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Local and systemic oxidative stress in oral leukoplakia: A Systematic Review.

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1. SUMMARY

Oral leukoplakia (OLK) is one of the most common oral potentially malignant disorders and carries a risk of transformation into oral squamous cell carcinoma (OSCC). While its exact etiology remains unclear, oxidative stress has been proposed as a key factor in its pathogenesis. This systematic review aimed to evaluate the presence of local and systemic oxidative stress in patients with OLK through the analysis of specific oxidative and antioxidant biomarkers. A comprehensive search was conducted across PubMed, Web of Science, and Scopus databases, including studies published between 2000 and 2024. 15 studies met the inclusion criteria, involving a total of 1,315 patients and analyzing samples from serum, saliva, and oral tissue. The results demonstrated a consistent increase in oxidative markers such as MDA and 8-OHdG in OLK patients compared to healthy controls. In parallel, significant decreases in antioxidants like GSH, GPx, SOD, CAT, and vitamins C and E were observed. Several studies also reported a correlation between the degree of epithelial dysplasia and oxidative damage, suggesting a potential link between oxidative imbalance and malignant transformation. These findings support the hypothesis that oxidative stress plays a significant role in the development and progression of OLK. Furthermore, the identification of reliable oxidative biomarkers could contribute to improved early diagnosis and clinical management. Future research is needed to validate these markers and explore antioxidant-based therapeutic strategies.

2. ABSTRACT

Introduction: Oral leukoplakia (OLK) is a common potentially malignant disorder in the oral mucosa, with a multifactorial etiology. Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and antioxidant defenses, is increasingly linked to the development and progression of OLK. Understanding this relationship may aid in early diagnosis and risk assessment.

Materials and methods: This systematic review was conducted following the PRISMA guidelines and registered in PROSPERO (CRD42025644565). Studies were retrived from PubMed, Web of Science, and Scopus, including observational and interventional studies published between the year 2000 and 2024. Oxidative and antioxidant biomarkers were evaluated in serum, plasma, saliva, and tissue samples from OLK patients and compared to healthy controls.

Results: 15 articles met the inclusion criteria, involving a total of 1,315 patients. OLK patients exhibited elevated levels of oxidative stress biomarkers such as MDA and 8-OHdG, alongside a marked decrease in antioxidants like GSH, GPx, SOD, CAT, and vitamins C and E. A correlation was observed between oxidative damage and the severity of epithelial dysplasia.

Conclusion: This review supports an association between OLK and both local and systemic oxidative stress. Oxidative biomarkers could be valuable tools for early diagnosis and assessing malignant transformation risk. Further research is needed to validate these findings and explore antioxidant-based therapeutic strategies.

3. KEY WORDS

I. Oral leukoplakia
II. Oral premalignant lesions
III. Potentially malignant disorders
IV. Oxidative stress
V. Oxidant biomarkers
VI. Free radicals
VII. Reactive Oxygen Species (ROS)
VIII. Reactive Nitrogen Species (RNS)
IX. Nitrative stress
X. Cellular apoptosis
XI. Antioxidant defense system
XII. Antioxidant biomarkers
XIII. Antioxidant levels

4. ABBREVIATIONS

8-ISO – 8-isoprostane

8-OHdG – 8-hydroxy-2-deoxyguanosine

AIDS – acquired immunodeficiency syndrome

E-SOD - erythrocyte superoxide dismutase

EBV – Epstein Barr Virus

GLRX2 – Glutaredoxin 2

GPx – glutathione peroxidase

GR – glutathione reductase

GSH – reduced glutathione

GSSG – oxidized glutathione

HPV – Human Papilloma Virus

HIV – Human Immunodeficiency Virus

HOBr – Hypobromus acid

HOCl – Hypochlorous acid

H_2O_2 – hydrogen peroxide

MDA – malondialdehyde

$NO2^-$ – nitrite

$NO3^-$ – nitrate

OSSC – Oral Squamous Cell Carcinoma

OCSCC – Oral Cavity Squamous Cell Carcinoma

OLK – Oral Leukoplakia

OHL – Oral Hairy Leukoplakia

ROS – Reactive Oxygen Species

RNS – Reactive Nitrogen Species

SOD – superoxide dismutase

SOD2 – Superoxide Dismutase 2

TAC – Total Antioxidant Capacity

$TNO2^-$ – total nitrite

UA – uric acid

TXN2 – Thioredoxin 2

tGSH – total glutathione

OH• – hydroxyl radical

• $\mathbf{O_2^-}$ – superoxide anion

$\mathbf{^1O_2}$ – singlet oxygen

ROO• – peroxyl radical

$\mathbf{O_3}$ – ozone

• **NO** – nitric oxide

$\mathbf{ONOO^-}$ – peroxynitrite anion

TBARS – thiobarbituric acid reactive substances

iNOS – inducible nitric oxide synthase.

IR – immunoreactivity

TAS – Total Antioxidant Status

TAC – Total Antioxidant Capacity

5. INTRODUCTION

5.1 Oral leukoplakia

Oral leukoplakia (OLK) is the most frequently occurring (pre)malignant or potentially malignant lesion in the oral mucosa (1). Thus, it is classified as part of a group of conditions referred to as oral potentially malignant disorders (OPMDs) (2). It is characterized by the presence of a white patch or plaque that cannot be scraped off and that cannot be classified as any other specific lesion (3). In 2012 van der Waal proposed this definition "A predominantly white lesion or plaque of questionable behaviour having excluded, clinically and histopathologically, any other definable white disease or disorder" (4).

The plaque is located in the oral mucosa which includes the tongue, cheeks, gingiva as well as the floor of the mouth. Generally, the lesions appear painless and present a rough, hard, and thickened texture (5). Although OLK is generally benign, it has the risk of a malignant transformation into oral squamous cell carcinoma (OSCC), particularly the leukoplakia with dysplastic changes (5). OCSCC is the most frequent head and neck cancer and its classified among the most aggressive malignant tumors due to its metastatic behaviour and its high recurrence rate (6).

Histologically OLK is characterized by hyperkeratosis of ortho- or parakeratotic type and acanthosis of the epithelium, with different extents of chronic inflammatory infiltrates in the lamina propria. Thus, varying levels of epithelial dysplasia which also influences the risk of malignant transformation. Some microscopic features of dysplasia include: loss of basal cell polarity, an elevated nuclear-to-cytoplasmic ratio, irregular epithelial layering, an increased number of abnormal mitotic figures, their presence in the superficial epithelium, cellular and nuclear pleomorphism, and keratinization of isolated cell clusters (7).

5.1.1 Types of OLK

OLK can predominantly be divided into two groups, homogenous and non-homogenous (4). Furthermore, hairy leukoplakia and proliferative verrucous leukoplakia also exist. There is disagreement among various articles regarding whether the latter belongs to the category of non-homogeneous lesions or should be considered a distinct group (3). In this systematic review it will be considered a separate disease (8,9).

5.1.1.1 Homogenous leukoplakia

The homogenous type is presented uniformly with a thin white area that can alter or not the normal oral mucosa (4). Some authors apply the term homogenous leukoplakia to thin and flat leukoplakia, whilst others also include a thick variant of homogenous leukoplakia (10). As subgroups of homogenous leukoplakia, *velvetlike type* and *pumice-stone type* are among those described (10). Nevertheless, it represents the most common type of leukoplakia and is asymptomatic in most cases.

5.1.1.2 Non-homogenous leukoplakia

The non-homogenous OLK are those lesions that deviate from the homogenous description, although it may similarly present a flat surface. Normally, it has a mix of red and white colour («erythroplakia») and a speckled or nodular surface. As well as superficial focal ulceration joined by diffuse borders. Though OLK is usually not accompanied by pain, the red or ulcerated areas may be symptomatic. Besides the red and white erythroplakia there are also the wart-like and proliferative verrucous type, which according to some studies, can also be considered in the category of non-homogenous. These lesions with presence of redness or nodularity should be regarded with great suspicion, as they statistically carry a higher risk of malignant transformation compared to the homogenous type (2,11).

5.1.1.2.1 Speckled leukoplakia

Speckled leukoplakia is considered a rare form of leukoplakia with a high risk of malignization. According to the World Health Organization (WHO) the speckled type of leukoplakia are those lesions with a mixture of leukoplasic white and erythroplasic red plaques (12,13). Lesions are described as erythroleukoplakia, leukoerythroplakia, or

speckled leukoplakia when they exhibit a combination of red and white areas, or when white patches overlay a red plaque (12).

5.1.1.2.2 Nodular leukoplakia (erythroplakia)

The nodular leukoplakia presents a white surface, and as in its non-homogenous nature, the plaque is verrucous, nodular, ulcerated, or erythematous in character, similar to the speckled type. Thus, it has a greater risk of malignant transformation when compared to the homogenous variant. The term erythroplakia is also used due to its erythematous mucosa (14).

5.1.1.2.3 Proliferative verrucous leukoplakia

Over the past few decades, a distinct third clinical subtype has been introduced in the literature, different from the classifications of homogeneous and non-homogeneous leukoplakia. This subtype, known as proliferative verrucous leukoplakia (PVL), has generated significant confusion since its initial description, largely due to the absence of a clear and standardized definition (10). Although, some authors may suggest it as a subtype of the non-homogenous leukoplakia (8). Proliferative verrucous leukoplakia is considered a rare type of OLK. It is known for its multiple recurrences, its refractoriness to treatment, and its rapid malignant transformation. Thus, it is considered a true premalignant lesion (4). Due to its appearance in the early stage, some research studies imply that it may resemble oral lichen planus which can lead to misdiagnosis, this can be critical given its possibility to transform into OSCC (8). Although oral lichen planus is also a premalignant lesion it has a lower risk of transforming into OSCC than OLK (15,16).

5.1.1.3 Hairy leukoplakia

Oral hairy leukoplakia (OHL) is a benign, asymptomatic white lesion characterized by hyperkeratosis. Typically placed on the lateral borders of the tongue, rarely found elsewhere, either unilaterally or bilaterally. The surface of the lesion can appear flat or elevated, vertically corrugated, or distinctly hairy. It predominantly affects individuals with severe immunosuppression, particularly those living with *human immunodeficiency virus* (HIV), although not limited to these patients (17,18). If the patient presenting OHL has a HIV diagnosis it may cause a rapid onset of acquired immunodeficiency syndrome (AIDS). Despite this, it is not classified as a premalignant lesion and is unlikely to cause OSSC as

it is benign (17). A causal relationship has been suggested between *Epstein-Barr virus* (EBV) and OHL, as EBV DNA and viral proteins encoded by EBV genes have been detected in affected cells. OHL is believed to result from active EBV replication within the oral mucosal epithelium, primarily along the lateral borders of the tongue. In some cases, EBV-driven OHL may represent the initial clinical sign of an HIV infection (12).

