruca vulgaris, condyloma acuminatum), rarely multiple, irregular in shape and with a variable degree of keratinization. There are over 200 subtypes of HPV, but some of these are

capable of triggering malignant tissue transformations, including HPV 16. Surgical excision and histological evaluation of the removed piece represent the treatment of choice. Sometimes these lesions can reach considerable dimensions, of several centimetres, sometimes they are placed in sites that are difficult to reach and can be strongly recurring. These reasons have led some clinicians to try photodynamic therapy also on the oral cavity, in a patient with a large squamous papilloma in the soft palate. In this case, 10% of 5-aminolevulenic acid was used as a photosensitizer, injected at the perilesional level and activated with wavelengths of 630 nm at a fluence of 100 J/cm^2 and at 300 mW/cm^2 of power density for 6 min. After 2 sessions the lesion healed [46].

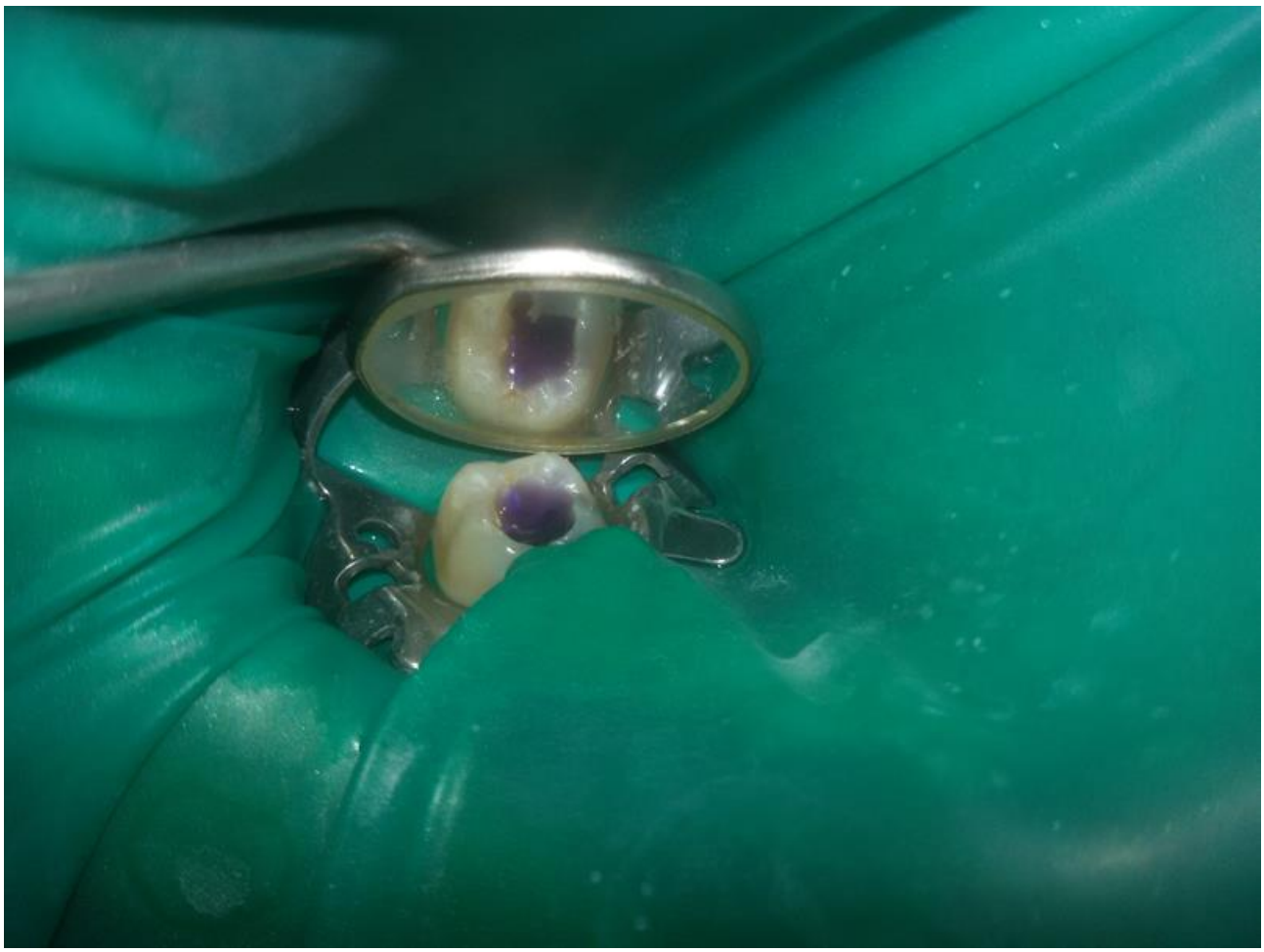


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Fig 20. Photosensitizer application inside a tooth decay. The photosensitizer used is

Toluidine Blue at 1%.

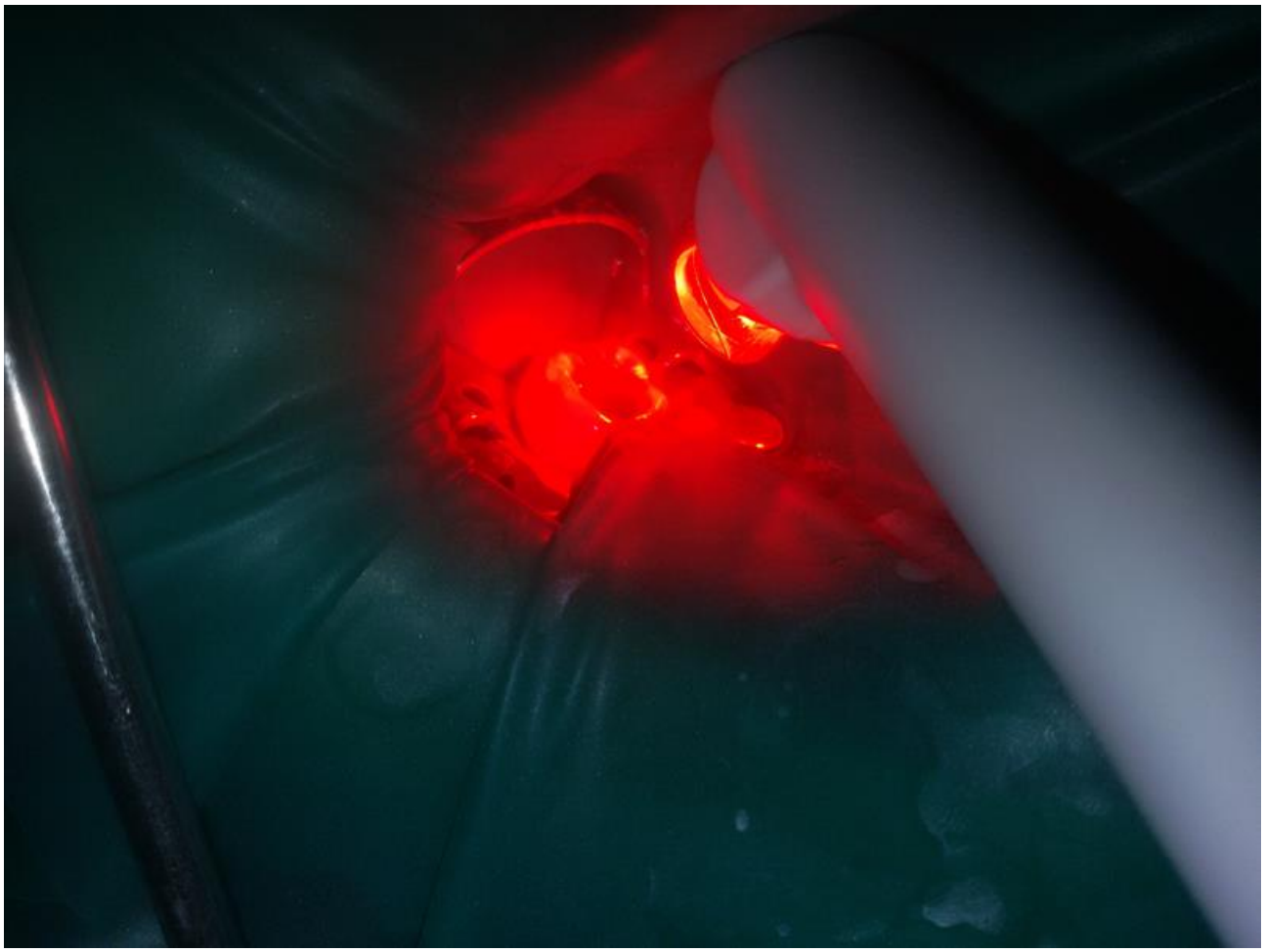


Fig 21. Toluidine Blue activated by LED light at 630 nm, 4 Watt of power, to reduce bacterial load in a tooth with decay.

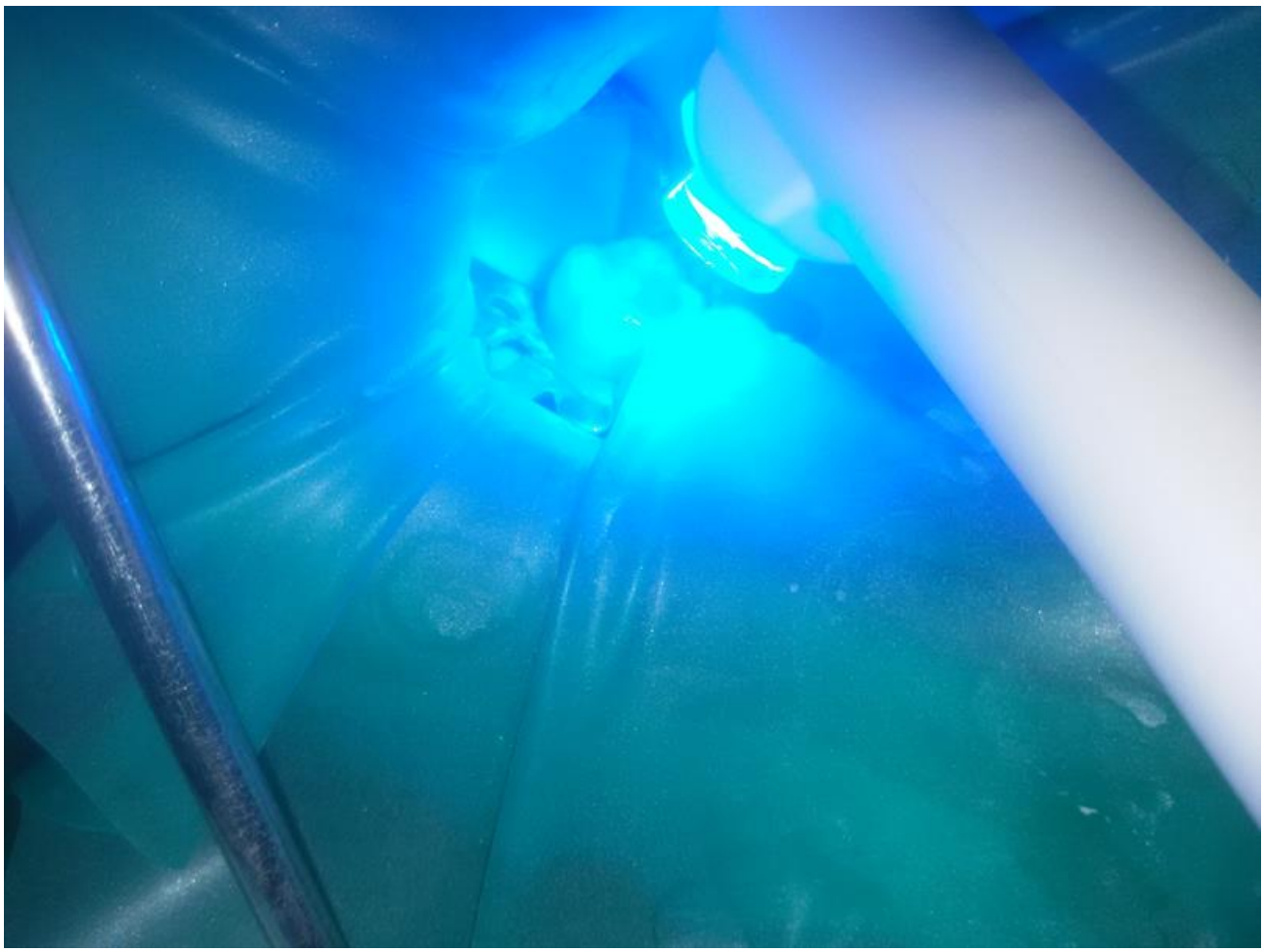


Fig 22. Photodynamic therapy performed with LAD at 460 nm, 7 watts of power.

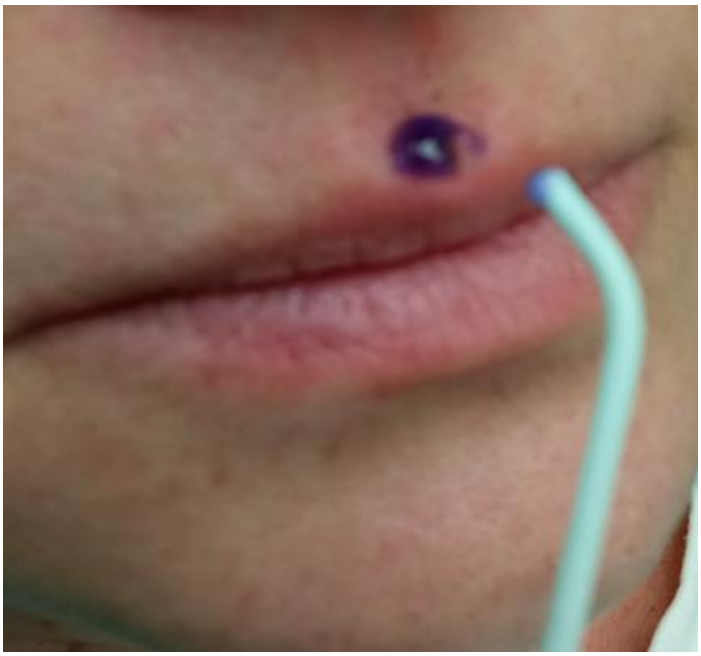


Fig 23. Application of photosensitizer, in this case Methylene Blue at 1%.

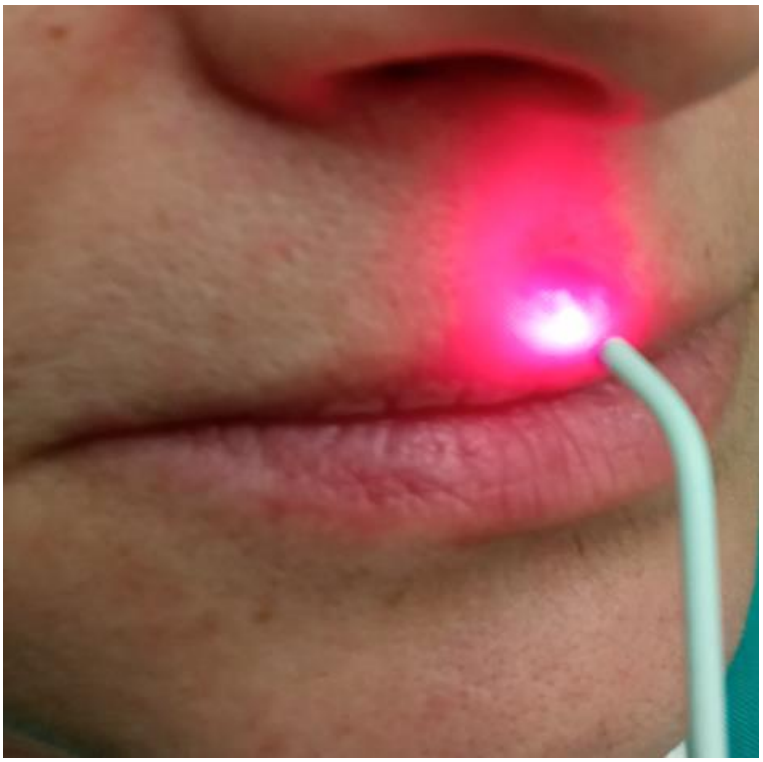


Fig 24. Illumination of the photosensitizer with diode laser at 660 nm, 100 mW of power, to treat recurrent herpes simplex infections.

Photodynamic Therapy for Oral Candidiasis

Oral candidiasis, as previously mentioned, is an infection that increasingly shows resistance to the main antifungals. Photodynamic therapy already 15 years ago, was proposed as an alternative especially in relapsing cases of oral candidiasis. Among the most tested photosensitizers we have methylene blue, which associated with laser and non-red laser lights, has demonstrated excellent antifungal properties at different concentrations [46-49].

Toluidine blue has also been widely proposed, with different concentrations in the treatment of Candida infections in the oral cavity. A recent review on the subject has highlighted that most of these are *in vitro* studies, where the parameters used are very different between the various studies, even if almost all report the efficacy of photodynamic therapy. In addition to the more common *C. albicans*, it has also been

tested against different species of Candida in some studies, such as *C.glabrata*, *C. krusei*, *C. parapsilosis, C. tropicalis*. Toluidine blue has been shown to be effective against all types of *Candida spp.* mentioned [35]. Most of the *in vitro* studies were studies in which both the activity of PDT in its planktonic form and in the biofilm of the fungus were evaluated. The prevailing method was that of microplates [35].

Subsequently, other photosensitizers were also proposed, including Photodithazine, a chlorine derivative, activated by red lights around 660 nm, which have also demonstrated efficacy *in vivo* in clinical trials [49-53].

Among the most recent photosensitizers, indocyanine green activated by a wavelength of 808 nm has also been proposed. In a recent study it was compared with the pharmacological treatment based on Nystatin, and the PDT showed a significant reduction in the CFU on the various *Candida spp*. tested, greater than the group of control patients [54]. In another *in vivo* study on refractory subprosthetic candidiasis, PDT with indocyanine green was used as an adjunct to conventional pharmacological therapy, showing better results than the group treated only pharmacologically [55]. Furthermore, in another study it was observed that it proved to be more effective *in vitro* than PDT performed with Methylene Blue [56].

Other photosensitizers tested *in vitro* against Candida are erythrosine and Rose Bengal activated by green diode lights around 532 nm [57,58].

In particular, erythrosine was also evaluated against a species of Candida rarely studied in PDT, *C. dubliniensis*, demonstrating good efficacy in the planktonic form but less activity on the biofilm [59].

Among the natural photosensitizers we find 5-aminolevulenic acid [60] and derivatives from *Curcuma Longa*. The first work carried out using this photosensitizer was published in 2013 [61]. Subsequently, other studies evaluated its safety in terms of cytotoxicity, its antimicrobial activity, which was also compared with other synthetic photosensitizers [62-63]. In a study, activated by LED light at 460 nm, it showed an antifungal activity in vitro, superior to Methylene Blue activated by red light at 660 nm

[63]. Animal studies have also evaluated efficacy with polymer nano-particle enhancement [64]. A recent study evaluated the ability to prevent fungal infections in 108 radio-treated patients for head and neck cancer through two compared photodynamic therapy protocols. In this case the Methylene Blue activated by red lights was the most effective in reducing the fungal load compared to the protocol with curcumin [65].

Photodynamic Therapy for Oral Potentially Malignant Disorders

Photodynamic therapy has also been proposed for potentially malignant lesions of the oral cavity, in particular leucoplakia and oral lichen planus, which we have already mentioned previously. Despite the strong diversity of the protocols used, the best performing photosensitizer from the analysis of the literature is alphalevulenic acid known as ALA, in various concentrations, but mainly used between 4 and 10%. Both laser lights and LED lights have been used for the activation of this photosensitizer, at wavelengths between 460 and 660 nm. The various studies have mainly observed the size of the lesions before and after photodynamic therapy, while specific studies on the reduction of the degree of dysplasia of these lesions are not present [66].

Compared to photodynamic therapy performed in an antimicrobial sense, the results are less striking. Some studies have highlighted a 40% healing of the lesions, others report lower percentages, while the data on the clinical improvement of the lesions is highly variable but around 50% of the cases, expressed above all as a reduction in size. However, we do not currently have any protocols either on the total number of sessions and on the frequency. In some cases, more than ten sessions were carried out, in others there was resolution after only 4 sessions. The frequency of the sessions varied from 2 times to 1 time per week [66].

Other studies report interesting data with other more convenient photosensitizers such as 5% Methylene Blue and 1% Toluidine Blue. Refractory cases, in which no type

of clinical improvement is seen, are a non-negligible part. There is currently no data in the literature on PDT performed on other potentially malignant oral lesions such as submucous fibrosis and erythroplakia. The selective binding mechanism through which the photosensitizer binds to the altered cell is not known, however the almost total absence of side effects of PDT in the treatment of these lesions can be confirmed [66]. More studies on mycotic superinfection of these lesions should be carried out, also to understand how important the role of fungal infections can be in the refractoriness of the lesions and in the strong tendency to recurrence and the connection with the greater tendency to carcinogenesis.

Chapter 7-Aim of the study

The aim of this work was to evaluate the efficacy of different tools for photodynamic therapy in the treatment of patients affected by oral candidiasis refractory to pharmacological treatments through first *in vitro* studies and then *in vivo* studies.

The work was therefore divided into three parts:

I. *In vitro study* to evaluate the effectiveness of 3 different wavelengths (660, 630, and 460 nm) in combination with 3 different commercial photosensitizers (Methylene Blue derivative, Toluidine Blue, and Curcumin+ Hydrogen Peroxide) on Candida colonies (performed within 1° year) in collaboration with Oral Biotechnology Laboratory, University of Cagliari (*in vitro study 1*)
II. *In vitro* evaluation of the effectiveness of other at least 2 natural product extracts, activated also with new wavelengths, against Candida colonies (performed within the 2° year). This part was realized in partnership with electronic engineering staff to choose the best type of wavelength to activate the new natural photosensitizers proposed (*in vitro study* 2). I evaluated more than two

natural photosensitizers. This step also comprised a model in human cell culture infected with *C. albicans* (*in vitro study 3*).

III. Clinical evaluation of the healing time of oral Candidiasis treated with PDT realized with the lights and photosensitizers evaluated in the *in vitro* studies (3° year), in cooperation with a statistician (clinical study).

The main objectives are:

- To find the best parameters possible for PDT effectiveness in the treatment of oral Candidiasis
- To find photosensitizer that can be ingested by the patient treated with PDT on the oral mucosa to avoid any minimal toxic effect.

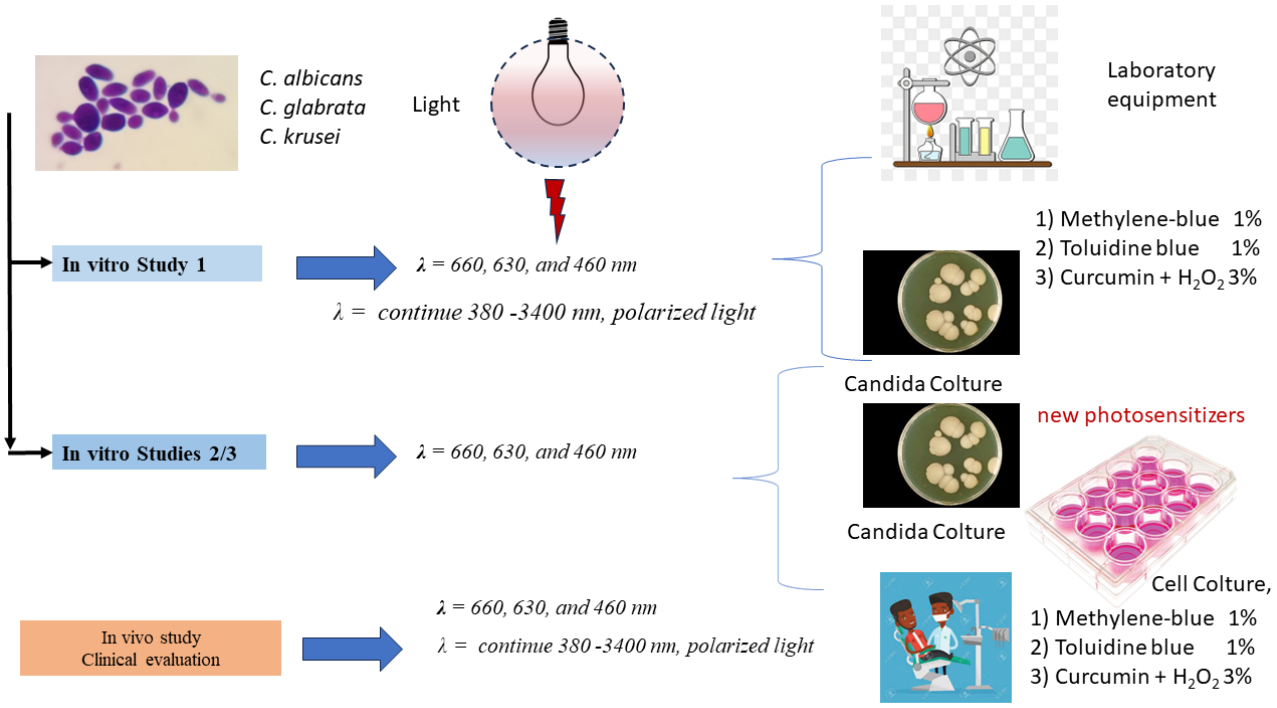


Fig. 25. Operative diagram of the thesis work.

Chapter 8- Materials and methods

For the *in vitro* study on the effectiveness of commercial PDT we used *Candida spp.,* multidrug-resistant isolates from refractory patients. It was used to perform the antimicrobial test. This strain was previously characterized for its response to different antifungal drugs. We have characterized it in our previous studies both by cultural and molecular procedures.

Candida strains used in this study

In this work, three different oral clinical isolates of *Candida spp*. (*C. albicans* CA97, *C. glabrata* CA96, and *C. krusei* CA95) were tested using a PDT sensitivity test. For example, *C. albicans* strain (CA97) was found to be resistant to three different azoles (Fluconazole, Voriconazole, and Ketoconazole), especially due to a mutation in the ERG11 gene [13].

These strains were plated in Sabouraud glucose agar for 48 h at 35°C (Microbiol, Uta, Cagliari, Italy). The colonies were identified with an API ID32C system (Biomerieux, St Louis, MO) and maintained at -20°C in skimmed milk (Oxoid, Basingstoke, UK) with a concentration of $1*10^8$ CFU/mL.

Materials and General Description

All antimicrobial procedures used in this work were based on the CLSI and EUCAST protocols (Link 3, Link 4). As described later, for each *Candida spp*., we used an inoculum of $1*10^7$ CFU/mL in the Kirby Bauer assay and $1*10^5$ CFU/mL for the growth curve assay in liquid medium. The Kirby Bauer experiments were performed in Petri dishes of 90 mm in diameter that contained 15 mL of Sabouraud agar (Microbiol, Uta, Cagliari).

***In vitro* study 1**

To perform this part of the work (*in vitro* study 1) are used 3 different photosensitizers:

- Methylene Blue 1% (Bredent, Senden, Germany);
- Toluidine Blue 1% (CMS Dental, Copenhagen, Denmark)
- Curcumin+ Hydrogen Peroxide 3% (CMS Dental, Copenhagen, Denmark).

To activate these photosensitizers are used 4 different lights:

- Diode laser at 660 nm of wavelength, 100 mwatt of power (Helbo system, Senden, Germany)
- Diode light at 630 nm of wavelength, 4 watt of power (CMS Dental, Copenhagen, Denmark)
- Diode light FlashMax P7 at 460 nm of wavelength, 7 watt of power (CMS Dental, Copenhagen, Denmark)
- Polarized light with wavelength 380-3400 nm (Bioptron, Zepter, Switzerland)

The groups tested on the first part of this in vitro experiment (In Vitro Study 1) are:

- Methylene Blue 1% + Diode Laser 660 nm (A)
- Toluidine Blue 1% + Diode Laser 660 nm (B)
- Curcumin and H_2O_2 + diode light at 460 nm (C)

The groups tested in the second part of this in vitro study (*in vitro* study 1) are:

- Toluidine Blue 1% + diode light at 630 nm (D)
- Curcumin and H_2O_2 + diode light at 460 nm (E)

- Curcumin and H_2O_2 + Polarized light (F)

The aim of this first experimental group was to perform a standardized procedure to evaluate the antimicrobial activity of photodynamic therapy against the pathogenic fungus *Candida albicans*. In this operative set, we have used two different approaches: (i) in solid medium and (ii) in liquid medium, (iii) in acrylic oral prosthesis (simulating a mucositis). These experiments were based on modified, already-described protocols published on the Clinical and Laboratory Standards Institute (CLSI) web page (Link-3). Therefore, the experimental procedure considered 3 types of PDT protocols based on different photosensitizers: Methylene Blue, Toluidine Blue and Curcumin + H_2O_2 respectively.

First part

In the first part of the *in vitro* experiment, the Kirby-Bauer procedure has been used. 15 mL of agarized medium (Sabouraud agar Microbiol, Uta, Cagliari, Italy) at 55°C was put into a Ø 90 mm Petri dish and, prior to agar solidification six sterile iron rivets, Ø 10 mm in diameter and 2 mm thick (Firm, Milan, Italy), were put into the agar hot solution and then removed from the medium when it solidified (about 30°C). A *C. albicans* log-phase suspension, after 15 hours of growth in Sabouraud Broth, was used as an inoculum with the previous described, microbial concentration of $5*10^7$ (CFU/mL). The yeast was inoculated onto the plate surface using a sterile swab. 0.05 mL of photosensitizer solution was put in each well positioned inside the agar thickness.

For each Petri dish, 2 wells contained a solution of 100 µl of Methylene Blue 1% (Group A); 2 wells with 100 µl of Toluidine Blue 1% (Group B); and 2 wells with 100 µl of Curcumin + H_2O_2 (Group C). At this point, the light was irradiated on one of the 2 wells surface with the specific light for each group, as previously mentioned, for 60 seconds

at a distance of 0.5 cm from the wells. Petri dishes were incubated in the air at 37°C for 48 h. After incubation, the diameter of the possible inhibition halo was measured. The final value was represented as the geometrical mean of three different experimental repetitions, *Figure 26.*

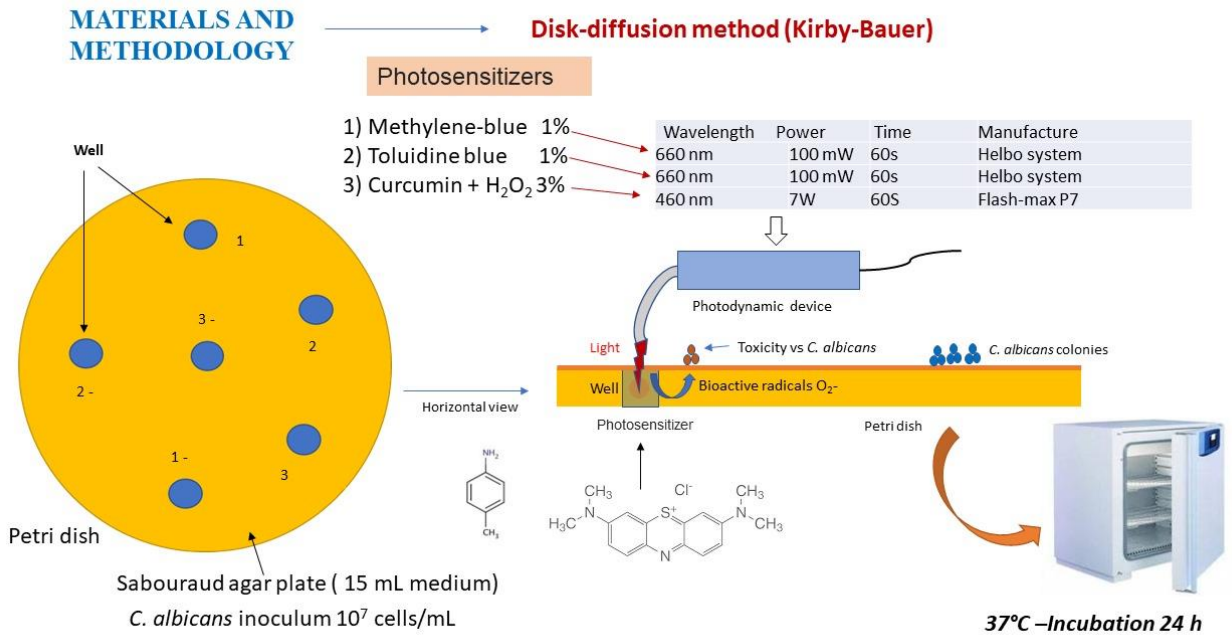


Fig 26. Materials and Methodology of the first part of the *in vitro study 1*.

Second part

In this part only the mixture contained curcumin and hydrogen peroxide 3% and Toluidine Blue 1%, were used for a subsequent experiment in liquid medium, this was performed in a 96-well microplate. In the first instance, we have considered the UV-visible spectra of Curcumin/H_2O_2 and Toluidine blue. In fact, normally, the maximum adsorption wavelength represents the respective λ with the best antimicrobial activity [61-64]. The spectral properties of the Curcumin/H_2O_2 base product, were studied by using a UV– visible spectrophotometer JASCO V600 – Bio, (JASCO Europe, Cremella, Italy) in the range of 200-700 nm, by using an optical path of L = 10×10 and mm glass

cuvette (Hellman Analytics, Munich, Germany), following the manufacturer's instructions. We have investigated the absorption spectra of commercial formulations as such, and concentrations diluted in water 1/10-fold. In practice, calculating the UV-vis spectra was required to assess the perfect assonance between light emission profiles and photosensitizer excitation motifs for use optimally in photodynamic therapy.

For the Microplates Assayed Methods, a specific experimental design was used, which includes 96 microplates of 10 mm in diameter each. For each well were added: the inoculum, in this case *C. albicans* 10^5 CFU/ml dissolved in 180 μl of Sabourad broad, and 20 μl of the photosensitizer substance, activated or not by light. The turbidity is tested before and after the incubation time, which is therefore an indication of the antimicrobial capacity of the tested solution.

Another aim of this experiment was to also evaluate the activity of large range λ polarized light in comparison with LED light at 460 nm, Figure 27.

This experiment was performed in triplicate.

The groups tested are:

Controls:

- 9 microplates with only inoculum, C. *albicans* 1x 10^5 CFU/ml dissolved in 180 μl of Sabourad broad (positive control test).
- 9 microplates with only medium, 180 μl of Sabourad broad (negative control test)

Photodynamic group D

- 3 microplates with 20 μl of Toluidine Blue 1% (only photosensitizer).
- 3 microplates with only activation of 60 seconds of diode light at 630 nm (only

light).

- 3 microplates with 20 µl of Toluidine Blue activated by 60 seconds of 630 nm diode light (photodynamic therapy with Toluidine Blue).

Photodynamic Group E

- 3 microplates with 20 µl of Curcumin+ H_2O_2 3% alone.
- 3 microplates with only the activation of 60 seconds of 460nm diode light.
- 3 microplates with 20 µl of Curcumin+H_2O_2 3% and 60 seconds of 460 nm diode light.

Photodynamic Group F

- 3 microplates with 20 µl of Curcumin+ H_2O_2 3% alone.
- 3 microplates with activation of 60 seconds of Polarized light alone.
- 3 microplates with 20 µl of Curcumin+ H_2O_2 3%, activated by polarized light.

The plates were incubated at 37° C for 48 hours and then the *C. albicans* growth level, were evaluated by spectrophotometric analysis, by multiscan spectrophotometer at 600 nm, Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific , USA.

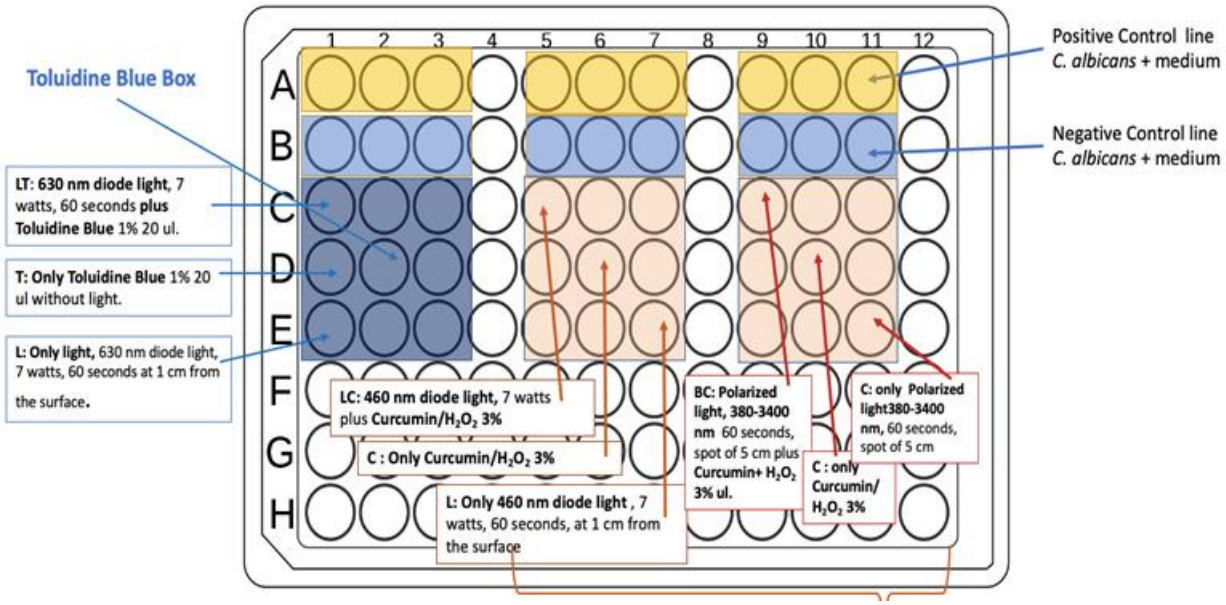


Fig. 27. Schematic representation of microplate assay used in the second part of the

Biofilm evaluation

In oral microbiology, the production of biofilms—during which the bacterial cells are immobilised in a tissue or extracellular polymeric matrix (i.e., an oral denture)—is a key factor in fungi pathological activity. Biofilms are one way that microbial cells adapt to multicellular lifestyles, according to *Candida spp*. Biofilm matrix are normally waterproof against antifungal agents as well as host immunological cells and molecules. In this *in vitro* work stage, we have measured the biofilm amount in the same microplate used for growth evaluation (described before) through the Centre for Biofilm Engineering protocol of Montana University, https:// biofilm.montana. edu/index.html.

Third part.

For the third part of the in *vitro study*, I performed a simulation of fungal infection on an acrylic resin prosthesis with a particular clinical isolate of *Candida glabrata* MDR, a

strain resistant to drugs and to some photodynamic therapies with other commercial photosensitizer, this aspect was described in the “results experiment session”.

A polyacrylate denture in vitro infected with a MDR clinical isolate of *C. glabrata* (BF3) was used for this study to simulate a subprosthetic infection. This dental prosthesis was suitably disinfected with alcohol at 90° and then rinsed with distilled water to deprive it of previous environmental contamination. It was then infected in a homemade bioreactor by using a concentration of 10^5 colonies of *C. glabrata* and placed in a liquid culture medium with Sabouraud Broth (Microbiol, Uta, Italy) containing 2% human saliva, this is to introduce salivary mucins *Figure 28*. The box containing medium-infected and polyacrylate dentures has been vortexed to better spread the colonies over the entire surface of the prosthesis. The vital *C. glabrata* cells number in a cm^2 of the prosthesis were evaluated following the subsequent formula: $[Nx = (0.5*N1)/mL_1]$

were:

Nx = represents the *C. albicans* CFUs in a cm^2 of prosthesis.

N1 = total CFUs counted in the agar plate.

mL_1 = suspension volume put down in the petri dish.

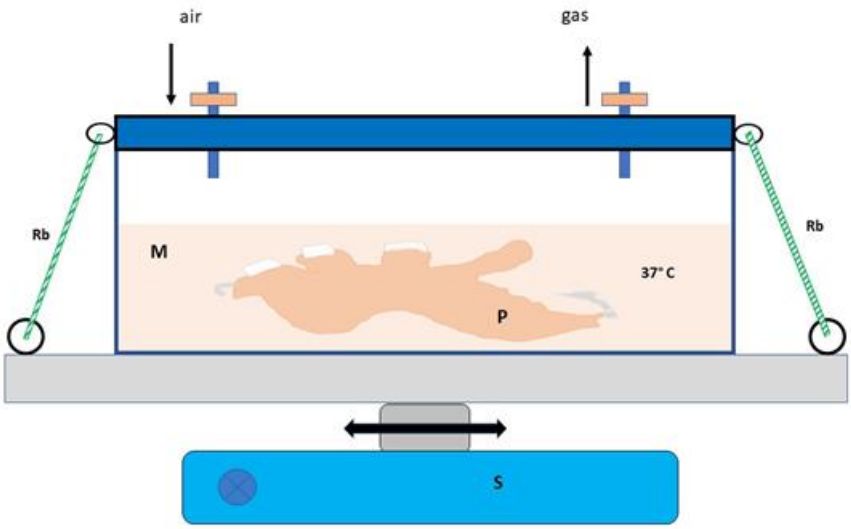


Fig 28. Schematic reproduction of the bioreactor containing culture medium and the infected prosthesis.

Legend:

M= 2% saliva cultural Medium

P = prosthesis used in this experiment.

Rb = Rubber bands used to secure the Bioreactor to the

Mixer.

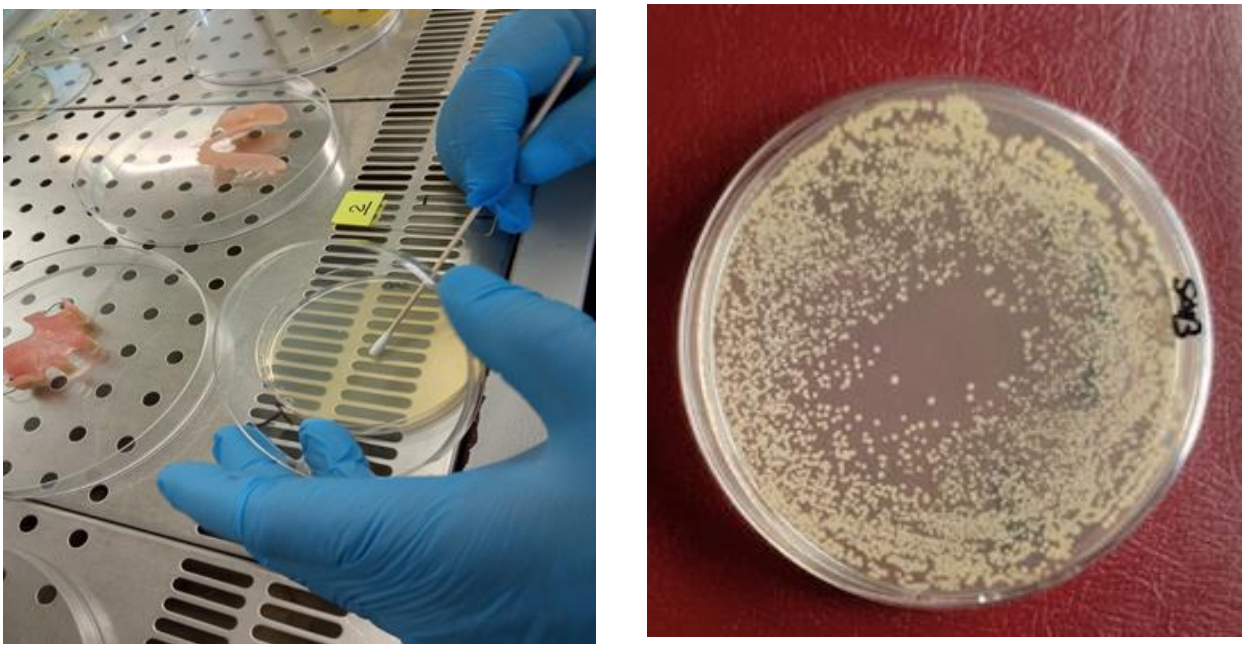
S = Vertical mixer.

The sterile box was placed in an incubator for 48 h at 37° C.

The Candida titre on the prosthesis surface was evaluated by biofilm scraped by Cytobrush (DOC, NY, USA), rubbed on the 1 cm^2 surface of the prosthesis for 30 seconds.

The sample was then dissolved in 1 mL of 0,9% saline sterile solution on a 100 µl disposed surface of 90 mm Sabouraud agar medium (Microbiol, Uta, Cagliari).