5.1.2 Epidemiology: Prevalence of OLK

OLK is widely recognized as one of the most thoroughly studied PMODs, and its epidemiology has been extensively documented (3). The prevalence of leukoplakia varies across different scientific studies, with a global review indicating a prevalence of 2.6% (3). Leukoplakia is more frequently observed in middle-aged and elderly males compared to other groups, with its prevalence rising as age increases (3). It is rarely seen in the two first decades of life, which can be a helpful parameter for diagnosis (11). Studies suggest that the condition primarily affects men over the age of 40 (4).

5.1.3 Risk factors and aetiology

Even though the etiological factor or causal agent of OLK is not completely agreed on, it is thought to be multifactorial (3,10). The most researched and well-established risk factors include areca nut, tobacco, and alcohol consumption. In addition, there is a clear link to chronic irritation or inflammation of the oral mucosa. This could be from various sources, such as tobacco and alcohol consumption, but also poor oral hygiene, chronic irritation from badly adjusted dental restorations or prosthesis, viruses e.g. *human papillomavirus* (HPV) (5,19), fungal infections e.g. candidiasis, bacterial infections, sexually transmitted lesions e.g. syphilis, combined micronutrient deficiency, viral infections, hormonal disturbances, and ultraviolet exposure (3). Furthermore, radiation and anticancer therapy are sources of exogenous free radicals (20). Lastly, though it may be considered controversial, oral galvanism due to restorations (3,21).

5.1.4 Physiopathology

When cells are exposed to carcinogenic factors, they often attempt to adapt to the damaging stimulus (3). In the case of chronic mucosal irritation, this may result in an

increase in cell proliferation and a diminished capacity for the cell to manage stress, as part of an adaptive response. This heightened proliferation can manifest as mucosal hyperplasia. If the irritant persists, the oral epithelium may exhibit signs of cellular degeneration, such as apoptosis or atrophy, which are further indications of the body's attempt to adapt. Once cellular damage becomes irreversible, the outcome may be either apoptosis or malignant transformation. The accelerated cell proliferation during the early stages of this process can drive the progression toward cancer (3).

5.1.5 Diagnosis and treatment

As previously mentioned, OLK is diagnosed once other similar lesions have been excluded. Considering this, the diagnosis can be challenging. Differential diagnosis includes oral lichen planus, leukoedema, lupus erythematosus, white sponge nevus, morsicatio buccarum, candidiasis, psoriasis and chemical burns. Nevertheless, as with other precancerous lesions in the body an early diagnosis is crucial. Therefore, efficient and precise diagnostic tools are of utmost importance (3). Recent studies state that, in many parts of the world, dentists are likely to require the assistance of a specialist to confirm or rule out the clinical diagnosis of OLK, as well as to guide further patient management, including providing appropriate patient education (11). The diagnosis of leukoplakia is established through expert clinical evaluation and histopathological examination (22).

For diagnosing OLK, the gold standard remains obtaining a biopsy from the lesion site. However, this method is invasive, painful, costly, and time-consuming (2,23). For smaller lesions an excisional biopsy is advised, while for larger lesions the incisional biopsy including healthy adjacent tissue is performed, both for histopathological examination. When examined histologically, the primary cellular changes include keratinization of the epithelium as either hyper ortho-keratinization or hyper para-keratinization, increased epithelial thickness, acanthosis, thinning of the basement membrane, and changes in the cellular layer (10,11). Other findings include an increased nuclear-cytoplasmic ratio, hyperchromatic nuclei, nuclear hyperplasia, abnormal mitotic figures, increased mitotic activity, pleomorphic nuclei, basilar hyperplasia, drop-shaped

rete pegs, and loss of cell polarity (10,22). Additionally, an inflammatory component may be observed in the connective tissue. In addition to biopsy, other diagnostic tools are available, such as toluidine blue dye, oral brush biopsy kits, salivary diagnostics, and optical imaging systems. Over the past few years, new light sources and chairside diagnostic instruments have been promoted to dentists as easy-to-use methods for diagnosing OLK (3).

Table 1 presents various parameters that can be considered when establishing a clinical diagnosis of OLK. However, factors such as gender, ethnic background, delineation of the lesion and solitary versus multiplicity have no clinical significance. Oral site as well is not relevant for diagnosis as the lesion can occur anywhere in the mouth with buccal mucosa, floor of mouth, ventral and lateral of tongue and soft palate as most common sites (1,11,24).

Table 1: Parameters for diagnosing OLK

Parameter	Relevance
Age	The occurrence of OLK in the first two decades of life is rare, this can be helpful knowledge when diagnosing a lesion that looks like OLK.
Medical history	Medical history of genodermatoses, syphilis and HIV-infection can be connected to the diagnosis of OLK.
Profession	Glassblowers have been found to have an increased risk of developing OLK, with no other occupations showing similar relevance. This can be an indicator when diagnosing OLK.
Smoking habits	OLK lesions are commonly seen in tobacco users.
Symptoms	Normally asymptomatic, but it may show symptoms such as pain or itching. Non-homogenous types are more prone to be symptomatic.

Onset of disease	Most, if not all leukoplakias are of slow onset, usually over several months or years.
Course of the disease	Regarded as a stable condition, with no periods of remission or exacerbation.
Size	Historically, a minimum size of 0.5 cm in diameter was required for a lesion to be diagnosed as OLK. However, this criterion has since been removed, and lesion size is no longer considered a relevant factor in the current diagnostic approach.
Colour	The coloration of OLK lesions can range from white to a combination of red and white. This coloration is essential for the diagnosis.
Texture	Textures can range from smooth to wrinkled and may even appear wart-like in cases of proliferative verrucous leukoplakia. Indurations upon palpation are typically observed in non-homogeneous lesions, while homogeneous lesions usually lack this feature. The presence of ulceration is not typical for leukoplakia and could indicate the potential for malignancy.

In early stages, the first step in treatment of OLK is eliminating its contributing factors. Moreover, in cases with moderate to severe dysplasia and in signs of carcinoma development, surgical excision or laser should be the elected treatment, specifically in lesions on ventral and lateral borders of tongue, soft palate, floor of mouth and oropharynx (1,24). Therefore, surgical removal is the treatment of choice in erythroplakia and proliferative verrucous leukoplakia as they show more dysplasia compared to the homogenous lesions (3).

5.1.6 Potential for Malignant Transformation

OLK is considered particularly dangerous and clinically significant due to its potential for malignant transformation (2). A study from 2017 by Carrard VC et al. (11)

states that if leukoplakia is not malignant at the initial visit, the annual risk of malignant transformation is approximately 2-3%, whereas, a study from 2023 by Mohammed et al. (3) suggest that the overall malignancy transformation rate ranges from 0.1% - 17.5% (3,11). Recent studies have identified specific parameters that can help predict factors increasing the risk of malignancy (2).

First of all, the size of the lesions influences the risk of malignization. Lesions with a size >200 mm² has a 4.10 higher chance of malignization (24). Furthermore, the non-homogenous clinical type including speckled and nodular (erythroplakia), as well as the verrucous proliferative variant has a higher risk of malignancy, as it includes the typical malignancy signs like undefined borders and symptoms such as pain (24). Research shows it has a 6.52 higher chance compared to the homogenous type (24).

Another important factor is the age of the patient. As mentioned, the lesion predominantly occurs in men at the age of 40 (4). Similarly, the risk of malignant transformation increases progressively with age. Yet, gender is also a significant factor. Females are more prone to malignant transformation; thus, they are more prone to the non-homogenous variant of leukoplakia. Research from 2022 (24) shows that, specifically in cases of verrucous proliferative leukoplakia, gender plays a significant role, considering that females are 2.50 more exposed to malignant transformation (24).

Although smoking is considered a risk factor of OLK appearance, and the lesions are more often seen in smokers, paradoxically, studies show that malignization is more frequent in non-smokers. One study reported a 3.20 time higher likelihood of malignancy in non-smokers (24). However, it is important to note that the study conducted by Rubert et al. (25) included a higher proportion of women, and they also established that smoking is more prevalent among men (25). In addition, the lesion sites that have shown a higher malignization prevalence are floor of the mouth, lateral and ventral tongue, and soft palate. Conversely, buccal mucosa is at lower risk. Specifically, floor of mouth and tongue has 4.48 higher chance of malignant transformation (24).

Among the key factors we also find symptomatic lesions. Studies suggest that non-homogenous are both more commonly symptomatic and more commonly prone to malignization. Nonetheless, the homogenous clinical type is the most common as well as its asymptomatic in 88,3% of the cases (25). Furthermore, it is also important to mention the histology. The higher the grade of epithelial dysplasia shown in histological samples, the higher the risk of malignancy. No dysplasia, mild dysplasia and moderate or severe dysplasia. Studies show that the presence of dysplasia was linked to malignant transformation. Thus, in the same study stated that their research revealed that the majority (65.7%) showed no epithelial dysplasia. Among those lesions that exhibited dysplasia, mild dysplasia was the most prevalent, accounting for 23.8%. Moreover, the proportion of lesions with moderate dysplasia (18.4%) and severe dysplasia (5.3%) was notably higher in the non-homogeneous OL lesions. Severe dysplasia was notably more common in patients with non-homogeneous lesions, occurring in 5.3% of cases ($p = 0.00$) (25). As a conclusion there is no fully reliable predictor of malignant transformation established yet, although these parameters can be useful in malignization prediction (25).

Table 2 displays nine clinical warnings to consider in a patient diagnosed with OLK (according to NICE guidelines (2,26):

Table 2: Signs of lesion malignization

1.	Non-homogenous OLK, specifically erythroplakia
2.	Exophytic growth or lumpy appearance
3.	Non healing ulcers lasting >2 weeks, yellow presentation with red rolled borders
4.	Lesion induration
5.	Adhesion of tissue planes
6.	Tooth mobility (with absence of periodontal disease)
7.	Impaired socket healing after tooth extraction

8.	Pathological fractures
9.	Cervical lymphadenopathy

5.2 Oxidative stress and antioxidant defense mechanisms

Free radicals are a reactive species derived from oxygen, nitrogen, or sulfur, characterized by an unpaired electron in their outer shell (27). The free radicals are generated through normal metabolic processes such as respiration, food digestion, drug and alcohol metabolism, and fat breakdown for energy production (20). These physiological activities serve as natural sources of free radicals in the body (27). These species fall into two categories: radical or non-radical (molecules or ions), and they exhibit varying degrees of reactivity (28). These molecules play a crucial role in the body facilitating signal transmission and supporting the immune system.