The Petri dish was then placed in the incubator using the same methods described previously. After 48 hours, the values of CFUs represented the yest title on the prosthesis surface (Figures 29a and 29b).



A) B)

Fig. 29 (a-b). (a) Sample deposition in agar medium, (b) Petri dish showed CFUs of *Candida glabrata* taken from the surface of the prosthesis infected after 24 h of incubation.

Photodynamic treatment procedure

The surface of the denture that normally adheres to the palate was exposed to 1.5 ml of a photosensitizer with a mixture of 3% of curcumin and hydrogen peroxide and illuminate with 460 nm diode lamp (7 watts) for 5 minutes, at distance of 1 cm from the surface treated (Figure 2). To avoid microbial contamination, all experiments were

performed in a multiple biological safety cabinet class II (KW Apparecchi Scientifici S.r.l. Siena, Italy), *Figures 30, 31.*

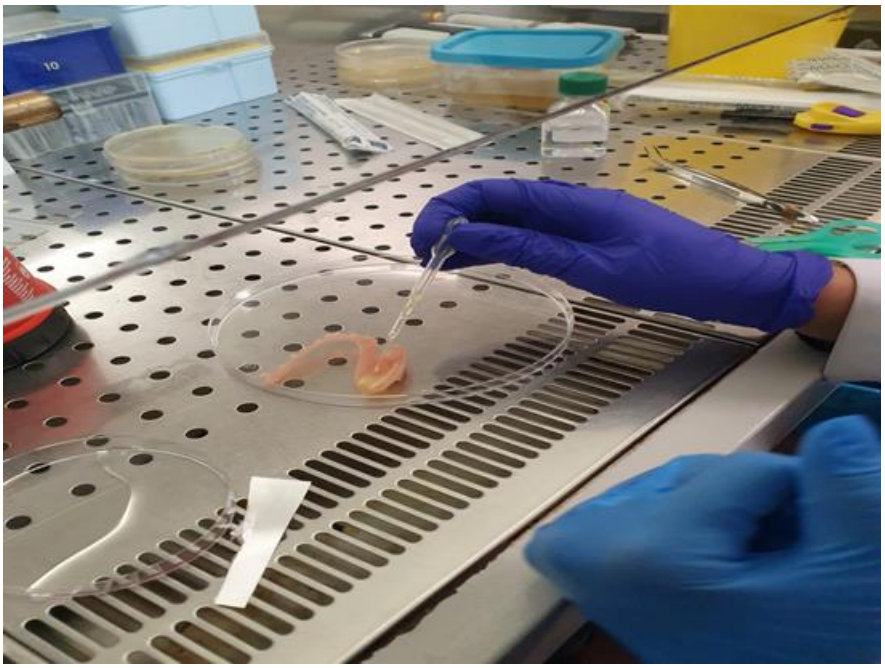


Fig. 30. Application of photosensitizer on the surface of the denture.

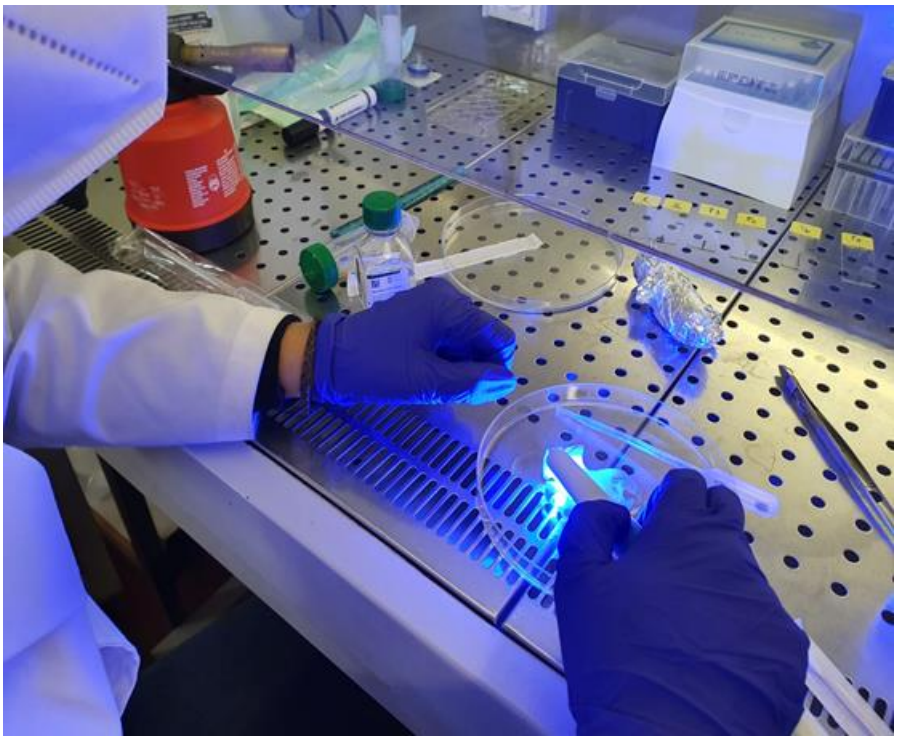


Fig 31. Photo-activation of curcumin-based photosensitizer on the denture surface.

As described before, after photodynamic therapy, a sterile brush was rubbed on the surface of the freshly treated prosthesis for 30 seconds and then used to contaminate a Petri dish containing Sabouraud gel as a culture medium. This second plate will be incubated under the same previous conditions. The residual CFUs after the incubation period will be compared with those of the previous plate and will therefore be an expression of the antimicrobial activity of PDT.

Statistical analysis

Significant values were defined as those having a standard deviation (SD) smaller than 10% of the mean value for the same experimental condition. The Fisher test was used to compare the variations across the tested formulas, using a 0.05 significance level.

***In vitro* study 2**

The aim of this experimental session was evaluating new photosensitizers able to inhibit *Candida* growth after light stimulation.

Materials

Oral already described MDR isolates of *C. albicans, C. krusei and C.glabrata* are used for this part of the work.

New types of photosensitizers evaluated:

- Extra-Virgin Olive Oil (EVOO) Coratina Cultivar,
- Mixture of 20 mg of bovine Lactoferrin in Sabouraud Broth
- Saffron solution 0,3% in distilled water
- Pompia Juice
- Strawberry Juice

- Hydrogen peroxide 3% to activate natural compounds

Lights used in this part of the work:

- Diode light FlashMax P7 at 460 nm of wavelength, 7 watt of power (CMS Dental, Copenhagen, Denmark)
- Polarized light with wavelength 380-3400 nm (Bioptron, Zepter, Switzerland)

Evaluation of absorption spectra for new natural photosensitizers candidates

The maximum absorption was evaluated for each natural element tested by evaluation of the absorbance value. The spectral properties of these formulations, were studied by using the already described procedure by a UV– visible spectrophotometer JASCO V600 – Bio, (JASCO Europe, Cremella, Italy) in the range of 200-700 nm, by using an optical path L = 10×10 and mm glass cuvette (Hellman Analytics, Munich, Germany), following the manufacturer's instructions, *Figure 32*, Table I.

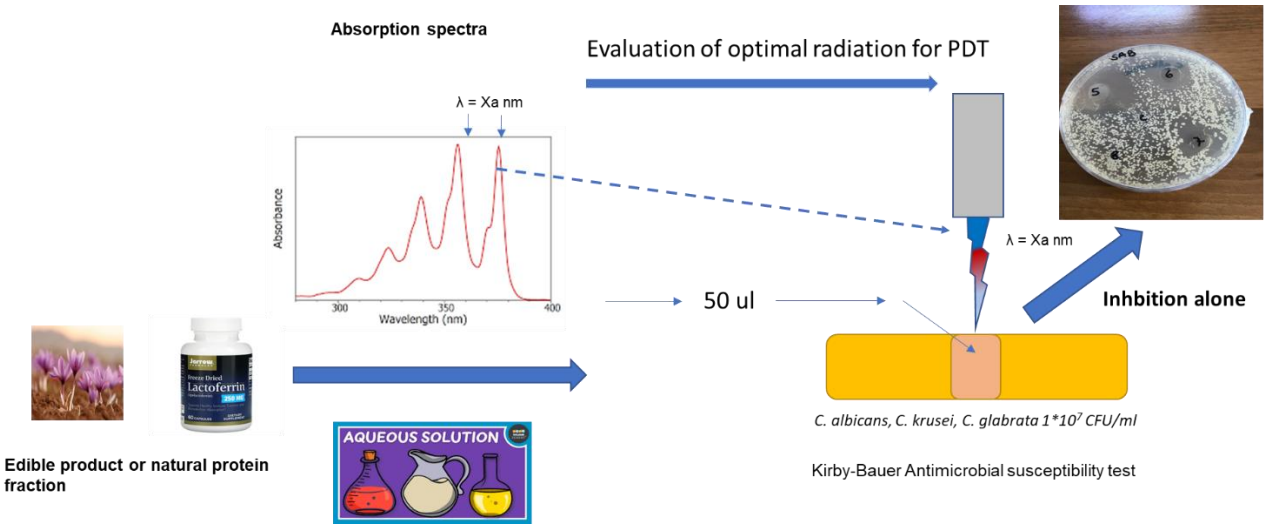


Fig. 32. Operational workflow indicating the different work steps (max light absorption frequency assessment, PDT irradiation, and antimicrobial profile).

Tab. I. UV-vis adsorption spectra of the PT candidates.

Candidate photosensitizer	Max absorption λ (nm)
Olive oil (EVOO)	390-490 and 650
Bovin Lactoferrin	280; 350; 460 (low)
Saffron solution 0,3%	320; 440
Pompia Juice	200 - 350
Strawberry Juice	320; 500
Hydrogen peroxide 3%	270; 445 (low)

To evaluate the antimicrobial profile of these natural elements against oral MDR isolates of *C. albicans, C. kruseii* and *C.glabrata*, a Kirby Bauer test was performed in accordance with EUCAST protocols, Figure 32. For antimicrobial profile assessment, each Petri dish was infected with $1x10^7$ CFU of the aforementioned Candida species by cotton brush smeared from the centre of the well to the circumference of the Petri dish, as shown in figure 33.

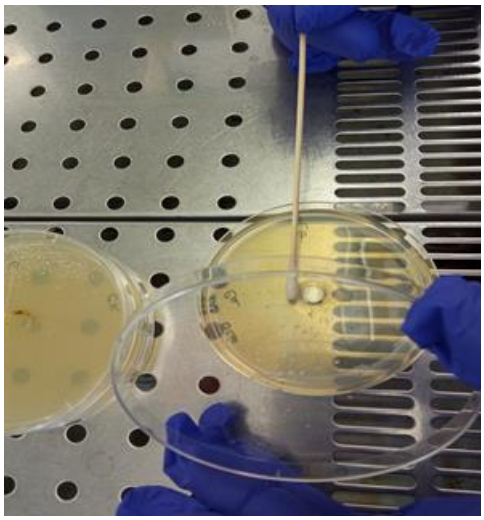


Fig 33. Detail of the infection of Petri dishes by using MDR *Candida spp.*

Olive Oil test.

For each strain, six different combinations were evaluated: (i) 100 µl EVOO oil; (ii) 100 µl EVOO oil previously activated with 3% H_2O_2 that was mixed with the vortex in a ratio of 1:1, and then re-prepared the oil phase called "(EVOO-H)"; (iii) 100 µl EVOO oil + 5 minutes of polarized light (380-3400 nm, 25 Watt), and (iv) 100 µl of (EVOO-H) irradiated with the same light; (v) 100 µl of EVOO oil + 5 minutes of 660 nm diode laser light irradiation (100 mW); (vi) 100 µl of (EVOO-H), + 5 minutes of 660 nm diode laser light irradiation (100 mW), *Figures 34, 35,36.*

Fig. 34. Mixing EVOO with hydrogen peroxide, before separating the oil fraction.

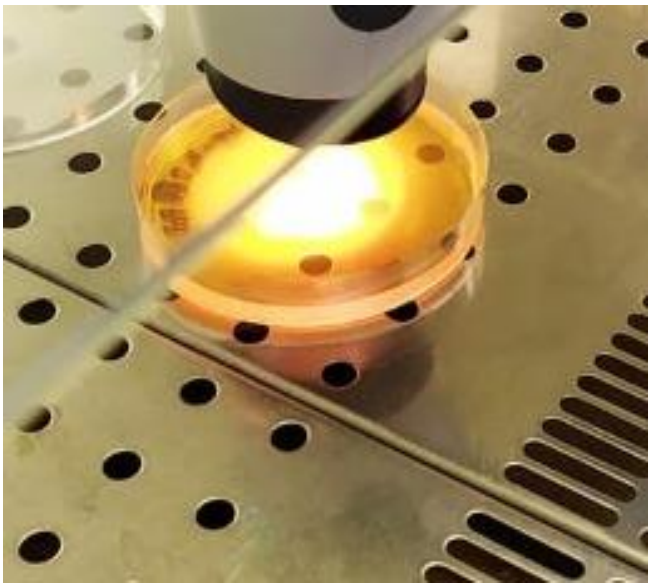


Fig. 35. Activation of EVOO with Polarized Light.

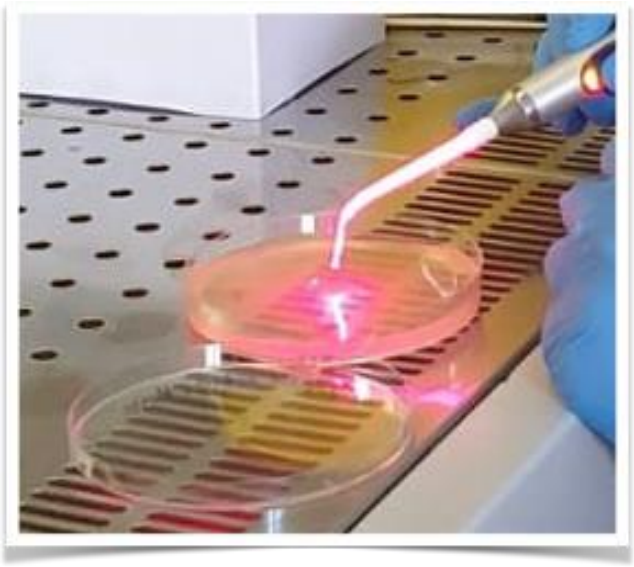
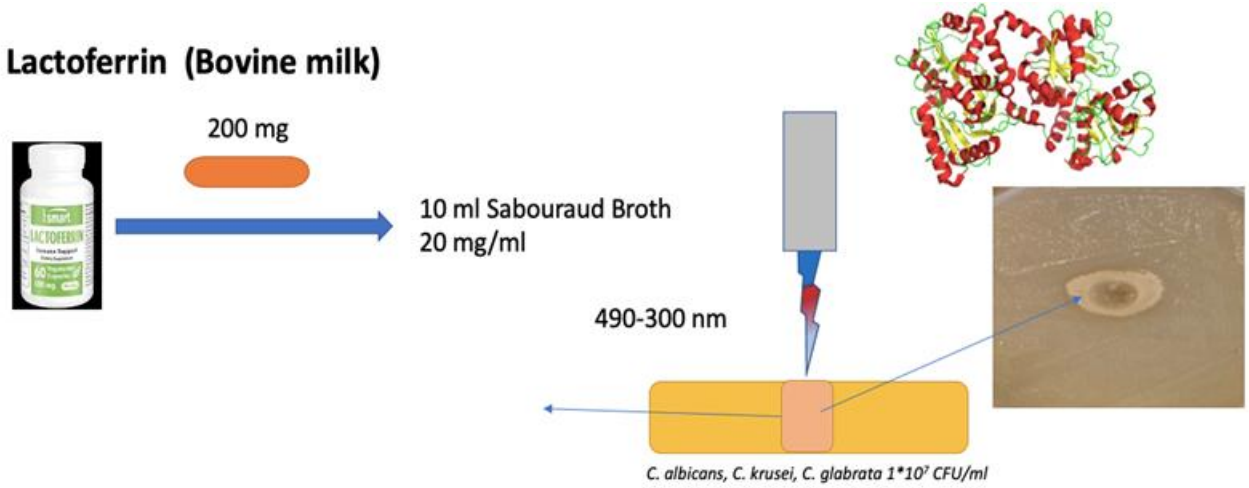


Fig 36. Activation of EVOO with laser light at 660 nm.

Lactoferrin test.

20 mg of bovine lactoferrin was prepared and dissolved in 1 mL of Sabouraud broth; this solution was used in four different combinations and tested against each strain: (i) 50 µl of solution as it is; (ii) 50 µl of solution activated with 3% H_2O_2; (iii) 50 µl of solution activated by light at 310-350 nm; (iv) 50 µl of solution activated both with 3%

H_2O_2 and light at 310–350 nm (the maximum light absorption was previous evaluated by spectrophotometer). For this part of the experiment on the lactoferrin mixture, another particular strain of *Candida albicans* was also used, called CC1. This strain was clinically isolated from a patient suffering from subprosthetic candidiasis, particularly refractory also to other types of commercial photodynamic therapy previously



described, *Figure 37.*

Fig 37. Scheme of the preparation of the lactoferrin mixture and its photo-activation by blue light.

Saffron test.

A solution of 0,3% of Saffron, dissolved in distilled water was used, the maximum light absorption evaluation was at 430 nm diode light. For each strain, two different combinations were evaluated: (i) 100 µl of saffron solution alone; (ii) 100 µl of saffron solution activated by a diode light at 460 nm, 7 watts of power for 1 minute.

Pompia Juice test and Strawberry Juice test.

Fresch juice of Pompia, *Figure 38*, and Strawberry were obtained from fruit collected in Siniscola Town (Sardinia, Cagliari) in February–March 2023.

A squeezing process was performed on ice to avoid high temperatures and the biodegradation of the juice biomolecules. Immediately, these compounds were used for PDT treatment by using the already described PDT olive oil assay through the Kirby-Bauer method (using 100 µl of each sample irradiated with only polarized light to 380–3400 nm, 25 Watt). The test strain was *C. albicans.*

Fig.38.Pompia is a citrus hybrid grown for its edible fruit. Its scientific name is Citrus medica var. tuberosa. It is taxonomically equivalent to *Citrus medica*. Pompia is indigenous to and exclusively found in Sardinia, particularly the eastern half, where it can be grown in backyards and orchards as well as in the wild in citrus groves.

In vitro study 3

This experimental session was performed to study the impact on PDT on the production of cellular signalling of inflammation, in this case the gene expression of two microRNA molecule called miRNA144a and miRNA 155.

The role of miRNA in Candida oral infection

Following the data and considerations published on the web sites miRBase (Link-5) and miRDB (link-6), there is growing evidence pointing to miR-144a and miR155's as criticals function in regulating physio pathological processes in various fungi-infected cells, including apoptosis, the recruiting of macrophages, production of pro-inflammatory cytokines. In addition to these roles, miRNAs can indicate the effectiveness of clinical treatment, drug sensitivity, and patient prognosis. In this study, we provide a measurement of these two miRNAs in cell cultures infected with *C. albicans* CA97 and then treated with PDT with curcumin/H_2O_2 based photosensitizer.

Cell cultures & infection

Human embryonic kidney (HEK)-293 cells were grown in MEM medium supplemented with 10% FBS, 1% penicillin/streptomycin (100 iU/mL; 100 μg/mL). The cells were incubated in atmosphere of 5% CO_2 at 37°C, until the confluency (about 48 hours). In this step the plates were infected with a suspension of *C. albicans* CA97 with a multiplicity of infection (MOI) of 1/1. The experimental design is showed in *Figure 39*, all experiments were performed in triplicate. After 2 hours of incubation, cells of each experimental variant, were used for RNA extraction by using the viral RNA extraction spin kit (Anatolia, Istanbul, Turkey), following the manufacture instruction.

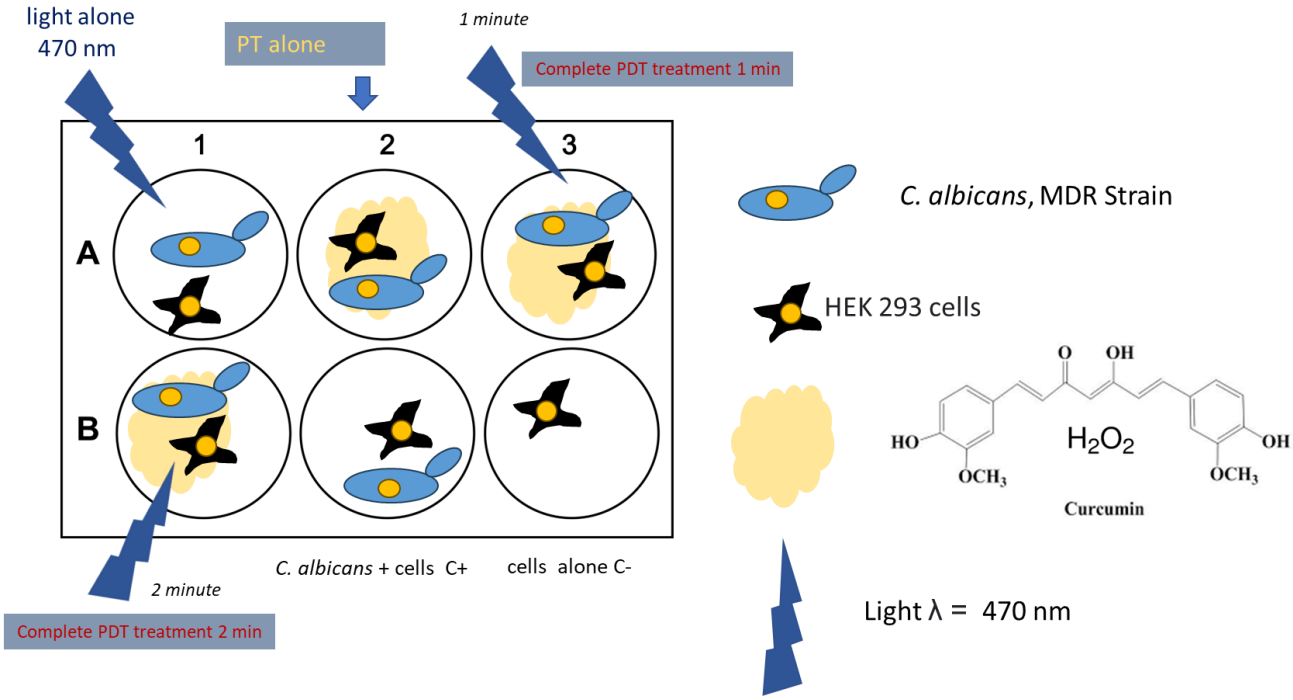


Fig.39. Schematic representation of the *in vitro* experiment.

In this experiment was used a lab-made 470 nm light emitter (200 mW), to avoid *Candida* cell contamination on commercial devices.

Pri- miRNAs measurement

Forward and reverse primers were designed on the genome sequences respectively: accession number MN298232.1 (Pri-miRNA 144a) and NC_000021.9 (Pri-miRNA 155), Table II

Tab.II. RT pCR Primers used to detect the miRNAs by real time RT PCR

Nome	Sequence : 5'----------3'
OG Pri-Mir 146 F	TTTACAGGGCTGGGACAG
OG Pri-Mir 146 R	TCAGGATCTACTCTCTCCAGG

nome	Sequence : 5'----------3'

OG Pri-Mir-155 F	AGGAAGGGGAAATCTGTG
OG Pri-Mir-155 R	TCATGCTTCTTTGTCATCCT

Possible oligos dimers formation and/or self-complementarity and theoretical melting temperatures of primers (Tm) were calculated using Oligo program version 6 (MedProbe, Oslo, Norway). Oligos hybridization were set with the following parameters: monovalent cation concentration of 0.05 mol/L, Mg2+ at 0.002 mol/L, a concentration of probe and target of 100 mM, initial hybridization temperature of 37 °C. The miRNAs were quantified by relative quantification ($2^{-\Delta\Delta Ct}$ method) by using beta-actin as a reference housekeeping gene; in this case, the oligos used are: OG650 (Beta-act) F 5'-GCATGGGTCAGAAGG-3' and OG651 (Beta-act) R 5'-AGGCGTACAGGGATAG-3'. The real-time PCR conditions for amplifying the reference gene were the same as described below for miRNA, (link-8).

Real time RT PCR conditions

The TaqPathTM 1-Step RRT-PCR MasterMix (Life Technologies, USA) was used to perform the SYBR®Green Real-time PCR experiment. A final volume of 20 µl contained 5 µl of the master mix solution, 2 µl of the SYBR®Green solution 1/1000, (Sigma-Aldrich, St. Louis, MO, USA), 1 µl of each primer (Table II) 5 µl of RNA extract and 7 µl of DNase RNase free water. The PCR profile, conducted by using a CFX 96 apparatus (Bio-Rad laboratories USA), was as follows: (i) an initial uracil–DNA–glycosylase (UNG) incubation at 25 °C for 2 min, (ii) RT incubation at 50 °C for 15 min and (iii) 40 cycles of 3 s at 95 °C, 30 s at 60 °C and 2 s at 81 °C, Fluorescence was detected at the end of the 81 °C segment. The experiment was executed in triplicate; the standard deviation (SD) of the threshold cycles Ct for each sample were comprised between ±0.8 Ct. The Pri miRNAs amplicons fold structure, evaluate by The UNAFold Web Server are showed in *Figure 40* and *Figure 41* for miRNA 146a and 155 respectively.

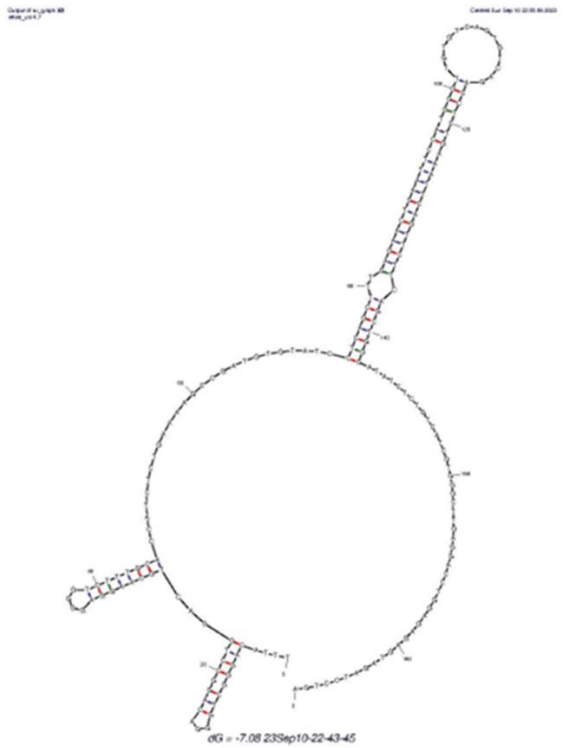


Fig.40. Folding structure of Pri-miRNA 146a PCR amplicon.

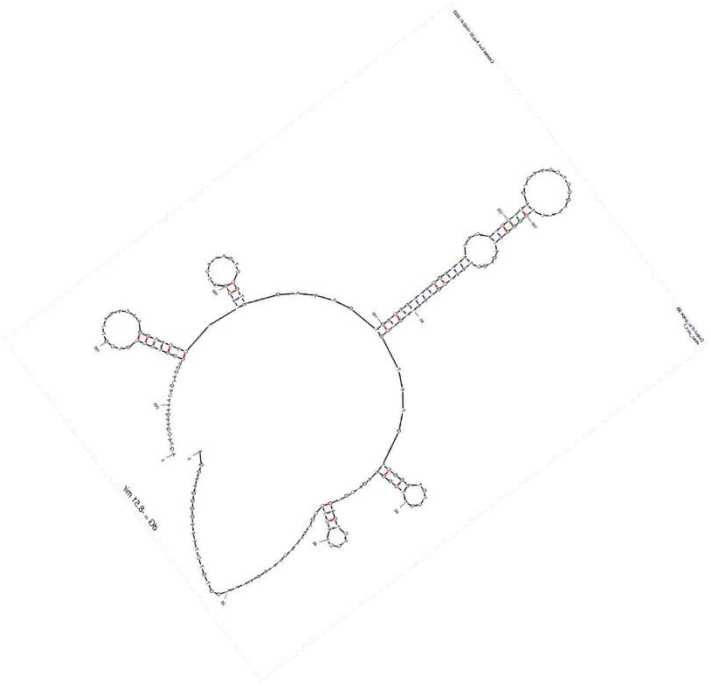


Fig.41. Folding structure of Pri-miRNA 155 PCR amplicon.

Methods in Clinical studies

In the Methods session of *in vitro* study 1, I have experimented some commercial devices used to perform photodynamic therapy. In this session there is a concise description of the methodology used in the *in vivo* evaluation on patients, the photodynamic procedure (Photosensitizers, light wavelength and power) is already described in the study 1 session. This is an observational study and the described procedures are the same routinely used in the clinical protocols.

This study was approved by the Ethic Committee of Cagliari University Hospital (approval number: NP/2023/2551 del 31/05/2023, Comitato etico indipendente AOU Cagliari). In any case, the Helsinki guidelines have observed and complied and a written informed consent was obtained by the patients.

Patients with refractory candidiasis (clinical diagnosis accompanied by any laboratory tests) to the drug therapy considered gold standard will be treated with the different photodynamic therapy protocols described, without a preference for one device over another, only on the basis of the personal preference of the patients, to the use of one photosensitizer rather than another. Those who have difficulty swallowing manoeuvres or have lesions in very posterior areas of the mouth will tend to prefer, for example, the curcumin-based Photosensitizer. Those who find the taste of curcumin unpleasant will tend to prefer methylene blue or toluidine blue as a photosensitizer. Both the operator and the patient wore the protective goggles foreseen and suitable for each wavelength, immediately after placing the photosensitizer on the affected oral tissues and before illumination.

Protocol with Toluidine Blue

The Toluidine Blue at 1%, dispensed via sterile disposable applicator, in 1.5 ml syringes, has been used to cover the entire surface of the mucosa affected. Subsequently, after

about 3-5 minutes, the red light at 630 nm, 4 watts of power, is activated to illuminate the area covered by the Toluidine Blue, with applications of 1 minute per cm^2, at a distance of 0.5 cm from the tissue, *Figure 42*. The device foresees the insertion of specific and disposable transparent probes, with a blunt tip of 8 mm in diameter. During the entire period of photosensitizer injection and light activation, an aspirator is used to prevent accidental ingestion of the photosensitizer and to reduce the salivary flow produced. The amount of saliva produced during the manoeuvres to perform PDT could determine a dilution of the photosensitizer or create phenomena of obstruction of the illumination, with consequent reduction of the antimicrobial activity of the photodynamic. After the period of illumination, the photosensitizer will be removed by vacuuming and rinsing with water.

Fig 42. FotoSan 630, device used to activate Toluidine Blue.

Protocol with Methylene Blue 1%

The Methylene Blue at 1%, dispensed via sterile disposable applicator, in 1 ml syringes, has been used to cover the entire surface of the mucosa affected. Subsequently, after about 3-5 minutes, the laser light at 660 nm, 100 milliwatt of power, is activated to illuminate the area covered by the photosensitizer, with applications of 1 minute per cm^2, at a distance of 0.5 cm from the tissue, *Figure 43*. In this case, the excess can also be removed using the water supplied by the dental unit's air and water syringe. The thickness of the photosensitizer in this case is infinitesimal. The tip provided by the mother house company is a 13 mm long transparent tip, which generates a 1 cm^2 spot of coherent and collimated light. As regards the use of the aspirator and the considerations on its use, the same recommendations previously described apply.

Fig 43. Helbo system device used to activate Methylene Blue.

Protocol with Curcumin-based photosensitizer

The Curcumin+ Hydrogen Peroxide at 3%, has been dispensed via sterile disposable

applicators. The syringes of the commercial photosensitizer contain powder which must be hydrated with distilled water at a quantity of 1.5 ml, shaken and left to act for a few minutes before the thick liquid solution is ready as a photosensitizer. It has been used to cover the entire surface of the mucosa affected. Subsequently, after about 3-5 minutes, the blue light at 460, 7 watts of power, is activated to illuminate the area covered by photosensitizer, with applications of 1 minute per cm^2, at a distance of 0.5 cm from the tissue. The type of disposable tips used are the same as described for the device used to activate Toluidine Blue. As regards the use of the aspirator and the considerations on its use, the same recommendations previously described apply.

The patients were monitored, to evaluate the presence or absence of recurrent fungal infection lesions; any residual presence using a millimeter probe (parodontal probe, Hu-Friedy, Chicago, USA) and recording of the measured quantity. Any laboratory tests may be carried out to establish the residual credits, in cases where the clinical improvement may be doubtful. Any side effects and discomfort will be recorded and taken into consideration.

Fig. 44. Flashmax P7 used to activate Curcumin-based photosensitizer.

Among the clinical studies reported, a device created as a caries detector, that emits violet light (Detecta, Precision Tech, Dentalica, Milan, Italy), was also used. The use of which in the oral cavity is considered free of side effects by the manufacturer. This is a device used in an emergency case in which it was not possible to have the other lighting systems provided and already described, available. The Company does not specify the exact wavelength, but it is comprised between 380 and 420 nm, but even in this case, the use of appropriate protective glasses is mandatory.

Fig. 45. The violet diode light "Detecta", used in an emergency case.

Chapter 9- Results

In vitro study 1

The results on the inhibition halo of the three commercial photodynamic therapy systems showed that Toluidine Blue and Methylene Blue, alone or activated by the appropriate lights, did not produce inhibition on fungal growth, the *C. albicans* colonies had grown also inside the wells, as can be seen in figure 46. The mean values of the inhibition halo were 46 mm for the group of plates with Curcumin+H_2O_2

activated by light at 460 nm, 36 mm for the Curcumin+H_2O_2 alone group. Subsequently, an evaluation of the inhibition halo of only 3% H_2O_2 was performed in triplicate, with the same Kirby Bauer method already described, with the aim of verifying whether the result obtained was linked exclusively to the presence of H_2O_2 in the photosensitizer based of curcumin as a catalyst. The mean inhibition values in the plates treated only with H_2O_2 was 21 mm, therefore much lower than the values of curcumin-based photosensitizer, alone or activated with light at 460 nm (Figure 47).

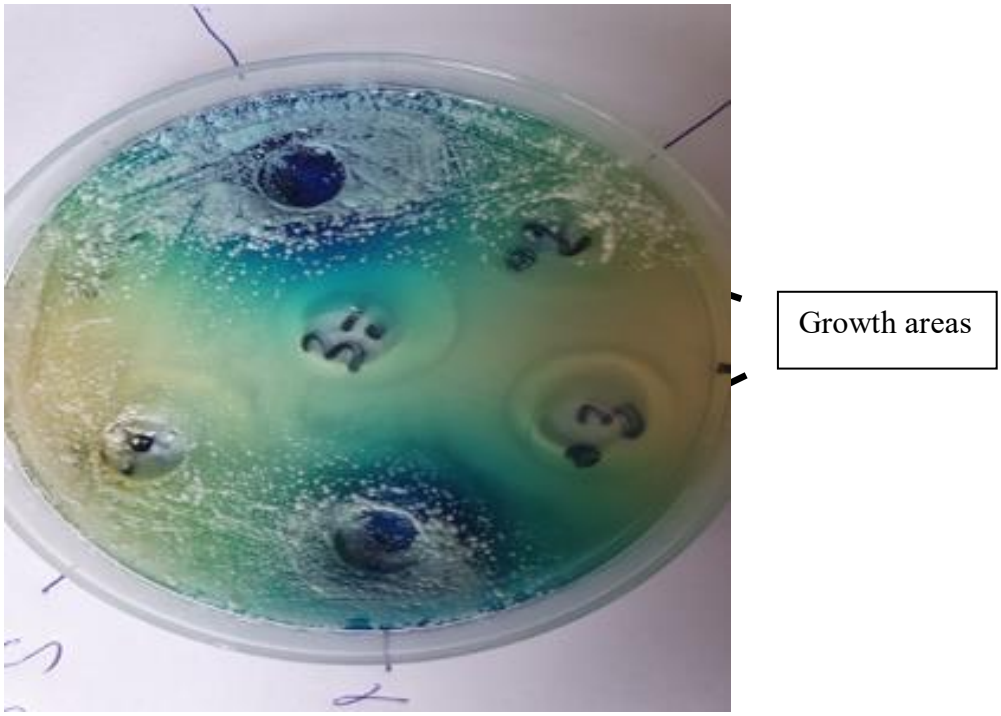


Fig 46. Toluidine Blue and Methylene Blue didn't induce inhibition; as you can see the colonies of *C. albicans* grew inside the wells.

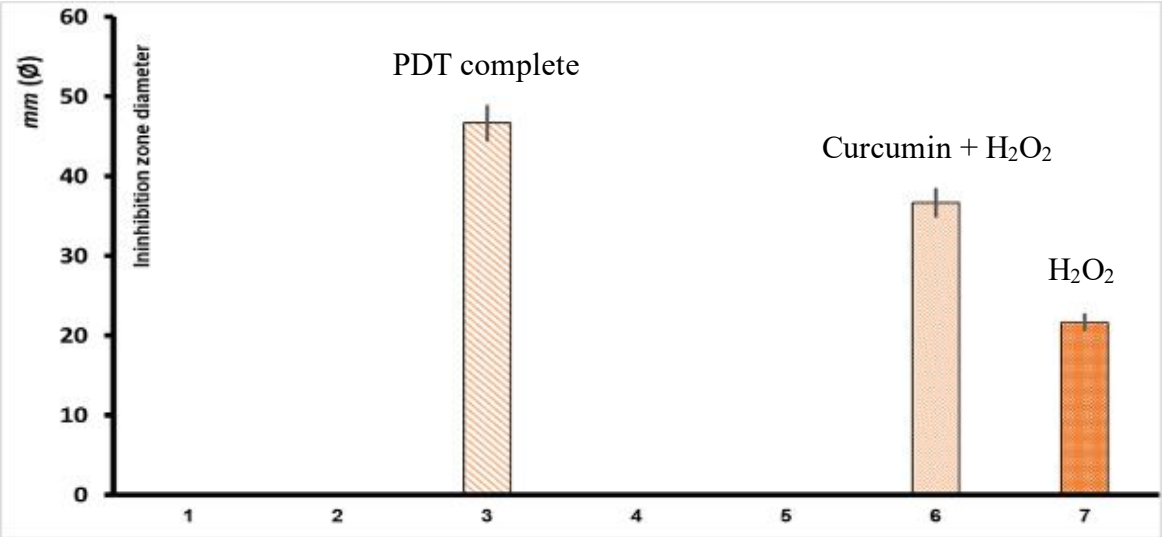


Fig 47. Halo of inhibition for Curcumin + H_2O_2, alone and activated with 460 nm diode light.

Microplate assay

For the second part of in vitro study 1, carried out with the Microplates Assays method, as previously described, the Candida growth after incubation time was proportioned to the 600 nm absorbance (ABS) values evaluated in every well.

A reduction in the yeast growth of the 1% Toluidine Blue group only occurred in the presence of activation with diode light at 630 nm. While the light at 630 nm and Toluidine blue 1% alone did not determine any ABS decrease, instead, Curcumin+H_2O_2 determined Candida reduction growth alone, but it increased when activated by the light at 460 nm. The light alone at 460 nm did not cause any reduction of the growth inside the microplates. The ABS values were even lower when the curcumin-based photosensitizer was activated by polarised light at 380–3400 nm. The latter determined the lowest ABS value with respect to all groups tested (Figures 48,49,50).

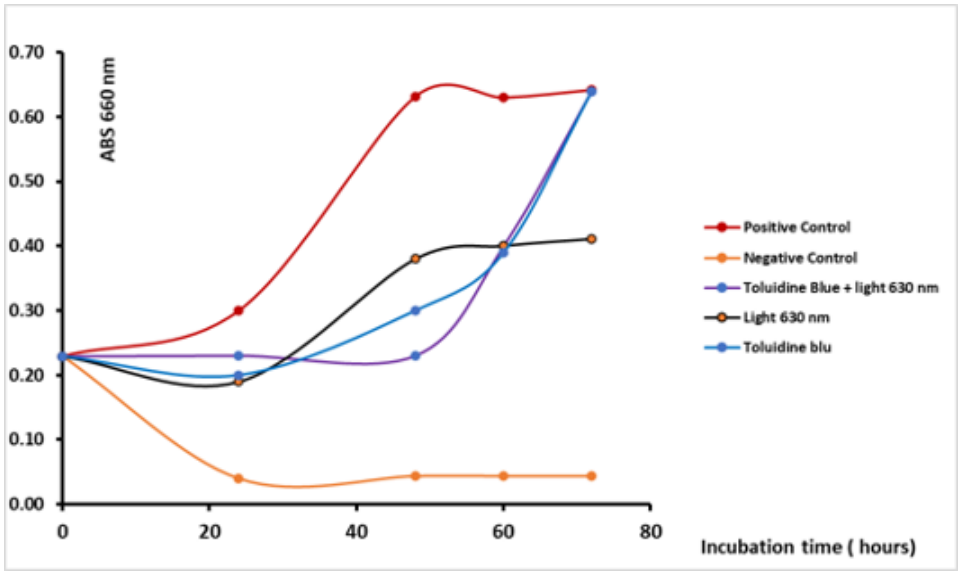


Fig 48. Log phase graph of the results on Toluidine Blue groups tested (Group D).

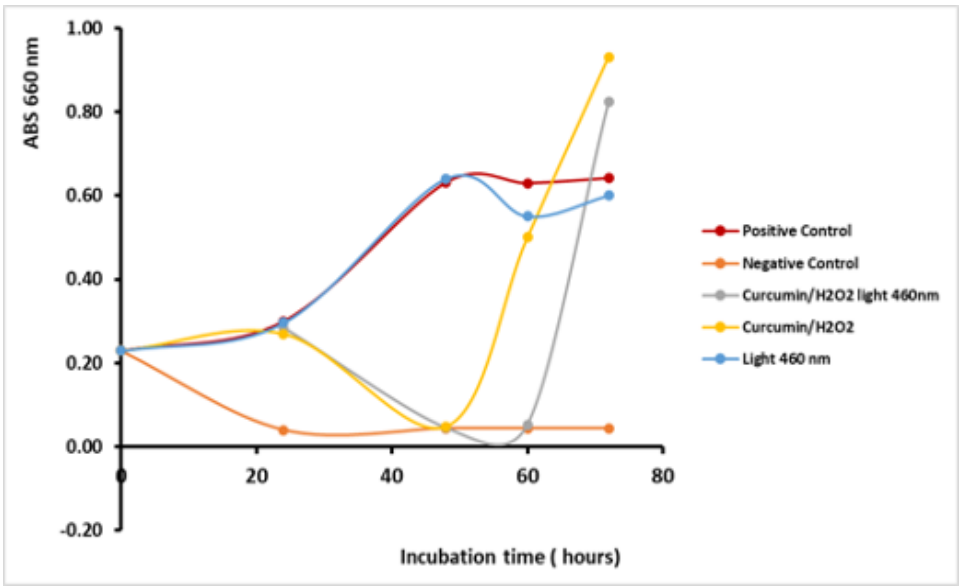


Fig 49. Log phase graph of the results on Curcumin+H_2O_2 3% (Group E).

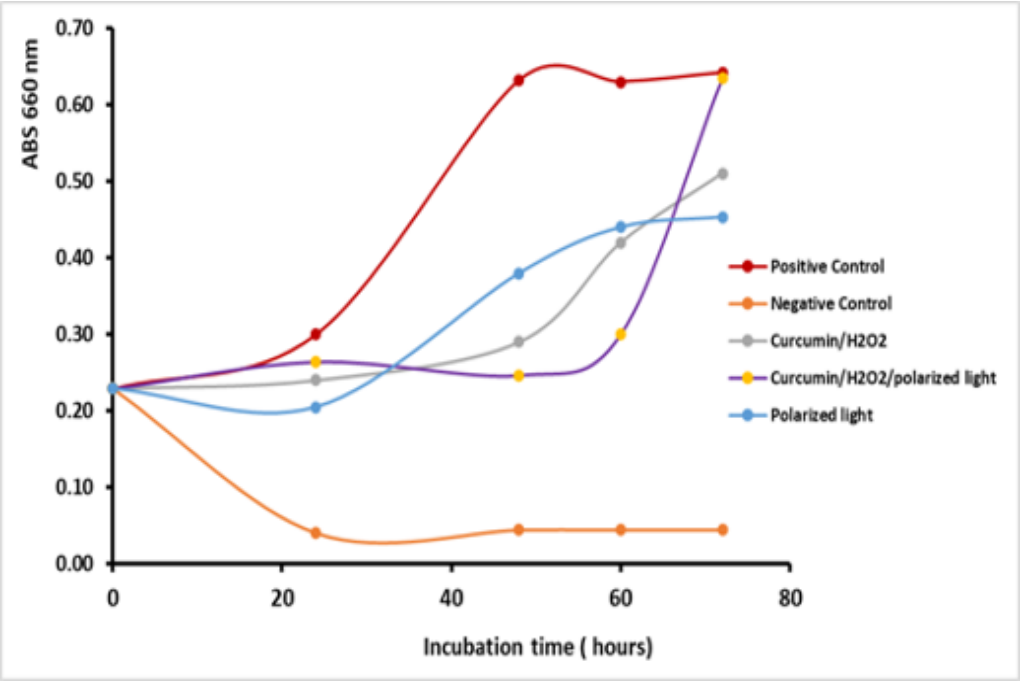


Fig 50. Log phase graph of results on Curcumin based photosensitizer (Group F).

These experiments in microplates and the respective graphical data suggested a re-growth of the strain after 48–72 hours; in practice, the figures showed an inhibition window of about 25–30 hours. This aspect is evaluable considering the biofilm amount after 96 hours of incubation. The Candida sessile status was evaluated by Montana University protocol (Centre for Biofilm Engineering) (link 9). In fact, the fungistatic effect of PDT and the relative yeast regrowth determine the formation of a structured biofilm for all tested PDT combinations (*Figures* 51, 52, and 53).

The plates were observed for 24, 36, 72, 96 hours, to verify if the effect of photodynamic therapy and photosensitizers was still present. While in the case of Toluidine Blue 1% there was a recovery of the fungal growth activity already after 6 hours, this occurred at 24, 36, 48 hours in the group treated with Curcumin + H_2O_2 alone, Curcumin + H_2O_2 activated by light at 460 nm, and the same activated by polarized light.

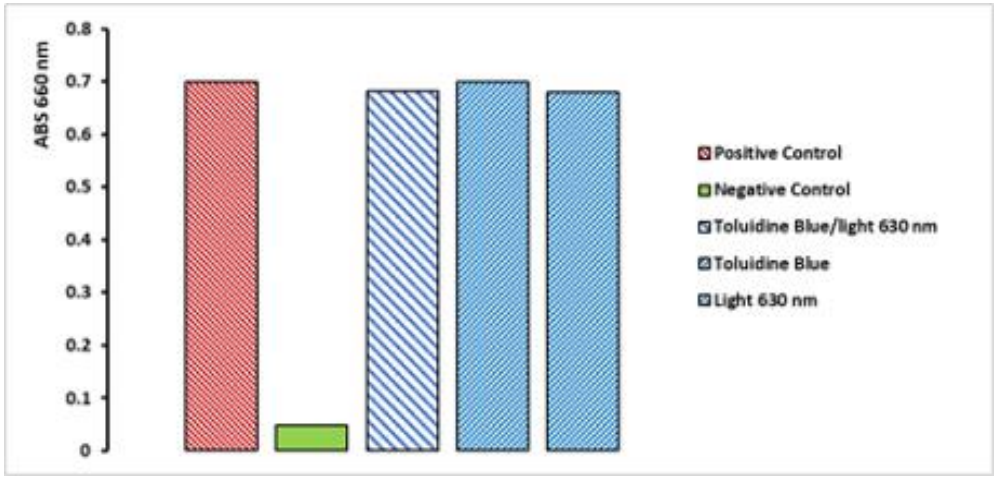


Fig 51. *Candida albicans* Biofilm at 96 hours for Group D

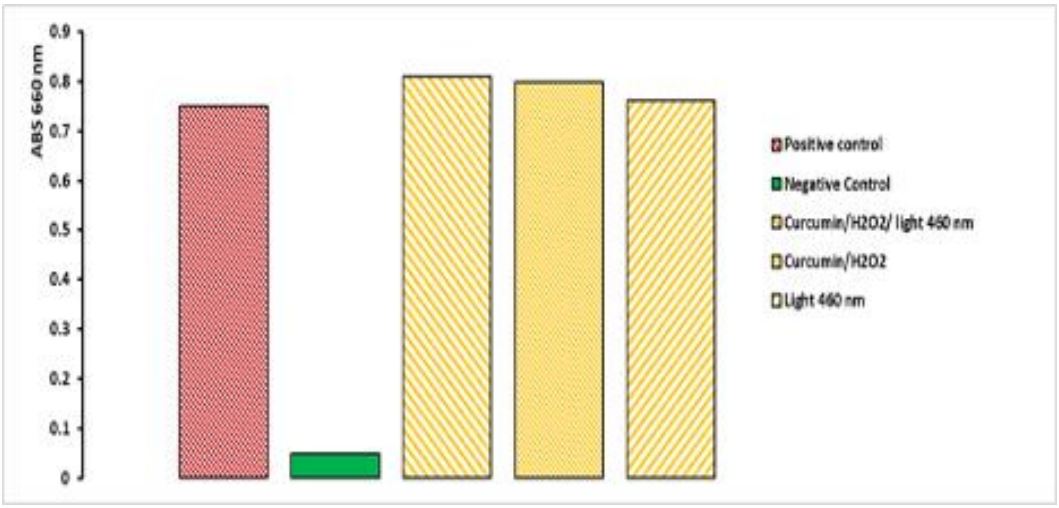


Fig 52. *Candida albicans* Biofilm at 96 hours for Group E

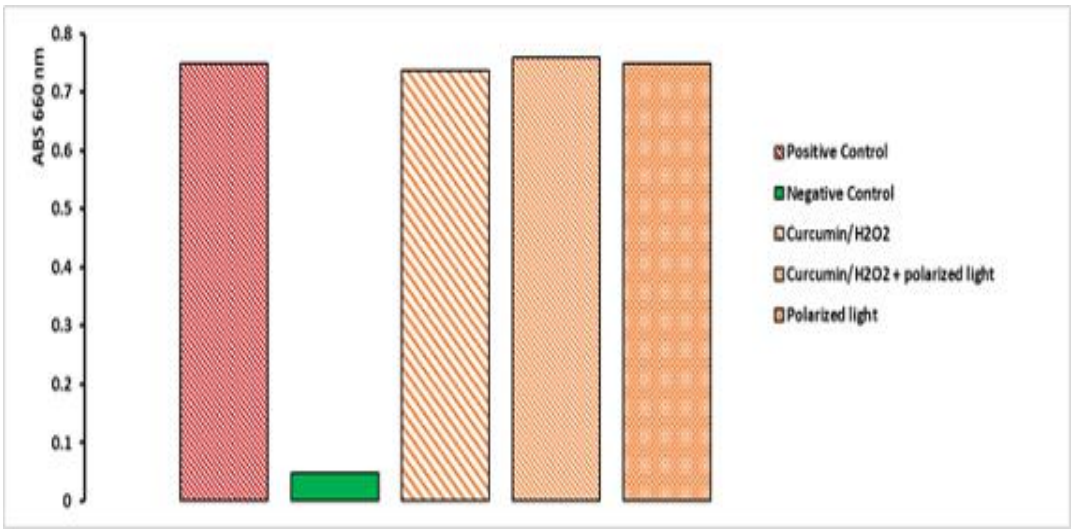


Fig 53. *Candida albicans* Biofilm at 96 hours for Group F

Photodynamic therapy *in vitro* on oral Prosthesis

The results of the third part of *in vitro* study 1 showed that the plate seeded with *C. glabrata* taken after photodynamic therapy with Curcumin+ H_2O_2 and activated with light at 460 nm presented a significant reduction in CFUs.

In practice, from 1500 CFU/cm^2 of the first plate inoculated with the sample taken before the photodynamic experiment to 20 CFU/cm^2 of the second plate after prosthesis treatment, *Figure 55 and Figure 55.*

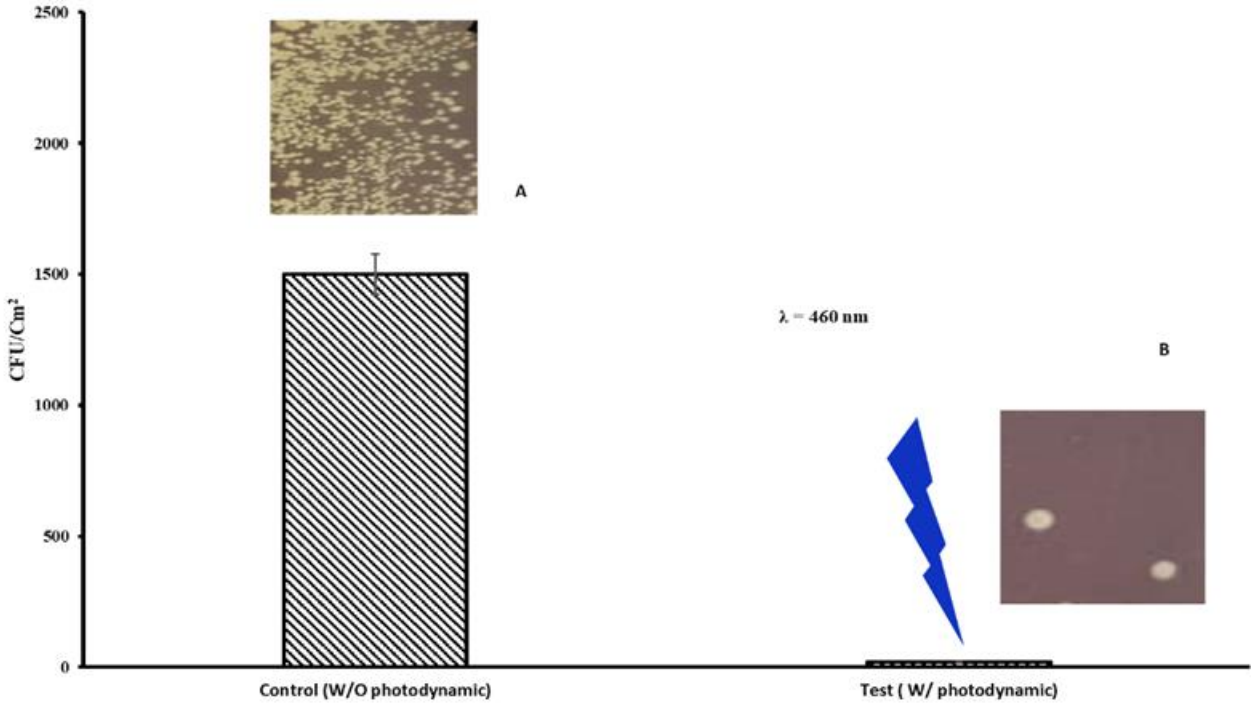


Fig 54. Values of CFU/cm^2 the acrylic resin surface of the prosthesis, before PDT, and after PDT.

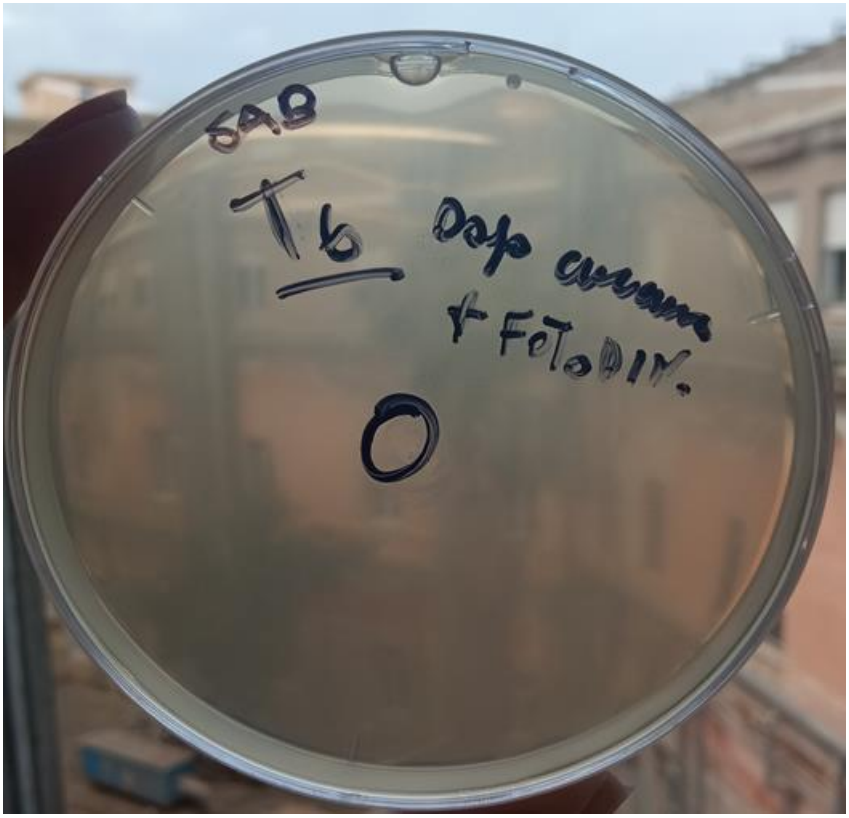


Fig 55. Effect of antimicrobial activity of Curcumin based PDT on the surface of oral dentures: no Candida colony is observed in the Sabouraud agar plate after 48 hours of incubation.

***In vitro* study 2.**

Olive oil test.

The clinical isolates of *Candida spp.* have shown different behaviour with the different assayed experimental groups. *C. kruseii* was insensitive. *C. albicans* was inhibited only with EVOO-H light activation, with a halo of inhibition of 42 millimetres for polarized light and 30 mm when activated by laser light at 660 nm. *C. glabrata* was sensitive to all combinations, with a 50% increase in inhibition by using polarized light. The halo of inhibition is: EVOO alone was 32 mm, EVOO+ polarized light was 45 mm, EVOO+

laser light was 42 mm, EVOO-H was 35 mm, EVOO-H + polarised light was 62 mm, and EVOO-H + laser light was 45 mm, *Figures 56 and 57*.

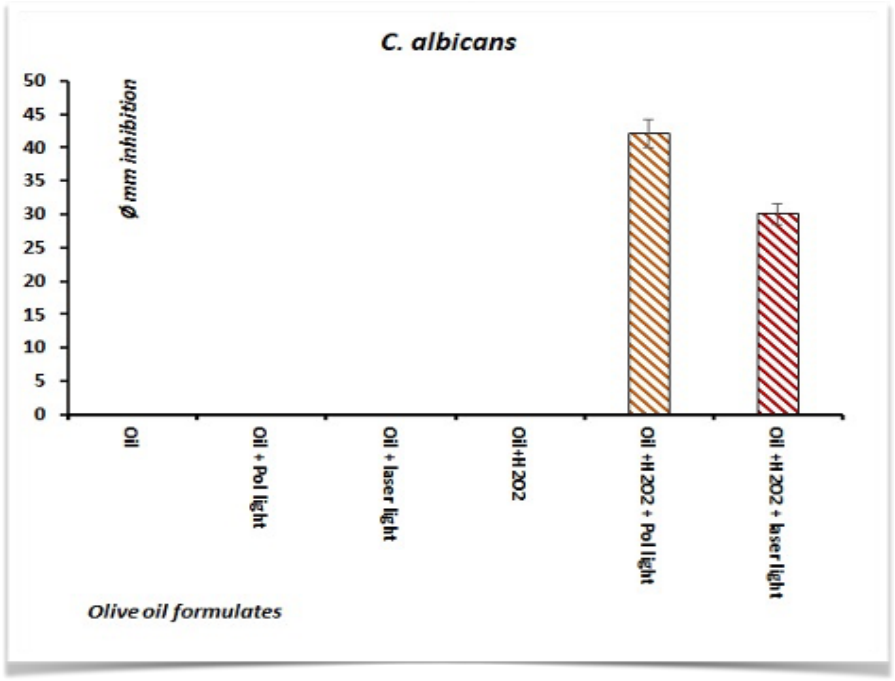


Fig 56. Inhibition halo of C.*albicans* MDR

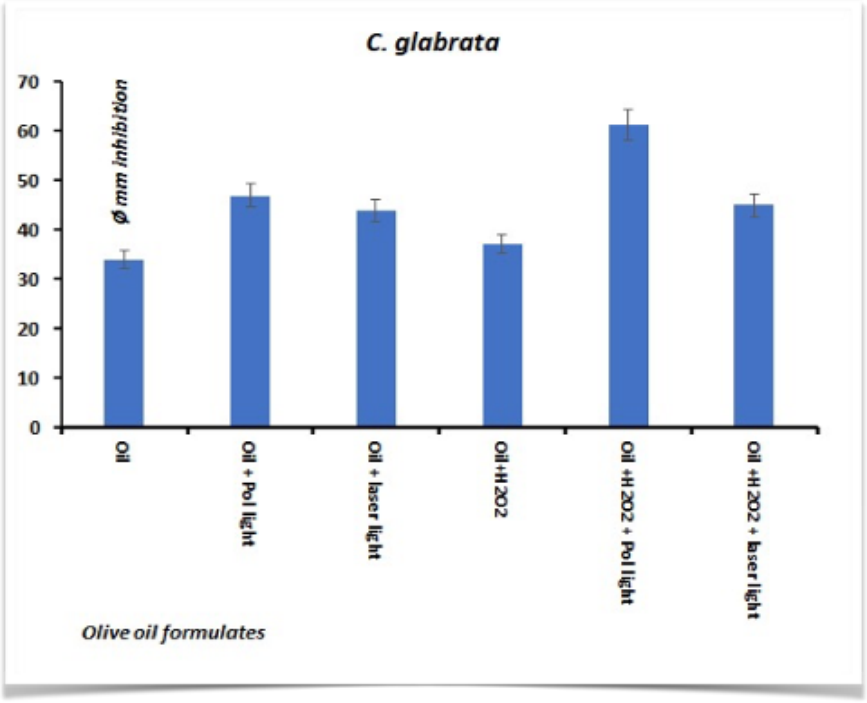


Fig 57. Inhibition halo on *C. glabrata* MDR.

Lactoferrin mixture test.

Lactoferrin alone or combined with light have an antimicrobial activity against all types of *Candida spp* previously described. Lactoferrin mixture evaluated by Kirby-Bauer test was active alone against *C. glabrata*, with a halo of 20 mm, Lactoferrin+Light produced an inhibition halo of 22 mm on *C. albicans*, 30 mm on *C. glabrata*, 20 mm on CC1 our clinical isolate. Lactoferrin +H_2O_2+ light produced an inhibition halo of 40 mm versus *C. albicans*, 35 mm on *C. glabrata*, 30 mm on C. krusei, 30 mm on CC1 clinical isolate. Mean values of inhibition halo with H_2O_2 alone were 23 mm on *C. albicans*, 28 mm on *C. glabrata*, 12 mm on *C. krusei*, 18 mm on CC1 clinical isolate, *Figure 58,* Table III.

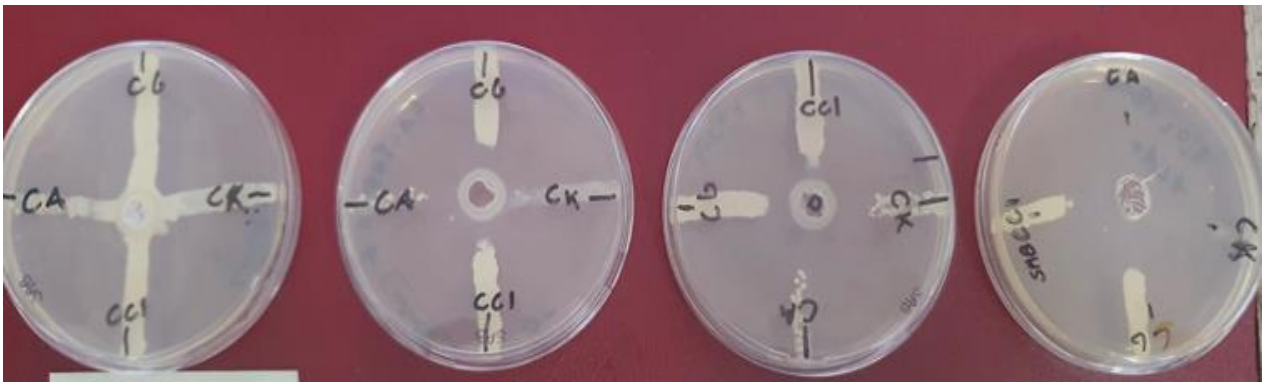


Fig 58. Petri dishes with the 4 types of *Candida spp* tested with the different groups of Lactoferrin test of in vitro study 2.

Tab.III. Inhibition halos obtained with lactoferrin-based PDT.

Strain	Negative Control	Lactoferrin	H_2O_2	Lactoferrin +light	H_2O_2 +light	Lactoferrin H_2O_2+light
			Inhibition halos Ø +/- 1-2 mm			
C. albicans	0	10	23	22	30	40
C. glabrata	0	20	28	30	30	35
C. krusei	0	10	12	10	30	30
CC1	0	10	18	20	18	30

Saffron test.

0,3% of Saffron solution was effective only when activated by the 460 nm diode light against *C. krusei.* No antimicrobial activity has been shown against *C. glabrata, C. albicans* and *CC*1 clinical isolate, *Figure 59.*

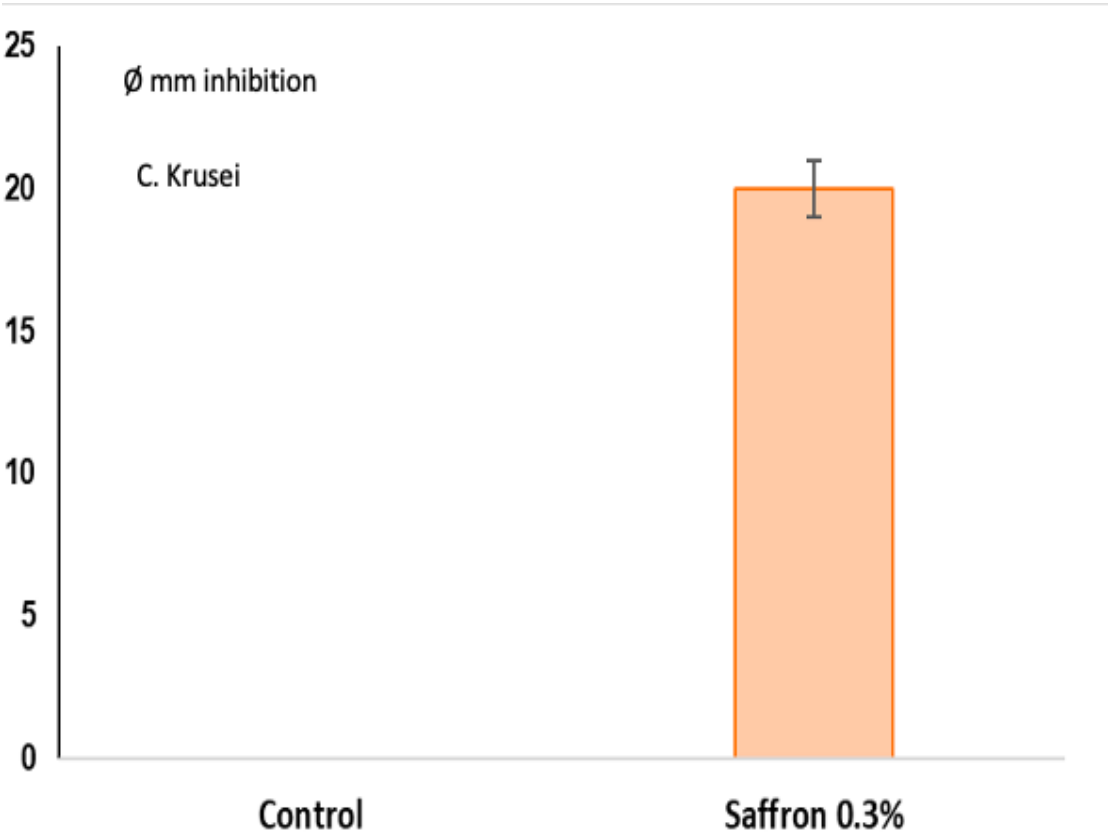


Fig 59. Inhibition halo of 0,3% Saffron solution against MDR *C. krusei* strain.

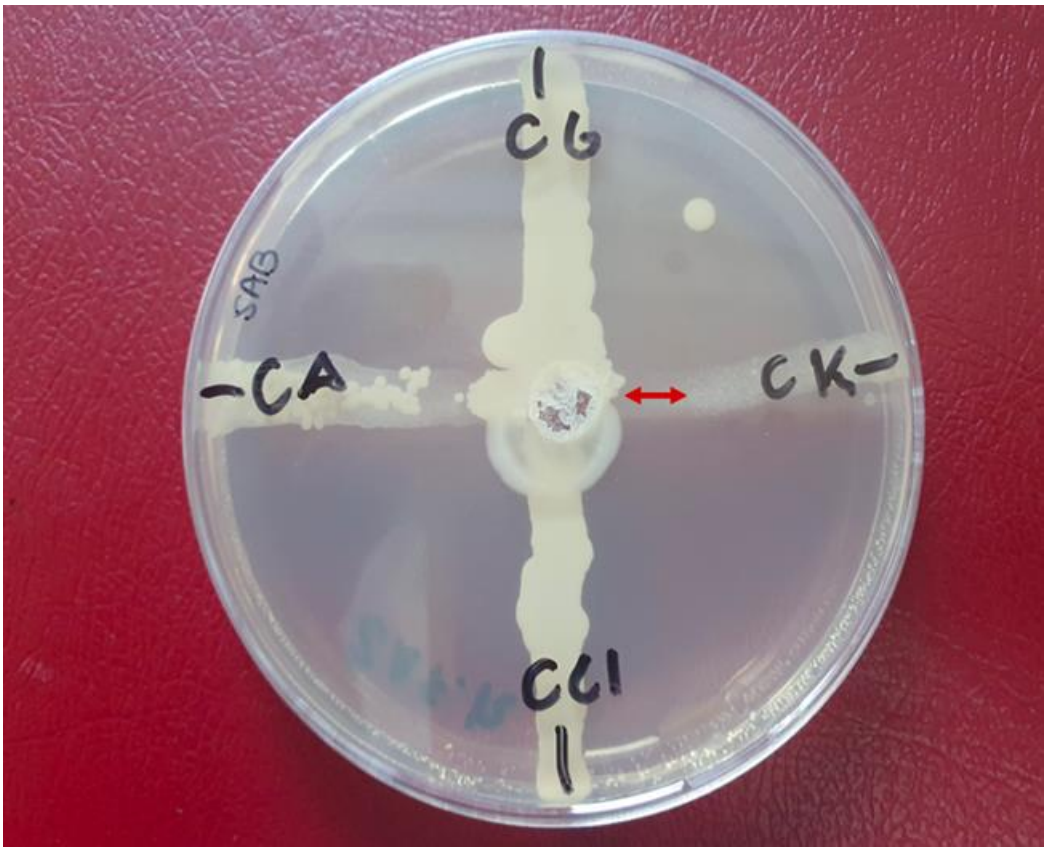


Fig 60. Petri dish treated with 0,3% saffron solution activated by the light. A partial small inhibition was observed. It has been effective only against *C. krusei.*

Pompia juice & Strawberry Juice

No activity was observed with these fruit juices, inhibition halos = 0 for all combinations, data no shown.

In vitro study 3

This work was performed by using an in vivo infection on HEK cells cultured in a 6-well microplate. To avoid any *Candida albicans* contamination risk, a 470 nm lab-made light emitter (Figure 61).

The results for Pri-miRNA 146a and Pri-miRNA 155 expression in HEK 293 cells are illustrated in Figures 62A and B. This experimental stage has reported that the entire photodynamic treatment was more effective in comparison with the photosensitizer

or light alone. The expression levels of miRNA 146a and miRNA 155 decreased by about 50% after 2 minutes of treatment, from: 2 to 0,9 for miRNA 146a and from 3,4 to 1,5 considering miRNA 155. Values measured after 2 minutes of PDT irradiation. This in vitro assessment also showed that the decrease in miRNA processing is related to the 470 nm light exposition time.

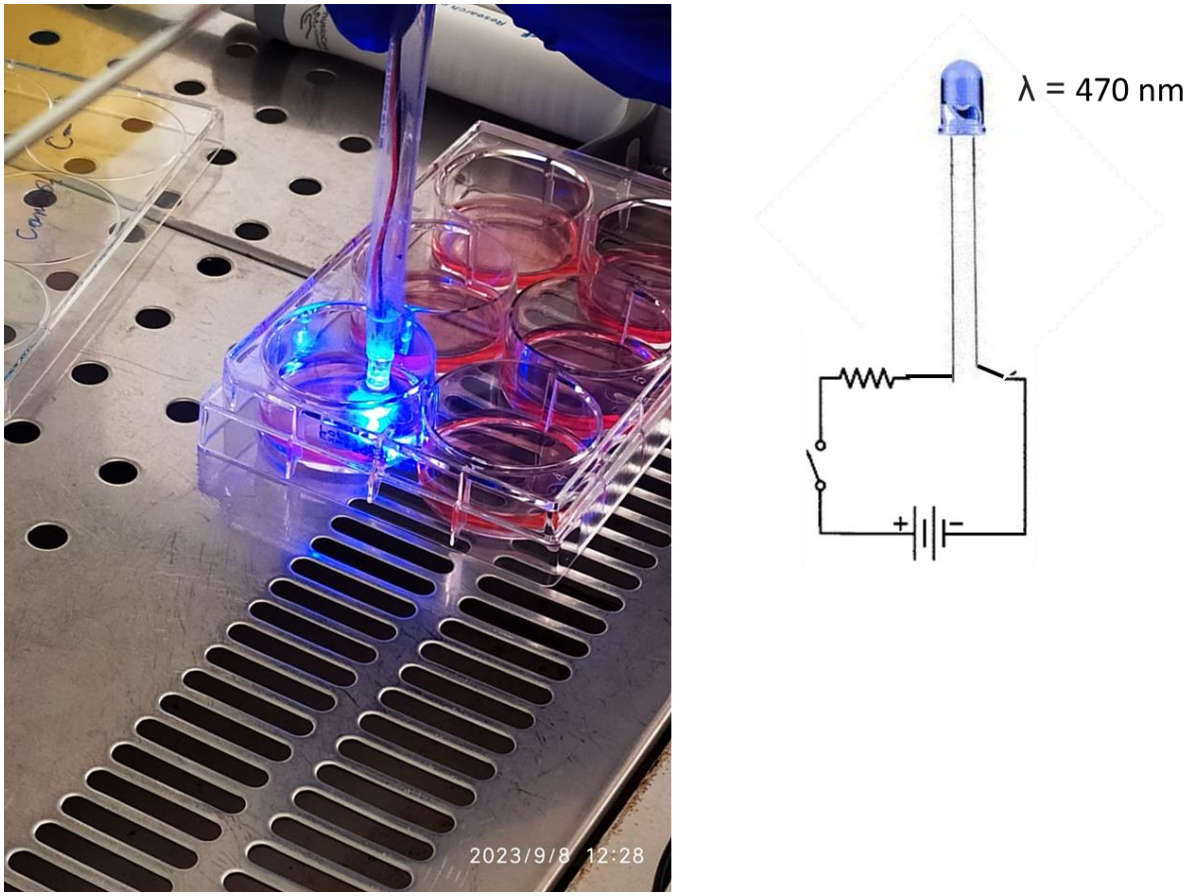
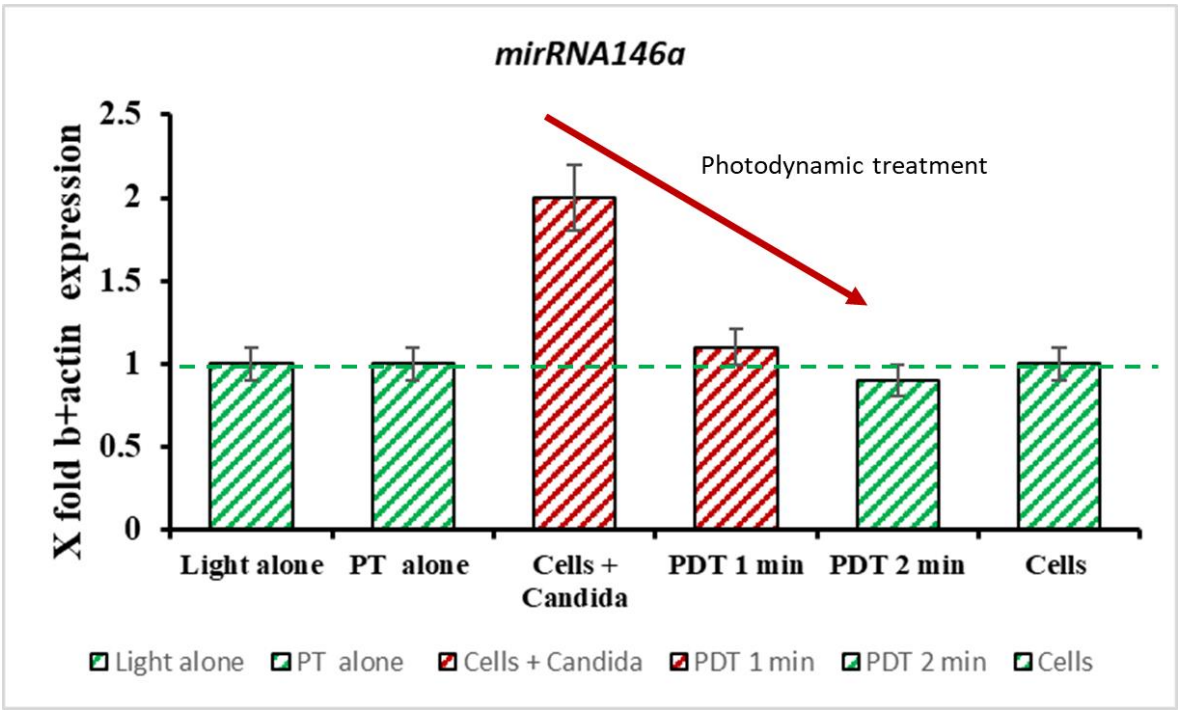
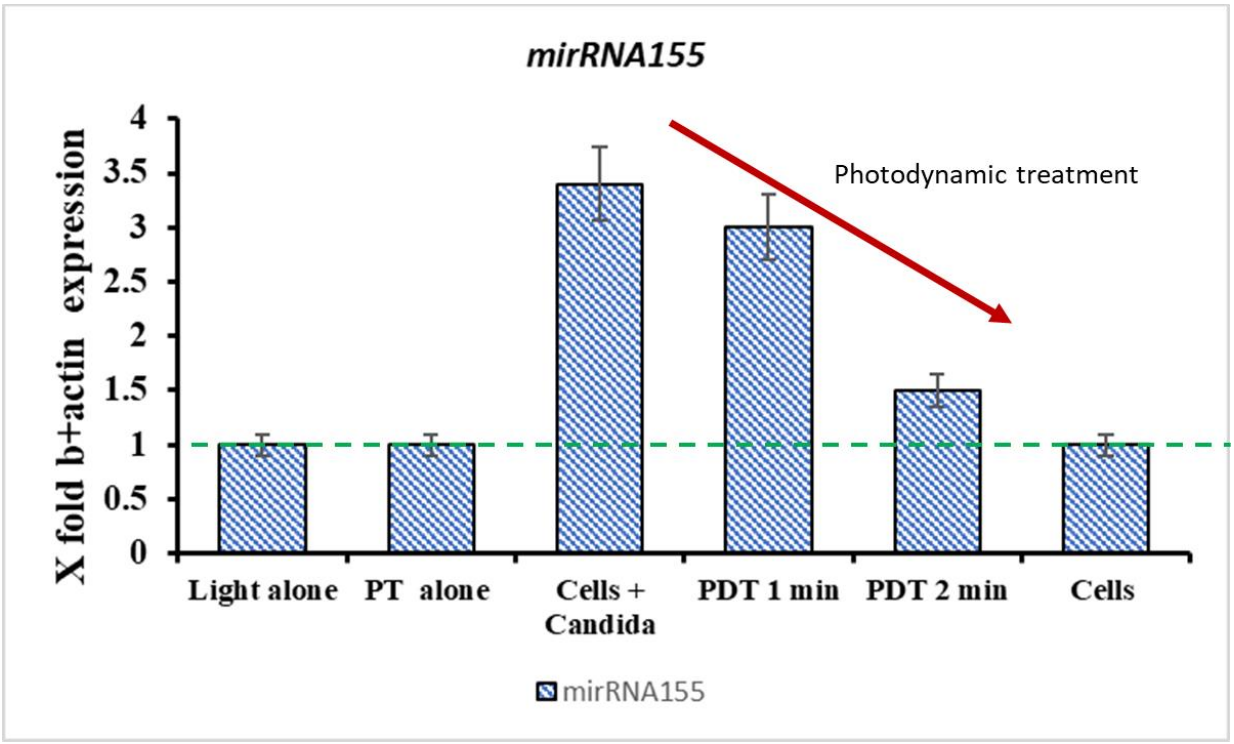


Fig.61. The system cell HEK 293 culture, and the lab-made device used to emit light 470 nm radiation.


mirRNA146a
X fold b+actin expression
2.5
2
1.5
1
0.5
0
Photodynamic treatment
Light alone
PT alone
Cells + Candida
PDT 1 min
PDT 2 min
Cells
Light alone
PT alone
Cells + Candida
PDT 1 min
PDT 2 min
Cells

A


mirRNA155
X fold b+actin expression
4
3.5
3
2.5
2
1.5
1
0.5
0
Photodynamic treatment
Light alone
PT alone
Cells + Candida
PDT 1 min
PDT 2 min
Cells
mirRNA155

B

Fig. 62. A strong decrease in the Pri-miRNA 146a (A) and Pri-miRNA 155 (B) expression levels (compared β-actin = 1) was observed during PDT with a light intensity of 470 nm

Results on Clinical Studies

1st case.

The first case treated is a 64-year-old male patient, with a negative general history, smoker, about 40 cigarettes a day. He had a slight burning sensation in the labial commissures and on the back of the tongue. A few years ago he had developed a relapsing angular cheilitis with the presence of a whitish patina on the tongue, the diagnosis of which was relapsing Candida infection (*Figure 63*). The presence of an altered vertical dimension facilitated the invagination of the labial mucous membranes and therefore the continuation of the infection. The patient had been treated with photodynamic therapy performed with 1% Toluidine Blue as photosensitizer and with diode light at 630 nm, with the same modalities described in the previous session. The one-week check-up showed a good improvement in the condition *Figure 64*, as well as the symptoms.

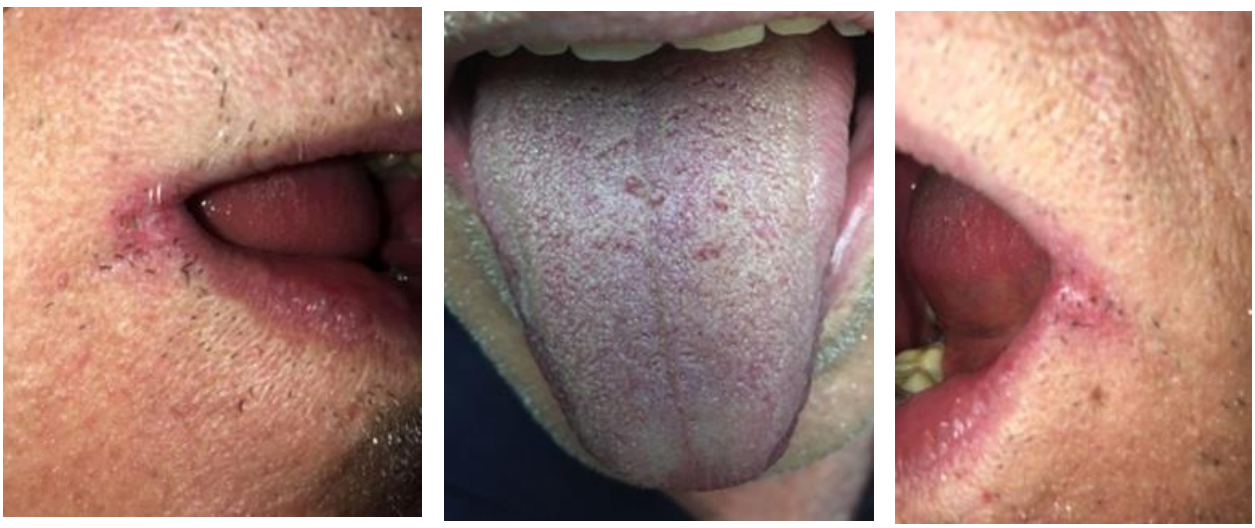


Fig 63. First Clinical presentation of chronic oral Candidiasis.

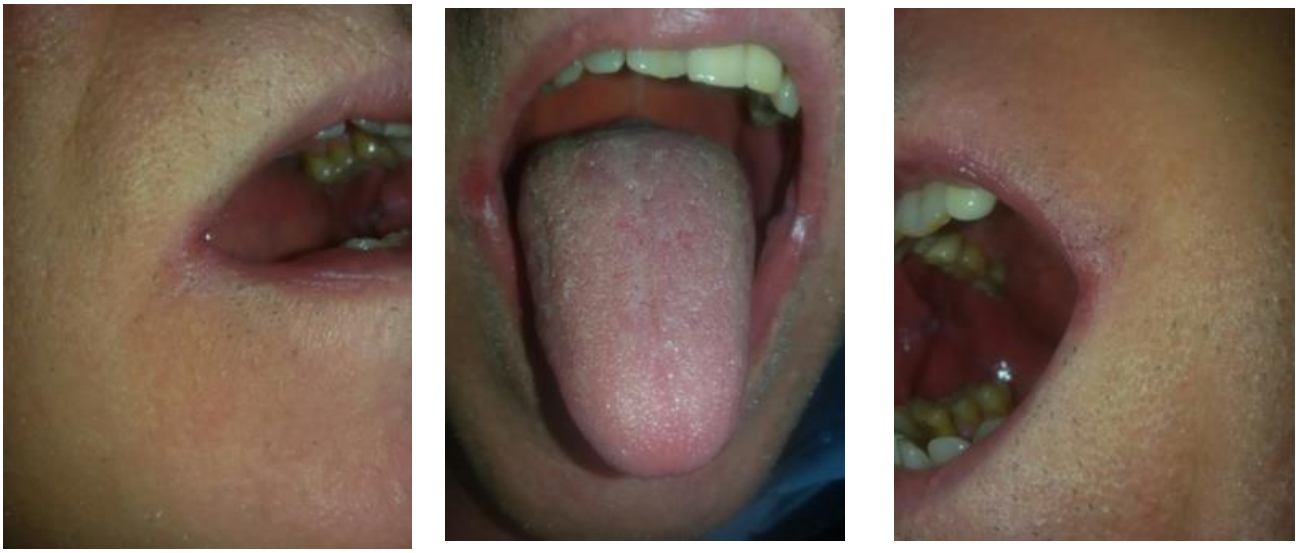


Fig. 64. Clinical presentation one week after PDT.

2nd case

The second patient was a 46-year-old female with no systemic disease. He complained of a slight burning in the right labial commissure. From a clinical point of view, there was a mildly erosive area attributable to unilateral angular cheilitis, which is generally linked to the presence of fungal infection *Figure 65*. The patient had already tried the application of various ointments, without concrete results. A photodynamic therapy session was performed with 1% Methylene Blue and activation with laser light at 660 nm, as described in the previous Materials and Methods session *Figure 66*. The one-week follow-up showed complete clinical and symptomatic recovery *Figure 67*. The lesion did not recur after more than 2 months.