In physiological concentrations reactive oxygen species (ROS) contributes beneficially by acting as signalling molecules in redox signalling. They also play a crucial role in intracellular destruction of bacteria through phagocytosis, specifically granulocytes and macrophages. However, at high concentrations the homeostasis between ROS and antioxidants is disrupted leading to a condition called oxidative stress (29), in which the ROS can cause oxidative damage to various biomacromolecules including DNA, lipids, proteins, and carbohydrates (28,30). They cause damage to macromolecules in cells, triggering a harmful cascade of events, disrupting cell membranes, inactivating major enzymes, interfering in important cellular processes, and inhibiting cell division. Which can all lead to disease development. Oxidative damage is frequently connected to various diseases (27), as well as cancer, senescence and neurodegenerative disorders (28). Therefore, the elimination and generation of ROS must remain balanced with the antioxidant defense system, to avoid the occurrence of oxidative damage (28).

Measuring ROS directly is nearly impossible due to their short lifespan (28,29). Therefore, products of oxidative stress are often measured instead, these are usually referred to as biomarkers. Thus, measuring the concentrations of ROS by-product and

protective antioxidants can be helpful in determining specific diseases as well as maintai ning healthy conditions of the human body (20,28,30).

ROS include superoxide anion (• O_2^-), hydroxyl radical (OH•), singlet oxygen ($^1O_2$), peroxyl radical (ROO•), ozone (O_3), hydrogen peroxide (H_2O_2), nitric oxide (• NO), peroxynitrite anion ($ONOO^-$), and hypochlorous (-bromous) acid (HOCl, HOBr). Although • NO and $ONOO^-$ are technically classified as reactive nitrogen species (RNS), they are often considered ROS due to their oxygen-containing nature. H_2O_2 is a non-radical molecule and is a more stable and diffuse form of ROS. It displays selective reactivity towards cysteine residues in proteins and, at low nanomolar concentrations, it plays a role in cellular signalling pathways (31). More specifically, ROS is produced by the mitochondrial respiratory chain and by enzyme catalysed reactions involving NADPH oxidase (NOX), xanthine oxidase, nitric oxide synthase (NOS), arachidonic acid and metabolizing enzymes including cytochrome P450 enzymes, lipoxy genase and cyclooxygenase (20).

Due to the absence of an electron in their outermost shell, ROS exhibit high reactivity and actively seek electron donors. Antioxidants function as electron donors, without being destabilized in the process, thereby maintaining their structural integrity (31). Oxygen is the most common oxidizing agent and plays a crucial role in energy production during cellular respiration. Its reduction is essential for life, enabling the efficient conversion of nutrients into energy through processes such as the electron transport chain in mitochondria. When ROS acquire electrons from antioxidants or cellular components, a redox reaction occurs, wherein the ROS undergoes reduction while the donor molecule is oxidized (20,27,30).

The body produces intrinsic antioxidant enzymes e.g. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione reductase (GR) and peroxiredoxins (PRDXs) and antioxidant molecules such as glutathione (GSH), coenzyme Q, ferritin and bilirubin. While we also acquire dietary antioxidants such as vitamin A, C and E from sources such as vegetables, herbs, spices and fruits, which are known for

their high polyphenol content (28). SOD are considered the first antioxidant enzymes able to dismutate two $O_2^{\cdot -}$ anions into H_2O_2 and O_2. In human cells there are three forms of SOD expressed: copper-zinc SOD (CuZnSOD) located in the cytoplasm, manganese SOD (MnSOD) in the mitochondria and extracellular SOD (29,31). A study by Li et al. (32) states that animals lacking CuZnSOD or MnSOD are at elevated risk of various cancer types (31,32).

CAT is a heme enzyme that catalyses the reaction that converts two molecules of H_2O_2 to O_2 and two molecules of H_2O which is accountable for the detoxification of various phenols, alcohols and H_2O_2 (31). Several epidemiological studies have researched the relation between mutations of CAT and human cancer cells, but the results are contradictory. Decreased CAT activity has been found in both blood and tissue samples of different cancer types, namely breast, oral and pancreatic cancers. Whereas other studies found a correlation between higher levels of CAT and other cancer types such as breast cancer and colorectal carcinoma. Therefore, the role of CAT is complex and not fully understood (31).

Moreover, PRDXs are considered among the most important antioxidant enzymes as they balance the production of cellular H_2O_2 which is crucial for cell signalling and metabolism. Several studies have shown that the overexpression of PRDXs could either inhibit or promote cancer development (27). On one hand, PRDX1 and PRDX5 has tumour-suppressive roles in breast cancer and on the other hand, it is also associated with the promotion of oral, esophageal, lung, hepatocellular and pancreatic carcinoma. Several studies have demonstrated that the elevated expression of PRDX1, PRDX2 and PRDX3 plays a crucial role in many cases of drug resistance, thus its commonly studied as a treatment for cancer. In contrast, PRDX3, PRDX4 and PRDX6 participate in tumor promotion in cancer (31,33).

GSH plays a key role in antioxidant defense in which it detoxicates xenobiotics and participate in many metabolic processes such as the protein and nucleic acid synthesis (20,34). A loss of GSH or a decrease in the glutathione/glutathione disulphide

(GSH/GSSG) ratio leads to increased susceptibility to oxidative stress and carcinogenesis. Elevated GSH levels enhance the antioxidant capacity of many cancer cells, boosting their resistance to oxidative stress (34).

5.2.1 Relation between oxidative stress and oral leukoplakia

Studies suggest a significant relation between OLK and oxidative stress. Although the aetiology of OLK is not completely agreed upon, there are various factors that are thought to be the cause (3,22). Well-documented risk factors include tobacco use, alcohol consumption, and areca nut, which are all external sources of ROS(33). Additionally, other factors have been identified, such as chronic irritation of the oral mucosa. Chronic irritation can stem from poor oral hygiene, ill-fitting dental restorations, or prostheses, as well as infections. For example, HPV, fungal infections (e.g., *Candida*), bacterial infections, and sexually transmitted lesions are all associated with leukoplakia. Nutritional deficiencies, viral infections, hormonal disturbances, and ultraviolet exposure also contribute to the risk. Lastly, oral galvanism, though considered controversial due to a lack of scientific evidence supporting its significance (3,21).

Chronic irritation, often a source of pro-oxidants, combined with nutrient deficiencies, which reduce the availability of antioxidants, can result in oxidative stress due to an imbalance between these opposing systems. Antioxidants act as electron donors to neutralize pro-oxidants, maintaining cellular homeostasis (28). While pro-oxidants are naturally produced during normal physiological processes, such as immune defense mechanisms, excessive exposure to external pro-oxidant sources can overwhelm the antioxidant defense system, leading to oxidative damage (33).

Oxidative damage affects lipids (lipid peroxidation), proteins, and nucleic acids, causing structural and functional alterations. Damage to cellular DNA may result in mutagenesis, while protein oxidation can impair enzymatic and structural protein functions. Similarly, lipid peroxidation compromises cell membrane integrity, which can lead to cellular dysfunction or apoptosis (21). These processes collectively contribute to pathological changes and may increase the risk of malignant transformation in

conditions such as OLK.

The accumulation of ROS creates a favourable environment for the development of premalignant lesions, which, if continuously exposed to high ROS levels, may progress to malignant transformation, resulting in OSCC. One of the risk factors for malignant transformation is a lesion size exceeding 200 mm^2 (35) and long-standing lesions, indicating that prolonged exposure to external ROS not only increases the risk of OLK but also its potential for malignant transformation. Therefore, oxidative stress appears to play a crucial role in the onset of OLK, suggesting that early antioxidant intervention could be effective in both prevention and treatment.

6. JUSTIFICATION AND HYPOTHESIS

6.1 Justification

Oxidative stress is an important factor in the appearance and progression of various critical diseases concerning the nervous, cardiovascular, and respiratory system, as well as cancer development and more (28,30,36).

Early diagnosis of cancer is crucial to limit the progression of the disease and improve the patient's prognosis. Early detection enables targeted treatment interventions that not only increase the chance of cure, but also reduce the burden of the disease on the patient's quality of life and health. This underscores the importance of regular screening and vigilance for early symptoms to intervene at a time when treatment options are most effective. Therefore, research into how cancer develops and the chemical mechanisms that cause precancerous lesions to occur is very important, to be able to diagnose them as early as possible.

Although various studies have been done on OLK (23), there remains a lack of clear definition regarding the condition, as well as limited knowledge about the biomarkers that may contribute to the development of the lesion. Diagnosis of OLK is based on exclusion of other similar presenting lesions together with the experience of the clinician, unexperienced clinicians are encouraged to derivate the patient to oral pathology specialists (1,4,7,23).

Identifying OLK patients at risk of developing OSCC is challenging due to the lack of biomarkers that can predict malignant transformation, hindering effective clinical management (1,37). Because the diagnosis can be challenging, broader knowledge of oxidative stress biomarkers would be highly valuable for both the medical and dental communities. This systematic review will provide a summary of various biomarkers and explore the relationship between the precancerous lesion OLK and oxidative stress, locally and systemically.

Considering the points discussed, a systematic review of the literature evaluating the effects of oxidative stress in patients affected by OLK compared to healthy subjects and analysing its action at the systemic and local levels, was regarded as necessary and reasonable.

6.1.1 SDG, objective 3 – Good health and well-being

This systematic review aligns with the United Nations Sustainable Development Goal number 3 (Good Health and Well-Being) through its focus on oral health, which is an integral component of overall health and quality of life. By addressing the relevance of oxidative stress and antioxidant treatments in relation to the premalignant lesion OLK. The review contributes to advancing preventive and therapeutic strategies that promote better health outcomes. This aligns with the goal's emphasis on reducing disease burden and ensuring access to effective, evidence-based healthcare solutions. Furthermore, by synthesizing knowledge to improve understanding and treatment of oral diseases, the review supports a more sustainable and efficient way to diagnose and apply preventative measures.

6.1 Hypothesis

Although the exact aetiology of OLK is not fully understood, it is believed to be strongly influenced by factors such as tobacco smoking and long-term alcohol consumption, both of which generate free radicals (5). Based on this understanding, the following hypothesis is proposed: OLK is associated with increased levels of oxidative stress.

7. OBJECTIVES

The objective of this study is to generate comprehensive and robust evidence, not only substantiating the presence of oxidative stress in patients with OLK but also advancing research focused on the development of therapeutic interventions to prevent and counteract oxidative stress.

7.1 General objectives

To evaluate the presence of oxidative stress by measuring its biomarkers in saliva, blood and tissues in patients affected by OLK in comparison with healthy control patients.

7.2 Specific objectives

1. To evaluate the increase of oxidative markers in patients with OLK.
2. To evaluate the decrease in antioxidant defenses in patients with OLK.