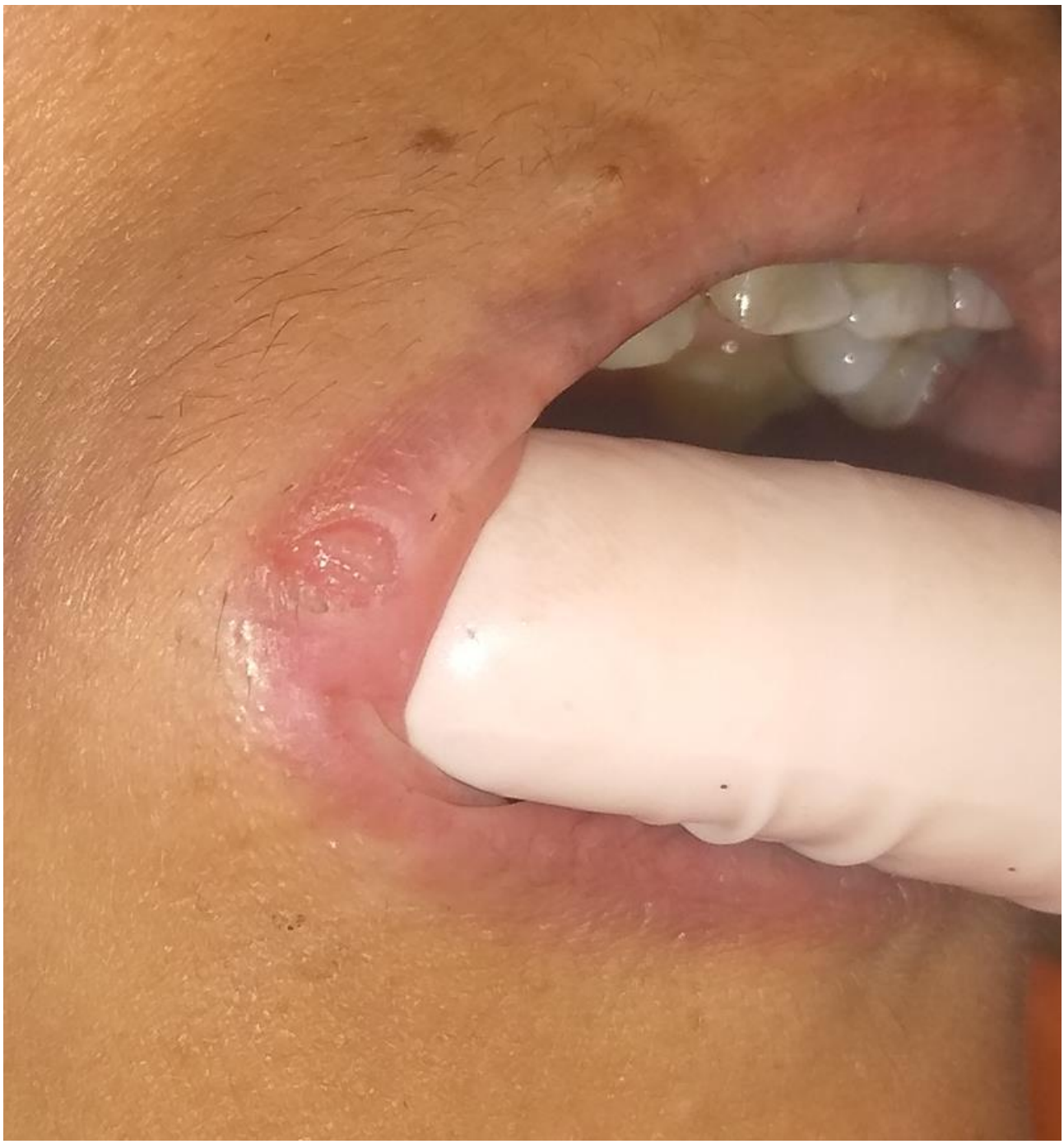


Fig. 65. First clinical presentation of the lesion on the upper lip.

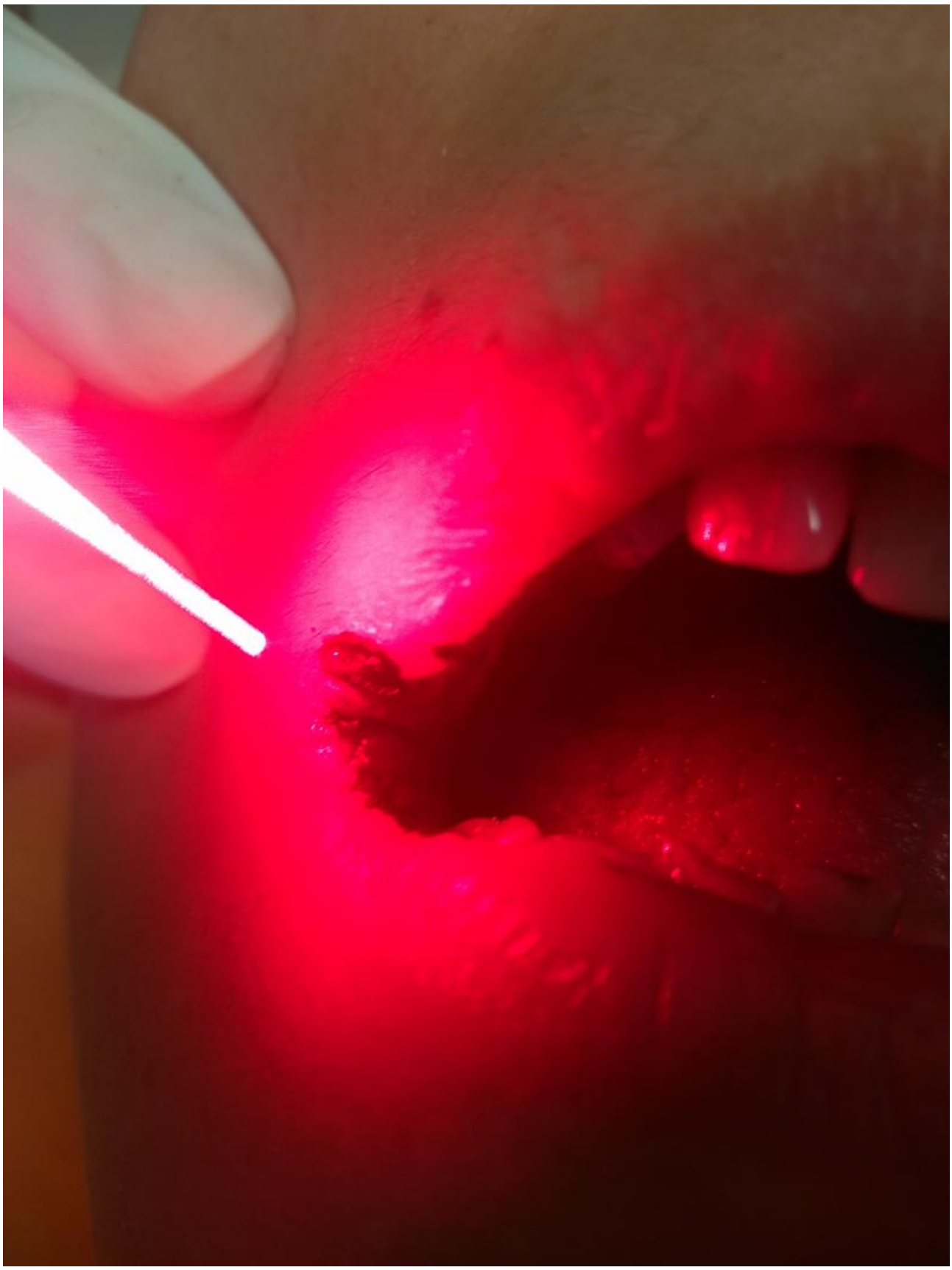


Fig.66. PDT session with Methylene Blue activated by Laser light 660 nm.

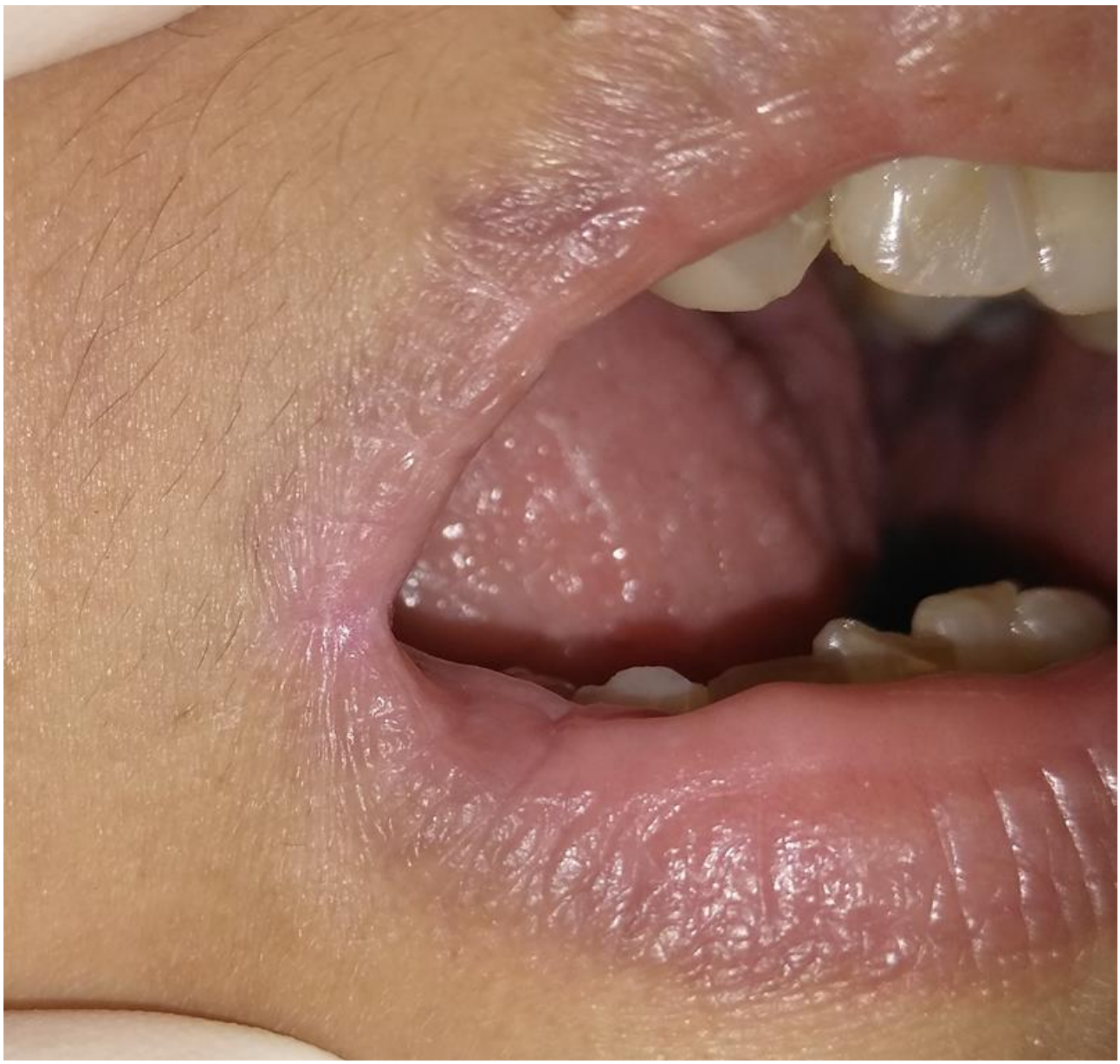


Fig.67. 1 week follow up after PDT. The lesion healed.

3th case.

The third case is from a 45-year-old woman with a positive history of atopic dermatitis, who had developed a symptomatic lesion of the right labial commissure about 9 months ago. The patient reported a state of intense physical debilitation in the last year, also due to various cortisone therapies performed. The lesion had been treated with various ointments previously, without success. The clinical appearance was sufficient to diagnose unilateral angular cheilitis, *Figure 68*. She was first subjected to

topical therapy with 1% miconazole for 15 days, without any clinical success. A session of photodynamic therapy with 1% Methylene Blue and laser light at 660 nm was then carried out, with the protocol already described in the previous session. The one-week follow-up showed a complete regression of the lesion *Figure 69*, a result which was also maintained at the subsequent one-month follow-up, *Figure 70*.

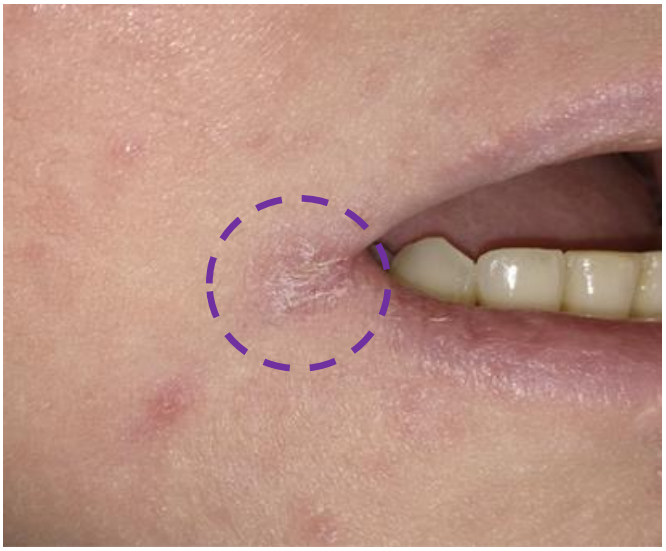


Fig 68. First Clinical presentation of angular cheilitis

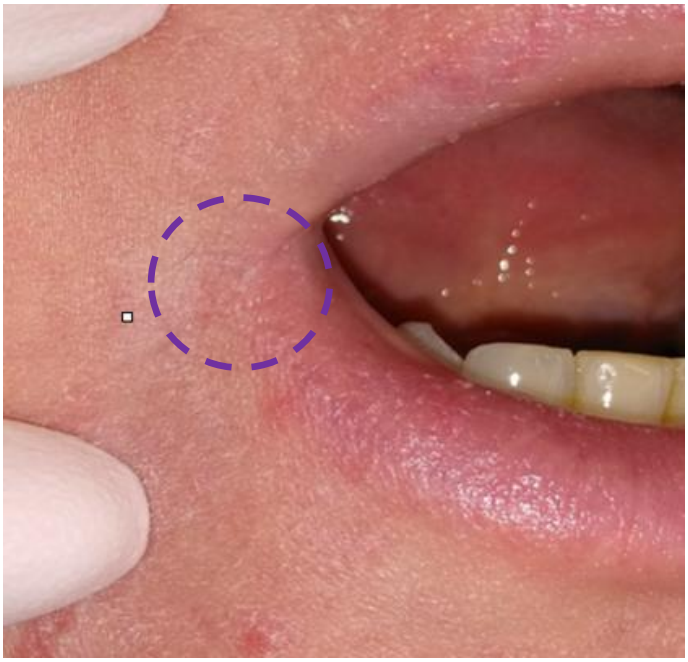


Fig. 69. 1 week follow up after PDT. The lesion healed.

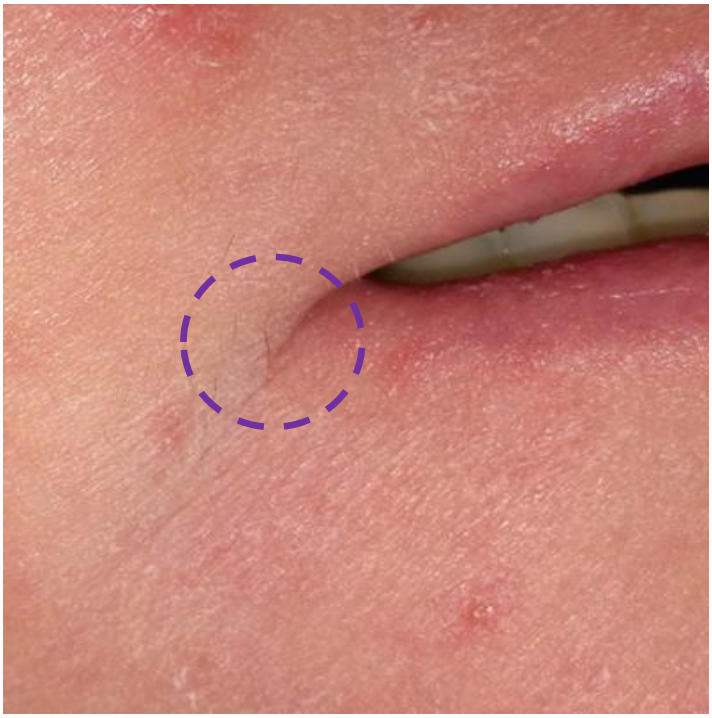


Fig. 70. 1-month follow up after PDT session.

4th case.

The fourth case treated concerns a woman of 47 years, with the presence of relapsing angular cheilitis, previously diagnosed by other clinicians and treated with topical antifungals without success, *Figure 71*. She then underwent a session of photodynamic therapy with 1% Methylene Blue activated by laser light at 660 nm, with the modalities previously described, *Figure 72 a/b*. A clear clinical improvement could be appreciated at a one-week and one-month follow-up, *Figure 73a, 71b.*

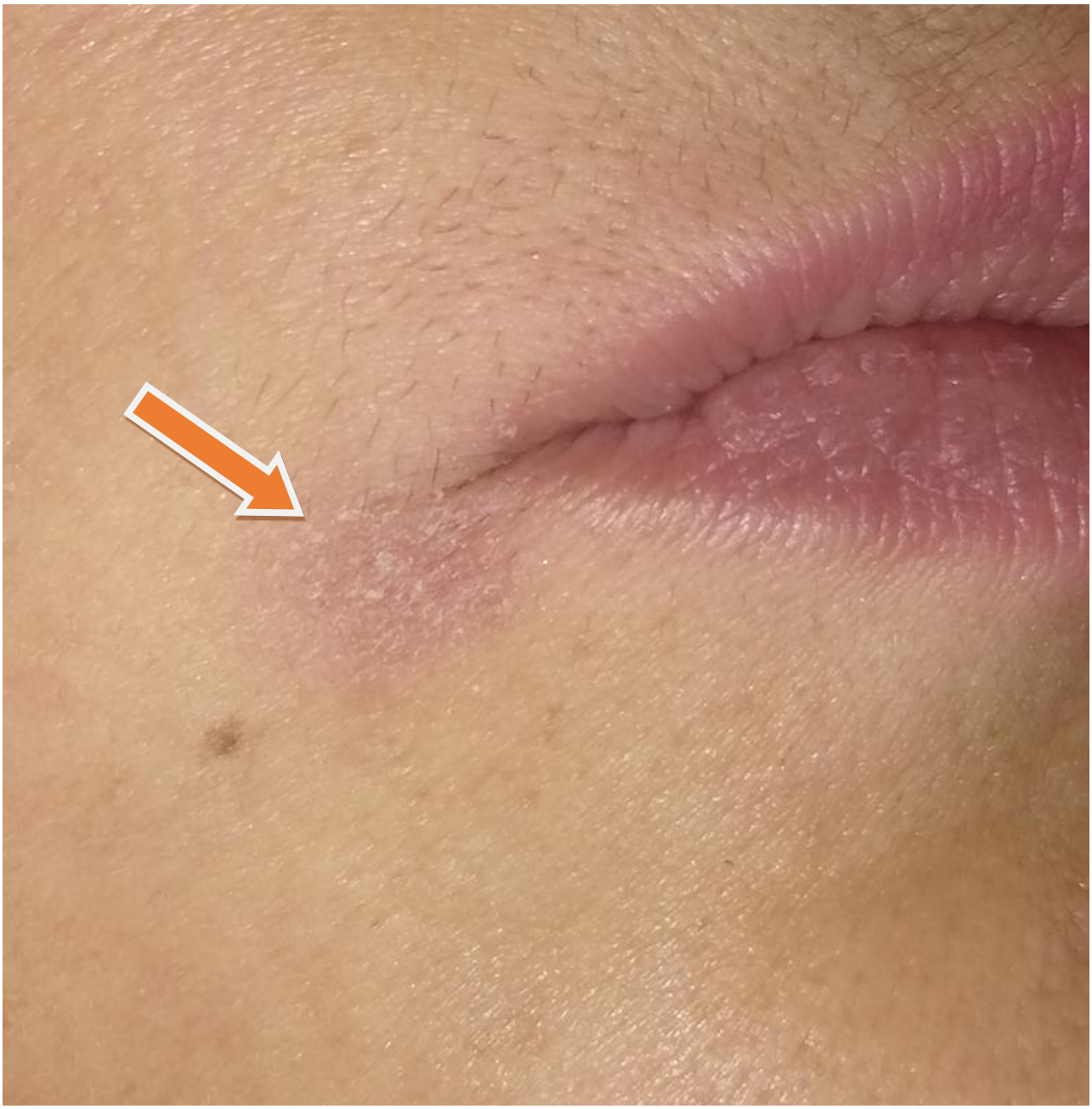


Fig. 71. First clinical presentation of the angular cheilitis of the case number 4. The lesion was treated with topical antifungals without success.

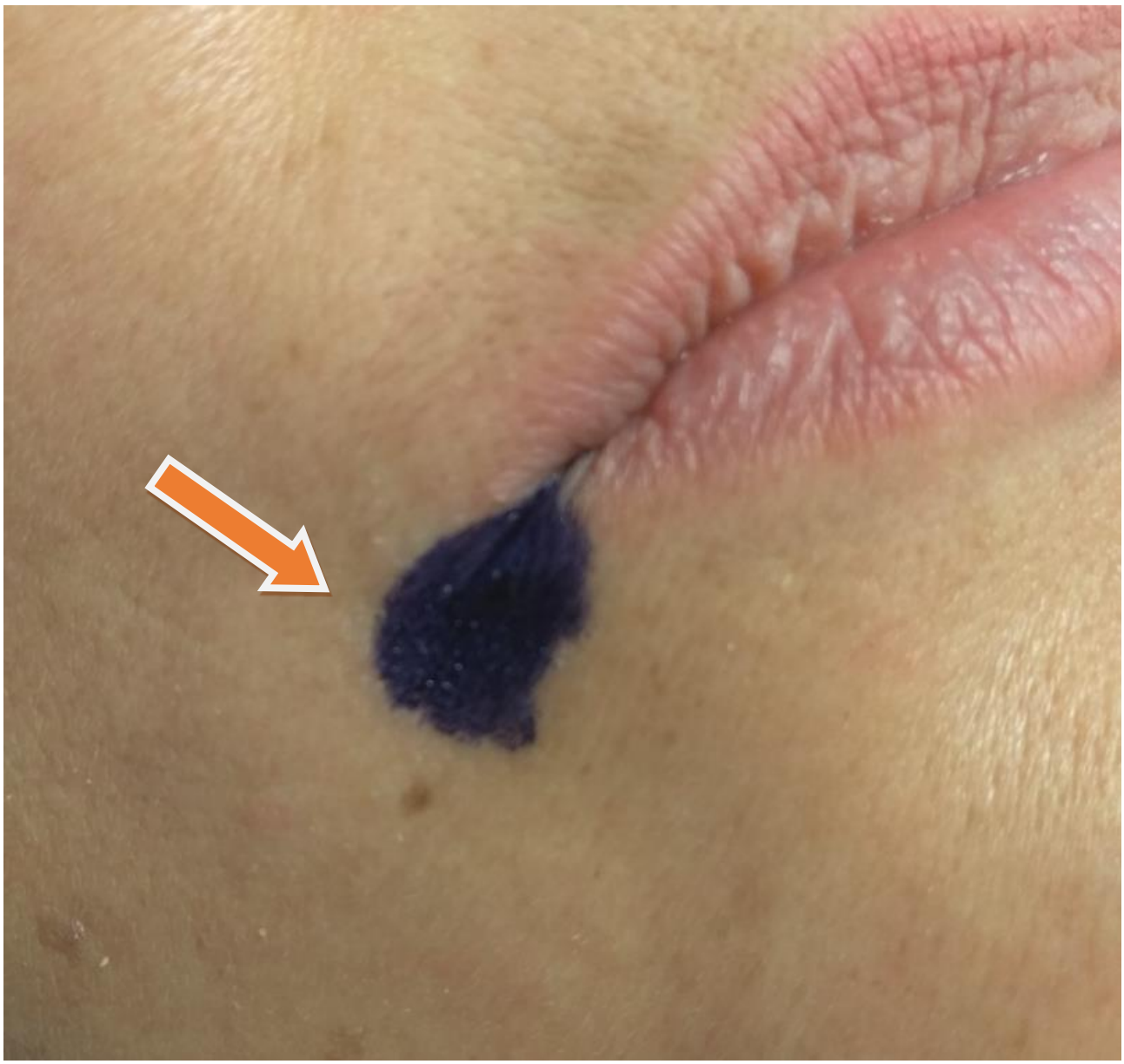


Fig.72 a. Application of photosensitizer Methylene Blue 1% on the lesion.

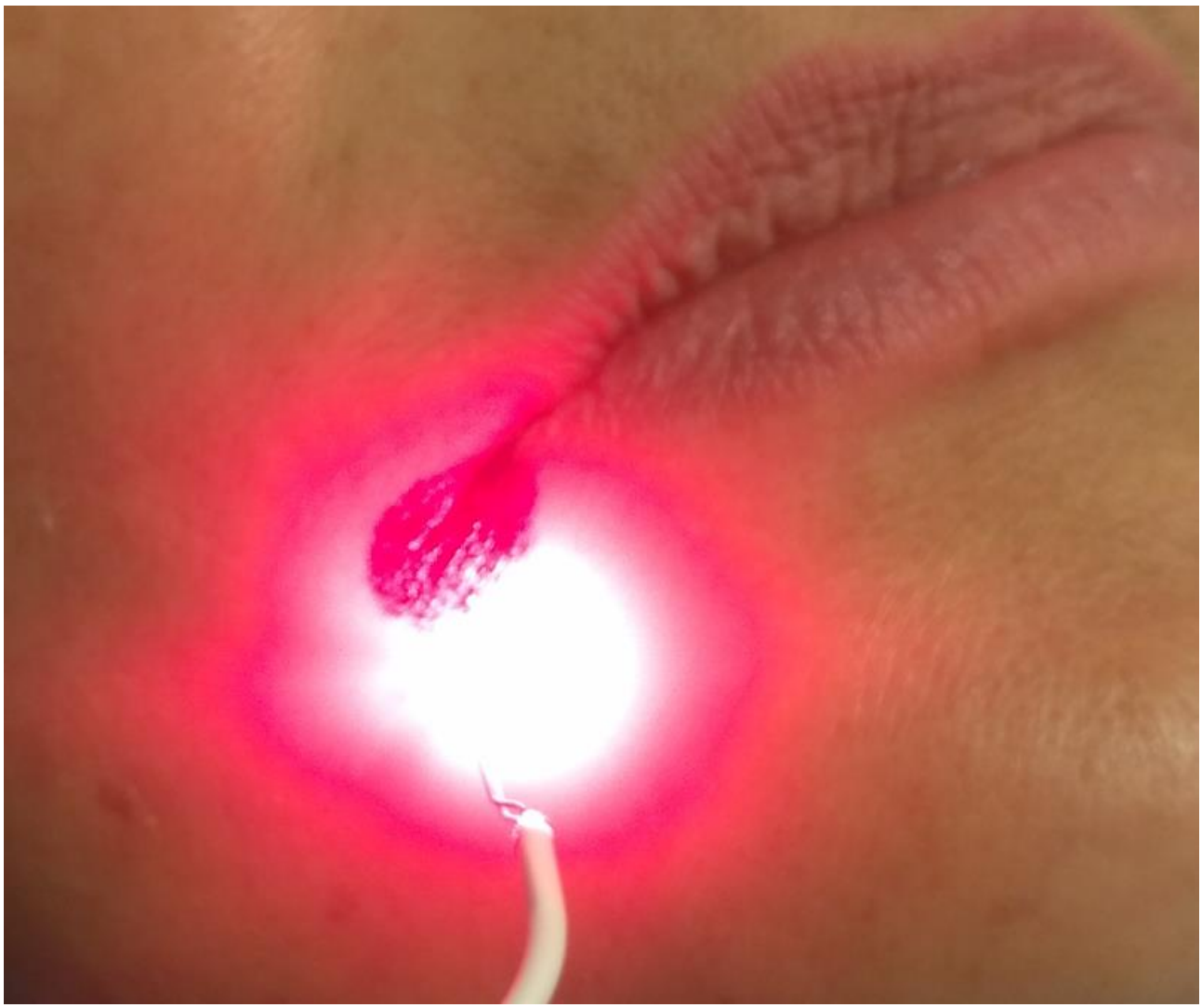


Fig.72 b. Activation of Methylene Blue with Laser Light at 660 nm.

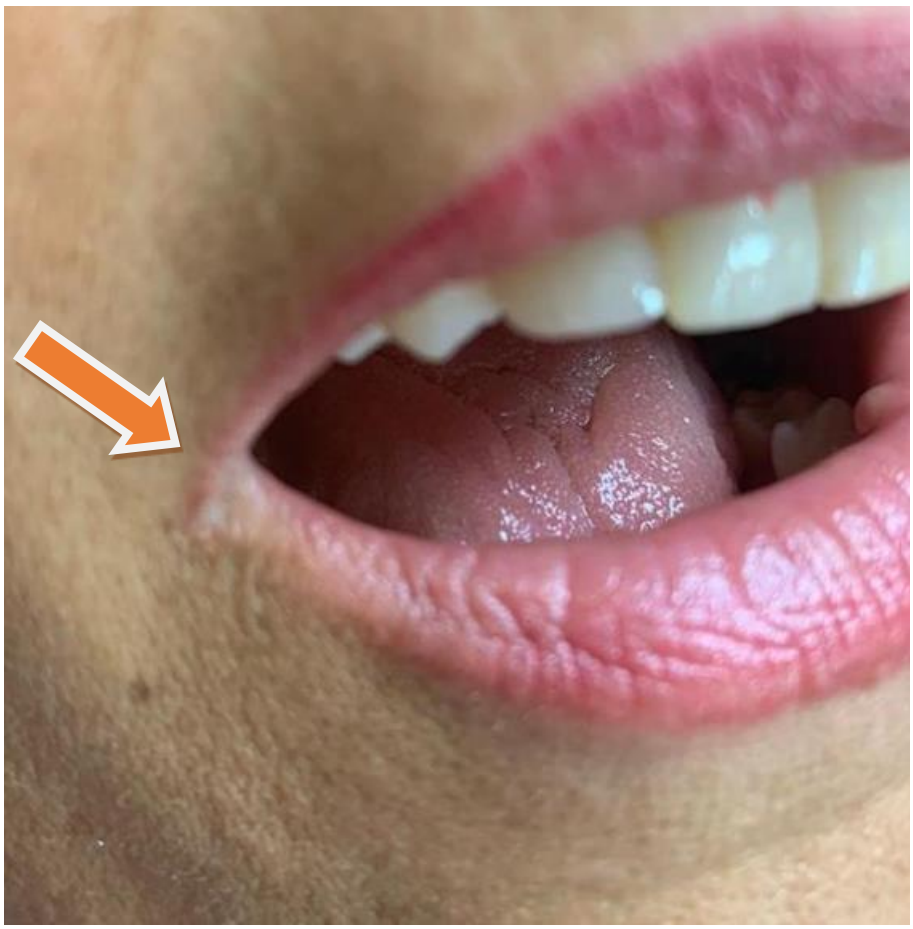


Fig. 73a. Angular cheilitis at 1 week follow up. The lesion improved.

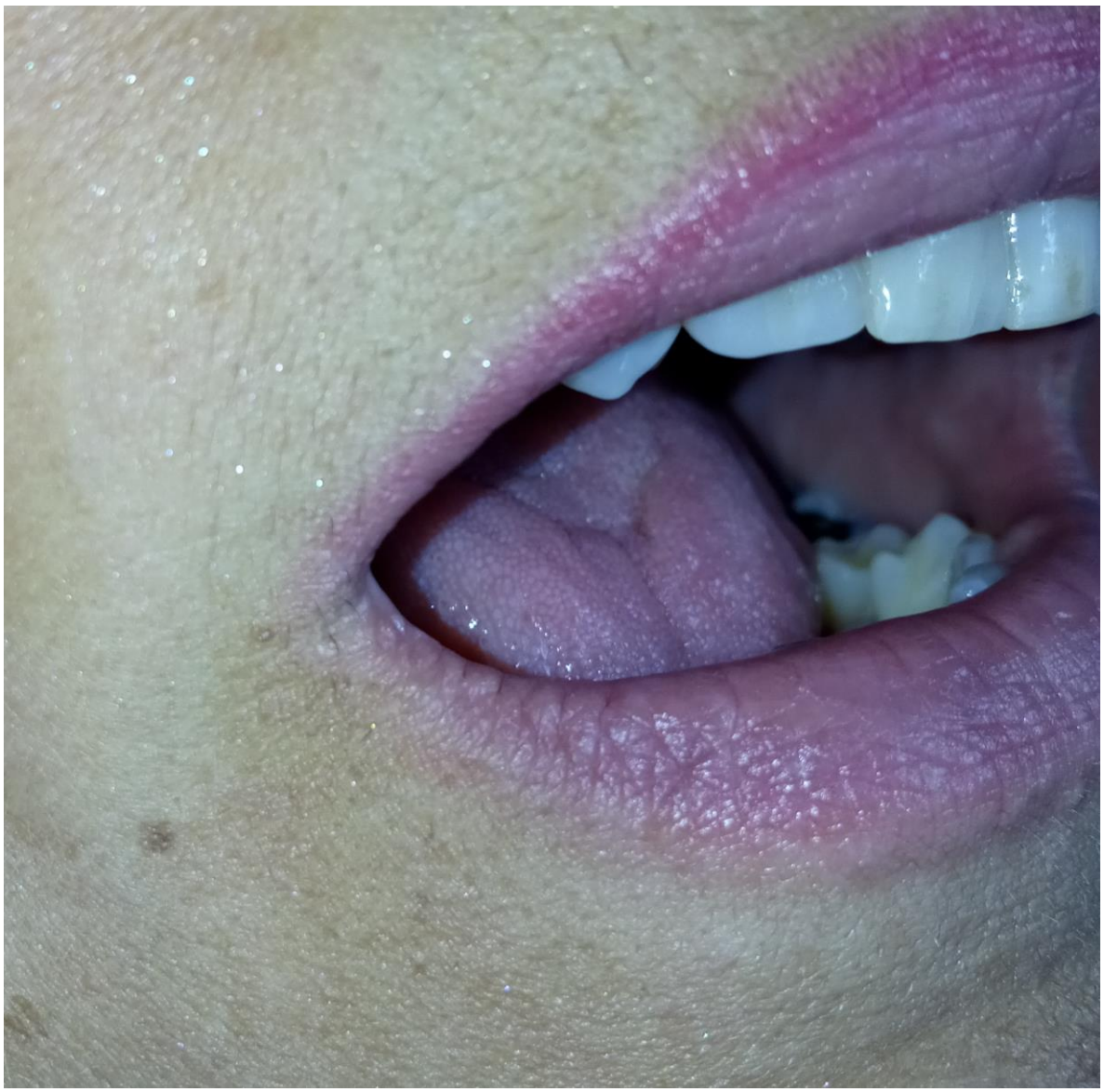


Fig 73b. Patient at 1 month follow up. The lesion healed.

5th case

A 46 years old female patient went to our observation for the presence of a symptomatic bilateral angular cheilitis, *Figure 74,75*. She had 2 small daughters and was very stressed, but not particular systemic health problems. She refers us that in the past she has had recurrence of this type of condition and healed in 8-10 days. Because she prefers to avoid administration of systemic drugs, I had treated the cheilitis with PDT with curcumin-based photosensitizer activated by 460 nm diode light, *Figure 76*. After the application of photosensitizer, each side has been illuminated for 5 minutes and then the photosensitizer removed.

After one day, the lesion was asymptomatic and after 4 days completely healed, *Figure 77* and *Figure 78.*

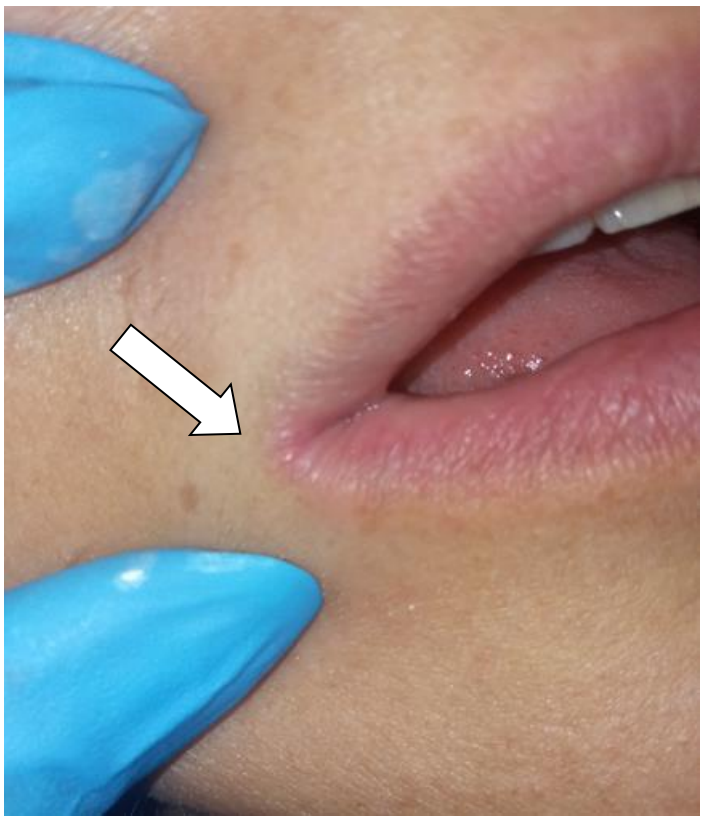


Fig. 74. Angular cheilitis in a 46-year-old female patient, right side.

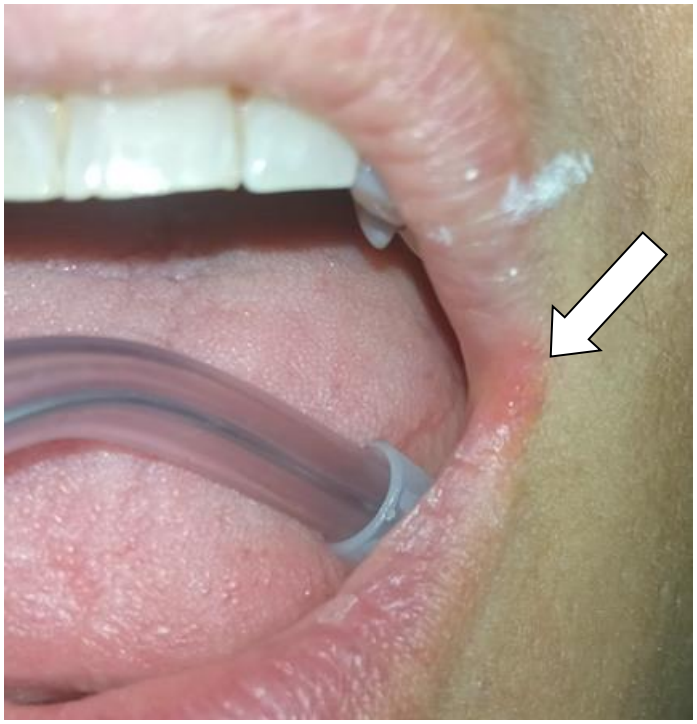


Fig.75. Angular cheilitis in a 46 year-old patient, left side.

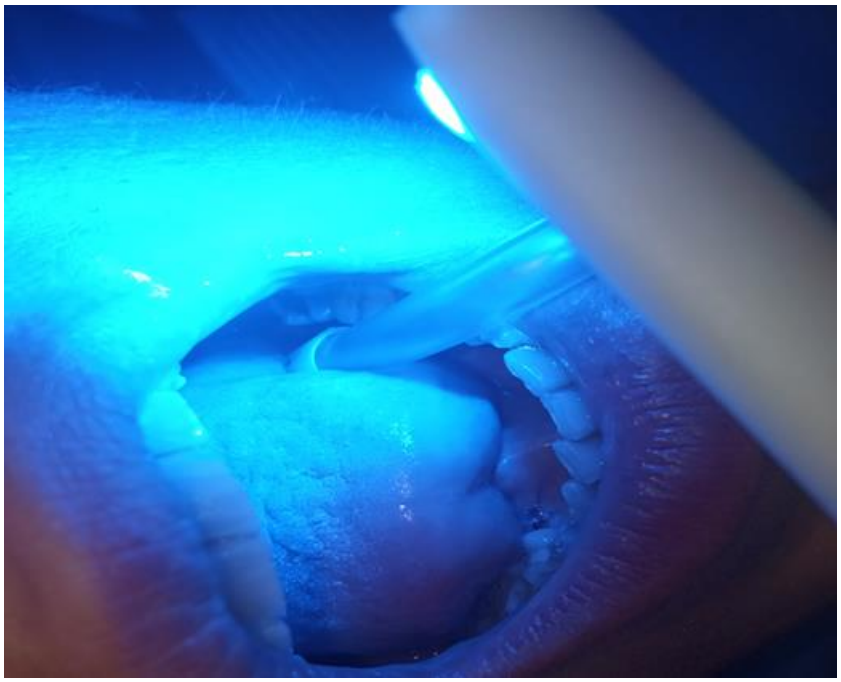


Fig.76. Photodynamic Therapy with curcumin-based photosensitizer, activated by 460 nm diode light.

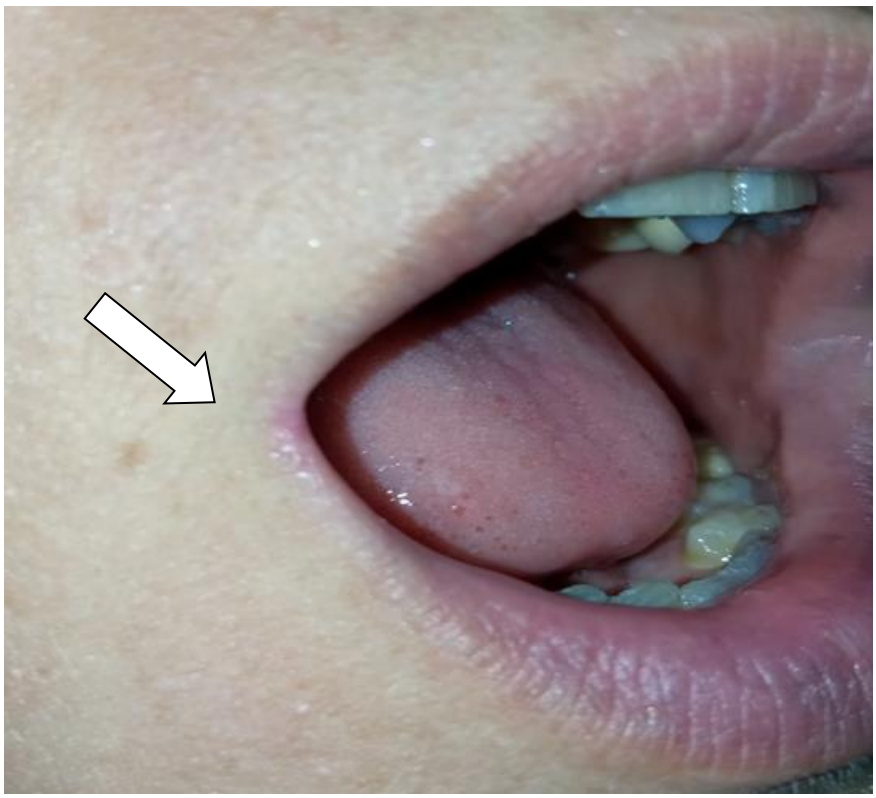


Fig. 77. The lesion healed, right side.

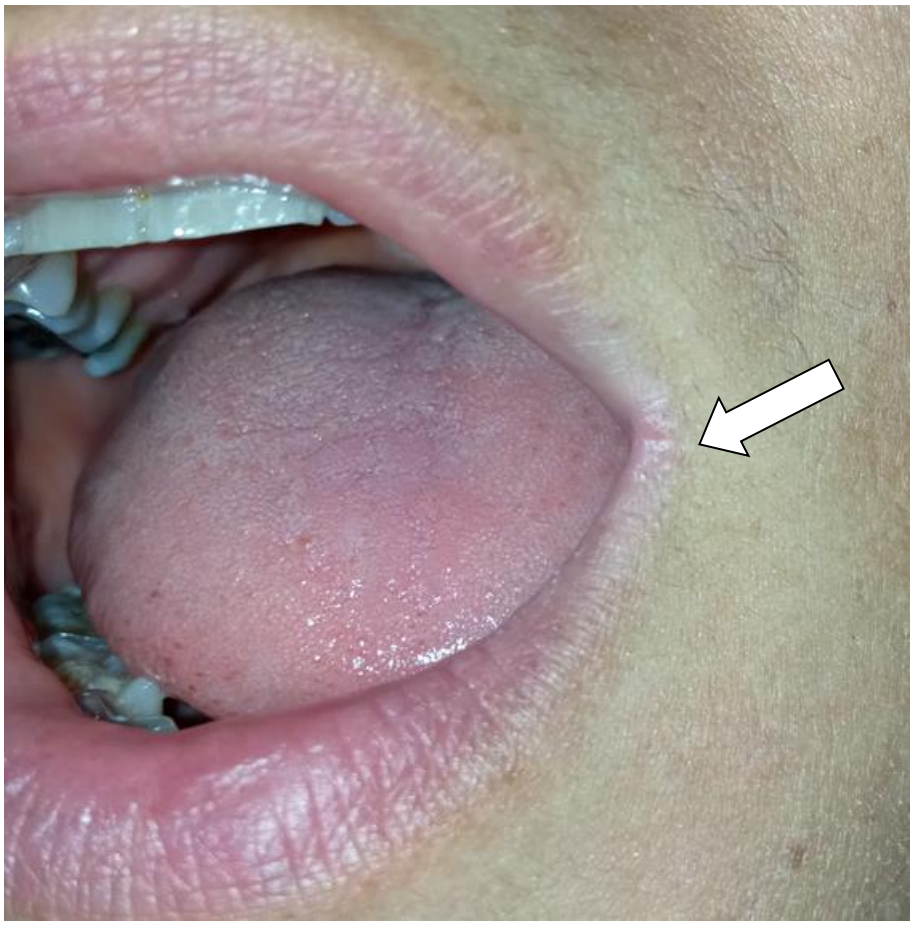


Fig.78. The lesion healed, left side.