8. MATERIALS AND METHODS

This systematic review was conducted through the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analysis) guideline statement (38). This checklist provides a comprehensive set of recommendations aimed at enhancing the quality and transparency of systematic review and meta-analysis reports. It comprises 27 key checkpoints that address critical sections such as the title, abstract, introduction, methods, results, and discussion.

Additionally, it includes a flowchart illustrating the process of selecting studies for inclusion in the review. The primary objective is to support the critical evaluation and reproducibility of systematic reviews and meta-analyses by readers, editors, and reviewers. Furthermore, the review has been registered in the international prospective registry of systematic reviews, PROSPERO, under the registration number CRD42025644565.

8.1 Identifying the investigation question (PICO)

To carry out the search for articles about oxidative stress related to OLK, published from 2000 until 2024, the following databases were used: Medline-PubMed (United States National Library of Medicine), Scopus and Web of Science.

Based on the chosen information sources, the aim was to address the following research question: “Is there any association between variations in oxidant and antioxidant levels and OLK?”

Based on the objectives of this systematic review, the research question focuses on the following components:

- **P** (Population): Patients with OLK
- **I** (Intervention): Measurement of oxidative and antioxidant biomarkers
- **C** (Comparison): Healthy controls

- **O** (Outcome): Concentrations of oxidative stress and antioxidants biomarkers from patients with OLK and healthy controls

8.2 Eligibility criteria

Inclusion criteria

- Type of study: Observational studies (Cohort, Case-Control studies, and Cross-sectional studies), experimental studies (Randomized control trials), studies written in Spanish or English, articles published from 2000 to 2024.
- Type of patients: Patients with OLK and healthy patients, human adult patients
- Type of intervention: Measurements of oxidative stress compared to antioxidant levels, biomarkers of oxidative stress present in presence of OLK lesion.
- Type of control: Healthy controls without OLK or other premalignant lesions.
- Type of result variables: Measurement of biomarkers, oxidants, and antioxidants, the association between oxidative stress and OLK, oxidative stress as an etiopathogenic factor in OLK.

Exclusion criteria

- Type of studies: Clinical trials, systematic reviews, articles that does not distinguish between the oral precancerous lesions, preclinical studies, studies that are not done in humans, animal or in vitro studies, studies published before year 2000, studies done in other languages than English or Spanish, studies with less than 5 patients.
- Type of patients: Paediatric patients, patients without a confirmed diagnosis of OLK or those with other oral lesions unrelated to premalignant conditions, patients with underlying HIV diagnosis or other immunosuppressive diseases.

8.3 Information sources and search strategies

A comprehensive search was performed across databases including PubMed/MEDLINE (Medical Literature Analysis and Retrieval System Online), Web of Science, and Scopus to identify relevant studies. The Boolean operator "AND" was

applied to ensure the inclusion of both oxidative stress and oral leukoplakia in the results, while "OR" was used to integrate synonyms and related terms, broadening the search scope. The search strategies for the different databases are presented in table 3.

Table 3: Search strategy

Database	Search strategy
PubMed	("leukoplakia, oral"[MeSH Terms] OR ("leukoplakia"[All Fields] AND "oral"[All Fields]) OR "oral leukoplakia"[All Fields] OR ("oral"[All Fields] AND "leukoplakia"[All Fields]) OR "OLK"[All Fields]) AND ((("oxidative stress"[MeSH Terms] OR ("oxidative"[All Fields] AND "stress"[All Fields]) OR "oxidative stress"[All Fields]) AND ("biomarker s"[All Fields] OR "biomarkers"[MeSH Terms] OR "biomarkers"[All Fields] OR "biomarker"[All Fields])) OR ("oxidability"[All Fields] OR "oxidable"[All Fields] OR "oxidant s"[All Fields] OR "oxidants"[Pharmacological Action] OR "oxidants"[MeSH Terms] OR "oxidants"[All Fields] OR "oxidant"[All Fields] OR "oxidate"[All Fields] OR "oxidated"[All Fields] OR "oxidates"[All Fields] OR "oxidating"[All Fields] OR "oxidation"[All Fields] OR "oxidations"[All Fields] OR "oxidative"[All Fields] OR "oxidatively"[All Fields] OR "oxidatives"[All Fields] OR "oxide s"[All Fields] OR "oxides"[MeSH Terms] OR "oxides"[All Fields] OR "oxide"[All Fields] OR "oxidic"[All Fields] OR "oxiding"[All Fields] OR "oxidisability"[All Fields] OR "oxidisable"[All Fields] OR "oxidisation"[All Fields] OR "oxidise"[All Fields] OR "oxidised"[All Fields] OR "oxidiser"[All Fields] OR "oxidisers"[All Fields] OR "oxidises"[All Fields] OR "oxidising"[All Fields] OR "oxidization"[All Fields] OR "oxidize"[All Fields] OR "oxidized"[All Fields] OR "oxidizer"[All Fields] OR "oxidizers"[All Fields] OR "oxidizes"[All Fields] OR "oxidizing"[All Fields]) OR ("react oxyg species apex"[Journal] OR "ros"[All Fields]) OR ("reactive oxygen species"[MeSH Terms] OR ("reactive"[All Fields] AND "oxygen"[All Fields] AND "species"[All Fields]) OR "reactive oxygen species"[All Fields]) OR (("antioxidant s"[All Fields] OR "antioxidants"[Pharmacological Action] OR "antioxidants"[MeSH Terms] OR "antioxidants"[All Fields] OR "antioxidant"[All Fields] OR

	"antioxidating"[All Fields] OR "antioxidation"[All Fields] OR "antioxidative"[All Fields] OR "antioxidatively"[All Fields] OR "antioxidatives"[All Fields] OR "antioxidizing"[All Fields]) AND "status"[All Fields]) OR ("ieee trans affect comput"[Journal] OR "tac"[All Fields]))
Web of Science	(oral leukoplakia OR OLK) AND (oxidative stress biomarkers OR oxidants OR ROS OR reactive oxygen species OR antioxidant status OR TAC) (All Fields) and 2024 or 2023 or 2022 or 2021 or 2020 or 2019 or 2018 or 2017 or 2016 or 2015 or 2014 or 2012 or 2010 or 2009 or 2008 or 2007 or 2006 or 2005 or 2004 or 2002 or 2001 (Publication Years) and English (Languages)
SCOPUS	TITLE-ABS-KEY ("oral leukoplakia" AND ("oxidative stress" OR oxidants OR ros OR "reactive oxygen species" OR "nitrosative stress"))

8.4 Process of selecting studies

The articles to be studied in this systematic review were selected through a three-stage process. The selection of studies was carried out by two reviewers (UPCU, CEN). The first stage involved selecting articles based on their titles to exclude any publications unrelated to the research. In the second stage, studies were filtered by reviewing abstracts and selected based on study type, patient characteristics (type and number), oxidative marker measurements, intervention type, sample types assessed, and outcome variables. For the third stage, we selected the eligible articles for our review by reading them in full and conducted data extraction using a pre-established collection form to confirm study eligibility. There were no disagreements among the reviewers at any stage of the process.

8.5 Extraction of data

General variables:

- Oxidative stress: The amount of oxidative stress levels in patients with oral leukoplakia when compared to healthy patients who does not present OLK lesions. The measurements were collected using tissue samples, blood and/or saliva, with values expressed as mean ± standard deviation.

Specific variables:

- Oxidative markers: All the main biomarkers of oxidative stress were collected in order to measure the presence of oxidative stress in patient with and without oral leukoplakia lesions (8-ISO, 8-OHdG, MDA, NO2-, NO3-, TNO2-, UA, ROS, RNS)

- Antioxidant markers: All the key biomarkers of antioxidant defense system, crucial for detecting the presence of oxidative stress (E-SOD, GPx, GR, GSH, GSSG, SOD, SOD2, TAC, tGSH, GLRX2, TXN2, Vitamin C (ascorbic acid), Vitamin E (tocopherol)

8.6 Bias assessment

The Newcastle-Ottawa guidelines (39) were used to assess the quality of case-control and cohort studies. These guidelines were developed through a collaboration between the University of Newcastle in Australia and Canada. The assessment is done through a “star system” that evaluate the studies through three check points, which are the following: selection of the study groups, the comparability of the groups and the ascertainment of either the exposure or outcome of interest for case-control or cohort studies, respectively. In total there are eight questions to be answered and the maximum possible score is nine stars. The publications were considered as «low risk of bias» if they met a star score >6 and «high risk of bias» in the cases with scores ≤ 6.

In addition, the AXIS tool was used to critically appraise cross-sectional studies (40). AXIS (Appraisal tool for Cross-Sectional Studies) is a standardized instrument designed to assess the quality and reliability of observational cross-sectional research. It focuses on key elements such as clarity of study objectives, appropriateness of study design, risk of bias, and the validity of the conclusions. The tool consists of 20 questions that can be answered with "yes", "no", or "don’t know", accompanied by optional comments. Although the full tool contains 20 items, in this review a selection of the most relevant criteria was applied to evaluate aspects such as study design, sample representativeness, and risk of bias. Unlike other tools, AXIS does not provide a numerical

scoring system; rather, the overall assessment is based on a qualitative judgment of the answers to the checklist.

For non-randomized studies that were not case-control, cohort, or purely cross-sectional, the ROBINS-I tool (Risk Of Bias In Non-randomized Studies of Interventions) was applied (41). This tool was developed by the Cochrane Bias Methods Group and is structured to assess risk of bias across seven domains: confounding, selection of participants, classification of interventions, deviations from intended interventions, missing data, measurement of outcomes, and selection of the reported result. The final judgment for each study is categorized as “low”, “moderate”, “serious” or “critical” risk of bias, depending on the level of concern in each domain. The ROBINS-I tool is particularly suitable for evaluating studies that aim to assess the effect of interventions without using randomization.

8.7 Synthesis and certainty assessment

In order to analyse the collected data, a methodological approach combining qualitative and quantitative methods will be used. In addition, a flowchart has been developed in line with the PRISMA guidelines , showing the results based on the inclusion and exclusion criteria for each search in the scientific databases. Furthermore, a table will be summarised with the selected studies, specifying the PICO strategy and the results are synthesised.

9. RESULTS

9.1 Study selection: Flow chart.

During the initial search in total 288 articles were obtained. Medline-PubMed (n= 155), Web of Science (n=70), SCOPUS (n= 63) and manual search (n=2). Out of these, 95 were duplicated. Furthermore, 153 articles were excluded based on title and 26 were excluded based on abstract. Then the full text of the remaining 22 articles was obtained and evaluated for its eligibility. Out of these seven were excluded, four of them for not dividing between the different premalignant lesions (PML), one because it was an in vitro study which does not align with the inclusion criteria of this systematic review. Furthermore, one article was excluded because it only measured the effect of the treatment and lastly one was excluded because it didn't include comparison to healthy controls. The excluded articles are found in table 4, with author and year together with their reason for exclusion. Finally, 15 of the articles met the inclusion criteria and were included in the systematic review (Figure 1).