6th case.

A 55 years-old female patient came to our observation for a symptomatic pain on the left side of angular mouth. Her anamnesis was positive for Systemic Lupus Erythematosus (diagnosed 20 years earlier) and skin lichen planus. She was treated with topical and systemic corticosteroids and evaluation on the liver condition (the part most affected by SLE) with blood examination has been performed each 6 months. In this general clinical condition, the use of other topic or systemic drugs is not recommended.

At a clinical observation it could be possible to see the presence of erythema and

erosions and a diagnosis of angular cheilitis has been made. The patient referred us that this condition is very frequent in period of immunodepression linked to the main disease, about a recrudescence each 2-3 months. It was decided to perform a photodynamic therapy session using the protocol with curcumin-based photosensitizer and LED light at 460 nm. After the positioning of the photosensitizer on the surface of the lesion, 5 minutes of illumination has been performed. After that the photosensitizer has been removed with a gauze. The 1 week follow up showed a good improvement of the lesion.

A 1 month follow up showed a complete healing of the lesion, and at 3 months follow up the patient had not relapse.

The patient is still monitored, also because the general health condition could generate other oral infection, also linked to the pharmacological therapy, *Figures 79, 80, 81, 82, 83.*

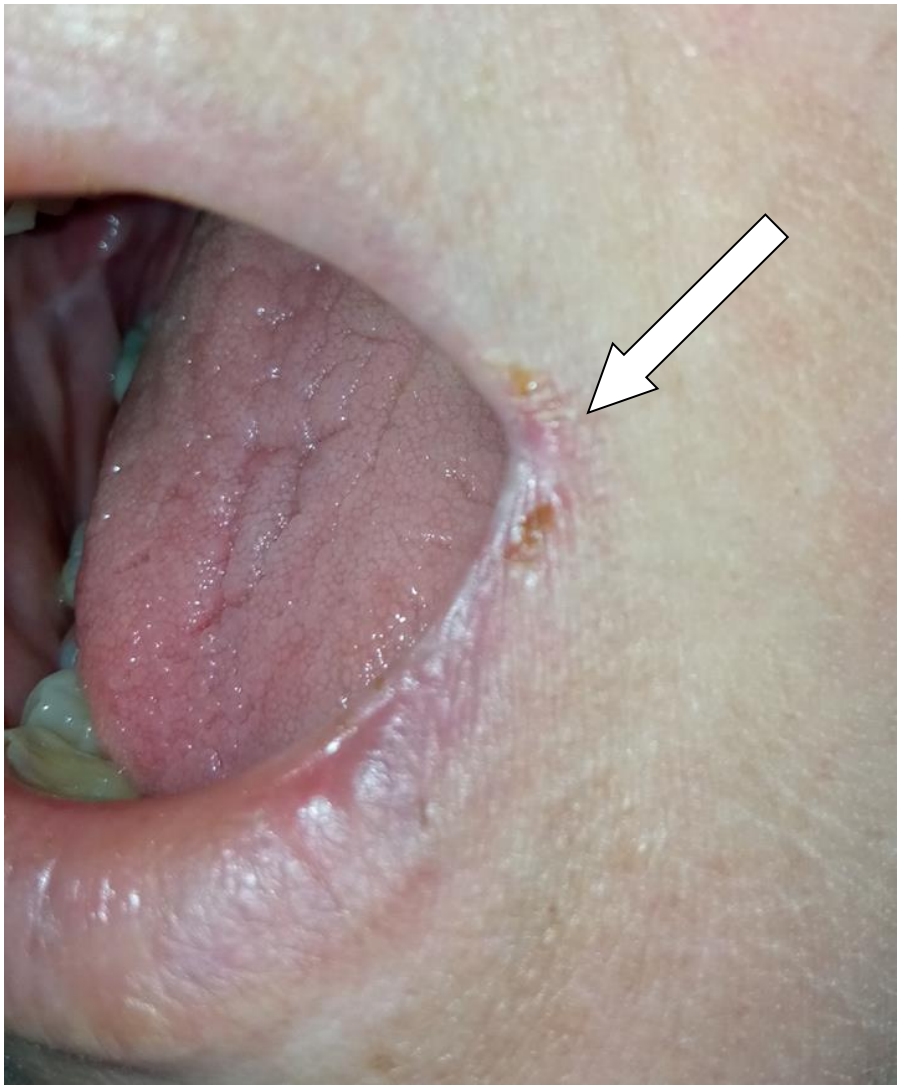


Fig. 79. First clinical presentation of angular cheilitis on the left side.

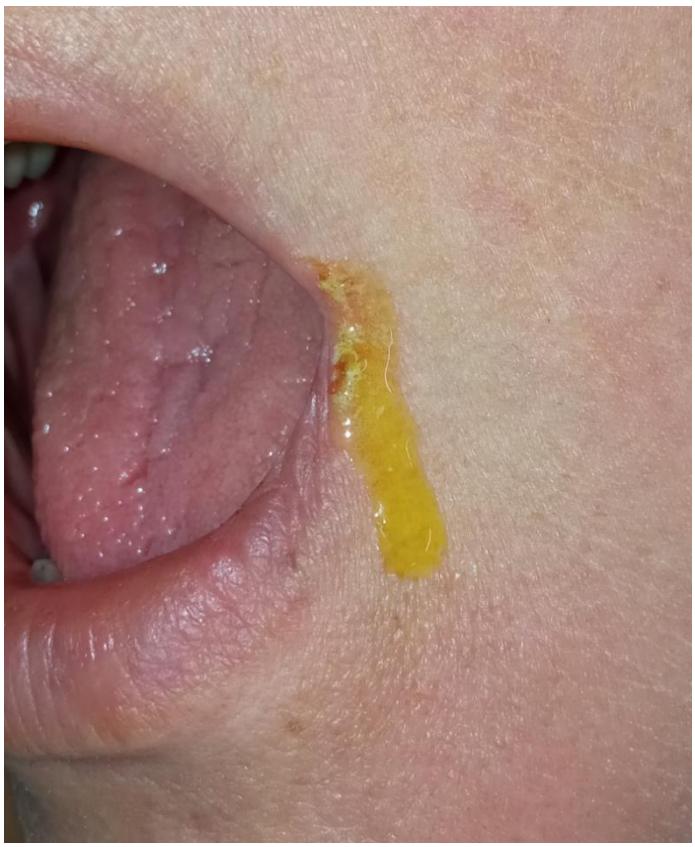


Fig.80. Application of the curcumin-based photosensitizer. It is possible to see the presence of little bubbles of the photosensitizer on the surface infected as sign of leakage of it with mycotic cells and starting release of free radicals.

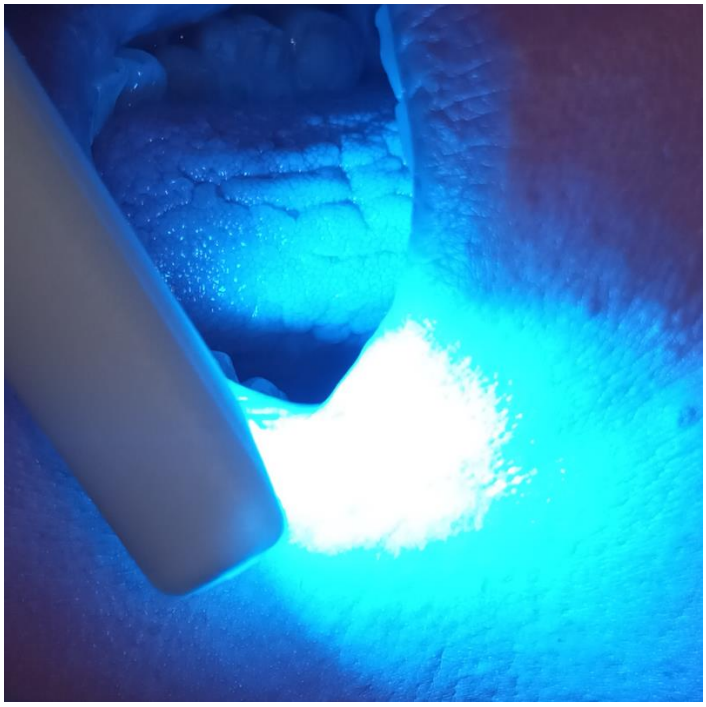


Fig. 81. Illumination with a LED lamp of 460 nm of wavelength for 5 minutes.

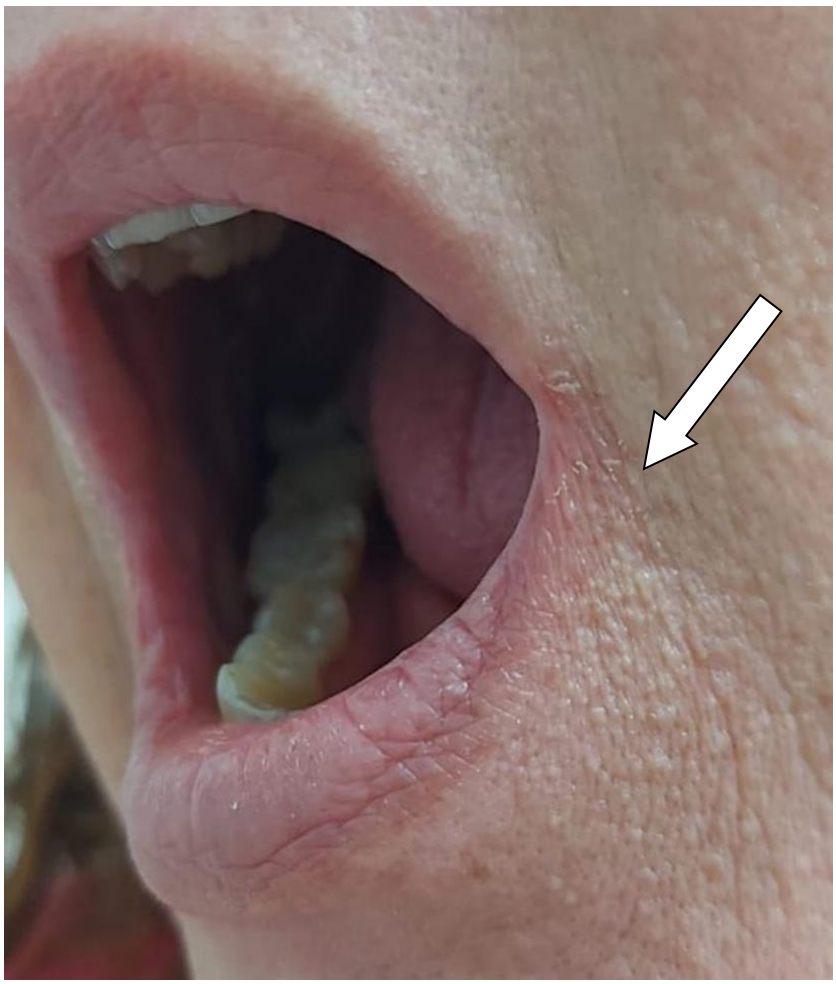


Fig. 82. Healing of the lesion at 1 week follow-up.

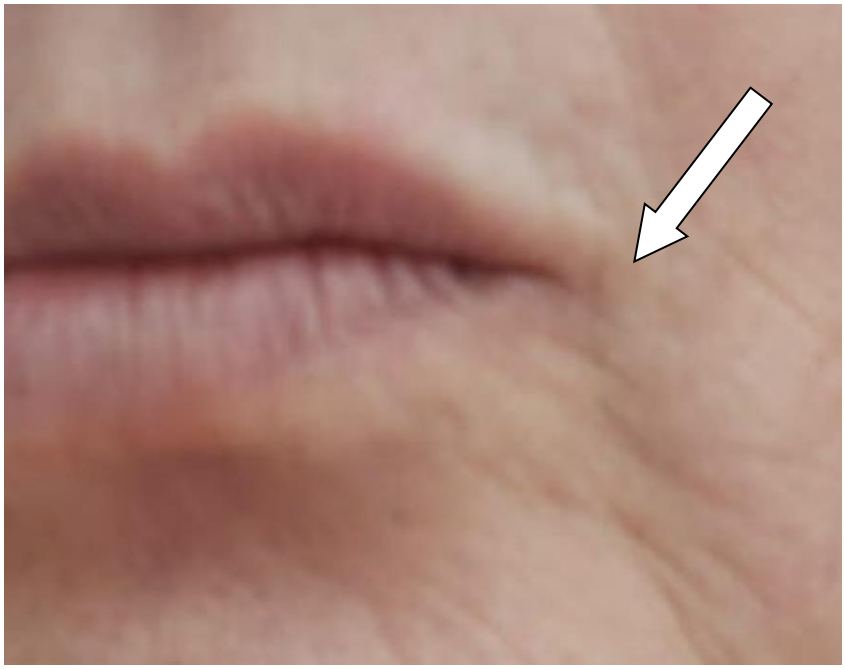


Fig 83. Healing at 1 month follow-up.

7th case.

A 64-year-old male patient went to our observation for the presence of light burning sensation on the palate. He declared a history of GERD (gastro-esophageal reflux disease) from several years. He wears an upper mobile denture, and at the clinical evaluation we could see a big red stain in the central part of the palate, corresponding to the major adherence of the prosthetic device. This last, was quite clean at the clinical observation, but we perform a provisional diagnosis of sub-prosthetic Candidiasis. A brush was performed on the patient's palate to evaluate the presence or absence of fungi in the site involved, to validate our diagnostic hypothesis. A brush was also performed on the internal surface of the prosthesis, and both, after being promptly sent to the OBL laboratory, were used to contaminate 2 petri dishes with Sabouraud gel culture medium and placed in an incubator at 37 degrees for 48 h. After the incubation period, numerous fungal colonies could be seen, both in the plate contaminated by the palatal sample and in the plate contaminated by the prosthetic sample, *Figure 84,85,86,87*. A diagnosis of subprosthetic candidiasis was made, and the patient was treated with miconazole 1% gel 2 times a day for 3 weeks, without any improvement. A PDT session was performed with 1% Toluidine blue and diode light at 630 nm. After the application of the photosensitizer on the entire affected palatal surface, it was activated with 630 nm diode light for a total duration of 5 minutes (10 activations of 30 seconds of the LED device). At one week it was possible to notice a symptomatic but not clinical improvement. The patient returned for checks after several months, for family reasons. The extremely acidic oral environment linked to GERD could facilitate sub-prosthetic fungal growth. A new session of PDT was performed, with curcumin-based photosensitizer and 460 nm diode light, with the same protocol described before. We have had a clinical

improvement but not completely resolution, *Figures 88*.

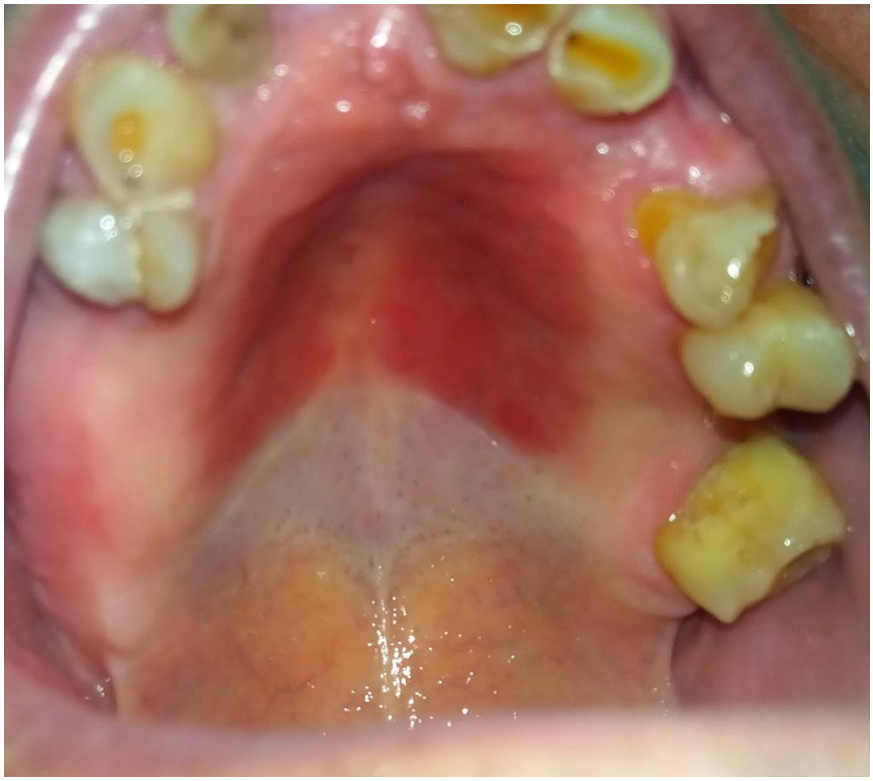


Fig. 84. Clinical presentation of the sub-prosthetic Candidiasis.

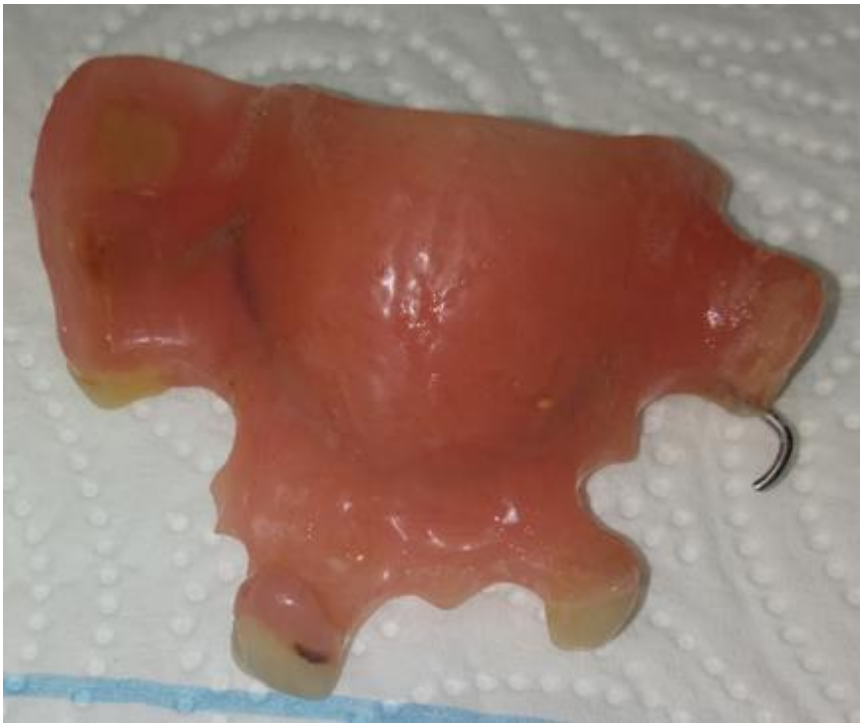


Fig.85. Prosthetic device worn by the patient.

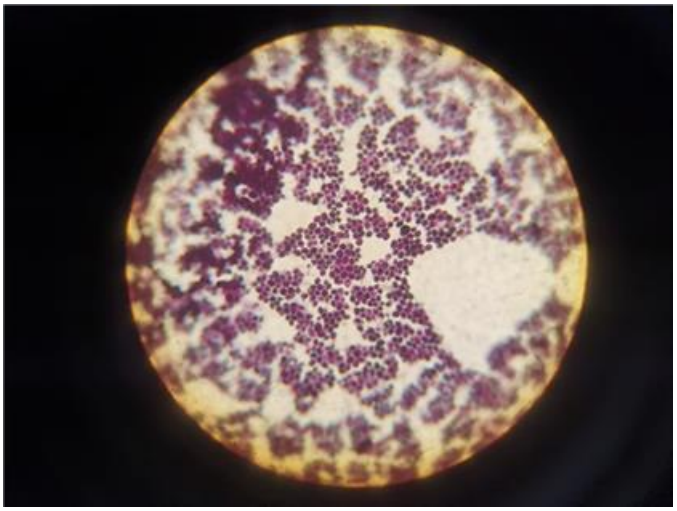


Fig 86. *Candida albicans* cells grown from the sample derived from the palate tissue of the patient, slide Gram stained and observed under optical microscope 1000X.

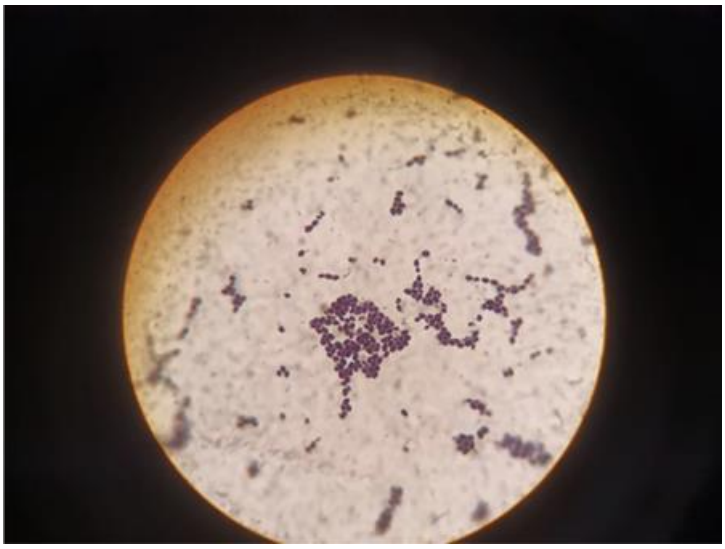


Fig. 87. *Candida albicans* cells grown from the sample derived from the denture surface, slide Gram stained and observed under optical microscope 1000X.

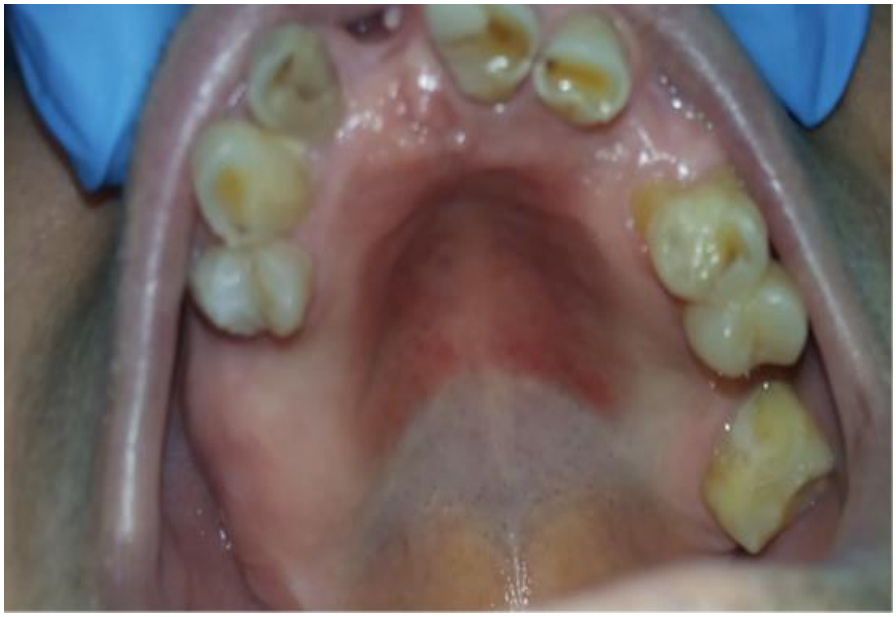


Fig. 88. Clinical improvement of chronic sub-prothesis Candidiasis, not a complete healing.

8th case.

A 9-year-old girl came to our observation for a check on her oral hygiene status following the placement of a fixed orthodontic appliance, specifically a palatal expander, about 2 months ago. The notable presence of plaque and the bad breath perceived by the little patient's parents had led the orthodontist to perform brushes to evaluate the total bacterial load and presence of fungal microorganisms, this time proceeding through a simple qualitative analysis of the Candida panel. The brushes were rubbed for 30 seconds each (one for quantitative bacterial analysis, one for qualitative fungal analysis) on each side where there was greater adhesion between the fixed expander-type device and the palatine mucosal surface (see *fig 89,90* for the sampling sites). The analysis highlighted a notable bacterial load, especially of *F.nucleatum, T forsithia, P.gingivalis, P.intermedia*; furthermore, two fungal species that were not very frequent in oral candidiasis were present: *C.parapsilosis* and *C.lypolitica*. With the aim of decontaminating the infected mucosal surfaces,

photodynamic therapy was performed using a curcumin-based photosensitiser and 460 nm light, activated for a total of 2 minutes for each side corresponding to the sampling area (*Figures 91,92*). After a week, two samples were taken with the same previous methods and for the same purposes. The results of the biomolecular tests showed a decrease in the total bacterial population, the disappearance of the fungal DNA of *C.parapsilosis* and *C.lypolitica*, and the presence of *C.tropicalis*. The transition from the presence of 2 fungal species to just one was interpreted as an improvement from a microbiological point of view *(Figures 93,94,95).*

The result at a clinical level was not visible due to the presence of the fixed appliance cemented between the teeth and palate. However, the study of this case made us suppose that photodynamic therapy can be used as a tool to reduce bacterial and fungal contamination in small, non-cooperating patients with fixed orthodontic appliances.

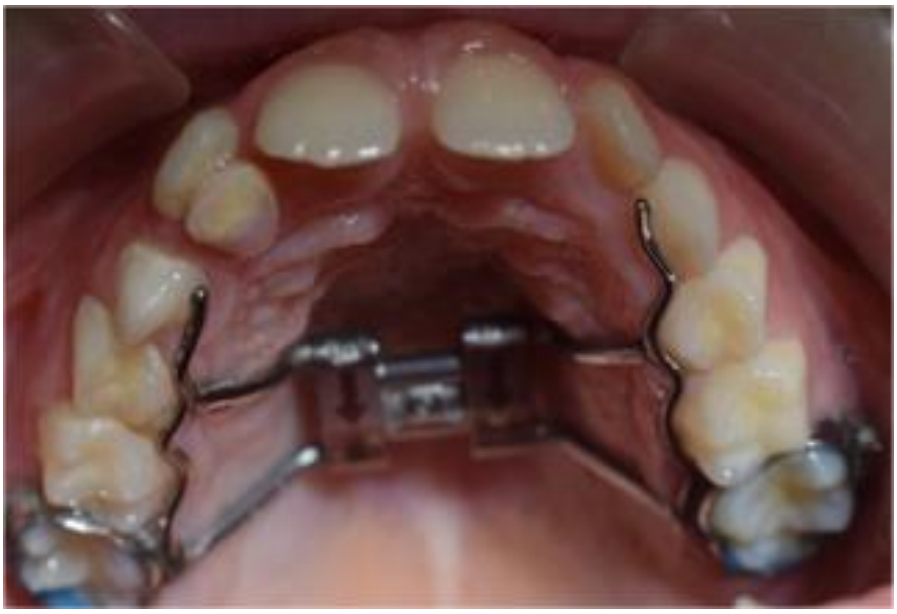


Fig. 89. Clinical presentation of the oral condition in the patient of 9 years old.

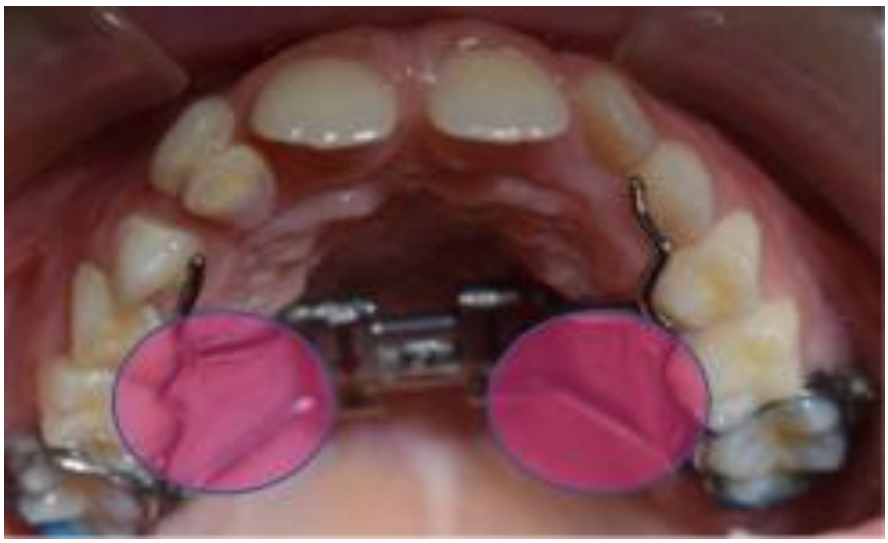


Fig.90. Sampling site for the bacterial and fungal evaluation.

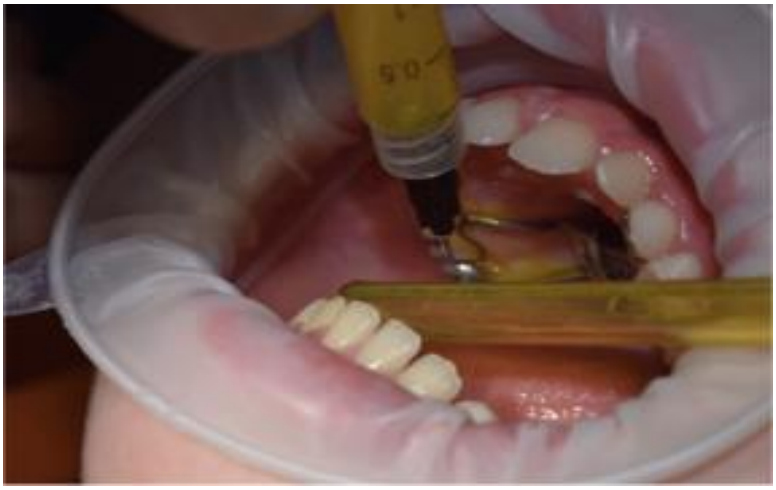
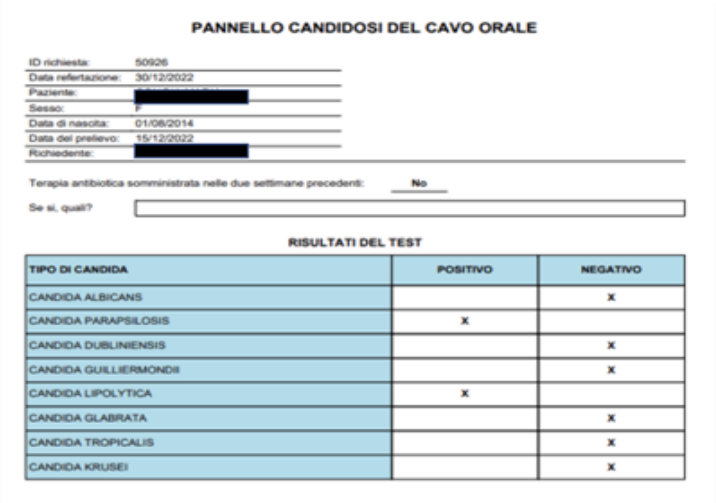


Fig 91. Application of the curcumin-based photosensitizer.

Fig.92. Light illumination with LED 460 nm for each side involved



PANNELLO CANDIDOSI DEL CAVO ORALE

ID richiesta: 50926
Data refertazione: 30/12/2022
Paziente: [redacted]
Sesso: F
Data di nascita: 01/08/2014
Data del prelievo: 15/12/2022
Richiedente: [redacted]

Terapia antibiotica somministrata nelle due settimane precedenti: No

Se si, quali?

RISULTATI DEL TEST

TIPO DI CANDIDA	POSITIVO	NEGATIVO
CANDIDA ALBICANS		X
CANDIDA PARAPSILOSIS	X	
CANDIDA DUBLINIENSIS		X
CANDIDA GUILLIERMONDII		X
CANDIDA LIPOLYTICA	X	
CANDIDA GLABRATA		X
CANDIDA TROPICALIS		X
CANDIDA KRUSEI		X

Fig. 93. Report of Candida panel before the PDT session

PANNELLO CANDIDOSI DEL CAVO ORALE

ID richiesta: 51394
Data refertazione: 11/02/2023
Paziente: [redacted]
Sesso: F
Data di nascita: 24/01/2005
Data del prelievo: 27/01/2023
Richiedente: [redacted]

Terapia antibiotica somministrata nelle due settimane precedenti: No

Se si, quali?

RISULTATI DEL TEST

TIPO DI CANDIDA	POSITIVO	NEGATIVO
CANDIDA ALBICANS		X
CANDIDA PARAPSILOSIS		X
CANDIDA DUBLINIENSIS		X
CANDIDA GUILLIERMONDII		X
CANDIDA LIPOLYTICA		X
CANDIDA GLABRATA		X
CANDIDA TROPICALIS	X	
CANDIDA KRUSEI		X

Fig 94. Report of Candida panel after PDT session

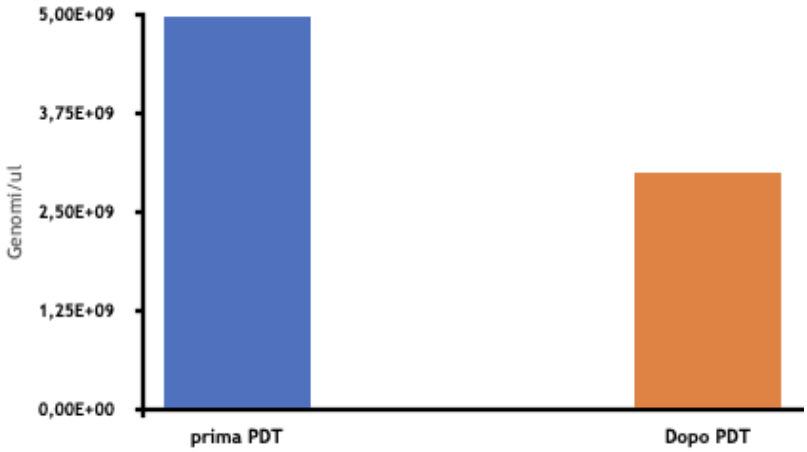


Fig. 95. Bacterial load before and after PDT session.

9th case.

An 84-year-old patient came to our observation due to the presence of a strong intraoral burning sensation beneath the lower prosthetic product. The patient's medical history reported allergies to various drugs, valve replacement surgery, mild osteoporosis, xerostomia and was taking Teriparatide for heart problems. The patient had already performed microbiological evaluations which highlighted the presence of a high *C.albicans* fungal load at an oral level. The highly symptomatic area corresponded to the attached gingiva and alveolar mucosa beneath a mobile prosthetic rehabilitation which was inserted into a metal bar supported by two implant elements. The mucosa appeared slightly red, and there was a whitish plaque above the metal portion *Figures 96a, 96b*, 96c. A therapy based on miconazole 1%, 2 times a day was administered for 2 weeks, without any symptomatic remission. It was therefore decided to carry out a PDT session with the protocol which involves the use of the curcumin-photosensitiser and LED light at 460 nm. Two sessions were performed 15 days apart and the whitish patina on the metal portion was removed using ultrasound. PDT was also carried out on the prosthetic product, in the internal portion that adhered to the symptomatic gingival and alveolar tissue, for a total of 5 minutes per session *Figures 97,98*. The patient improved greatly on a symptomatic

level, VAS values decreased from 6,5 to 3,5 before and after PDT sessions, while there was no significant difference on a clinical level between before and after the treatment.

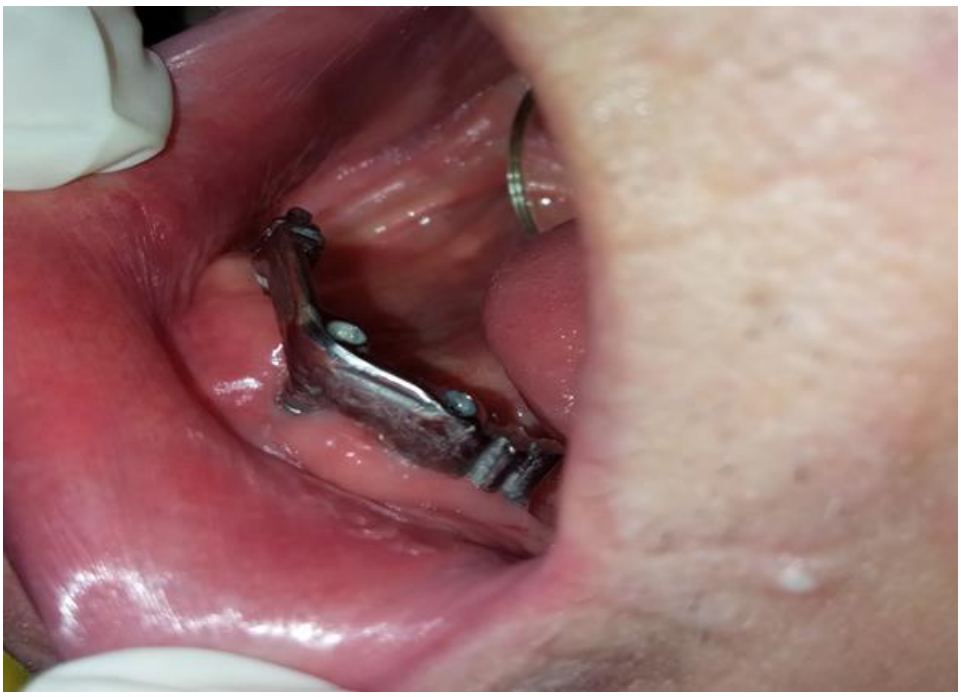


Fig. 96a. Clinical presentation of the area with Candida infection, left side.

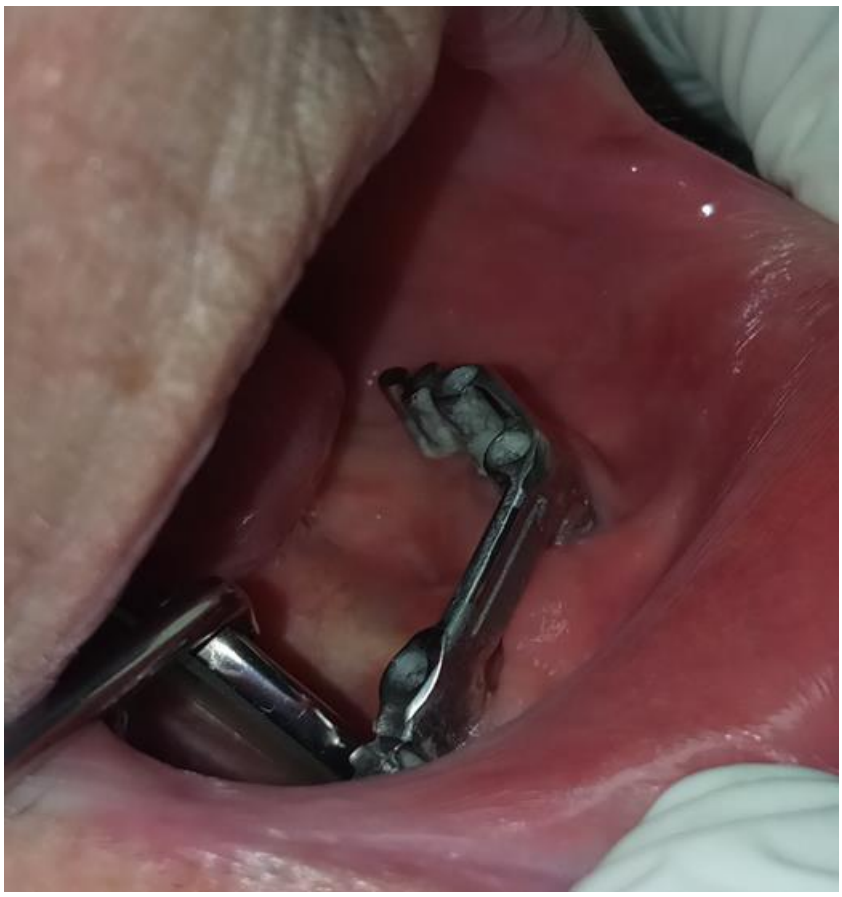


Fig 96b. Clinical presentation of the area with Candida infection, right side.

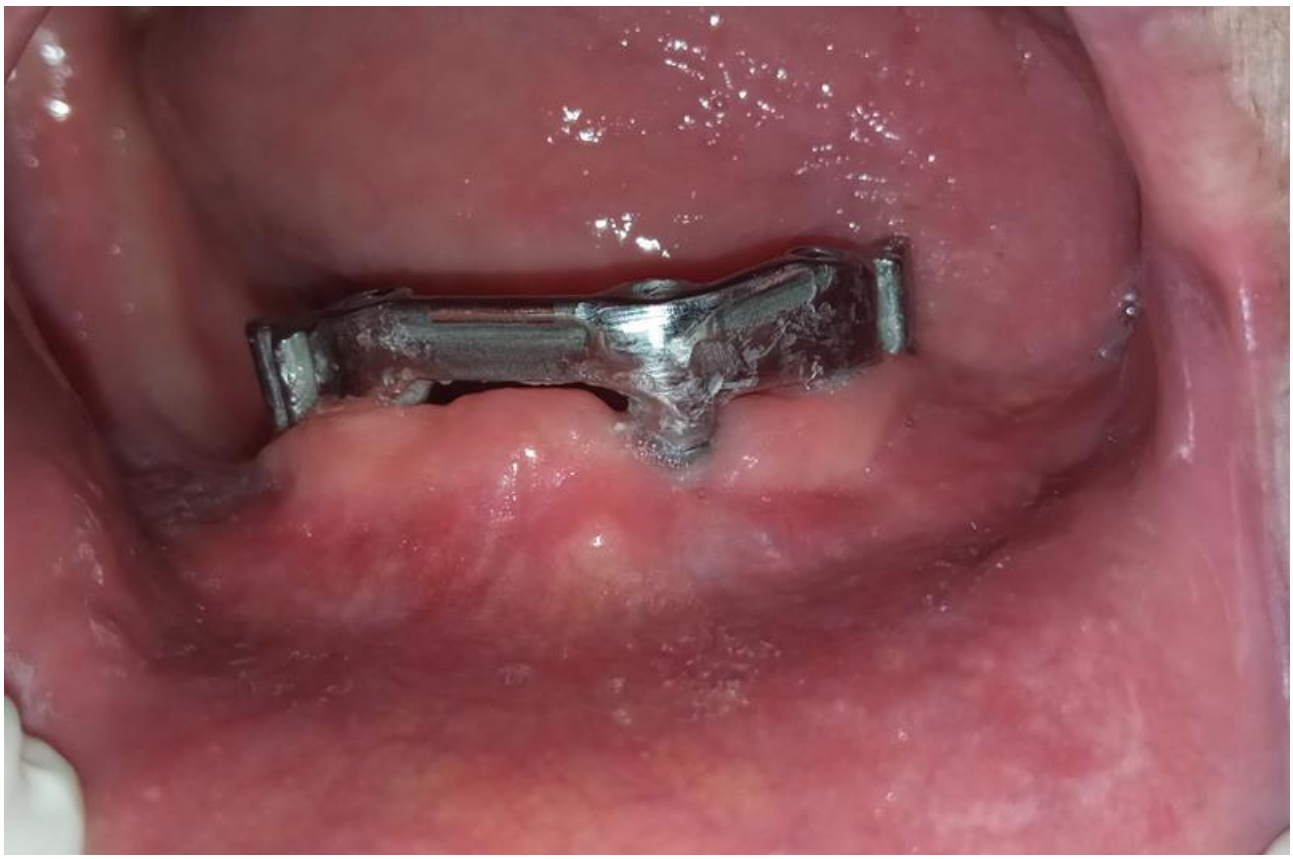


Fig 96c. Clinical presentation of the area with Candida infection, central view.