Agreement between reviewers concerning study inclusion yielded k-values of 0.92 for titles and abstracts, and 1.0 for full-text articles, indicating "substantial" and "perfect" agreement, respectively, based on the criteria by Landis and Koch (42).

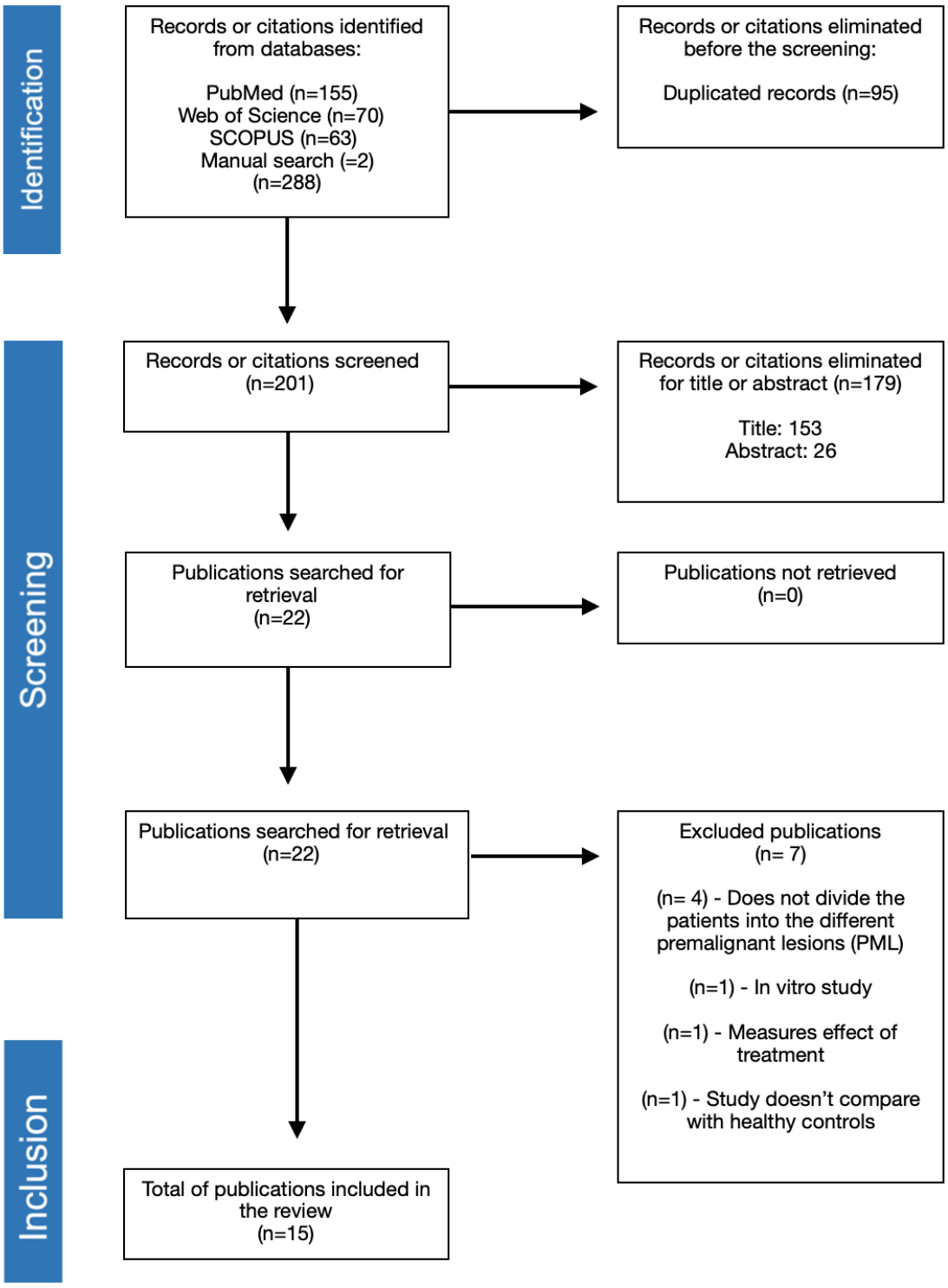


Figure 1: PRISMA flow chart, process of article selection for the systematic review.

Table 4: Excluded publications and their reasoning.

Author and year	Exclusion reason
(Vlkova et al., 2012) (43)	Does not differentiate between the different PML
(Shahi et al., 2020) (44)	Does not differentiate between the different PML
(Gregorczyk-Maga et al., 2019) (45)	Does not differentiate between the different PML
(Shetty et al.,2014) (46)	Does not differentiate between the different PML
(Yang et al., 2024) (47)	In vitro study
(Ding et al., 2022) (48)	Measures only the effect of treatment
(Srivastava et al., 2019) (49)	Study doesn´t compare with healthy controls

PML: Premalignant lesions

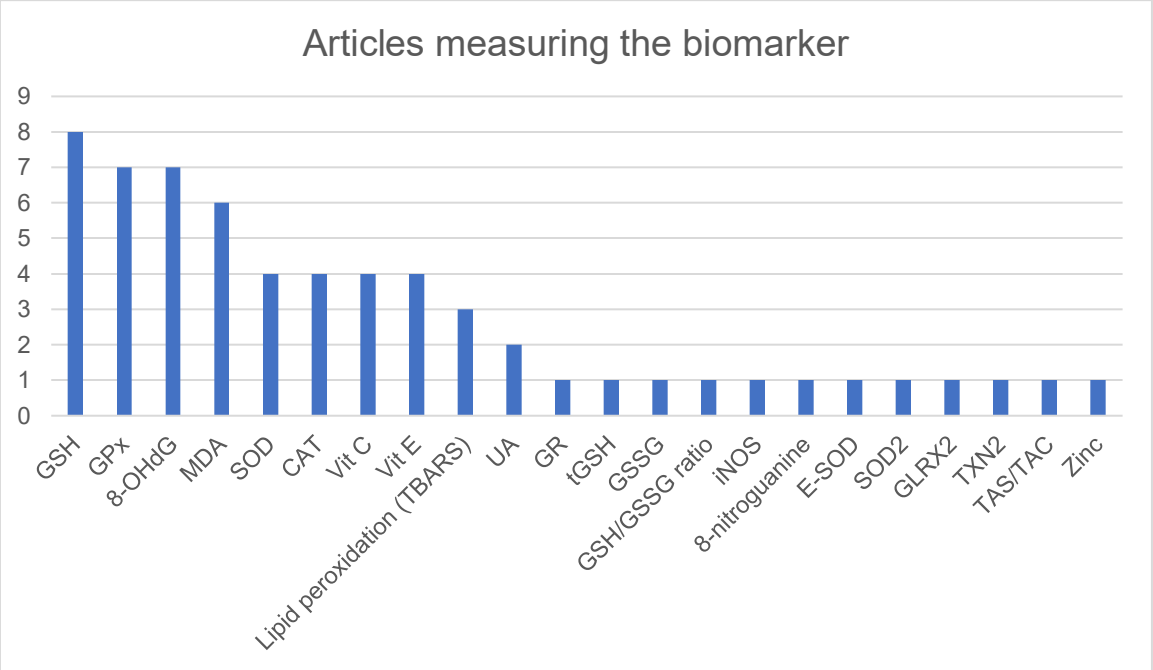
9.2 Analysis of the characteristics of the revised studies

Out of the 15 articles chosen four studies were using tissue samples (50–53), seven studies used serum samples (54–60), two studies used saliva samples (61,62) and lastly two articles used both saliva and blood samples (63,64). In total 1 315 patients, and 22 different markers were included in this systematic review. Considering the 15 articles utilized there were eight case-control studies, five cross-sectional studies, one interventional study and one cohort study.

Across the different sample types, the oxidative stress biomarker GSH was the most frequently assessed biomarker, measured in eight articles (51,52,54,57–59,62,63) four of them were using serum samples (54,57–59), two of them tissue (51,52), one saliva (62) and one used both serum and saliva (63). 8-OHdG was evaluated in six articles (19,50,53,61,62,64,65), while GPx (51,52,54,55,58,62) and MDA were each measured in six. SOD and CAT were assessed in four articles, as were vitamins C and E. Lipid peroxidation was reported in three articles, and uric acid in two. 8-nitroguanine was measured in one article (50). Several other biomarkers were also measured in only one article, including TXN2, GLRX2, SOD2, E-SOD, GSH/GSSG ratio, tGSH, iNOS and GR.

The table 5 illustrates how many articles each biomarker was measured in.

Table 5: Number of articles measuring the specific biomarker.



Six of the studies also took into consideration and noted the habits of the patients such as cigarette smoking and alcohol ingestion (19,55,59,60,62,65). Nevertheless, some articles also divided leukoplakia into stages, where the oxidative damage increased as the disease progressed as in the study by Kumar Chandan Srivastava et al. (2013) (51) where they divide the OLK patients into stages I-IV. In that specific study they used tissue samples and concluded that a significant decrease in thiobarbituric acid reactive substances (TBARS) levels ($P < 0.05$) was observed only in stage IV leukoplakia patients. Among the antioxidant enzymes, GSH and GPx were the only ones to show a significant reduction ($P < 0.001$) across the different disease stages.

In table 6 the 15 articles are plotted together with their type of study, sample type, measuring method and number of participants.

Table 6: Distribution of articles included in the systematic review with the corresponding author with year, sample type utilized, type of study, measured variables, their measuring method, and patient group size.

Author and year	Sample type	Type of study	Age	Biomarkers measured	Measuring method
(Ma et al., 2005) (50)	Tissue	Case-control study	Cases: (63.9 +- 9.6 years) Controls: (67.7 +- 10.1 years)	8-nitroguanine 8-oxodG	8-Nitroguanine and 8-oxodG were detected in oral epithelium using double immunofluorescence labelling.
(Srivastava et al., 2013) (51)	Tissue	Case-control study	Age 46,20 ± 11,08 years	Lipid peroxidation, SOD, CAT, GSH and GPx	Lipid peroxidation (TBARS) and antioxidant enzymes (SOD, CAT, GSH, GPx) were analysed using spectrophotometry.
(Srivastava et al., 2016) (54)	Plasma	Case-control study	Cases: 46.20±11.08 years Positive controls: 39.55±9.22 years Negative controls: 37±7.56 years	TBARS, GSH, SOD, CAT and GPx	TBARS, GSH, SOD, CAT, and GPx were measured using standard colorimetric assays based on spectrophotometric absorbance at specific wavelengths.
(Metgud et al., 2014) (63)	Saliva and serum	Case-control study	Cases: 51.7 years Controls: 48.3 years	Lipid peroxidation, MDA and GSH	MDA (as TBARS) and GSH were measured using standard colorimetric methods, with absorbance read at 532 nm and 412 nm, respectively.
(Kaur et al., 2015) (61)	Saliva	Cross-sectional study	Age 49 ± 5.9 years	8-OHdG, MDA, Vit C and Vit E	MDA, 8-OHdG, and vitamins C/E were analyzed by TBARS, ELISA, and HPLC, respectively. ROC analysis defined thresholds; histopathology was the gold standard.