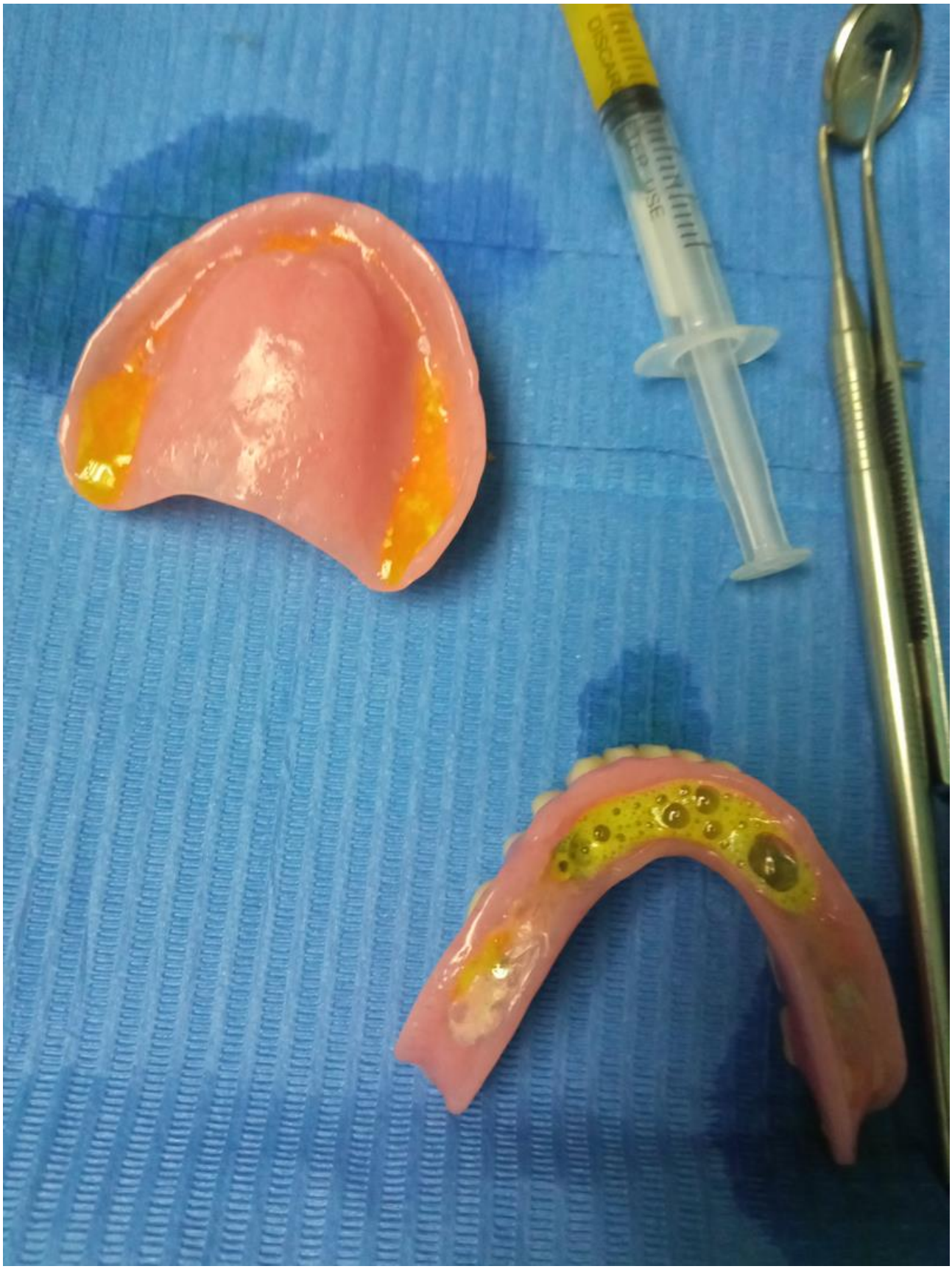


Fig. 97. Application of curcumin-based photosensitizer on prosthetic device

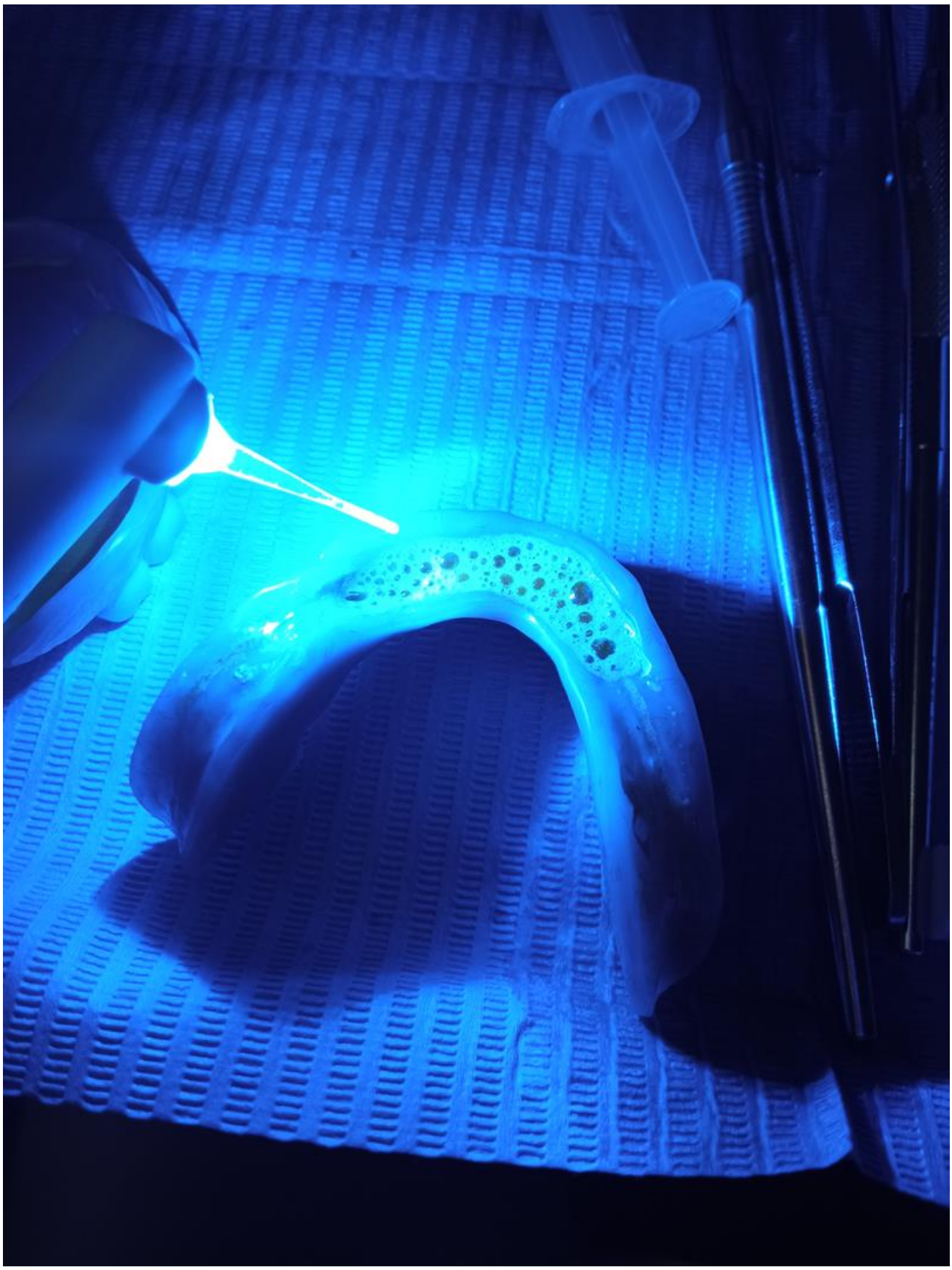


Fig. 98. Illumination with LED light 460nm after the application of photosensitizer in the prosthetic device.

10th case

A 55-year-old male patient came to our observation for the presence of whitish plaques on the cheek mucosa near the corner of the mouth, bilaterally, accompanied by a burning sensation. Based on his medical history, the patient states that he suffers from type 1 diabetes, mild arterial hypertension, reported that he has had allergies to artichokes, and that he is a smoker (over 40 cigarettes a day). On physical examination he showed fissures and erythema in the external part of the mouth attributable to bilateral angular cheilitis and whitish areas, which could not be removed with gauze or cotton pellets in the adjacent part of the buccal mucosa. On the right side the maximum diameter of the lesion was 12 mm, in the left side was 15 mm, measured with periodontal probe (Hu-Friedy, L.A., USA). Given the systemic condition that predisposes towards some types of chronic candidiasis, the fact that the patient was a heavy smoker and the clinical presentation of the lesions, a diagnostic hypothesis of chronic hyperplastic candidiasis was posed, *Figures 99, 100, 101*. Before proceeding with the therapy, an evaluation was carried out via autofluorescence examination with the Goccles device (Pierrel Pharma, Capua, CE, Italy) and illumination with a 460 nm ,4 watt LED lamp (Valo, Ultradent, MI, Italy), as suggested by the Company, on both the mucosal and cutaneous sides involved. The test, as shown in the photos, gave a negative result, no loss of fluorescence had been intercepted, therefore no suspicious areas of potential transformation, *Figures 102-105*. Topical antifungal therapy with miconazole 1% gel to be applied 2 times a day for 15 days was given, without success. Photodynamic therapy was performed with curcumin based-photosensitizer and 460 nm diode light on the right side and the same type of photosensitizer activated by polarized light, for 2 minutes for each side (1 minute on the skin surface and 1 minute on the oral mucosa), *Figures 106-109.*

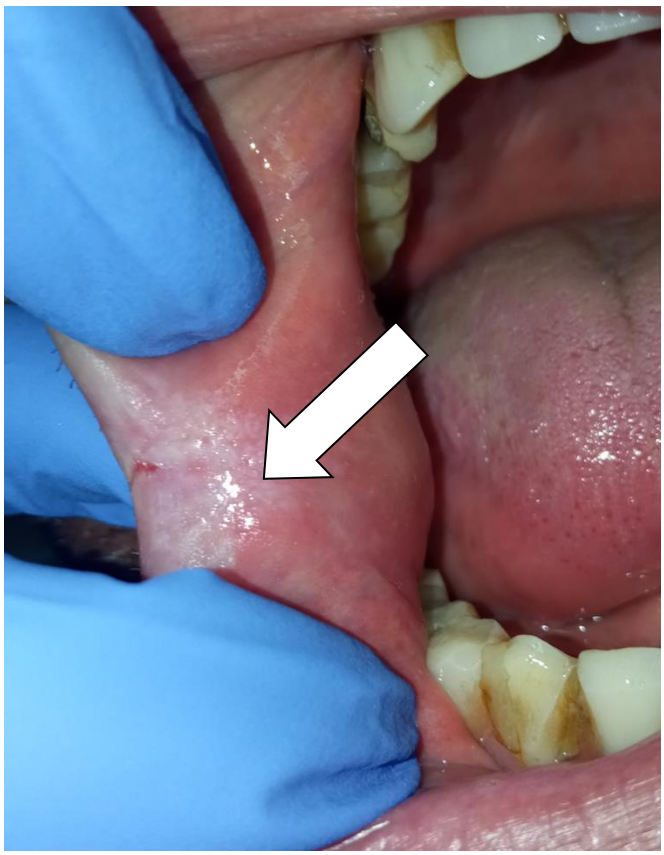


Fig. 99. Lesions on the right side of the mouth.

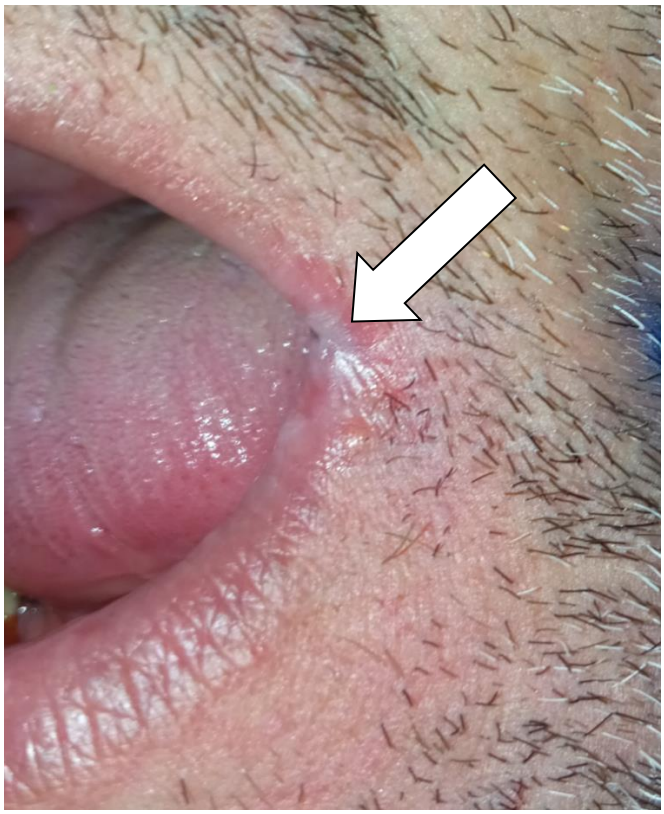


Fig 100. Angular cheilitis in the left side of the mouth

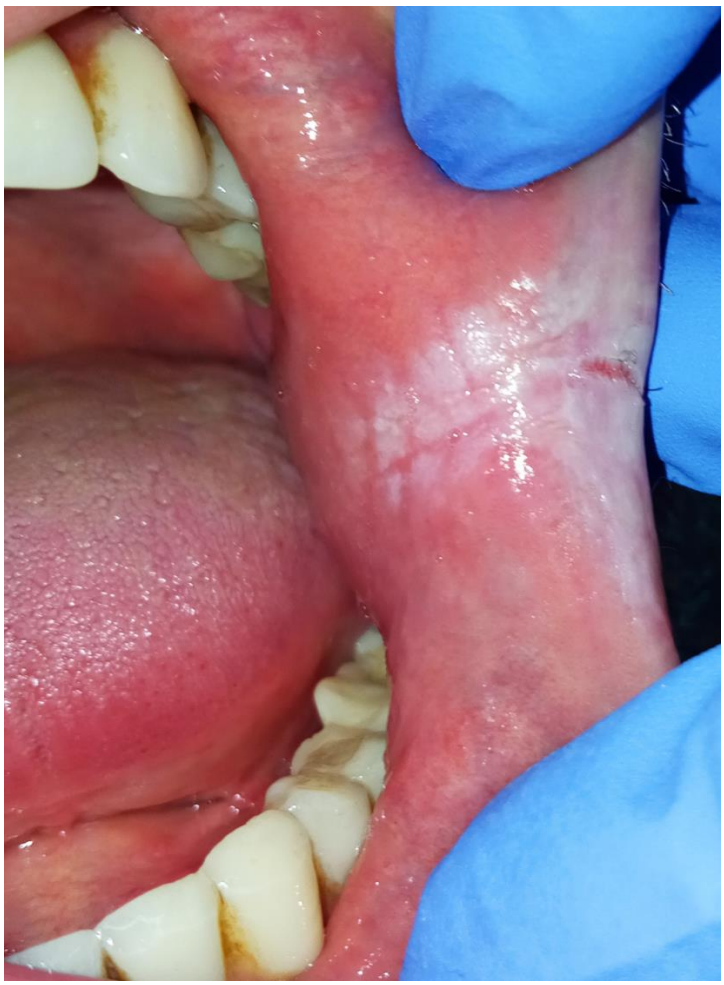


Fig. 101. Oral mucosa lesion in the left side of the mouth

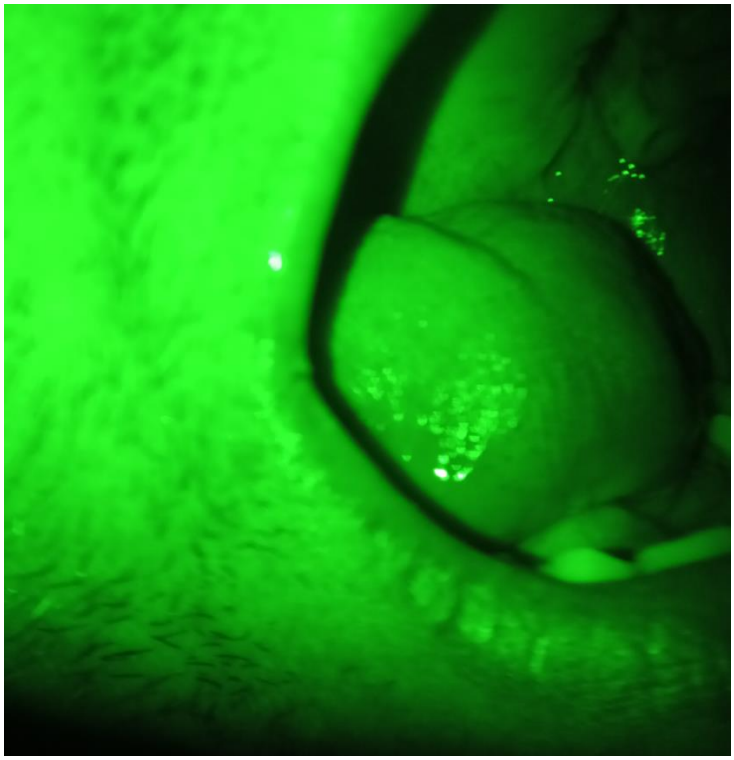


Fig 102. Fluorescence examination on the right skin side. No loss of fluorescence has

been detected.

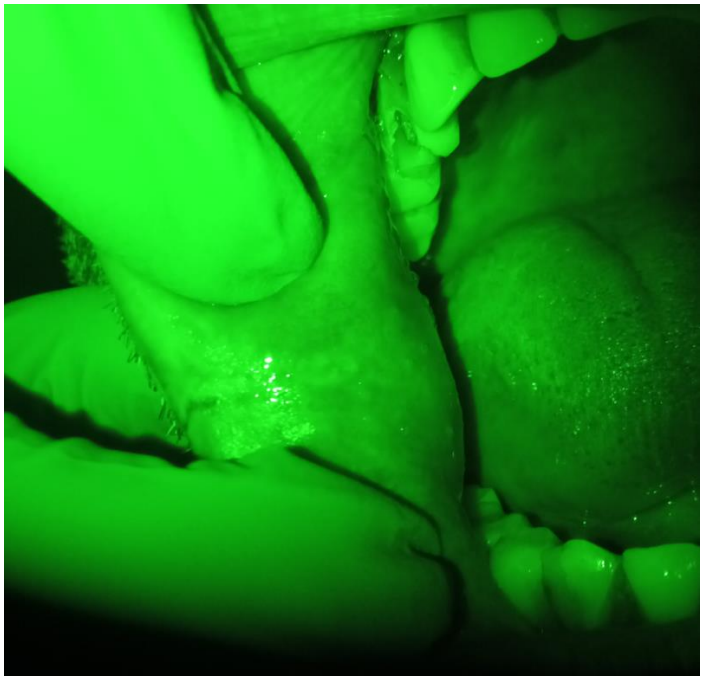


Fig 103. Fluorescence examination on the right oral mucosa. No loss of fluorescence has been detected.

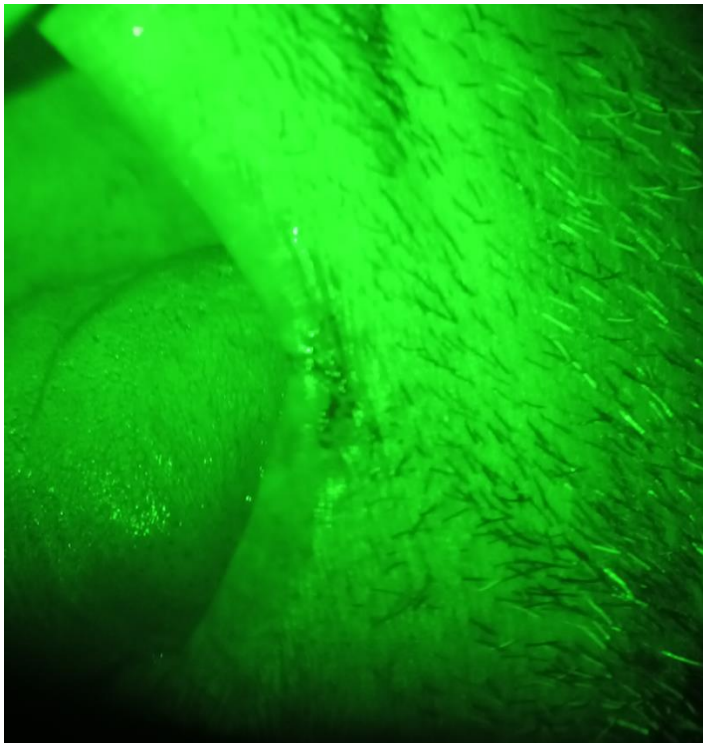


Fig 104. Fluorescence examination on the left skin side. No loss of fluorescence has been detected.

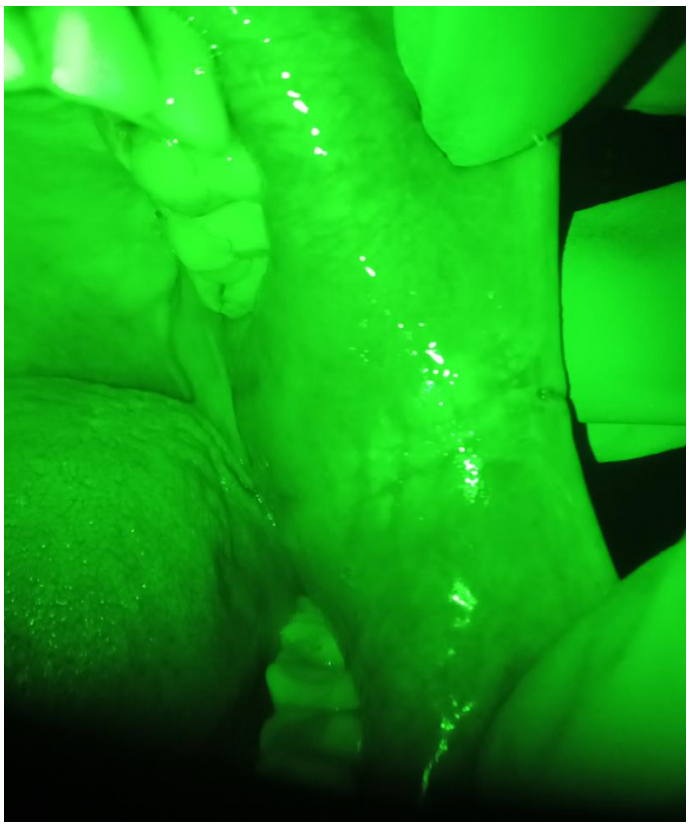


Fig. 105. Fluorescence examination on the left oral mucosa infected. No loss of fluorescence has been detected.

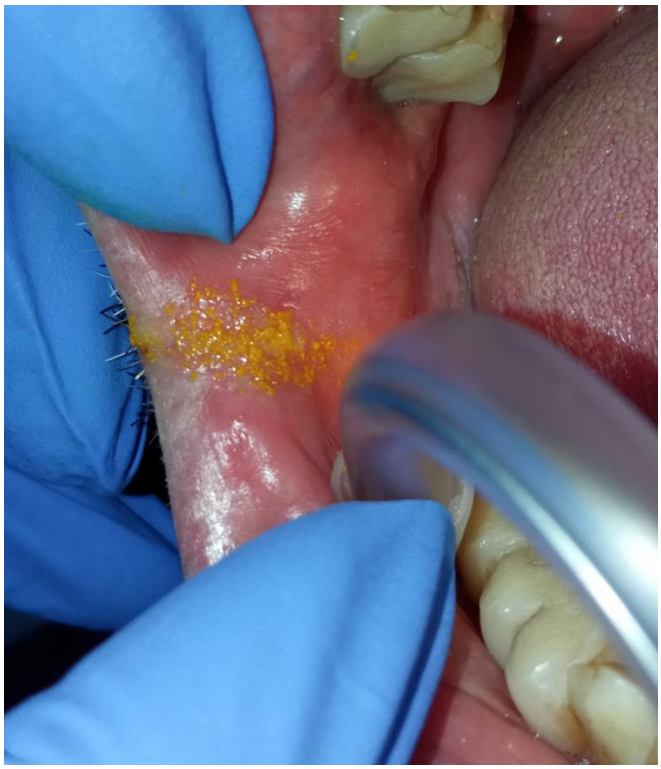


Fig. 106. Application of photosensitizer on the right side of the mouth.

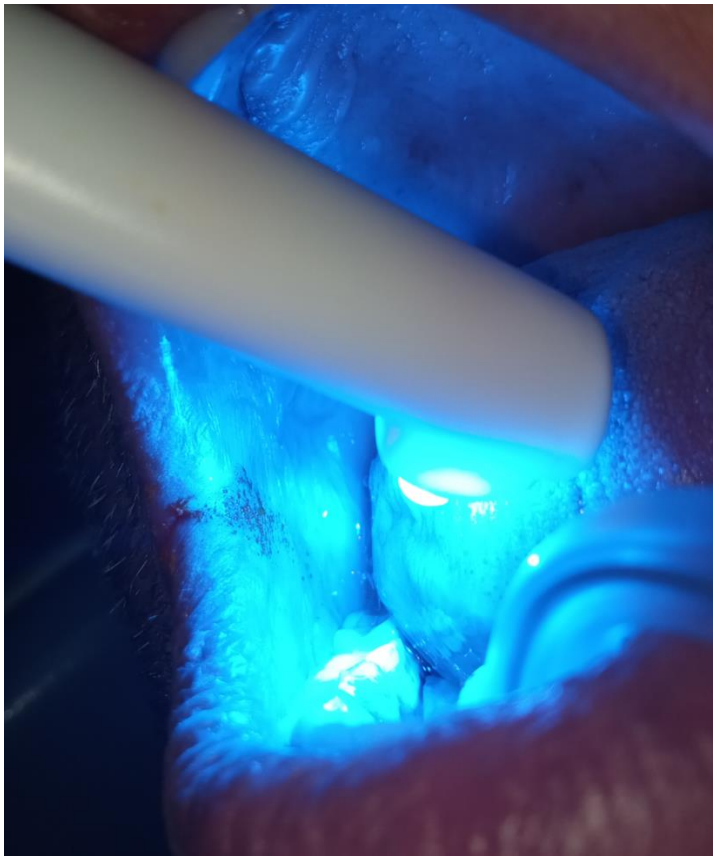


Fig.107. Illumination with LED diode lamp 460 nm on the right side of oral mucosa.

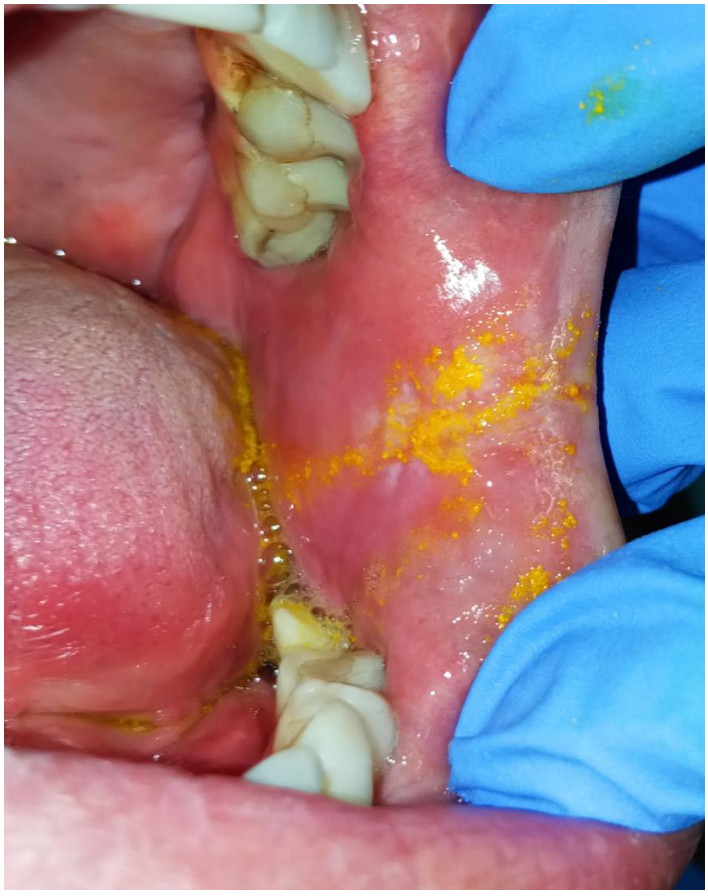


Fig 108. Application of photosensitizer on the left oral mucosa.

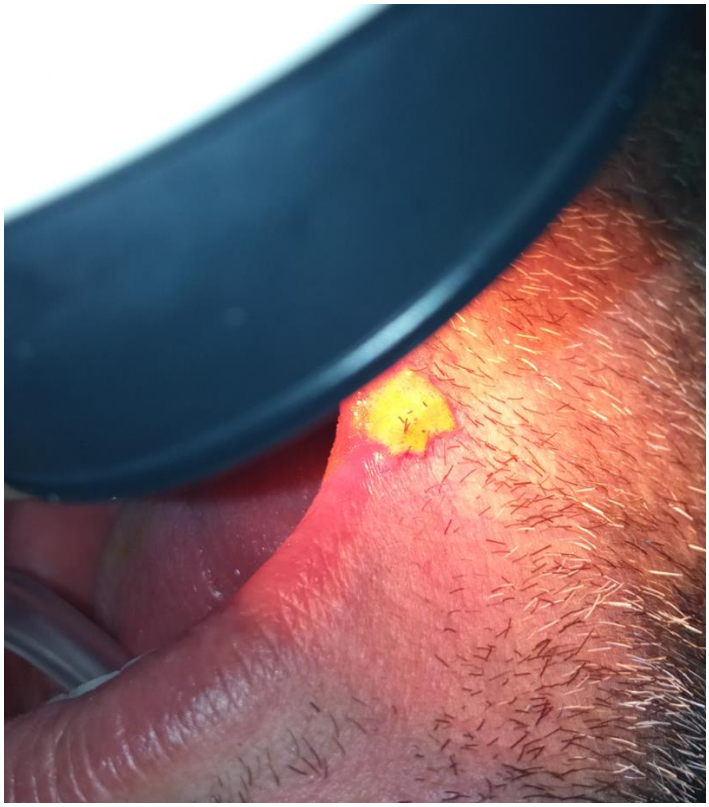


Fig.109. Illumination with polarized light (380-3400 nm).

At the 5-day check-up, the patient showed complete remission on the skin side on the right and on the left and an improvement in the mucosal lesion, which is clearly more marked on the left side (*Figure 110-113*).

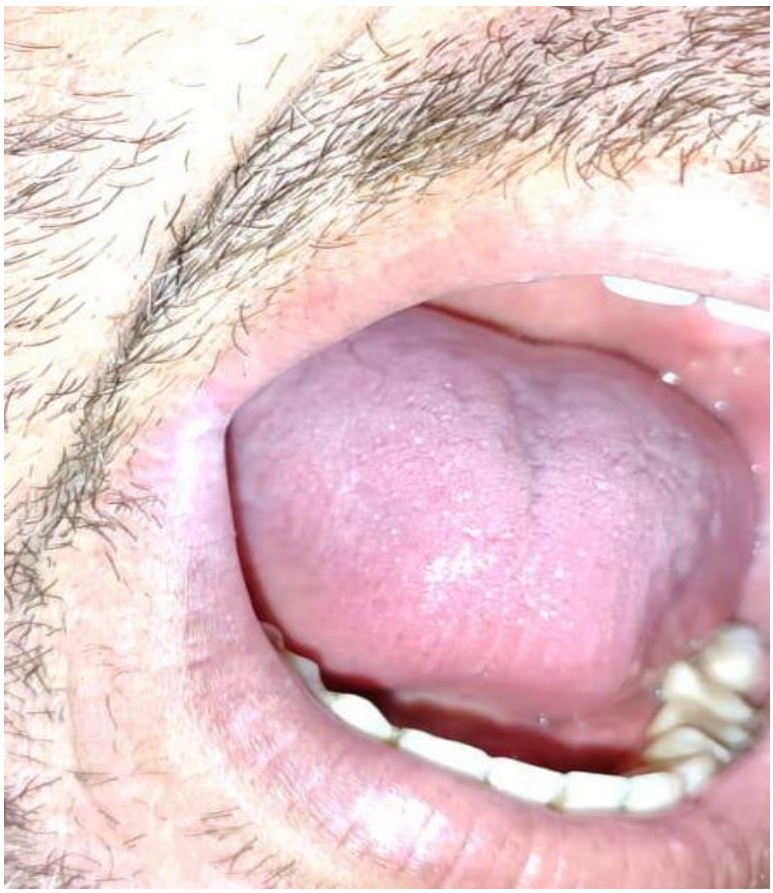


Fig 110. 5 days follow up after PDT session, healing on the right side of skin lesion.

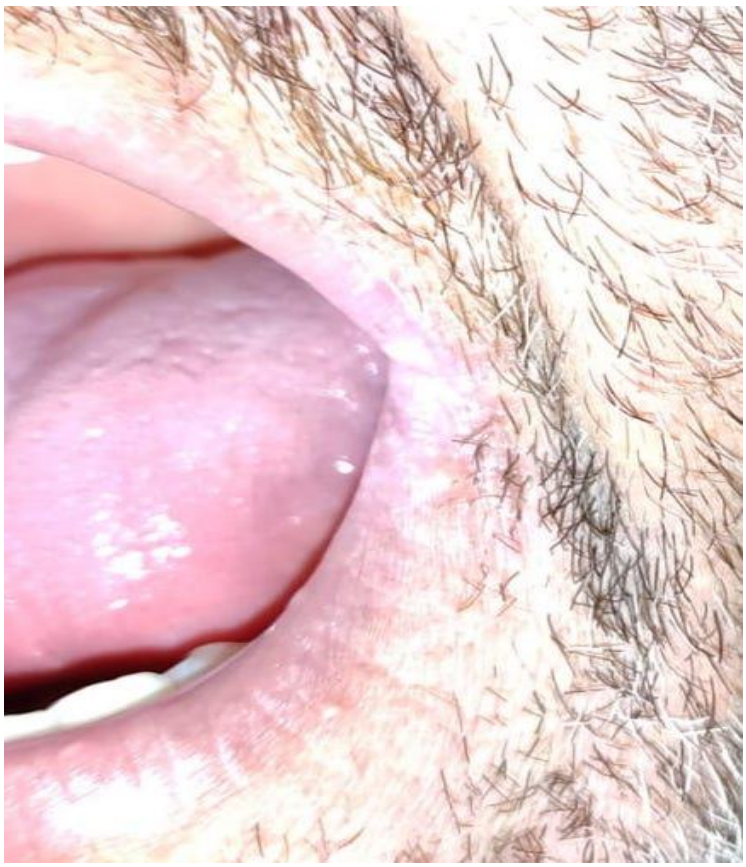


Fig 111. 5 days follow up after PDT session, healing on the left side of the skin lesion.

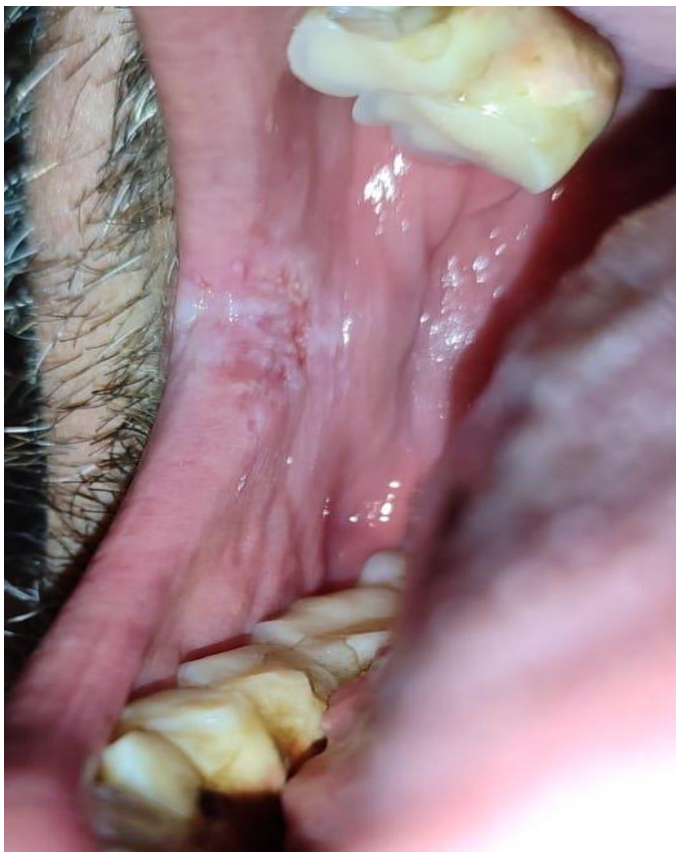


Fig 112. 5 days follow up after PDT session, right side of oral mucosa

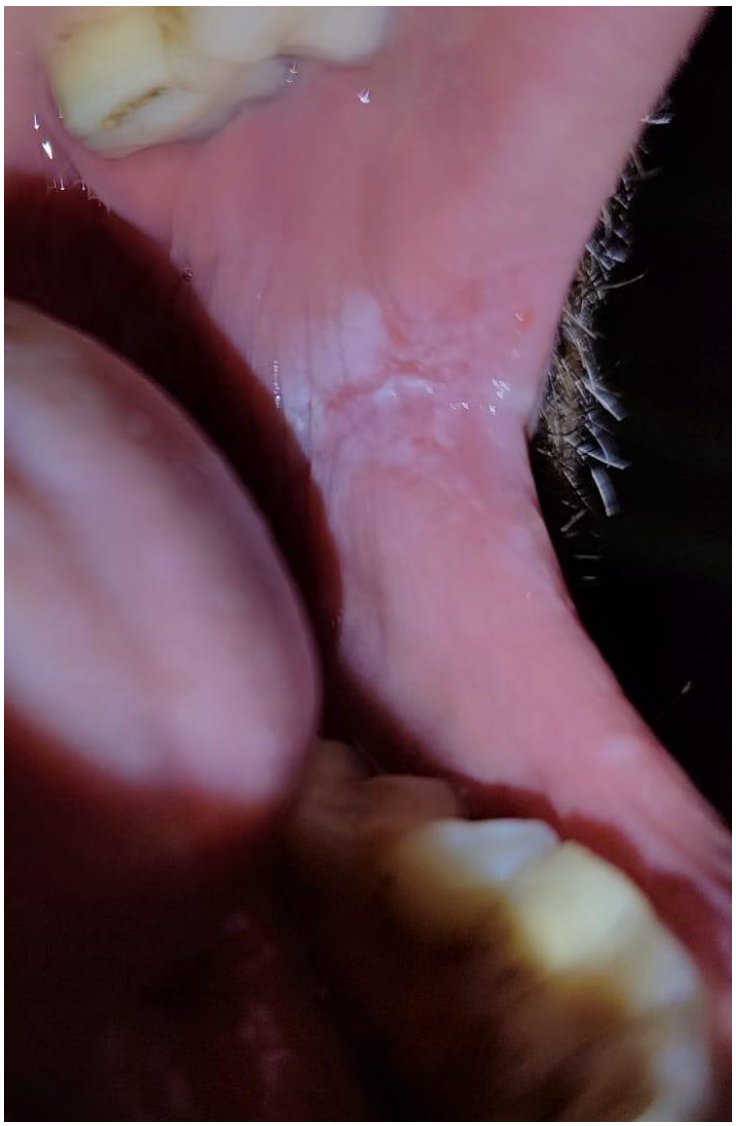


Fig. 113. 5 days follow up after PDT session, improvement of left side of oral mucosa.

11th case.

A 37-year-old man came to our attention with a suspected leukoplakia on the right side of the tongue of about 2 x 3 cm in dimension, *Figure 114, 115*. After signing the informed consent, the patient has been submitted to an incisional biopsy on the ventral right side of the tongue. The histological report confirms the suspected leucoplakia and describes the lesion as a flap of squamous epithelium with hyper-parakeratotic aspects, free from dysplasia but rich in microbial and fungal flora, with

the indication for the clinician to evaluate the load with a cultural investigation. A brush on the remaining lesion in order to detect what kind of microorganism could have colonized it, has been performed. The brush revealed a high presence of *C. albicans* tires. Due to what is reported in the literature about the association between Oral Candidiasis (OC) and Leucoplakia (OL) and its opportunity to become a potential malignant lesion, it has been decided to try to eradicate the microorganism using Photodynamic Therapy (PDT). The protocol used was the mixture of curcumin and H_2O_2 at 3% as a photosensitizer, activated with blue diode light at a wavelength of 460 nm and 7 watts of power (FlashMax P7, CMS Dental, Copenhagen, Denmark) for about 5 minutes, handling the light about one centimetre far from the lesion, like previous described, *Figures 116-117*. After that, the photosensitizer was washed away with water. Immediately after a brush (scraped for 30 seconds on the tongue mucosa) in order to evaluate *C. albicans* colonies, has been performed. Another brush, five minutes later in order to compare the two analyses and assess their effectiveness over time, has been taken with the same modality. Samples are immediately sent to the Molecular Biology Laboratory (University of Cagliari, Italy), for a cultural microbiological analysis, where both the two brushes were used to inoculate two different Petri dishes with Sabouraud agar that were successively put in incubation for 48 hours at 37°. After 48 hours of incubation, both samples revealed no presence of a fungal colonies load after the treatment, assessing the effectiveness of PDT with curcumin-based photosensitizer. We communicated the results to our patient, explaining to him that we drastically reduced the possibility of malignant transformation of his lesion, but we decided, according to him, to keep monitoring him with six-month controls in order to immediately recognize a possible change in the sense of malignant transformation in his lesion, *Figure 118*.

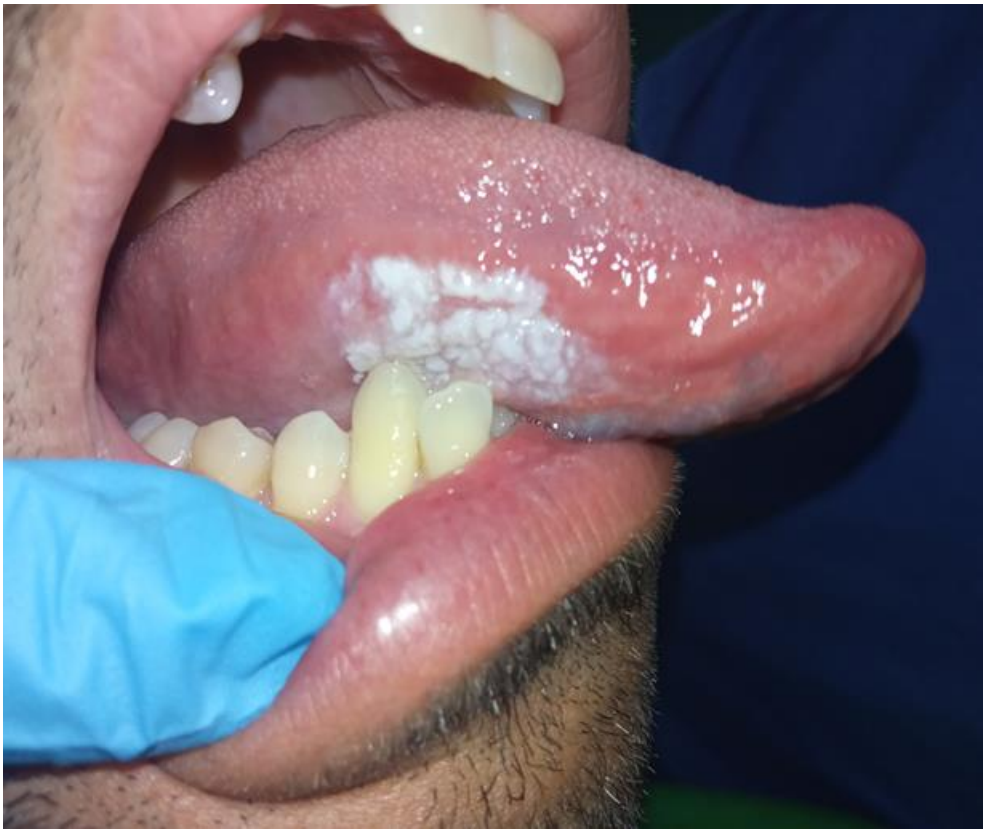


Fig. 114. Tongue Lesion of the patient before the biopsy.