(Gurudath et al., 2012) (55)	Whole blood, serum, and erythrocyte lysate	Case-control study	Cases: 40.73±9.65 years Controls: age/sex matched	E-SOD and GPx	E-SOD and GPx activities were measured spectrophotometrically using commercial kits.
(Yadav et al., 2019) (56)	Serum	Cross-sectional study	Cases: 42.8 years Controls: 37 years	UA	UA was measured using the uricase method.
(Shetty et al., 2013) (57)	Serum	Case-control study	Age 20-65 years	GSH	Serum GSH was measured colorimetrically using the Beutler method with DTNB at 412 nm. Data were analyzed using one-way ANOVA
(**Babiuch et al., 2018)** (62)	Saliva	Case-control study (pilot study)	The median age was 59 years for cases and 51 years for controls.	TAC, SOD, GPx, GR, tGSH, GSH, GSSG, GSH/GSSG ratio, 8-OHdG and MDA	TAC: Measured by FRAP assay based on reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. Absorbance was read at 593 nm. SOD: Assayed via inhibition of epinephrine autooxidation at pH 10.2. Absorbance measured at 480 nm. GPx:Measured indirectly by GSH oxidation and NADPH consumption at 340 nm. GR: Activity assessed by monitoring NADPH oxidation to $NADP^+$ at 340 nm. tGSH: Quantified using a colorimetric kit measuring both GSH and GSSG at 405 nm. GSH: Measured by DTNB reaction producing TNB, read at 412 nm. GSSG: Calculated by subtracting GSH from tGSH. UA: Determined via enzymatic oxidation with uricase and colorimetric detection at 546 nm.

					8-OHdG: Measured using sandwich ELISA with monoclonal antibodies and colorimetric detection at 450 nm. MDA: Determined by competitive ELISA using monoclonal anti-MDA antibody, read at 450 nm.
(Sachdev et al., 2022) (58)	Plasma, serum, and erythrocyte lysate	Cross-sectional study	Age 20-60 years	Lipid hydroperoxide, MDA, SOD, GPx, CAT, GSH, Vit C and Vit E	The levels of lipid peroxidation products, antioxidants, and NO products were determined by colorimetric methods.
(Bose et al., 2011) (59)	Plasma	Cross-sectional study	Age 28-40 years	Zinc, TAS, Vit A, Vit C, Vit E and GSH	Plasma levels of β-carotene, vitamins C and E, GSH, TAS, and zinc were measured using standard colorimetric methods. Absorbance was read at specific wavelengths, and group comparisons were analyzed using Student's t-test.
(Rai et al., 2010) (64)	Saliva and serum	Pre-post interventional study	Age 17-50 years	MDA, 8-OHdG, Vit. E and Vit. C	MDA was measured by the TBARS method, vitamins C and E by liquid chromatography, and 8-OHdG by competitive ELISA. Data were analyzed using ANOVA and Spearman correlation ($p < 0.05$).
(Kuthoor et al., 2023) (60)	Plasma	Case-control study	Cases: 50.6 ± 10.1 years Controls: 47.4 ± 10.9 years	SOD	SOD levels were measured and analyzed using one-way ANOVA, Student's t-test, and Pearson's Chi-square test, as appropriate.
(Banerjee et al., 2020) (52)	Tissue	Cross-sectional study	Cases: 52.69±5.35 years Controls: age/sex matched	SOD2, CAT, GLRX2, GSH, GPx and TXN2	Mitochondria were isolated from precancerous oral tissues by differential centrifugation and validated by immunoblotting with specific cellular and mitochondrial markers. Control tissue was obtained via vestibuloplasty.

(Barros et al., 2022) (53)	Tissue	Cohort study	Cases: 60 ± 13,3 years Controls: -	8-OHdG	8-OHdG expression in oral mucosa was assessed by immunohistochemistry.

GSH: glutathione; GPx: glutathione peroxidase; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; Vit C: vitamin C (ascorbic acid); Vit E: vitamin E (α-tocopherol); TBARS: thiobarbituric acid reactive substances; UA: uric acid; GR: glutathione reductase; tGSH: total glutathione; GSSG: oxidized glutathione; iNOS: inducible nitric oxide synthase; 8-nitroguanine: a nitrative DNA lesion; E-SOD: extracellular superoxide dismutase; SOD2: mitochondrial superoxide dismutase (Mn-SOD); GLRX2: glutaredoxin 2; TXN2: thioredoxin 2.

9.3 Evaluation of methodological quality and risk of bias

There were three different bias methods utilized in this systematic review, for the case-control and cohort studies the Newcastle-Ottawa (NOS) scale was used. This tool assesses studies based on three domains: selection of study groups, comparability between groups, and ascertainment of either exposure or outcome. In this systematic review there were eight case-control studies (50,51,54,63) and one cohort study (53). Each study can receive up to nine stars, with scores above six indicating a low risk of bias, and scores of six or lower considered high risk. In this review, all included studies asses by NOS were rated as low risk of bias, except for one study which received a score of six. Out of the articles evaluated in Newcastle-Ottawa almost all of them were assessed low risk, with the lowest rating being six (50), which indicates a higher risk compared to articles rating nine stars (Table 7 and 8).

Furthermore, for the five cross-sectional studies (52,56,58,59,61) Appraisal tool for Cross-Sectional Studies (AXIS) was utilized. AXIS is a critical appraisal tool developed to evaluate the quality and methodological rigor of cross-sectional research. While the full tool contains 20 items, a focused subset of seven key items was selected for this review to evaluate aspects most relevant to risk of bias. These included: clearly defined inclusion criteria, sample description, exposure measurement, use of objective criteria to measure the condition, identification and handling of confounders, and the use of appropriate statistical analysis. Each item was assessed qualitatively as “yes,” “no,” or “unclear.” The results indicate that while most studies reported inclusion criteria and sample descriptions adequately, several showed weaknesses in confounder control and statistical analysis, which may affect internal validity (Table 9)

Additionally, one non-randomized interventional study (64) included in the review was assessed using the ROBINS-I tool, which is designed for evaluating risk of bias in non-randomized studies of interventions. This study was found to have a serious risk of bias, primarily due to lack of a control group and absence of adjustment for confounding variables. While it contributes exploratory insight, its findings should be interpreted with caution and considered a limitation in the overall evidence synthesis (Table 10)

Table 7: Risk of bias assessment with Newcastle-Ottawa scale in case-control studies

Articles	Case definition	Representativeness	Selection of controls	Control definition	Comparability (most important factor)	Comparability (any other variable)	Ascertainment of exposure	Same method for both groups	Dropout rate	Total
Ma et al. (2005)	★	★	★	–	–	–	★	★	★	6
Srivastava et al. (2013)	★	★	★	★	–	★	★	★	★	8
Srivastava et al. (2016)	★	★	★	★	★	★	★	★	★	9
Metgud et al. (2014)	★	★	★	★	★	–	★	★	★	8
Gurudath et al. (2012)	★	★	–	★	★	–	★	★	★	7
Shetty et al. (2013)	★	★	★	★	★	–	★	★	★	8
Babiuch et al. (2018)	★	★	★	★	★	★	★	★	★	9
Kuthoor et al. (2023)	★	★	★	★	★	–	★	★	★	8

Table 8: Risk of bias assessment with Newcastle-Ottawa scale in cohort studies.

Article	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that the outcome of interest was not present at start	Comparability (main factor)	Comparability (additional factors)	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	Total
Barros et al. (2022)	★	★	★	★	★	_	★	★	_	7

Table 9: Bias assessment of cross-sectional studies with AXIS.

Article	Criteria clearly defined	Sample description	Valid and reliable exposure measurement	Use of objective criteria to measure condition	Identification of confounding factors	Strategies to deal with confounders	Appropriate statistical analysis
Kaur et al., (2015)	yes	yes	no	yes	?	no	no
Yadav et al., (2019)	yes	yes	no	yes	yes	no	no
Sachdev et al., (2022)	yes	yes	no	yes	yes	no	?
Bose et al., (2011)	yes	yes	?	yes	yes	no	no
Banerjee et al., (2020)	yes	yes	no	yes	yes	no	no

?:unclear

Table 10: Bias assessment of interventional studies with ROBIN-I tool (by Cochrane).

Study: Rai et al. (2010)

Domain	Risk Level	Justification
Bias due to confounding	Serious	No adjustment for smoking, age, sex, or other potential confounders.
Bias in selection of participants into the study	Moderate	Selection criteria described but unclear representativeness.
Bias in classification of interventions	Low	All patients received curcumin; no misclassification possible.
Bias due to deviations from intended interventions	Low	No deviations from planned intervention reported.
Bias due to missing data	Low	No relevant missing outcome data reported.
Bias in measurement of outcomes	Moderate	No mention of blinding; outcome measures could be influenced by knowledge.
Bias in selection of the reported result	Moderate	Full results reported, but no protocol registration noted.

9.4 Summary of results

9.4.1 Analysis of oxidative markers.

In this systematic review 15 articles were selected to study the presence of oxidative stress in patients affected by OLK. The article's utilized serum, saliva, and tissue samples to assess presence of oxidative stress. The biomarkers identified across the included studies were: GSH, GPx, 8-OHdG, MDA, SOD, CAT, Vit C, Vit E, Lipid peroxidation/TBARS, UA, GR, tGSH, GSSG, GSH/GSSG, iNOS, 8-nitroguanine, E-SOD, SOD2, GLRX2 and TXN2. A P-value of less than 0.05 was considered statistically significant in all comparisons and correlations to determine elevated levels of oxidative stress.