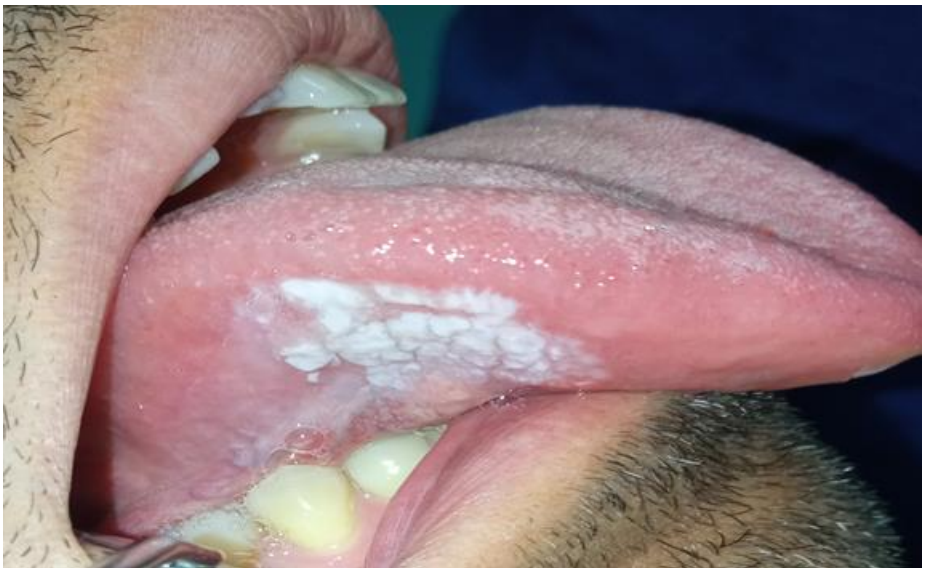


Fig.115. Tongue lesion 2 weeks after the biopsy

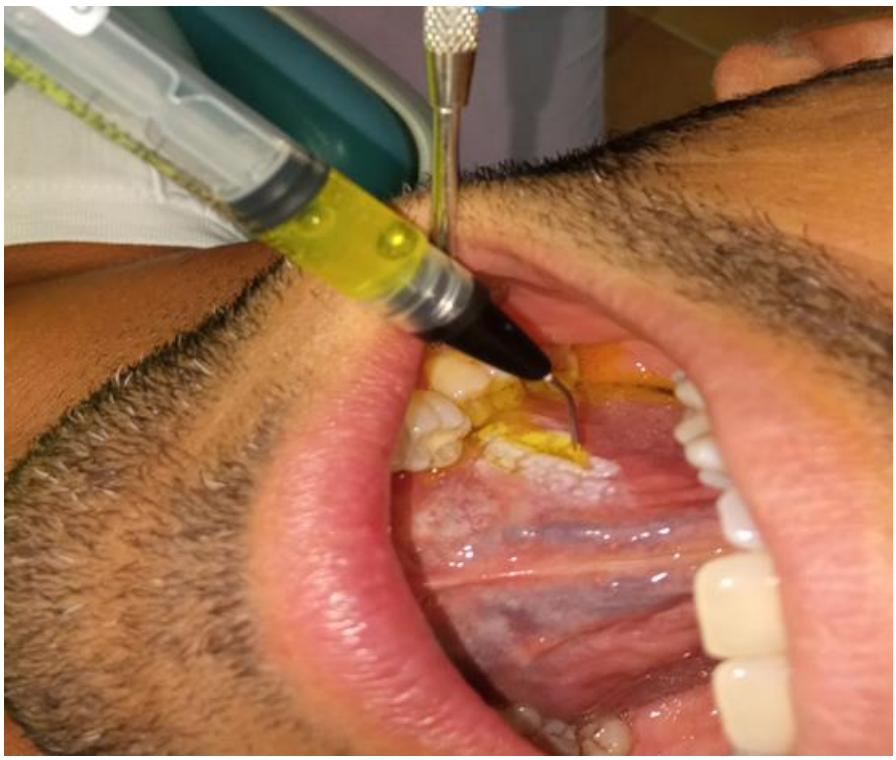


Fig 116. Application of the curcumin-based photosensitizer on the tongue lesion after a brush scraped for 30 seconds on the leucoplakia.

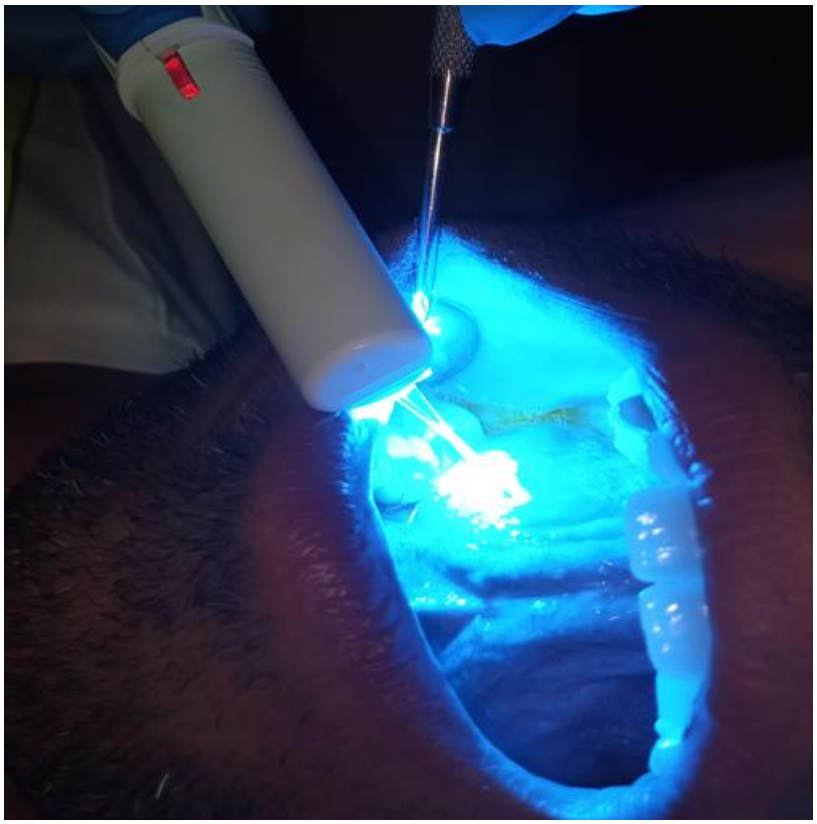


Fig 117. Illumination with diode light at 460 nm, for 5 minutes**.**

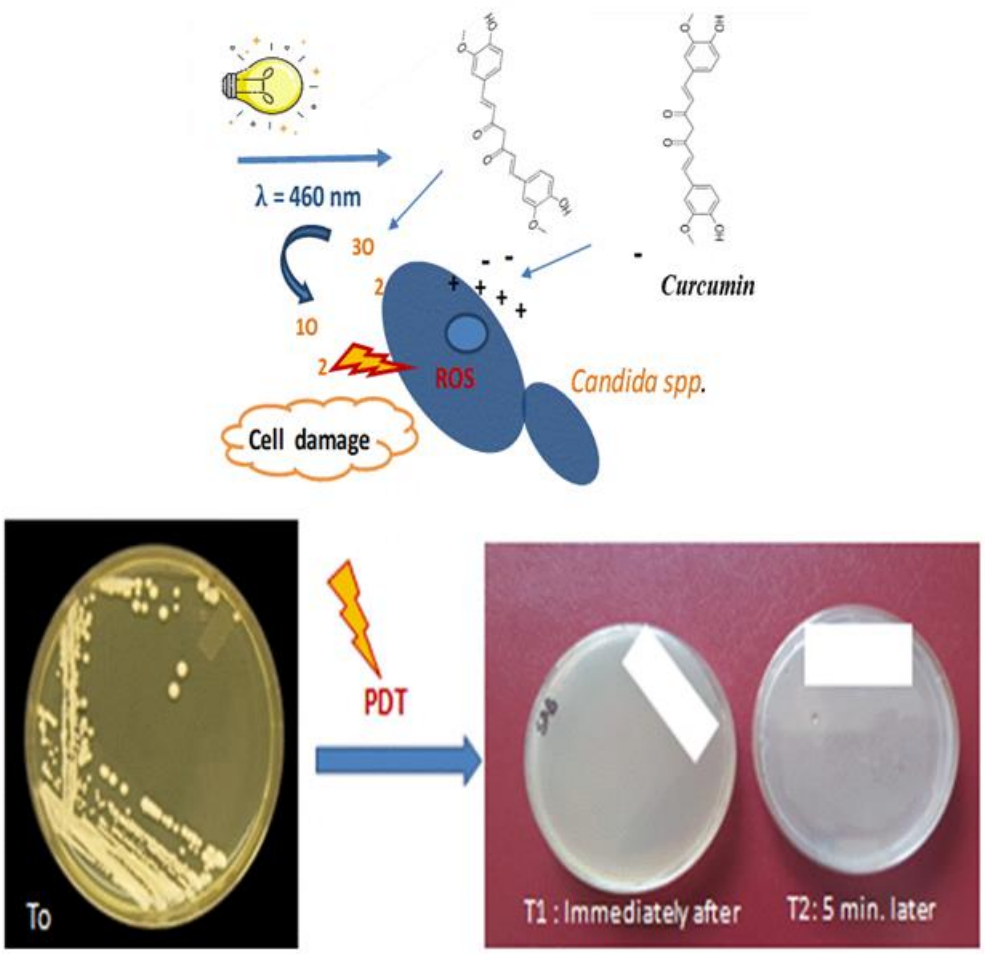


Fig 118. Comparison of sampling cultures from patient 11, before PDT and immediately after and at 5 minutes. After photodynamic therapy, no fungal colonies were present on the Petri dish with Sabouraud gel.

Case 12.

A 38-year-old woman came to our observation due to discomfort in the perioral region. His medical history reported only an allergy to fluoroquinolones, and she was in good general health. On physical examination, she showed bilateral erythematous areas associated with very small fissures on the right side of the labial commissures (figs 119,120). A diagnosis of angular cheilitis had been made. Since it was an

emergency visit during a holiday period, it was decided to proceed with a different natural photosensitizer among those provided: lactoferrin powder enriched with traces of biotin (Forhans, Rome, Italy), a product that can also be purchased as a supplement by the patient, and activation with violet (380-420 nm) diode light (Detecta, Precision Tech, Milan, Italy). After having spread the lactoferrin powder on the affected regions (*fig. 121*) the light was activated for 2 minutes, approximately 1 cm away from them, on each side (*fig 122*). The powder was removed with cotton balls, as it is a tested and ingestible commercial product. The patient informed us of her rapid relief shortly after the session, and at 36 hours, we could appreciate the total disappearance of the erythematous area and the symptoms reported by the patient (*Fig.123,124*).

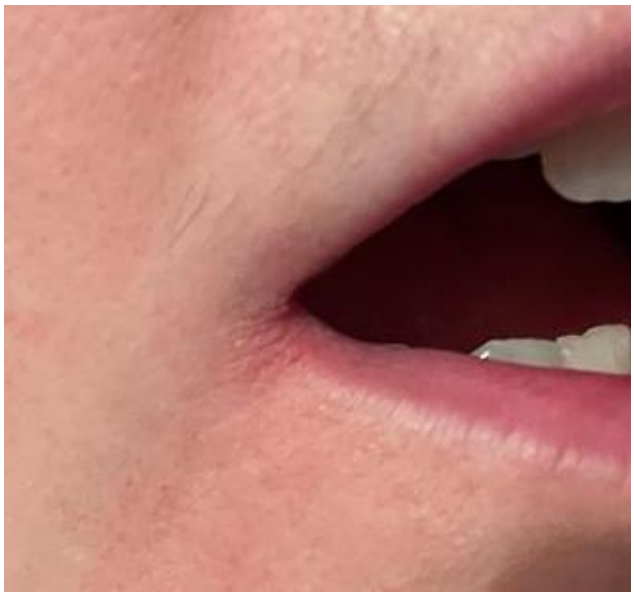


Fig 119. Angular cheilitis on the right side before PDT.

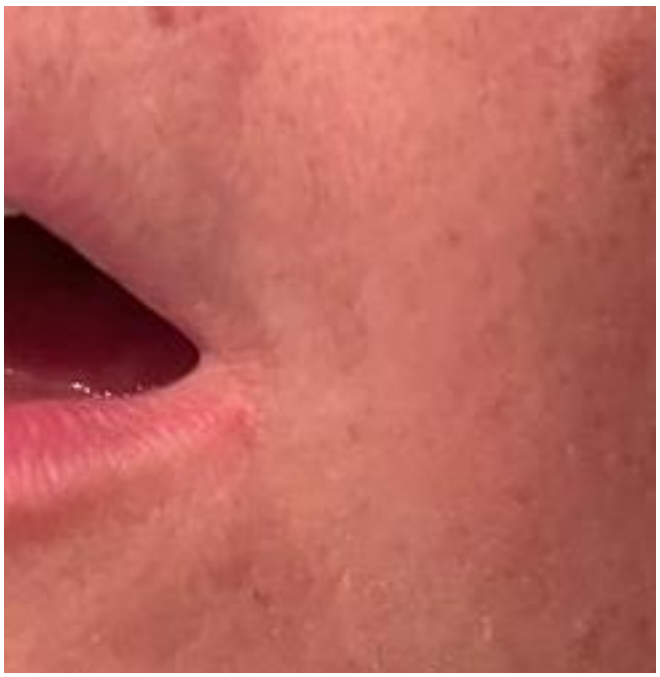


Fig 120. Angular cheilitis in the left side before PDT

Fig 121. Application of lactoferrin powder, used as a photosensitizer.

Fig 122. Illumination with the violet diode light “Detecta” (380-420 nm) for 2 minutes for each part.

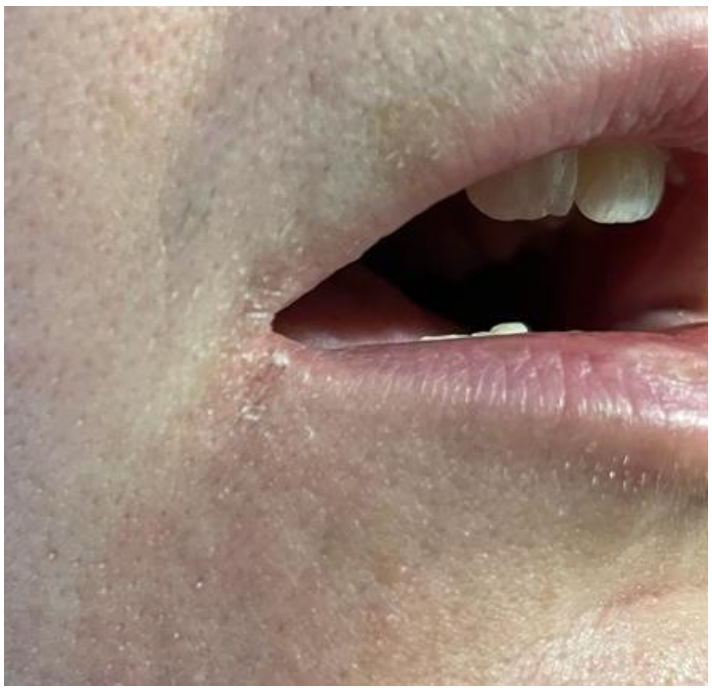


Fig 123. Right side treated after 36 hours after PDT.

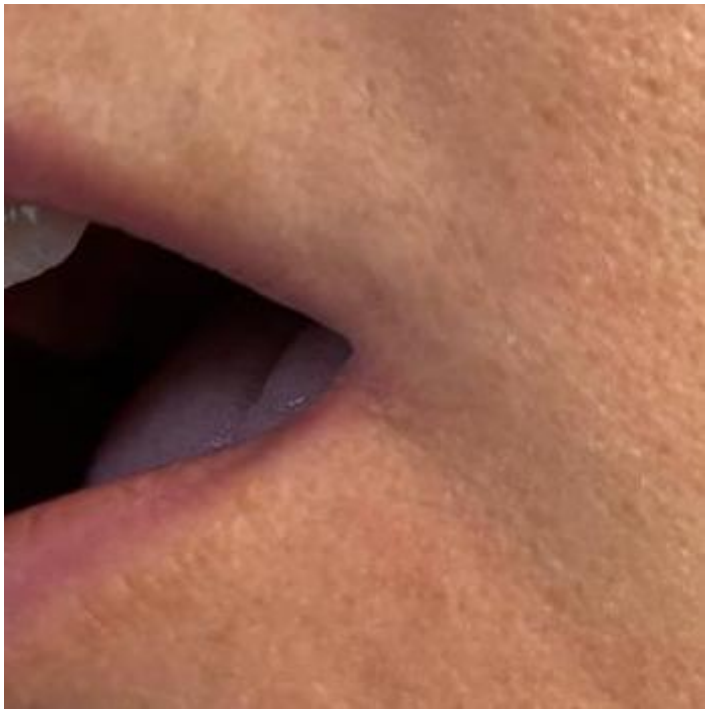


Fig 124. Left side treated 36 hours after PDT.

Chapter 10- Discussion

In the first part of the research project, we tested various photosensitizers already present on the market, but never compared to each other through in vitro studies in the treatment of MDR *Candida species*. The 3 types of photosensitizers used, Toluidine Blue, Methylene Blue, Curcumin and hydrogen peroxide, were chosen based on existing scientific literature.

Toluidine Blue, Methylene Blue, Curcumin and hydrogen peroxide

Toluidine blue at 1%, (*Figure 125*) has been considered one of the most important photosensitizers in the treatment of Candida infections in the oral cavity. A recent review on the subject has highlighted that most of these are *in vitro* studies, where the parameters used are very different between the various studies, even if almost all report the efficacy of photodynamic therapy. In addition to the more common *C. albicans*, it has also been tested against different species of *Candida* in some studies, such as *C.glabrata, C. krusei, C. parapsilosis, C. tropicalis*. Toluidine blue has been shown to be effective against all types of *Candida spp* mentioned [35]. Most of the *in vitro* studies were studies in which both the activity of PDT in its planktonic form and in the biofilm of the fungus were evaluated. The prevailing method was that of microplates [35].

In our work, *in vitro* study 1, no activity was detected against *C. albicans* MDR. The inhibition zone was not only 0 but fungal growth could be observed inside the wells. This result is in contrast with the results coming from international scientific literature, which predicts significant activity of Toluidine Blue 1% on various species of *Candida*. However, it must be considered that very few studies in the literature have been carried out on MDR *Candida species*, as in our case [35]. Furthermore, while in the first experiment of *in vitro* study 1 we used a wavelength of 660 nm, which may not

be as effective in activating Toluidine Blue as the 630 nm wavelength, in the second part of the in vitro study 1, where we wanted to evaluate the activity on the fungal biofilm, a wavelength of 630 nm was used for the activation of this photosensitizer. However, there were no interesting results even in the experiment performed in triplicate with the Microplates method. After only 6 hours fungal growth occurred, demonstrating a very modest fungistatic effect.

In our clinical experience we were able to observe partially satisfactory results in the treatment of case 1, with complete regression of the lingual lesions and discrete regression in the labial portion, while in case 7 the treatment with Toluidine Blue did not determine substantial changes in the subprosthetic erythematous candidiasis. We can conclude that our results on Toluidine Blue 1% as photosensitizer are not satisfactory.

This aspect has not described yet in literature considering an *in vitro* approach.

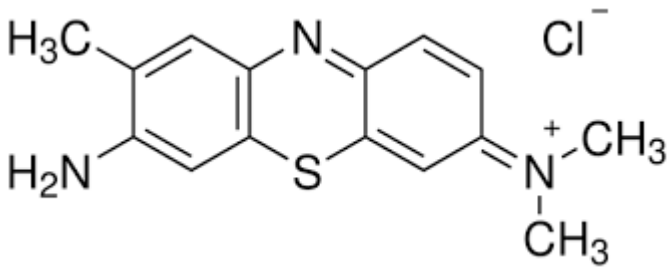


Fig.125. Chemical structure of Toluidine Blue.

Methylene blue has been considered one of the best performing photosensitizers against various microbial infections, extremely studied and among the most common used, demonstrating *in vitro* antifungal activity even greater than curcumin-based photosensitizers [34,65], (*Figure 126*). Generally the wavelength used to best activate this compound is 660 nm, the same used in both the first and second parts of our *in vitro* study 1. The inhibition zone of the experiment performed using the Kirby Bauer

method has been equal to 0. Also, in this case fungal growth could be observed inside the wells.

However, the results obtained with the Microplates Assay method are comparable to those obtained for Toluidine Blue 1%. This data is also in contrast with previous scientific literature. Also in this case, previous studies do not take into consideration the activity towards MDR *Candida* species, and this could partly justify the differences obtained in our work. The concentration of photosensitizer used appears to be the most documented in the scientific literature, and the one with the highest concentration, therefore tends to be the most performing. The power used, through the dedicated instrument, does not appear to be a parameter that can determine substantial variability in the activation of Methylene Blue, or at least it has not been documented before as a fundamental parameter in its activation. 100 mW appears to be among the powers used for the activation of Methylene Blue in PDT for the treatment of candidiasis [48,49]. The lighting times are extremely variable in the various studies already published, and range between 8 seconds and 10 minutes. Generally, they do not exceed one minute, therefore extremely similar to those used by us. The pre-lighting time is very limited and is reported between 1 and 5 minutes, in line with our exposure times [48,49]. A very recent scientific work has compared *in vivo*, through a randomized clinical trial, the antifungal effect of nystatin and PDT made with Methylene Blue, with comparable results between the two methods, demonstrating its effectiveness [67]. The patients examined had been diagnosed with erythematous candidiasis, but had not previously been treated with other measures, therefore they were not refractory infections as in the case of the fungal species analyzed by us. The aspect that we wanted to evaluate in vitro was precisely the activity towards this type of resistant species, the real emergency in the context of oral infections. From a clinical point of view, we have been able to observe the antifungal activity in the treatment of refractory and recurrent angular cheilitis, as can be observed in Case Report number 2,3,4.

Therefore, we can conclude that *in vivo* we obtained results very similar to previous scientific evidence. De Souto Medeiros et al 2023 [67] in their clinical trial evaluated erythematous candidiasis, which therefore exclusively concerns, by definition, the oral mucosa, while in the cases reported by us n. 2,3,4 they were infections mostly in the skin part of the lips. The humidity and general characteristics of these tissues could influence parameters such as the adhesion of the photosensitizer and the penetration of light. However, this aspect is not previous studied in the literature.

The first work in the use of photosensitizer curcumin-based dates 2013 [61]. Subsequently, other studies evaluated its safety in terms of cytotoxicity, its antimicrobial activity, which was also compared with other synthetic photosensitizers [62,63].

In a study activated by LED light at 460 nm it showed an antifungal activity *in vitro*, superior to Methylene Blue activated by red light at 660 nm [63]. Animal studies have also evaluated efficacy with polymer nano-particle enhancement [64]. A recent study evaluated the ability to prevent fungal infections in 108 radio-treated patients for head and neck cancer through two compared photodynamic therapy protocols.

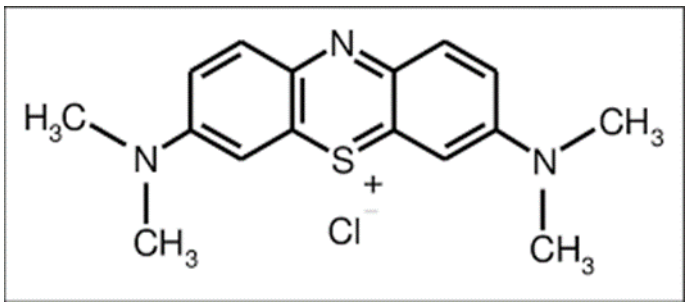


Fig.126. Chemical structure of Methylene Blue

In our study the photosensitizer based on Curcumin and H_2O_2 3%, *Figure 127*, was found to be the best performing of the three commercial products tested. In the Kirby Bauer experiment it determined, when activated by 460 nm diode light, an average inhibition zone close to 46 mm, and by evaluating the activity on the biofilm through the Microplates Assay method it was able to inhibit the fungal activity for 60 hours,

showing an excellent fungistatic effect. In scientific literature, the wavelength that most activates curcumin is around 460 nm. In the second part of the *in vitro* study 1, the curcumin-based photosensitizer was activated with polarized light with a wavelength spectrum varying between 380 nm and 3400 nm. The results on killing fungal biofilm showed better results than activation with 460 nm light. This type of electromedical device had not yet been used before as a light in photodynamic therapy against Candidiasis, but as a biostimulator tool. It is a device that was first created in the dermatological field, for example it was used successfully in the treatment of ulcers in diabetic subjects [68], but which has also found application in the oral field, both at a symptomatic level and in the evaluation of healing processes [69,70].

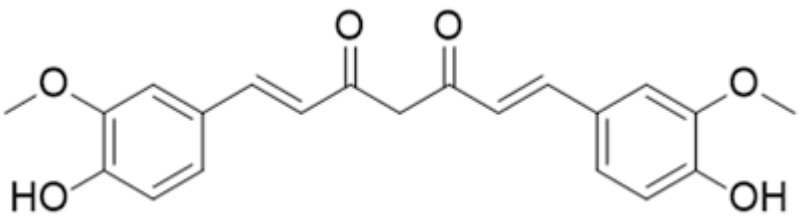


Fig. 127. Molecular structure of Curcumin.

The third part of the *in vitro* study 1 was carried out by proposing a fungal infection model with *Candida glabrata* on a prosthetic surface in acrylic resin. We have not found other papers in the literature that have reproduced the same type of infection *in vitro*. The choice to use *C. glabrata* MDR is dictated by the fact that this type of *Candida* is very often found in palatal fungal infections in denture wearers. The use of salivary mucins was important in promoting the adhesion of Candida to the acrylic resin, reproducing what happens in the mouth: the presence of a thin salivary film between the palate and the prosthetic resin provides the fungus with favorable humidity and proteins useful for its development [6]. Having noticed that the best

performing photosensitizer in previous experiments was the curcumin-based one, this was chosen to be used in the photodynamic protocol.

Also in this case, the use of the curcumin-based photosensitizer resulted in a drastic reduction in the CFU detected inside the plates infected with brushes applied to the prosthetic surface before and after photodynamic therapy. The reduction of the fungal load which went from 1500 CFU/cm^2 to 20 CFU/cm^2 confirms the effectiveness of this photosensitizer against *Candida spp* MDR. This third part of the *in vitro* study 1 is a fundamental part because despite being an *in vitro* study, we have a model that better simulates the dynamics that can occur in the mouth compared to previous experiments. The evaluation through use of a brush for the contamination of the plates could be the same protocol that a clinician can perform during a session of PDT. Notable decrease in the fungal load could also be influenced by the ability of the photosensitizer to adhere better to a rough surface such as the prosthetic one or by the micro-retention of the product in the prosthetic recesses. The exposure time to light was a total of 5 minutes for a surface of several cm^2, therefore an extremely limited time, close to illumination time reported in photodynamic therapy. Clinical case n. 5 and n. 11 also confirm the effectiveness of the protocol with curcumin + H_2O_2 *in vivo*. In case n. 11, the fungal infection present within the leukoplastic lesion disappears by applying the same PDT protocol and final evaluation method (brush used to infect plate with culture medium).

The use of ointments based on *curcuma longa* has already been successfully proposed in the treatment of denture stomatitis, with clinical and laboratory results very similar to topical clotrimazole 1% [71]. Photodynamic therapy protocols for the treatment of denture stomatitis that involve the use of curcumin-based photosensitizers have not yet been studied.

In our research project the main goal was to find new type of natural photosensitizers, we have tested olive oil, saffron and lactoferrin solution obtaining a good activity.

Olive Oil composition and systemic effect

Extra virgin olive oil (EVOO) is a typical product of the Mediterranean diet. It derives from the plant *Olea Europea L*. and all the products of the olive tree (leaves, roots, oil) are rich in polyphenols, it has been estimated that the concentration is between 200 and 800 mg/kg, perhaps the element richest in polyphenols. Among the latter the most studied are oleocanthal, oleuropein, oleocanthal, oleacein, hydroxytyrosol, and tyrosol, *Figure 128*. The activity of the various polyphenols depends on the absorption in the intestine of the individual. The activity of this type of molecule on human health is multiple [72]. For example, they have a demyelinating activity. Hydroxytyrosol and oleuropein seem to have the best neuroprotective properties. They are effects also in the immune cells. Researchers [73] demonstrated the efficacy of oleuropein in experimental autoimmune encephalomyelitis. Its application resulted in the downregulation of interferon-γ (IFN-γ) and IL-17 production during disease progression, counteracting pathogenic autoimmune responses that depend on Th17-, IFN-γ-, and IL-17-producing cells. In another study, the immunomodulatory effect of oleuropein was demonstrated with the suppression of T lymphocyte proliferation and a decrease in the autoimmune inflammatory process [72]. Another important activity is the antioxidant one. Especially, brain tissue is rich in polyunsaturated fatty acids (PUFA) and has low antioxidant defenses. Their activity could be very useful to increase the amount of PUFA and to prevent oxidative process. Oleuropein have demonstrated its antihypertensive activity. Olive leaf polyphenols have been shown to lower diastolic and systolic blood pressure in prehypertensive and hypertensive groups of patients [74].

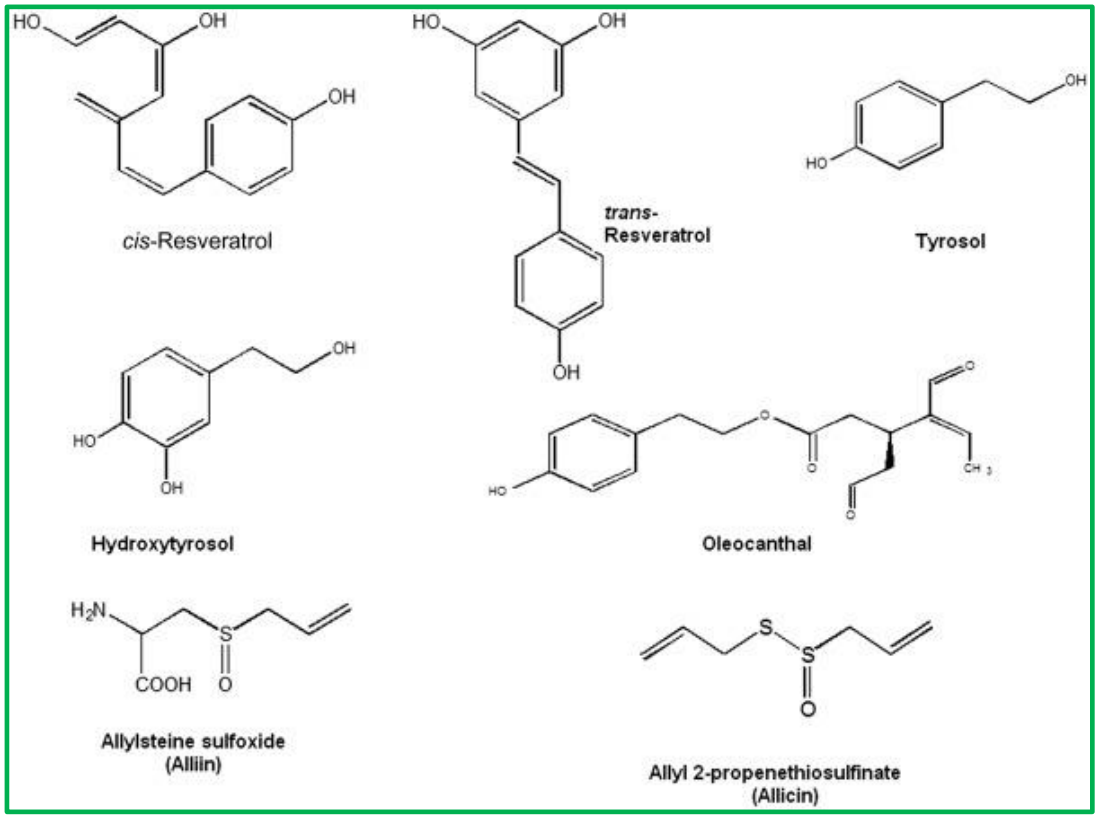


Fig. 128. Chemical structure of some functional components in olive oil.

The increased levels of ROS and pro-inflammatory factors, induced by EVOO and the decrease of NO secretion, could be considered very useful for endothelial dysfunction that is characterized by an imbalance between vasoconstrictor and vasodilator factors, modulated by EVOO [75].

EVOO have an interesting effect on reducing atherosclerosis, in fact researchers have demonstrated that consumption of polyphenol-rich olive oil reduces systemic LDL oxidation and pro-atherogenic genes in peripheral blood mononuclear cells, such as CD40-ligand, IL-23α, adrenergic β-2 receptor (ADRB2), oxidized LDL (lectin-like) receptor 1 (OLR1), and IL-8 receptor-α (IL8RA) in humans [76].

In the management of hyperglycemia, EVOO could be also considered very helpful, in fact regulation of hyperglycemia and insulin levels in the prediabetic and diabetic states by olive polyphenols intake might also reduce the pro-thrombotic risk for

cerebral insult. The cellular and animal studies clearly show that the polyphenols of extra virgin olive oil are able to inhibit platelet activation [77,78].

Oleic acid had showed a protective effect in liver dysfunction and gut inflammation, whereas phenolic compounds from EVOO protect colon cells against oxidative damage and improve the symptoms of chronic inflammation in IBD [79].

Olive Oil against Oral Candidiasis

Goel et al. conducted a research on over 1000 patients with systemic infection (fungemia) from *Candida spp*. Mainly 4 Candida species were extracted from the blood of affected patients: *C. albicans, C. tropicalis, C. parapsilosis, C.krusei*, resistant to fluconazole. The antifungal capacity of 50 µl of cinnamon extract and olive oil in each plate with culture medium infected with MDR species was evaluated using the Kirby Bauer method. After the 48 hours of incubation the inhibition haloes were recorded to evaluate results of the experiment. The results were interpreted as <9 mm (diameter of halo of inhibition)- inactive; 9-12 mm- partially active; 13-18 mm- active; >18 mm- very active. For olive oil, sensitivity of *C. albicans* was 53.65%, *C. tropicalis* was 48.83%, *C. krusei* was 55.55% and *C. parapsilosis* was 57.12%, Sensitivity to the two natural compounds was triple for *Candida albicans*, compared to fluconazole, and was much higher overall for others *Candida spp*. [80]. Researchers studied the efficacy of cinnamon oil and olive oil against *Candida spp* including *C. krusei* which is intrinsically resistant to fluconazole and discover that about 55.5% of *C. krusei* strains were sensitive to olive oil and cinnamon oil. EVOO could be an interesting way to beat the resistant Oral Candidiasis.

Olive Oil and PDT

There are still very few works on the use of olive oil in PDT. Recently some authors have tried to integrate oil derivatives into curcumin for photodynamic therapy for

breast cancer, and this mixture resulted in extension of drug release [81]. Some researchers have proposed the use of EVOO oil in the dermatological field to prevent erythema after photodynamic therapy sessions, obtaining good results [82]. In another study, EVOO derivatives were considered as emollients to facilitate the penetration of UVA and UVB light into skin tissues [83]. However, no studies have ever used EVOO as a photosensitizer in photodynamic therapy. Having already demonstrated antifungal effects on *Candida spp* MDR [80], also considering its anti-inflammatory properties which would bring benefits on the host tissues [72-81], we decided to use it as a photosensitizer in our research project, activated by an extremely variable wavelength spectrum.

In our study we used olive oil as a photosensitizing agent, activated by laser and non-laser lights, demonstrating antifungal activity on its own, particularly against *Candida glabrata*. The results against *C. glabrata* and *C. albicans* are very encouraging, while it is not active against *C. krusei*, which is notoriously much more difficult to eradicate. Different wavelengths are used because we have no data on the potential wavelength and type of light that can activate EVOO. We have therefore used both one of the most used wavelengths in antimicrobial photodynamic therapy for oral lesions (660 nm) and the polarized light device which produces a wide range of different wavelengths.

In our *in vitro* experiment *C. albicans* resulted inhibited only with (EVOO-H) light-activated, with a halo of inhibition of 42 millimeters for polarized light and 30 millimeters when activated by laser light at 660 nm. *C. glabrata* resulted sensitive with all combinations, with a 50% increase of inhibition halos by using polarized light. The halo of inhibition is: EVOO alone was 32 mm, EVOO + Polarized light 45 mm, EVOO + laser light 42 mm, (EVOO-H) 35 mm, (EVOO-H) +Polarized light 62 mm, (EVOO-H) +laser light 45 mm.

Polarized light appears more effective at activating EVOO than 660 nm laser light. The halos of inhibition on *C. albicans* using this device are very similar to those obtained from the use of the curcumin-based photosensitizer activated by diode light at 460 nm (42 mm vs 46 mm). EVOO also appears to be much more effective than the other commercial photosensitizers tested, Toluidine Blue and Methylene Blue, with the same method (Kirby Bauer). However, we do not have data comparing the effectiveness of EVOO on *C. glabrata*. This new natural photosensitizer activated by polarized light could be an ideal protocol for the eradication of MDR *Candida* spp.

Saffron

Saffron is the dried stigmas of *Crocus sativus L*. *Crocus sativus L* belongs to the family of Iridaceas, the line of liliaceas and is mainly cultivated in several countries characterized by a particular type of climate. Although the source of saffron is unknown, it apparently originated in the geographic area of Iran, Turkey and Greece, but now it is also successfully cultivated in such European countries as Spain, Italy, France and Switzerland, as well as in Morocco, Egypt, Israel, Azerbaijan, Pakistan, India, New Zealand, Australia and Japan. Iran produces about 90% of the total annual saffron, the total production in the world is 190 ton each year [84].

Crocin, an active ingredient derived from saffron, *Figure 129*, is one of the herbal components that has recently been considered by researchers. Crocin has been shown to have many anti-inflammatory and antioxidant properties, overall, for its ability to reduce ROS production, and therefore can be used to treat various diseases. It has been shown that Crocin has a positive effect on the prevention and treatment of cardiovascular disease, cancer, diabetes, and kidney disease [84]. Recently it has shown very interesting activity in reducing Lewy body, syn-nuclein protein and the process of demyelination, so proposed to treat Parkinson's and Alzheimer's diseases [85].

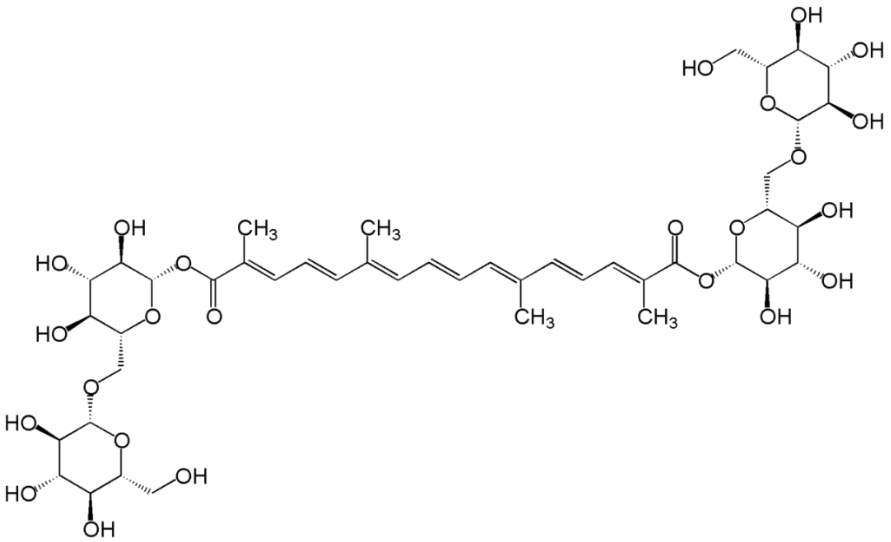


Fig.129. Molecular structure of Crocin.

Crocins are responsible for many of this valuable plant's pharmacologic effects. Crocins, unlike other carotenoids, have 20 carbons and several glycosides. Several earlier studies demonstrated that crocins, particularly alpha crocin, had radical quenching, antioxidant, and anti-inflammatory properties [86].

In addition to the anti-inflammatory, neuroprotective, blood sugar lowering action, an antitumor [84] and antifungal activity has been demonstrated [87]. Indeed, it demonstrated *in vitro* an activity against three mains important fungal pathogens: *Rhizopus stolonifer*, *Penicillium digitatum* and *Botritys cinerea*, which cause rot decay on the tomato, orange and apple fruits, respectively [87]. Saffron have shown an antiviral effect against EBV, HSV1 and HIV inactivation [88,89], and recently has been proposed in the management of COVID 19, not only against Sars CoV-2 but also for post-infection and for treatment of the disease [90].

Saffron in the oral disease

In the oral cavity, it has not yet been studied in depth, but it has been proposed *in vitro* studies as a natural element against oral squamous cell carcinoma, and for its neuroprotective properties in burning mouth syndrome in a clinical study [91,92]. A

group of researchers tested the extracts of four natural elements in cell cultures with malignant cells from oral cancer. In particular, were extracts of cinnamon, ginger, curcuma and saffron. The latter was the most active compared to the others in inducing apoptosis of tumor cells, and the researchers concluded that the mixture of saffron and cinnamon was the most active natural combination against oral cancer *in vitro* [91]. The minimum and maximum effective concentrations were respectively 10^8 and 217 mg/ml for curcumin with IC30 of 77mg/ml, 10^8 and 270 mg/ml for ginger with IC30 of 58 mg/ml, 2 and 10 mg/ml for saffron with IC30 of 1.9 mg/ml, and 5 and 40 mg/ml for cinnamon with IC30 of 3.3 mg/ml.

Another clinical study has shown an antidepressant-like activity for topical use of saffron. In this study, 20 patients diagnosed with Burning Mouth Syndrome (BMS) were taken into consideration, divided into two groups: one treated with citalopram and one treated with saffron extracts, for 11 weeks. The degree of anxiety and the degree of burning/pain of the patients were evaluated using the Hammiltom scales and VAS. In both cases there was a clear reduction of symptoms, with differences between the groups not statistically significant. The authors concluded that saffron may be a valid natural alternative to the treatment of BMS [92].

In another pilot study, saffron was used to implement a toothpaste, and to evaluate its efficacy compared to a placebo in the treatment of gingivitis, by analyzing some periodontal parameters before and after a month of use. Twenty-two patients with generalized marginal gingivitis were selected and randomly divided into two equal groups (test or placebo). In each group the pocket depth index (PD), gingival index (GI), plaque index (PI) and bleeding of probing index (BOP) were evaluated, before and after the period of observation. The comparison between gingival indices before and one month after toothpaste usage showed a significant decrease in some measured indices at the end of the study. Saffron stigma treated group had a significant difference in reducing GI and BOP indices in comparison with the placebo group [93].

Nobody has evaluated this natural element as a photosensitizer in photodynamic therapy. In our study saffron alone had no antifungal effect against MDR *Candida species*, but if activated by blue lights at 460 nm, 7 watt of power, it demonstrated, at a concentration of 0.3%, good activity against *C. krusei*, resistant to flucanozole, with an halo of inhibition of 22 mm. Other researchers have tried to evaluate effectiveness of PDT against *C. krusei*, using Toluidine Blue as photosensitizer, but the authors concluded that the CFU of *C. krusei* were not modified [94,95]. Our Results suggest that a solution of 0,3% of saffron could be used to improve the effect of other natural photosensitizer such as EVOO oil.

Lactoferrin and its properties

Lactoferrin (Lf) is a natural iron-binding protein that plays a significant role in the innate immune system, through the production of immunomodulatory proteins, in humans, *Figure 130*. This enzyme is contained in saliva, breast milk and some other biological fluids in lower concentrations [96,97]. Lf present in saliva has an important role in maintaining oral hygiene status. It exhibits protective function on mucosal surfaces, which constitute a barrier between the host and the external environment. Thus, Lf may be considered a very important protein that is associated with oral mucosal immunity balance [96]. Lf showed a lot of antimicrobial properties against bacteria (in particular *E. coli* and *P.aeruginosa*), viruses (for exemple HIV, EBV, CMV, Sars Cov-2), and fungi [96,97]. It is also important in the control of the oxidative stress in the body, linked to its high affinity for ferric (Fe^{3+}) ions and thus protects cells from oxygen injury [96]. It has been demonstrated that not only the whole protein but also its derived fragments possess antimicrobial peptide (AMP) activity, such as Lactoferrampin (Lfampin), and Lf (1-11). The first one comprising residues 268–284 in the N1 domain of Lf, is located in close proximity to Lfcin in the tertiary structure of Lf. The Lf (1–11) peptide is a piece of the first 11 amino acid residues of the N-terminal region

of Lf and exhibits antifungal activity against *C. albicans* and *A. fumigatus* [97]. Lf have a highly cationic N-lobe can interact with negatively charged cell wall components of Gram-negative and Gram-positive bacteria. These events produce cell wall destabilization, increase the membrane permeability, that can determine the cytoplasmic leakage, disruption of balance of solutes and ions within the cell and ultimately bacterium death [94,98].

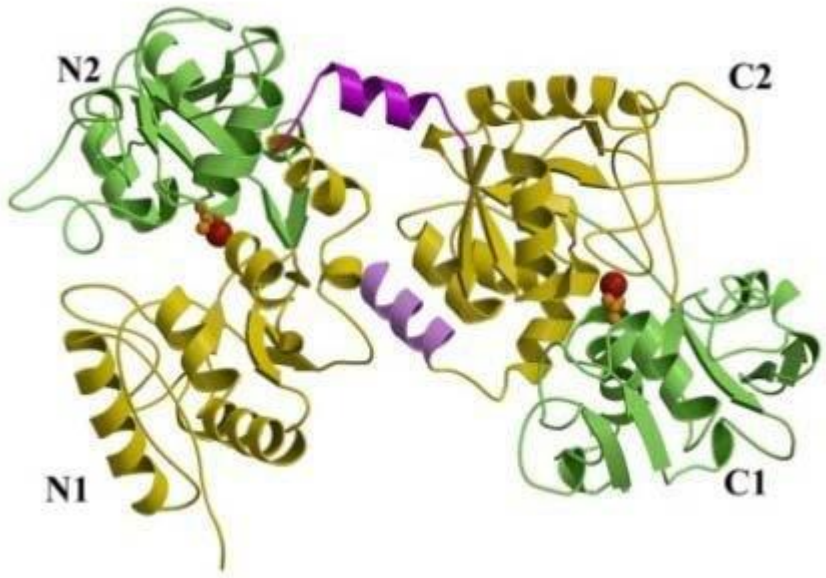


Fig.130. Secondary structure of Bovin lactoferrin

Lactoferrin and oral disease

Lf had shown *in vivo* studies, antimicrobial activity against *Aggregatibacter actinomycetemcomitans*, *Prevotella* and *Treponema* spp, involved in the parodontal diseases and also against *Streptococcus mutans*, the main responsible of dental decay [97]. Its antifungal properties it is mainly aimed at *Candida tropicalis, krusei* and *albicans*, while the minor antifungal activity is recorded against *Candida glabrata*. The ability of the molecule to subtract iron, and the activation of some cytokines involved in the immune process against *Candida spp*, such as Th17, are the basis of their fungistatic effect [98]. For this reason, it has been proposed as a molecule to be integrated in

prosthesis wearers with a tendency to prosthetic stomatitis [97]. Furthermore, to enhance the activity of some antifungals such as amphotericin B and some azoles, it has been used as an adjunct to these drugs, further reducing the minimum inhibitory concentration [99]. In a cell culture study, even better results were achieved by combining lactoferrin with lactoperoxidase. In fact, the union of the two enzymes is able to reduce the size and modify the shape of the fungal cell and to reduce its metabolic activity, more than other antifungal drugs, and much more than the single molecules, thus highlighting a powerful synergistic effect [100]. The study has been conducted on oral *Candida* spp.

Lactoferrin and PDT

Lf has been proposed in the treatment, through photodynamic therapy, for some types of malignant tumors, as an adjunct to other photosensitizers, in particular Chlorine6. Upon light exposure, the mixture proposed have shown a substantial decrease (> 4 times) in Ce6 requirement compared to free Ce6 in its ability to cause light mediated cell death in SK-OV-3 and MDA-MD 231 cells. Chlorine6 with Lf were shown to be non-toxic to the cells even at concentrations ten times that were used in the PDT study. Safety, efficient loading and significant uptake by cells and more importantly a significant decrease in IC_{50} values are the conclusions of these researchers [101]. The use of Lf to convey a photosensitizer has also been proposed in the treatment of brain tumors.

However, it has not yet been proposed as the only photosensitizer in photodynamic therapy, for infections of the oral cavity, despite its remarkable antimicrobial activity against oral bacteria and fungi.

In our *in vitro* study 2, we found very interesting results on lactoferrin as a photosensitizer.

In this part of the in vitro study, we have used, also a clinical isolate of *C. albicans* called CC1, taken from the mouth of a patient resistant to nystatin, miconazole and sessions of PDT realized with Toluidine Blue and 630 nm diode light. In this way we have tested a particular resistant type of *Candida*, refractory not only at the pharmacological treatment but also at Toluidine Blue 1%. Lactoferrin mixture alone was active against *C.glabrata*, with an halo of 20 mm, Lactoferrin + Light (310-350 nm) produced an inhibition halo of 22 mm on *C. Albicans*, 30 mm on *C.glabrata*, 20 mm CC1 isolate. Lactoferrin +H_2O_2+ light produced an inhibition halo of 40 mm on *C.albicans*, 35 mm on *C. Glabrata*, 30 mm on *C. Krusei*, 30 mm on CC1 clinical isolate. Mean values of inhibition halo with H_2O_2 were 23 mm on *C.albicans*, 28 mm on *C. glabrata*, 12 mm on *C. Krusei*, 18 mm on CC1 clinical isolate. These results are better than Toluidine Blue and Methylene Blue but not of EVOO oil and curcumin-based photosensitizer on *C.albicans* MDR, when lactoferrin solution was alone; while the presence of H_2O_2 produced an inhibition halo close to curcumin-based and EVOO oil. Results against *C.glabrata* when a lactoferrin solution with and without H_2O_2 are interesting but lower than EVOO. The most relevant effect of this part of the experiment was the halo of inhibition against *C.krusei* (30 mm), better than saffron solution however this last, was without the presence of H_2O_2 3%. Lactoferrin solution showed activity for each type of *Candida spp* MDR tested, for this reason we can consider it, among the natural ones tested, the most versatile against different species of *Candida* MDR. Furthermore, it also showed inhibitory activity against the new clinical isolate CC1 coming for the first time from a fungal infection resistant even to a previous photodynamic treatment. No scientific work has previously evaluated the anti-fungal effect of a natural product as photosensitizer on a clinical isolate that is also resistant to a previous PDT, constituting a novelty. An interesting element that emerged from these in vitro studies is that the use of H_2O_2 to emulsify EVOO oil and lactoferrin determines a significant increase in the activity of the photosensitizer. Even the commercial product based on curcumin contains traces of H_2O_2 at 3% and this could be interesting for designing

other natural photosensitizers to be emulsified or enriched with traces of H_2O_2. The comparison with 3% H_2O_2 alone on the inhibition zone highlights efficacy results, but which are very far from those of the natural products tested enriched with H_2O_2. At a clinical level, the use of lactoferrin has proven to be very useful for the management of a case of angular cheilitis, when activated by violet diode light. 36 hours after treatment with PDT, there was complete regression of the fungal lesions, providing further proof of its effectiveness already found *in vitro*.

Pompia Juice

Among Citrus species, Citrus limon var. pompia Camarda var. nova, that is also classified as Citrus x monstruosa, is a particular Sardinian citron-like tree called as "Pompia." It is an endemic species cultivated in Sardinia overall, in the north-eastern coast of the island. The tree has an ancient origin and its cultivation has recently known a revival because of the use of this particular fruit in Sardinian traditional sweets, and recently it has been classified as Slow Food. For centuries, the local tradition has attributed to Pompia flavedo and the essential oils derived important properties on health, even though no specific scientific evidence was available. It seems that the amount of Vitamin C is higher than other types of citruses [102].

Researchers found interesting results on its anti-inflammatory property. Authors in an in vitro study concluded that a low concentration of Pompia juice was very effective against reactive oxygen species (ROS) and has a moderate capacity to reduce ROS damages on cell membrane. This fruit has also an anti-tumoral effect, in fact Pompia juice treatment of in vitro colon cancer cell resulted in a significant reduction (20%) of the metabolic activity. The most important for our research is the antimicrobial effectiveness against pathogenic oral bacteria such as *Pseudomonas aeruginosa, Streptococcus aureus*, and *Enterococcus faecalis*. These results are evaluated with Kirby Bauer Method, the same used in our project to test the other natural product as new photosensitizers. For this reason, we decided to test it as a photosensitizing

agent, thinking that its antimicrobial activity could be extended to fungal species. The idea that it has anti-oxidant and anti-inflammatory effects would have made it an optimal candidate if we had decided to experiment it on patients too, in a subsequent step.

Unfortunately, no inhibitory activity was found in our in vitro study.

Strawberry tree juice

Very few works in the literature concern strawberry tree juice. Some authors have evaluated its antioxidant capacity and its properties on digestive enzymes, but there are no data in the literature on the antimicrobial effect. Being foods rich in flavonoids and having important nutritional properties, being an important source of vitamin C, we thought we could evaluate their antifungal effect possibly implemented by light activation [103]. Some evidence also reports direct antimicrobial effects especially in the treatment of urinary infections.

However, in our experiment we did not find any activity against *Candida* MDR.

Cell cultures and miRNA evaluation

As previously labelled in the materials and method session, an interesting and fast approach to evaluating the effectiveness of PDT against *C. albicans is* to measure some biochemical inflammatory signals due to infected tissue cells. The role of small highly conserved non-coding RNA molecules of about 20 nucleotides known as micro-RNA (miRNA) has been discussed in the medical scientific community in recent years. These RNA oligos are involved in thousands of biochemical processes that comprise the modulation of gene expression of pro-inflammatory end-inflammatory substances, such as cytokines and chemokines, and other protein pathways involved in innate and non-innate immunity. Normally, miRNAs are involved in gene silencing,

and for this reason, different expression patterns could show an increase or decrease motifs [104]

(link-6), *Figure 131.*

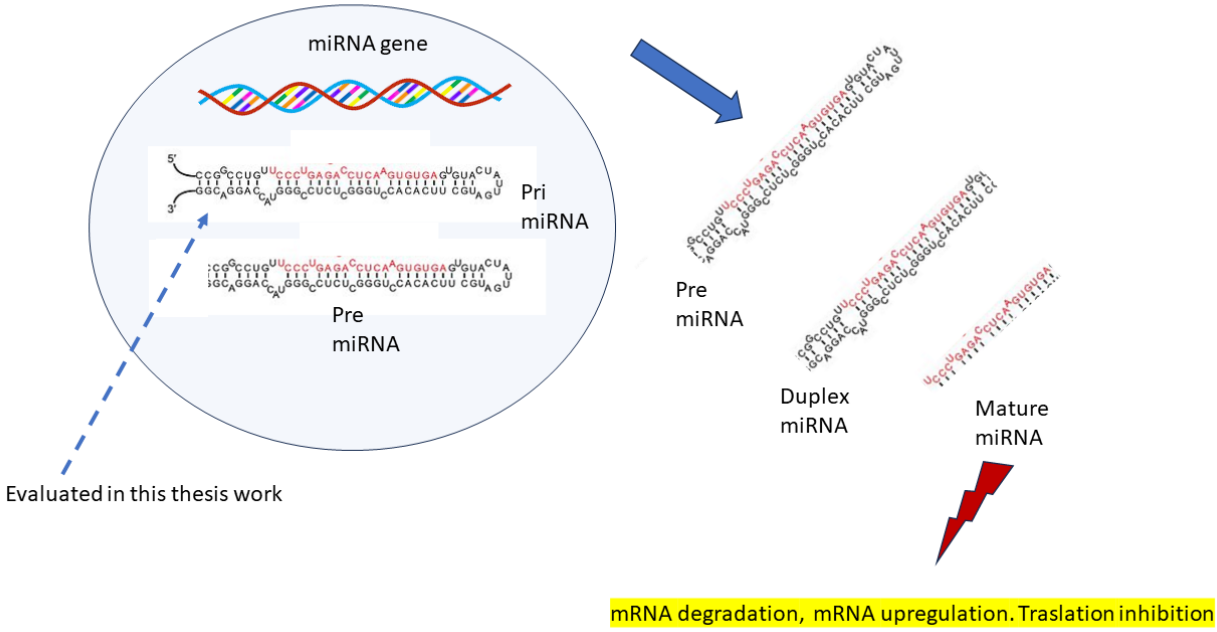


Fig.131. Different maturation stages for the miRNA.

Following recent literature on *C. albicans* infection and miRNA appears interesting 2 miRNA oligos, cited also in some chronic inflammatory disorders such as periodontal diseases, miRNA 146a and miRNA 155, these molecules are involved also in some neoplastic process in the oral mucosa and in other tissues. To simulate the infection-predictive function of these miRNAs, which is a possible use as an early predictive marker of infection before clinical signs, we have organized an *in vitro* simulation by using epithelial cells HEK 293 infected with a MDR strain of *Candida albicans*. The result is shown in Figure 60 A/B, suggesting a notable decrease in the expression pattern of these RNA oligos after PDT. These graphs also show a time-linked function of the expression level, in fact, especially for mRNA 155, at 50% in 1 minute. This indicates an interesting aspect; if this result is translated in vivo, that is, the Candida infection is detectable in a very short time (few minutes), this could be important in clinical

situations that are not clear, such as infection associated with a tissue base variation, i.e., Candida infection dentures associated.

Clinical evaluations

Out of 12 cases presented, in 7 cases there was a condition of angular cheilitis, while in two cases there was candidiasis at the lingual level, in 2 cases sub-prosthetic erythematous candidiasis, of which one at the palatine level and one at the lower gingival level, one case of suspected chronic hyperplastic candidiasis in the buccal mucosa, and a case of palatal fungal infection under orthodontic appliance. The cases of angular cheilitis were treated in one case with Toluidine Blue 1% and 630 nm, with clinical resolution after 7 days of chronic cheilitis; 3 cases were treated with 1% Methylene Blue photodynamic protocol and 660 nm laser light, 2 cases with photodynamic therapy with 3% curcumin+ H_2O_2 and 460 nm LED light; 1 case with lactoferrin powder activated by diode violet light. In all cases there was clinical and symptomatic success at 7-day follow-up, with clinical resolution already at 4 days in case 5, and at 36 hours in case 12. Previous works in the literature have reported clinical success with clinical and symptomatic improvement of angular cheilitis, 2 days after PDT, performed with Methylene Blue at a much lower concentration than the one we used (0.01%) and laser light at 660 nm. Our pre-illumination times were approximately 5 minutes in all treated patients, while in the cited work, the authors report application times of only 120 seconds [105].

No other protocols have been described in the scientific literature, but from our experience we can conclude that all three types of commercial photosensitizers tested have proven effective. The curcumin-based photosensitizer showed faster regression at a clinical level after the photodynamic therapy session compared to Methylene

Blue and Toluidine Blue. The use of a commercial product based on lactoferrin has proven particularly effective in managing a case of angular cheilitis, resulting in even faster healing than the other three commercial products. The rationale for choosing that type of light to activate lactoferrin is linked to the fact that in our *in vitro* study wavelengths in the ultraviolet spectrum, therefore 380-400 nm of wavelength, were evaluated as the best for the activation of the lactoferrin, evaluated with the spectrophotometer and confirmed by the result obtained on the inhibition of *Candida spp.* MDR. This case represents further confirmation of how important *in vitro* evaluations are before proceeding with the development of an *in vivo* protocol. Probably the type of fungal infection from which the treated patient was suffering was not caused by *Candida spp.* MDR, and this explains the even more exciting result *in vivo* compared to the results of lactoferrin alone *in vitro*. There are no other clinical cases in the literature in which lactoferrin has been used in vivo as a photosensitizer, nor cases in which the device used, developed as a caries detector, has been used in photodynamic therapy on the oral cavity. This treatment represents an absolute novelty both in terms of the photosensitizer and the type of light, opening new ways for the use of violet light in the treatment of oral candida infections.

In the fungal manifestations at the lingual level, in one case a PDT protocol was used which included Toluidine Blue 1% and 630 nm, and in another Curcumin+H_2O_2 and 460 nm. In case 1 there was a good clinical and symptomatic regression, in the case 11 at a microbiological level we could observe the reduction of the fungal load immediately after the photodynamic therapy. In the literature there are only a few *in vivo* works but on animal models in which induced lingual candidiasis was treated [52,106,107]. There are no data on antifungal treatment via PDT within potentially malignant lesions.

Treated subprosthetic candidiasis cases resulted in partial results. In fact, case no. 7 had already been previously treated with PDT performed with Toluidine Blue 1% and diode light at 630 nm, without having given a clinical remission of the candidiasis which was already refractory even to the topical drug. In the literature there are several studies that have taken this PDT into consideration for the treatment of sub-prosthetic candidiasis. However, there are few in vivo human studies, 2 RTCs in which the PDT protocol used did not include Toluidine Blue, but the PDT carried out with indocyanine green and PHOTOGEM proved to be as effective as nystatin in the treatment of sub-prosthetic candidiasis [108,109]. Some *in vitro* studies in which PDT carried out with Toluidine Blue on acrylic resin, i.e. the same material as removable prostheses, have given good results both against *Candida albicans, C.glabrata* and *C.krusei* [110]. It was possible to appreciate some improvements when PDT with curcumin-based photosensitizer was used in both patient 7 and patient 9. We have not found any scientific work in the literature that has used a curcumin-based photosensitizer for the *in vivo* treatment of subprosthetic candidiasis. Comparing the data obtained in experiment 3 of the *in vitro* study 1 with those *in vivo*, we realize that the effectiveness of the PDT protocol carried out with curcumin-based photosensitizer is lower *in vivo* than *in vitro*, probably linked to the fact that the oral environment is affected by various aspects that may or may not influence the antifungal activity: pH values (especially in subjects with GERD), salivary flow, the distance between the prosthesis and the mucosa of the palate. In a study on salivary Ph values in patient with oral Candidiasis authors found that salivary pH with acidic values (more in HIV+ patients) significantly favors Candidiasis development overall, for *C. albicans* and *C. glabrata* species and primarily the pseudomembranous and erythematous clinic types. They conclude that pH is not a determinant for Candida growth but could affect the adherence and invasiveness of the yeast [111].

Photodynamic treatment of Candida infections under palatal expander has never been documented before in scientific literature. The problem of oral pathologies linked to palatal orthodontic devices has been addressed by some authors, without however investigating the specific types of Candidiasis on the areas of palatine mucosa adjacent to the orthodontic artefact [112].

Among the species found in the microbiological sample of our patient we found the presence of *C.parapsilosis*. Very few studies have been carried out on the treatment of this type of *Candida* with photodynamic therapy. In a study, resistant species of *Candida*, including *C.parapsilosis*, were treated with PDT made with Methylene Blue activated by lights at 625 nm and lamps with a wavelength comprised between 400 and 700 nm, with or without the addition of Chlorhexidine 0.20%. Activation with the lamp and chlorhexidine determined the greatest antimicrobial effect, understood as a reduction in CFU [113]. In another *in vitro* study, the use of Methylene Blue enriched with silver nanoparticles as a photosensitizer determined an important effect against *C. parapsilosis*, when activated by very low power 660 nm lights [114]. No study has ever evaluated a curcumin photosensitizer in the photodynamic treatment of *C. parapsilosis*. However, there are no studies on the antimicrobial activity of PDT on C. *lypolitica*. In our case we had the disappearance of 2 species of Candida, namely *C.parapsilosis* and *C.lipolytica* and the appearance of *C.tropicalis*, upon sampling carried out after photodynamic therapy. We cannot give an answer to this phenomenon but it is possible that dietary factors have influenced it.

Another case presented was a patient with a particular Candida infection inside an oral leucoplakia. In the scientific literature, no case is currently reported of eradication of fungal load within lingual leukoplakia, diagnosed with histological examination on biopsy through PDT. Chronic hyperplastic candidiasis is a rare type of candidiasis that causes the formation of a non-removable white plaque, mono or bilateral, much

more frequent in the buccal mucosa than in other locations. Our case could be associated with a picture of somewhat atypical chronic hyperplastic candidiasis, although we cannot establish whether it was the fungal infection that caused the onset of the plaque or whether the fungal infection occurred after the formation of leukoplakia. Very few recent works report the use of PDT through 5-ALA as a photosensitizer in cases of chronic hyperplastic candidiasis, alone [115] or in association with topical anti-fungal [60]. In one case, the combined use of PDT and laser excision is reported on an 85-year-old patient refractory to pharmacology [116]. The use of a curcumin-based photosensitizer has not been documented in the in vivo treatment of this form of candidiasis, although it has proven to be effective on MDR *Candida spp* in our in vitro study. In no work has the fungal load within leukoplastic lesions been evaluated before and after photodynamic therapy. Even in works on the use of PDT in chronic hyperplastic candidiasis, the healing observed is essentially clinical. Evaluation through intraoral brush sampling and cooperation with a Molecular Biology Department could be protocols to be repeated in the case of leukoplakia and more generally in potentially malignant disorders. The association between Candida and Leukoplakia in fact highlights a greater predisposition of the patient to cancer. The control of the fungal infection within the leucoplakias and the management with PDT could be part of a prevention protocol that drastically reduces the percentage of cancer risk.

Case 10, a patient with suspected chronic hyperplastic candidiasis and bilateral angular cheilitis, following treatment with photodynamic therapy saw a complete regression of the angular cheilitis and an improvement of the mucosal part, greater on the left, testifying to a better effect of the photodynamic therapy carried out with the help of polarized light, which on the one hand probably activates the photosensitizer better than 460 nm light, as also demonstrated by our *in vitro* study 1, on the other hand it also has a bio stimulating effect on adjacent tissues, thus favoring a more rapid healing

speed. No work in the literature has reported a treatment of chronic hyperplastic candidiasis treated with this type of photosensitizer and more generally with the PDT protocols described. The use of the device for the evaluation of autofluorescence as a first level test to intercept any malignant lesions has already been documented in the literature. Sometimes it can be useful to avoid incisional biopsies and therefore unnecessary invasive interventions on the patient [117].

Chapter 11- Conclusions

The photodynamic treatment of candidiasis has given little or no results *in vitro* with regards to Toluidine Blue and Methylene Blue both activated by wavelengths around 630 nm and 660 nm, while excellent results both with respect to biofilm, both considering the inhibition halos were achieved with the curcumin-based photosensitizer activated by lights at 460 nm and activated by lights with wavelengths between 380 and 3400 nm. The in vivo photodynamic treatment has shown excellent results on chronic labial infections, both with Toluidine Blue, with Methylene Blue and with curcumin-based photosensitizer, and this would seem a bit in antithesis with the *in vitro* studies carried out. The use of lactoferrin powder as a photosensitizer is a novelty with interesting results. In intraoral *Candida* infections under prosthetic device and under the palatal expander we have had partial results. In the treatment of fungal infections associated with potentially malignant pathologies we have had a significant improvement at a clinical level. From the observation of this, some important considerations arise: *in vitro* studies cannot reproduce all the variables that can occur in the oral cavity, even if in vitro experiment number 3 there is a good simulation model of subprosthetic candidiasis not yet described in the literature. These variables depend first of all on the type of tissue, in fact, it seems that on the skin side all the protocols with

commercial devices have worked well. In fact, the degree of humidity and the almost total absence of saliva in that part of the oral district could have influenced both the adhesion capacity of the photosensitizer and the penetration of light. Another aspect to consider is that of the dilution of the photosensitizer itself in contact with saliva, and the palatal district and the inferior alveolar process are rich in minor salivary glands. On the lingual lesions during the photodynamic treatment, it was possible to better manage the quantity of saliva produced via aspirator without risking taking away the photosensitizer and this could have influenced the greater clinical success of the treated lesions. Some researchers have successfully tested saliva as a photosensitizer activated by blue lights, with or without curcuma addition, in the treatment of the corona virus [118]. The organic and inorganic part that represents approximately 1% of saliva could have antimicrobial properties that can be implemented by some lights, acting in a photodynamic sense. Some patients may have a greater or lesser salivary flow or a greater or lesser quantity of salivary corpuscular part and therefore influence the photodynamic process upon contact with the photosensitizer [119].

Another important parameter that could influence the antifungal activity of photodynamic therapy *in vivo* is pH value, already mentioned in the Discussion session. Variations in pH could affect the binding capacity of the photosensitizer and this parameter would vary from patient to patient also based on systemic problems such as gastro-esophageal reflux. Some authors, in a work on the use of PDT in tumor cells, have already demonstrated that the photosensitizer can have increased activity in the presence of a change in pH value [111].

The presence of some enzymes in the saliva could affect the activity of the photosensitizer and *in vivo* variables such as the production of interleukins could also be

important. In general, we can say that photodynamic therapy represents an effective tool both *in vivo* and *in vitro* for the control of MDR *Candida* infections.

The use of natural products such as olive oil (EVOO), saffron and lactoferrin also seem promising for *in vivo* protocols. The natural products activated by H_2O_2 appeared to perform better as photosensitizers, especially when considering the inhibition zone. The presence of a minimal quantity of hydrogen peroxide inside these natural molecules could be an interesting tool to make some photosensitizers more active, triggering the formation of free radicals. The use of a commercial device that emits polarized light, well documented in the literature and never before used against *Candida spp*. MDR in PDT, capable of increasing the antimicrobial activity of products such as curcumin and EVOO, could be the basis of home photodynamic protocols, as the use of this device does not require learning curves for its use, the use of protective glasses and also exists in a home version, also obtaining a biostimulator effect on the surrounding tissue. We hope that all the studies performed in this work are the basis for the definitive solution to such a widespread and important problem as MDR *Candida* infections.

Future perspective

The information obtained from the *in vitro* and *in vivo* studies carried out during this thesis work highlighted some fundamental aspects:

1) Natural photosensitizers, both commercial (curcumin) and new ones proposed (EVOO oil, lactoferrin), have shown greater antimicrobial activity when activated by hydrogen peroxide;

2) Polarized light with a wavelength spectrum between 380 and 3400 nm has been shown to more effectively activate some of the natural photosensitizers (curcumin and EVOO oil) than the wavelength that coincides with that recommended by the

parent company or with the maximum absorption of that natural substance evaluated with the spectrophotometer;

3) the in vitro evaluation of miRNAs 146a and 155 can be considered an excellent tool for evaluating the progress of the infection and the specific response of the host with a view to predictive and personalized medicine;

4) Various clinical cases referring to leukoplakia or chronic hyperplastic candidiasis, both successfully treated with PDT, pave the way for the use of this technique in the treatment of other lesions that may develop into malignancies or untreating candidiasis over time

These four points would lay the foundations for developing other *in vitro* and *in vivo* studies.

Natural photosensitizers and hydrogen peroxide.

The addition of hydrogen peroxide could also be evaluated on other natural compounds, evaluating the ideal minimum quantity of hydrogen peroxide to add to the photosensitizer so that it can determine an enhancement of a natural product already active against *Candida spp*. MDR, but which can be in such a concentration that it can even be accidentally ingested. The first natural photosensitizer that I would like to test with the addition of hydrogen peroxide is the 0.3% saffron solution, which has already proven effective in this thesis work against *C. krusei* MDR. A series of *in vitro* studies using the Kirby Bauer method could be further developed to study the effect of natural PSs enriched with H_2O_2. To evaluate this, one should focus on a bibliographic search aimed at finding previous works on the cytotoxicity of hydrogen peroxide on the mucous membranes of the oral and gastrointestinal tracts and then reproducing, with an *in vitro* model, the effect of hydrogen peroxide at the proposed

concentration, on cell cultures of oral, gastric or esophageal mucosas. Once the concentration of H_2O_2 to integrate with natural photosensitizers has been found, which makes them more effective but not toxic if accidentally ingested, the compounds could be proposed for human experimentation, after requesting approval from the ethics committee.

In summary: in vitro studies with Kirby Bauer Methods+ in vitro study on effect on oral, gastric and esophageal mucosa of H_2O_2+ in vivo evaluation of natural PS implemented with H_2O_2, *Figure 132.*

Polarized light as a new light to be used in photodynamic therapy

One of the future objectives will be to test this device with a certain variation of wavelengths and also in the activation of other natural photosensitizers that have proven effective against MDR *Candida spp*. species, in particular the lactoferrin-based solution with or without the addition of hydrogen peroxide and the saffron-based solution. Through in vitro studies performed with the Kirby Bauer method in triplicate, it will be possible to have an evaluation of the antimicrobial effect of the new natural PS and polarized light combinations, while with the Microplates assays method, it will be possible to see the effect on the biofilm. Furthermore, polarized light emits a wide beam of light and was created as a device for oral biostimulation. This could lead one to think that the success of the treatment performed *in vivo* (also refer to case n. 11) could be linked to a stimulation activity of the epithelial tissues around the infected ones, which could determine faster healing of the tissue following increased proliferation of uninfected mucosal epithelial cells. This effect should be studied through an *in vitro* experiment with cell cultures from oral mucosa, infected with MDR *Candida*

spp. treated with a PDT based on a natural photosensitizer and polarized light, compared with a natural photosensitizer and non-polarized light.

In summary: a) Performing in vitro experiments with the Kirby Bauer procedure to test other natural Ps activated by polarized light devices;

b) Performing an in vitro experiment on oral mucosa cell culture infected with Candida spp. and treated with PDT by using polarized light, compared to PDT in the same cell culture with the other types of lights previously tested, *Figure 132.*

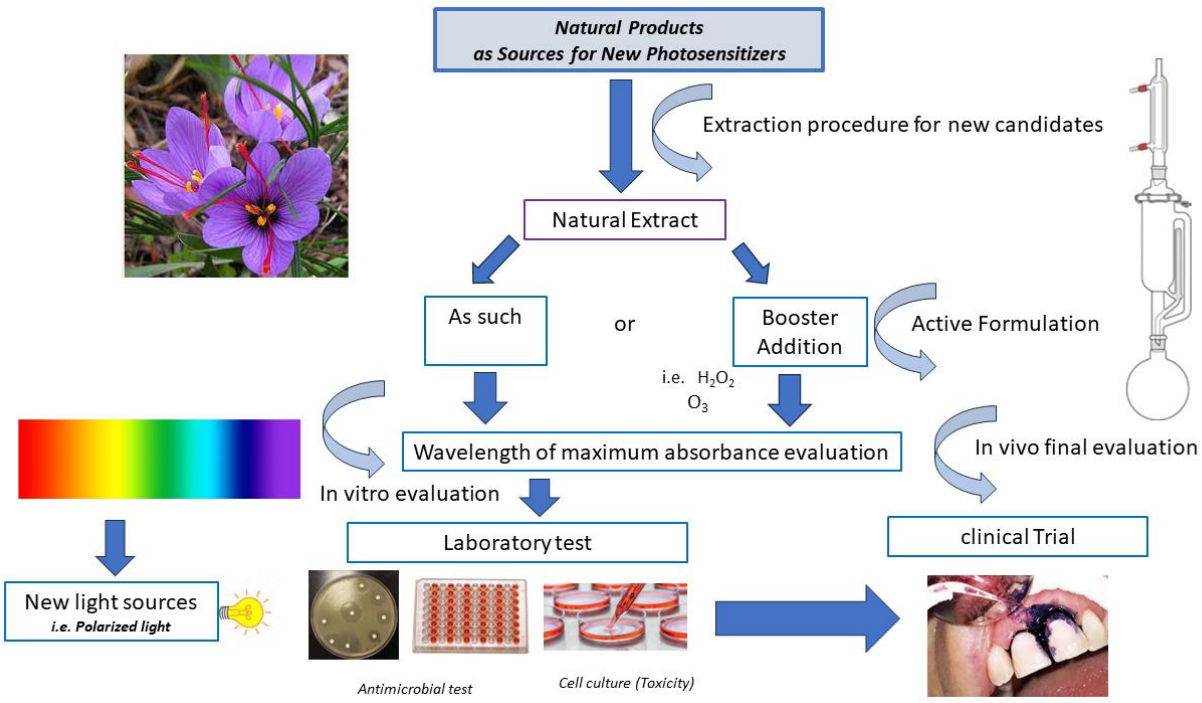


Fig.132. Schematic representation of flow work for new natural Photosensitizers.

<u>MiRNA 146a and 155 as an expression of PDT efficacy in vivo.</u>

We know that in *Candida spp.* infections, the load of the fungus *in vivo* does not always correspond to the severity of the infection which is determined by the imbalance between the host's defense capabilities (immune system, concentration of salivary enzymes, integrity of the mucous membranes) and the virulence of the fungus. This means that it is also necessary to evaluate the effectiveness of PDT in terms of the inflammation and damage produced by *Candida spp.* before, during, and after PDT treatment, regardless of the fungal load. The experiment proposed during this thesis work was intended to be a preliminary phase to understand whether the evaluation of miRNAs 146a and 155, already documented in international scientific literature as miRNAs expressed by Candida-infected host cells, could be a good method of evaluation of the progress of the treatment of fungal infection with PDT.
The miRNA values before and after PDT through our *in vitro* study on infected cell cultures were reduced by approximately 50%, which also reflected the decrease in the fungal load. The next step would be to perform this type of evaluation of salivary miRNAs, *in vivo*, by sampling with a brush and a container with dedicated transport liquid, after approval of the project by the ethics committee.
In summary: an *in vivo* study on the evaluation of PDT performed with natural PS, with miRNA 146a and 155 evaluations through saliva samples from patients (Ethics Committee required), *Figure 133.*

Use of PDT treatment on Oral potentially malignant disorders

We have seen how oral leukoplakia and chronic hyperplastic candidiasis have a very high rate of malignant transformation, approximately 9.5 and 12%, respectively. Especially with regards to chronic hyperplastic candidiasis, the reduction of the fungal load is connected with a reduction in the probability of cancerization. In addition, an *in vivo* study could be conducted to assess the fungal load and tissue inflammatory patterns before and after any treatment with PDT in the involved clinical area (i.e.,

miRNAs, Ig superfamily, cell adhesion molecules, integrins, etc.). This approach could clarify host cell behavior during the infection as well as early neoplastic biochemical signals. A further study could be conducted, in particularly refractory or relapsing cases, with sampling via liquid cytology or microbiopsy of tissues damaged by leukoplakia or chronic hyperplastic candidiasis and evaluation of the degree of dysplasia (none, mild, moderate, or high) before and after PDT sessions. This second part of the experiment would require further approval of the project by the ethics committee.

In summary, in vivo evaluation of Candida infection and the degree of dyplasia before and after PDT in oral potentially malignant disorders. *Figure 133*.

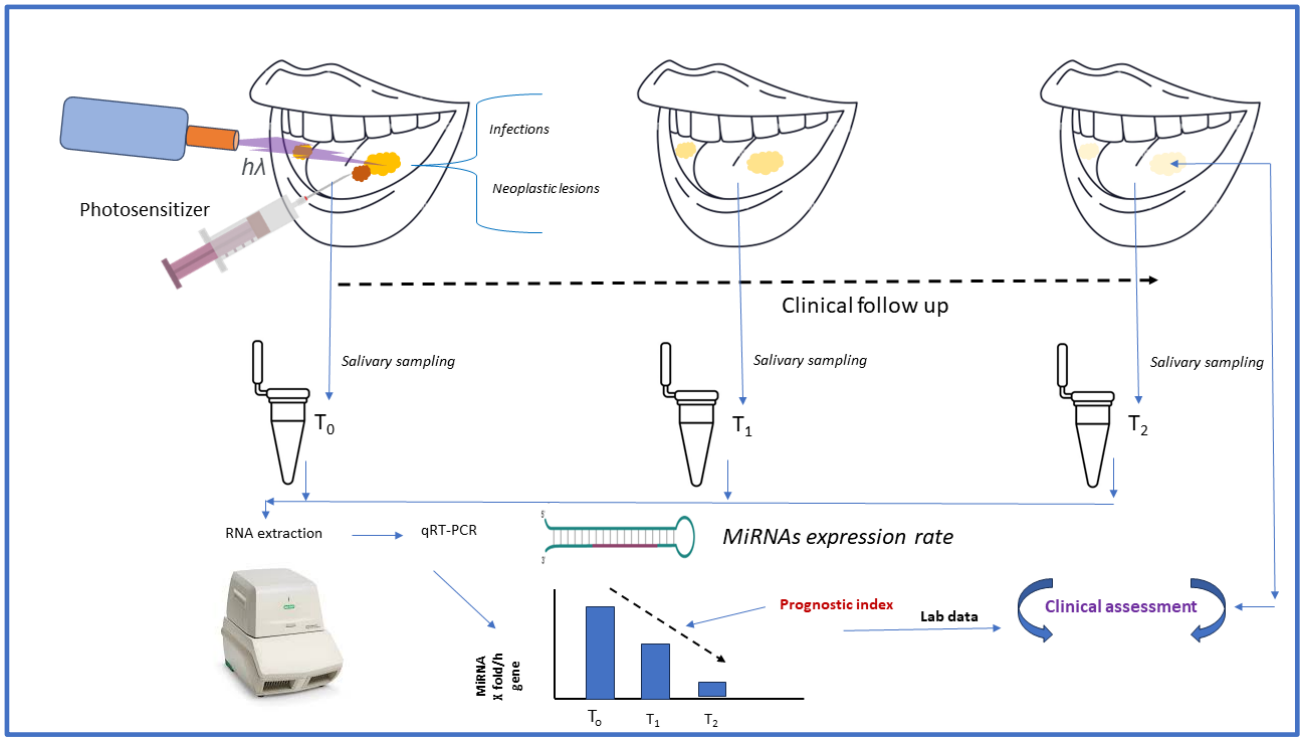


Fig.133. A hypothetical workflow in clinical laboratory diagnosis by using MiRNAs as predictive targets in the prognosis of main oral lesions.

Links/Footnotes

1. CDC Candidiasis report https://www.cdc.gov/fungal/diseases/candidiasis/index.html
2. CDC Candidiasis report 2 https://www.cdc.gov/fungal/diseases/candidiasis/invasive/statistics.html
3. The Clinical & Laboratory Standards Institute (CLSI). https://clsi.org/
4. The European Committee on Antimicrobial Susceptibility Testing – EUCAST https://www.eucast.org/
5. miRBase Sequence database and Registry (miRBase), https://mirbase.org/help/
6. miRNA target prediction and functional annotations (miRDB), http://www.mirdb.org/
7. ATCC HEK cells. https://www.atcc.org/products/crl-1573
8. Relative RNA quantitation: https://bitesizebio.com/29322/methods-relative-quantification-qpcr-data/

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Candidate's publications list on the topic of the thesis

(during Doctoral period, years 2020-2023)

Full-text indexed Journals

1. **Casu C**, Murgia MS, Orrù G, Scano A. Photodynamic therapy for the successful management of cyclosporine-related gum hypertrophy: A novel therapeutic option. J Public Health Res 2022; 11:22799036221116177. **(Q2)**
2. **Casu C**, Orrù G, Scano A. Curcumin/H2O2 photodynamically activated: an antimicrobial time-response assessment against an MDR strain of Candida albicans. Eur Rev Med Pharmacol Sci 2022 ;26:8841-8851. **(Q2)**

3. Scano A, **Casu C**, Orrù G, Coni P. Editorial - Epigenetic mechanisms in oral cancer: new diagnostic and therapeutic strategies. Eur Rev Med Pharmacol Sci 2022 ;26:7318-7320. **(Q2)**
4. **Casu C**, Murgia MS, Orrù G. Oral manifestations of monkeypox and potential tools for their management. Pan Afr Med J 2023 20;45:137. **(Q4)**
5. **Casu C**, Orrù G, Fais S, Mazur M, Grassi R, Grassi RF, Nardi GM. Efficacy of ozonated water as a PS in photodynamic therapy: A tool for dental caries management? An in vitro study. J Public Health Res 2023 Jun 24;12:22799036231182267. **(Q2)**
6. Casu C, Pinna M, Murgia MS, G. Orrù. Association between C.albicans and leukoplachia and its treatment with photodynamic therapy: a review of the literature and a case report. WCRJ 2023; 10: e2712
7. Casu C, Orrù G. The potential of photodynamic therapy in the management of infectious oral disease. World Journal of Experimental Medicine, Accepted on December 4th, 2023

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1. Cutuli O, Mosaico G, **Casu C**. PDT on a refractory gum pemphigoid in a non-Hodgkin's lymphoma patient, with MRONJ risk. Dental Cadmos Doi: 10.19256/abstract.cduo.15.2022
2. **Casu C**, Sinesi A, Scano A, Fais S, Orrù G. A natural photosensitizer against oral Candida MDR strains in Photodynamic Therapy. Dental Cadmos Doi: 10.19256/abstract.cduo.15.2022
3. **Casu C**, Piga A, Pinna M, Fais S, Orrù. G. Management of Oral Candidiasis using lactoferrin in Photodynamic Therapy: in vitro study. Dental Cadmos Abstracts Collegio dei Docenti Universitari di discipline

Odontostomatologiche ETS – La moderna odontoiatria tra tecnologie avanzate e umanizzazione pp.319-352 doi: 10.19256/abstract.cduo.13.2023

International Posters

1. Mara Pinna, Martina Murgia, Gloria Denotti, Germano Orrù, **Cinzia Casu**. Photodynamic Therapy against HSV-1 recurrent lesions in a pediatric patient. XXI Congresso Internazionale AIO, Chia, June 9-11, 2022.

2. **Cinzia Casu**, Martina Salvatorina Murgia, Alessandra Scano, Germano Orrù. Oral Herpes virus type 1 lesions: usefulness of a qPCR approach in a patient treated by Photodynamic therapy. XXI Congresso Internazionale AIO, Chia, June 9-11, 2022.

3. Martina Salvatorina Murgia, **Cinzia Casu**, Sara Fais, Mara Pinna, and Germano Orrù. Lesioni orali da Herpes Simplex Virus 1: utilizzo dell'approccio in qPCR in un paziente trattato con terapia Fotodinamica. Doctor Os Settembre 2022, XXXIII 07.
   The most clicked poster in the session Oral Pathology and Medicine at 4th International Congress "Technological Innovation and Experimental Protocols in Periodontology, Oral Surgery and Orthodontics", Bari, 8-10 September 2022

4. Luisa Ladu, Germano Orrù, Gloria Denotti, Alessandra Siotto-Pintor, **Cinzia Casu**. Viral, bacterial and fungal infections in pediatric patients in fixed orthodontic therapy treated with PDT: 2 case reports. **Honorable mention winner** in the session Oral Pathology and Medicine at 5Th International Congress "Technological Innovation and Experimental Protocols in Periodontology, Oral

Surgery and Orthodontics", Bari 14-16 September 2023

Oral Presentation/Speaker

1. **Cinzia Casu**, Martina Salvatorina Murgia, Valentino Garau, Lia Ester Argiolas, Germano Orrù. A particular photodynamic therapy for the treatment of refractory spontaneous maxillary ONJ: a case report. Qeios ID: GG5SAP · https://doi.org/10.32388/GG5SAP
2. Raffaello Viganò, Martina Salvatorina Murgia , Germano Orrù, Luca vigano, **Cinzia Casu**. B.P.F.C pure growth factor, PDT and LLLT for the treatment of severe MRONJ nonresponsive to prolonged antibiotic therapy. Qeios ID: 43BHBE · https://doi.org/10.32388/43BHBE
3. **Cinzia Casu**. La terapia fotodinamica per la Candidosi Orale. III Congresso AIRO, June 4 2022, Bari.
4. **Cinzia Casu**. Oral Candidiasis: new approach in diagnosis and therapeutic management. 5Th International Congress "Technological Innovation and Experimental Protocols in Periodontology, Oral Surgery and Orthodontics", Bari 14-16 September 2023

National Publications.

1. **Cinzia Casu**. Terapia fotodinamica per il trattamento di lesioni orali da HPV: 2 Case Report. Teamwork anno XXIII 2/2021
2. **Cinzia Casu**, Valentino Natoli, Luca Viganò. Ulcere traumatiche orali trattate con terapia fotodinamica. Dental Tribune Italian Edition April 2021.
3. **Cinzia Casu**. Ipertrofia gengivale non placca correlata in una paziente gravidica con Lupus Eritematoso Sistemico trattata con terapia fotodinamica: case report. teamwork clinic | year XXIII 4/2021
4. **Cinzia Casu**, Matteo Fanuli, Luca Viganò Viola Viganò. Gestione di un'

ipertrofia gengivale con l'ausilio della terapia fotodinamica. Dental Tribune, July 2022.

National Posters

1. Cutuli O, Casu C. Nuovo approccio clinico nell'utilizzo della terapia fotodinamica in campo parodontale come strumento di prevenzione sistemica: nuova proposta terapeutica. II Congresso AIRO " Oral-Systemic-Periodontal-Health", October 9, 2021, Saronno
2. Cinzia Casu, Roberta Musu, Sara Fais, Germano Orrù. Black Hairy Tongue Treated by Photodynamic Therapy. XX edizione Congresso della Commissione Nazionale dei Corsi di Studio in Igiene Dentale, June, 10-11, 2022, Napoli.

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