9.4.2 Analysis done in serum, plasma, erythrocyte lysate and blood samples.

A total of nine articles (54–60,63,64) used serum samples for biomarker analysis. Five of these nine articles measured GSH (54,57,59), and the results that were found for cases was the following 40.15±3.09 (54), 21.47 ± 3.35 (63), 01.04 ± 0.22 (57), 2.02±0.322 (58), 6.09±0.67 mg/L (59). In one study (54) controls were divided into positive and negative, the positive were the controls that did have tobacco chewing history and the negative were healthy patients with no history of such habits. The results for controls were the following 51.10±2.09 (negative control) and 48.93±0.86 (positive control) (54), furthermore the results in controls was 32.18 ± 5.53 (63), 1.88 ± 0.36 (57), 13.24±0.94 (58) and 10.09±0.89 mg/L (59). These findings collectively indicate that GSH concentrations are reduced in OLK cases relative to the controls.

Furthermore, three articles measured serum SOD levels (54,58,60). SOD is an antioxidant enzyme that provides cellular defense against toxic free radicals, similarly to GPx and CAT. The authors found the levels 2.09±0.08 (54), 188.45±8.54 (units/100 mg protein) (58) and 0.052±0.012 (U/ml) (60) for OLK cases and 4.70±1.26 (54), 233.64±11.89 (units/100 mg protein) (58), 0.074±0.014 (U/ml) (60) as the measurements for the healthy controls. All three studies reported decreased serum SOD levels in OLK patients compared to healthy controls, indicating a reduction in enzymatic antioxidant defense.

A total of three articles measured GPx (54,55,58), reporting levels of 19.09±0.56 (54), 2.67±1.34(58) and 21.55 (U/g Hb) (55) in the groups of cases. While controls showed significantly higher levels of 25.07±1.55 and 15.23±2.68. The latter study states that a normal level of GPx is in the ratio 27.5-73.6 U/g Hb (55). Furthermore, two of these same studies also measured the antioxidant CAT (54), gaining the results case/control: 1.37±0.08 / 3.46±0.85 (54) and 13.51±2.32/ 35.3±3.11 (58). The findings demonstrate a notable reduction in both GPx and CAT activity in leukoplakia patients in relation to healthy controls.

Out of the nine articles, one measured serum UA (56) with the results between cases and controls: 3.79±1.23 , 5.16± 0.98. A statistically significant association was observed only in the OSCC group (p=0.007). Among the three articles measuring MDA (58,63,64) the following levels were obtained for cases 3.31 ± 0.41(nmol/mL) (63), 5.68±0.322 (nmol/ ml) (58) and 1.23 (0.56) (µmol/l) (64). For controls 2.93 ± 0.79 (nmol/mL) (63), 1.96±0.145 (nmol/ ml) (58) and 0.98 (0.86) (µmol/l) (64). All of which were found statistically significant ($P < 0.05$) (63), ($P < 0.0001$) (58) and ($P < 0.001$) (64), respectively. Serum UA was measured in only one article, with lower values in OLK cases compared to controls, although statistical significance was only observed in the OSCC group. In contrast, all three studies measuring MDA reported higher levels in OLK patients than in controls, with statistically significant differences in each case.

Lipid peroxidation was measured in two of the articles (54,58), in one of them mentioned as TBARS which gave the results 2.20±0.44 for cases, with a P value < 0.001. For negative controls a 1.30±0.40 value was measured and 2.050±0.94 for positive controls. A progressive increase in mean TBARS levels was observed across the advancing stages of leukoplakia, with significantly higher values compared to both the positive and negative control groups (54). The other study got the value 467.65±17.43 for cases and 276.46±17.66 for controls. Both studies reported elevated levels of lipid peroxidation in OLK cases compared to controls, with one study also showing a progressive increase in TBARS levels across clinical stages of leukoplakia.

Additionally, three articles (58,59,64) measured both vitamin E (vit E) and C (vit C). The values found for vitamin C in the first study for cases was 0.41±0.162 (mg/dL) and for controls 2.78±0.31 (mg/dL) (58). Whereas, for vitamin E the value 0.73±0.211 (mg/dL) was found for cases and 11.74±0.566 (mg/dL) for controls (58). The two other studies found the following values for vit E cases 5.99±0.82 (mg/dL) (59) and 8.01 (1.23) (µmol/l) (64), whilst for controls the values were: 10.54±1.1 (mg/dL) (59) and 8.97 (2.34) (µmol/l) (64). For vit C the case values were 0.57±0.16 (mg/dL) (59) and 8.78 (3.12) (64) and for controls 1.08±0.16 (mg/dL) (59) and 9.05 (2.21) (µmol/l) (64). All three studies reported lower levels of both vitamin C and E in OLK patients compared to healthy controls.

Furthermore, only one article measured zinc (Zn) and the results found were statistically significant decrease in plasma Zn levels ($P<0.001$), when comparing the leukoplakia group to the controls (59). The values noted were 59.9±6.91 in leukoplakia cases and 91.2±11.8 in healthy controls. Thus, higher levels of Zn were seen in controls. One article analysed E-SOD (55), 91.52 ±19.45 (U/ml) was the value found in leukoplakia cases and the healthy controls were all in the range of 164-240 (U/ml) (55). Moreover, another article studied 8-OHdG and 2.13 (1.12) was the value for cases and 2.17 (1.45) for controls (64). Evidently, E-SOD and 8-OHdG showed reduced levels in cases.

In the table 11 and 12 the biomarkers are displayed with their corresponding values.

Table 11: Analysis results of serum/plasma/erythrocyte lysate and blood samples and their associated oxidative stress markers.

Author and year	N	GSH	SOD	CAT	GPx	E-SOD	UA	MDA
Srivastava et al. (2016) (54)	C: 20 HC: 20	C: 40.15±3.09** Negative control: 51.10±2.09** Positive control: 48.93±0.86**	C: .09±0.08* Negative control: 4.70±1.26* Positive control: 2.28±0.30*	C: 1.37±0.08** Negative control: 3.46±0.85** Positive control: 1.95±0.48**	C: 19.09±0.56** Negative control: 25.07±1.55** Positive control: 21.68±1.18**	–	–	–
Metgud et al. (2014) (63)	C: 20 HC: 30	C: 21.47 ± 3.35** HC: 32.18 ± 5.53**	–	–	–	–	–	C: 3.31 ± 0.41* HC: 2.93 ± 0.79*
Gurudath et al. (2012) (55)	C: 25 HC: 25	–	C: 91.52 (U/ml) *** HC: 199.35 (U/ml)***	–	C: 21.55(U/g Hb)*** HC: 60.46* (U/g Hb)***	C: 91.52 ±19.45 (U/ml)*** HC: 164-240 (U/ml)***	–	–
Yadav et al. (2019) (56)	C: 25 HC: 30	–	–	–	–	–	C: 3.79±1.23 HC: 5.16± 0.98	
Shetty et al. (2013) (57)	C: 25 HC: 25	C: 01.04 ± 0.22** HC: 1.88 ± 0.36**	–	–	–	–	–	–

Sachdev et al. (2022) (58)	C: 70 HC: 70	C: 2.02±0.322** HC: 13.24±0.94**	C: 188.45±8.54 (units/100 mg protein)*** HC: 233.64±11.89 (units/100 mg protein)***	C: 13.51±2.32** HC: 35.3±3.11**	C: 2.67±1.34** HC: 15.23±2.68**	–	–	C: 5.68±0.322*** HC: 1.96±0.145***
Bose et al. (2011) (59)	C: 23 HC: 23	C: 6.09±0.67* mg/L HC: 10.09±0.89 *mg/L	–	–	–	–	–	–
Rai et al. (2010) (64)	C: 25 HC: 25	–	–	–	–	–	–	C: 1.23 (0.56)** HC: 0.98 (0.86)**
Kuthoor et al. (2023) (60)	C: 29 HC: 25	–	C: 0.052±0.012 (U/ml)*** HC: 0.074±0.014 (U/ml)***	–	–	–	–	–

C: OLK cases; HC: healthy control; GSH: glutathione; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; E-SOD: extracellular superoxide dismutase; UA: uric acid; MDA: malondialdehyde; lipid hydroperoxide: lipid hydroperoxide; *:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

Table 12: Analysis results of serum/plasma/erythrocyte lysate and blood samples and their associated oxidative stress markers.

Author and year	N	Lipid peroxidation / TBARS	8-OHdG (ng/ml)	TAS	Vitamins	Zinc
Srivastava et al. (2016) (54)	C: 20 HC: 20	C: 2.20±0.44** Negative control: 1.30±0.40 Positive control: 2.050±0.94*	–	–	–	–
Metgud et al. (2014) (63)	C: 20 HC: 30	–	–	–	–	–
Gurudath et al. (2012) (55)	C: 25 HC: 25	–	–	–	–	–
Yadav et al. (2019) (56)	C: 25 HC: 30	–	–	–	–	–
Shetty et al. (2013) (57)	C: 25 HC: 25	–	–	–	–	–
Sachdev et al. (2022) (58)	C: 70 HC: 70	C: 467.65±17.43*** HC: 276.46±17.66***	–	–	**Vit E:** C: 0.73±0.211 (mg/dL)*** HC: 11.74±0.566 (mg/dL)*** **Vit C:** C: 0.41±0.162 (mg/dL)** HC: 2.78±0.31 (mg/dL)**	–

Bose et al. (2011) (59)	C: 23 HC: 23	–	–	C: 1.23±0.45*** HC: 2.47±0.43***	**<u>Vit E:</u>** C: 5.99±0.82 (mg/dL)** HC: 10.54±1.1 (mg/dL)*** **<u>Vit C:</u>** C: 0.57±0.16 (mg/dL)** HC: 1.08±0.16 (mg/dL)***	C: 59.9±6.91*** HC: 91.2±11.8***
Rai et al. (2010) (64)	C: 25 HC: 25	–	C: 2.13 (1.12)*** HC: 2.17 (1.45)***	–	**<u>Vit E:</u>** C: 8.01 (1.23) (µmol/l)*** HC: 8.97 (2.34) (µmol/l)*** **<u>Vit C:</u>** C: 8.78 (3.12) (µmol/l)*** HC: 9.05 (2.21) (µmol/l)***	–
Kuthoor et al. (2023) (60)	C: 29 HC: 25	–	–	–	–	–

C: OLK cases; HC: healthy control; TNO-2: total nitrite/nitrate; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; 8-ISO: 8-isoprostane; TAS: total antioxidant status; Vit A: vitamin A; Vit E: vitamin E; Vit C: vitamin C; antioxidant mineral zinc: zinc; GSH^{+}: total glutathione; *:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

9.4.3 Analysis done in saliva samples.

To asses oxidative biomarkers, in total four articles used saliva samples, two of them exclusively (61,62) and two articles used both saliva and serum samples, yet distinguishing their results (63,64).

MDA levels in OLK patients were measured in five articles, where the results in cases were 0.33 (0.07) (61) , 20.87 ± 1.23 (63), 8.30 (14.22) (62) and 0.36 (0.17) (64).The control groups presented the following values 0.08 (0.07) (61), 19.98 ± 0.81 (63), 2.32 (5.36) (62) and 0.11 (0.13) (64). Furthermore, three articles measured 8-OHdG, measured in (ng/ml), where the values in the group of cases were 0.36 (0.07) (61), 11.54 (8.22) (62) and 0.34 (0.24) (64). However the control group presented the following values 0.07 (0.07) (61), 8.58 (4.59) (62) and 0.11 (0.12) (64). In all the three articles, the values of the oxidative stress marker are increased compared to the control group.

Furthermore, two articles measured vitamin C and E, both measured in (µmol/l) (61,64). The numbers presented were case/control, vitamin C: 0.55 (0.13) / 1.2 (0.6) (61), 1.08 (0.98) / 1.46 (0.86) (64) and for vitamin E: 0.57 (0.16) / 1.4 (0.6) (61), 0.65 (0.31) / 0.91 (0.43) (64). Both studies showed lower levels of vitamins in OLK patients compared to healthy controls.

Yet, only one article measured TAC, SOD, GPx, GR, tGSH, GSSG, GSH/GSSG ratio and UA (62). The values were case/control, TAC: 0.74 (0.44) [mmol/l] / 0.51 (0.34) [mmol/l], SOD: 3.40 (3.92) [U/ml] / 2.36 (2.42) [U/ml] , GPx: 81.34 (22.56) [U/l] / 90.60 (18.65) [U/l], GR: 17.7 (27.48) [U/l] / 7.68 (6.47) [U/l], tGSH: 0.27 (0.26) (µmol/ml) / 0.25 (0.23) (µmol/ml), GSSG: 0.26 (0.25) (µmol/ml) / 0.23 (0.22) (µmol/ml), GSH/GSSG ratio: 0.21 (0.64) / 0.27 (0.43) and UA: 386.36 (235.96) (µmol/ml) / 256.79 (185.20) (µmol/ml). Two articles measured GSH, the values for cases were 0.01 (0.02) (µmol/ml) (62) and 8.67 ± 1.20 (63). On the other hand, the values for controls were 0.02 (0.01) (µmol/ml) (62) and 9.74 ± 0.53 (63), respectively. Compared to controls, cases showed higher TAC, SOD, GR and UA levels. Whereas for the antioxidants GPx and GSH, the levels were

lower in cases.

Significant correlations were observed among the salivary oxidative stress biomarkers. A strong positive correlation was found between 8-OHdG and MDA ($R = 0.79$, $p < 0.001$), indicating that higher levels of DNA damage were associated with increased lipid peroxidation (61). In contrast, 8-OHdG showed strong negative correlations with both vitamin E ($R = –0.79$) and vitamin C ($R = –0.77$), suggesting that antioxidant levels decline as oxidative damage increases. Similarly, MDA was negatively correlated with vitamin C ($R = –0.66$) and vitamin E ($R = –0.65$). An additional moderate negative correlation was found between vitamin E and vitamin C ($R = –0.67$). All correlations were found statistically significant ($p < 0.001$) (61).

The combined use of 8-OHdG, MDA, vitamin C, and vitamin E as salivary biomarkers demonstrated significantly higher sensitivity and specificity for distinguishing between healthy tissues and both precancerous and cancerous oral lesions, compared to the diagnostic performance of each biomarker used individually (61).

The results of the biomarker analysis done in saliva samples are presented in tables 13 and 14.

Table 13: Analysis results of salivary samples and their associated oxidative stress markers.

Author and Year	N	8-OHdG (ng/ml)	MDA (µmol/l)	Vitamins (µmol/l)	TAC (mmol/l)	SOD (U/ml)
Rai et al. (2010) (64)	C: 25 HC: 25	C: 0.34 (0.24)*** HC: 0.11 (0.12)***	C: 0.36 (0.17)*** HC: 0.11 (0.13)***	**Vit C:** C: 1.08 (0.98)*** HC: 1.46 (0.86)*** **Vit E:** C: 0.65 (0.31)*** HC: 0.91 (0.43)***	-	-
Metgud et al. (2014) (63)	C: 20 HC: 30	-	C: 20.87 ± 1.23* HC: 19.98 ± 0.81*	-	-	-
Kaur et al. (2015) (61)	C: 40 HC: 40	C: 0.36 (0.07)** HC: 0.07 (0.07)**	C: 0.33 (0.07)** HC: 0.08 (0.07)**	**Vit C:** C: 0.55 (0.13)** HC: 1.2 (0.6)** **Vit E:** C: 0.57 (0.16)** HC: 1.4 (0.6)**	-	-
Babiuch et al. (2018) (62)	C: 20 HC: 20	C: 11.54 (8.22) HC: 8.58 (4.59)	C: 8.30 (14.22) HC: 2.32 (5.36)	-	C: 0.74 (0.44) HC: 0.51 (0.34)	C: 3.40 (3.92)** HC: 2.36 (2.42)**

C: OLK cases; HC: healthy control; 8-OHdG: 8-hydroxy-2-deoxyguanosine; MDA: malondialdehyde;Vit C: vitamin C; Vit E: vitamin E;TAC: total antioxidant capacity; SOD: superoxide dismutase; *:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

Table 14: Analysis results of salivary samples and their associated oxidative stress marker.

Author and Year	N	GPx [U/l]	GR [U/l]	GSSG / GSH [µmol/l]	GSH/GSSG ratio	UA [µmol/l]
Rai et al. (2010) (64)	C: 25 HC: 25	-	-	-	-	-
Metgud et al. (2014) (63)	C: 20 HC: 30	-	-	**GSH** C: 8.67 ± 1.20*** HC: 9.74 ± 0.53***	-	-
Kaur et al. (2015) (61)	C: 40 HC: 40	-	-	-	-	-
Babiuch et al. (2018) (62)	C: 20 HC: 20	C: 81.34 (22.56), HC: 90.60 (18.65)	C: 17.7 (27.48), HC: 7.68 (6.47)	**GSH:** C: 0.01 (0.02)*** HC: 0.02 (0.01)*** **GSSG:** C: 0.26 (0.25) HC: 0.23 (0.22) **tGSH:** C: 0.27 (0.26) HC: 0.25 (0.23)	C: 0.21 (0.64)** HC: 0.27 (0.43)**	C: 386.36 (235.96), HC: 256.79 (185.20)

C: OLK cases; HC: healthy control; GPx: glutathione peroxidase; GR: glutathione reductase; tGSH: total glutathione; GSH: reduced glutathione;GSSG: oxidized glutathione;UA: uric acid; *:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

9.4.5 Analysis done in tissue samples.

In total, there were four articles using biopsied tissue samples (50–53), to measure the oxidative stress biomarkers included in this systematic review. One article measured 8-nitroguanine and 8-OHdG, and their results showed that immunoreactivities (IR) were strongly observed in epithelial cells and inflammatory cells in leukoplakia patients, whereas these IR were negative in normal mucosa (50). This article also measured inducible nitric oxide synthase (iNOS) and found its expression was strongly observed in inflammatory cells, yet weakly in epithelial cells. However, in normal mucosa little or no 8-nitroguanine formation and iNOS expression were observed. Thus, another article also measured 8-OHdG and found the results strongly positive IR in cytoplasm and negative IR in cytoplasm of controls (53).

Lipid peroxidation/TBARS was measured by one article and found the results 91.99±2.97 for cases and 127.93±2.97 for controls (51), it is important to note that the decrease in lipid peroxidation in tissue samples of OLK patients is a sign of oxidative damage. The same article also measured SOD and got the results 14.48±1.05 for the cases and 18.54±0.54 for controls (51). This article together with one other article measured the antioxidant CAT finding the corresponding results for cases: 6.36±1.10 (51) and 75.35 ±0.56 (52). Yet, for controls the results were 10.46±0.79 (51) and 98±0.32 (52). Overall, lipid peroxidation, SOD and CAT levels were consistently lower in cases compared to healthy controls.

In relation to GSH, two studies (51,52) reported the values for cases 30.43±2.90 (51) and 12.4 ± 0.432 mM (52). Nonetheless, the values for controls were 22.90±1.10 (51)and 11.3 ± 0.716 mM (52). Both studies reported slightly higher GSH levels in cases in comparison to controls.

GPx levels (51,52) were assessed in two studies, reporting values of 22.99 ± 3.43 for cases and 15.16 ± 0.48 for controls (51). Another study differentiated between GPX isoforms, with cases showing 48.58 ± 0.46 for GPX4 and 25.28 ± 0.55 for GPX1, compared to 95 ± 0.43 (GPX4) and 85 ± 0.32 (GPX1) in controls (52). Additionally, the study examined the expression of SOD2, GLRX2, and TXN2. SOD2 was significantly

reduced in cases (40.8 ± 0.44) compared to controls (85 ± 0.20), while both GLRX2 and TXN2 were elevated in cases, with values of 146.17 ± 0.43 and 146.11 ± 0.87 versus 90 ± 0.57 and 102 ± 0.70 in controls, respectively (52). GPx and related antioxidant enzyme levels showed a variable pattern, with GPx and GLRX2/TXN2 elevated in OLK cases, while GPX isoforms and SOD2 were markedly reduced compared to controls.

The corresponding values are shown in the table 15 and 16.

Table 15: Analysis results of tissue samples and their associated oxidative stress markers.

Author and Year	N	8-nitroguanine (nitric stress)	8-OHdG	Lipid peroxidation / TBARS	SOD	CAT	iNOS (nitric stress)
Ma et al. (2005) (50)	C: 19 HC: 4	C: Strongly positive (IR)** HC: Negative (IR)**	C: Strongly positive (IR)** HC: Negative (IR)**	-	-	-	C: Strongly and weakly positive (IR)*** HC: Negative (IR)***
Srivastava et al. (2013) (51)	C: 20 HC: 20	-	-	C: 91.99 ± 2.97 HC: 127.93 ± 2.97	C: 14.48 ± 1.05 HC: 18.54 ± 0.54	C: 6.36 ± 1.10 HC: 10.46 ± 0.79	-
Banerjee et al. (2020) (52)	C: 12 HC: -	-	-	-	-	C: 75.35 ± 0.56 HC: 98 ± 0.32	-
Barros et al. (2022) (53)	C: 44 HC: 10	-	C: Strongly positive (IR in cytoplasm)* HC: Negative (IR in cytoplasm)*	-	-	-	-

C: OLK cases; HC: healthy control; IR: immunoreactivity:TBARS:thiobarbituric acid reactive substances;SOD: superoxide dismutase;CAT:catalase;iNOS: inducible nitric oxide synthase*:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

Table 16: Analysis results of tissue samples and their associated oxidative stress markers.

Author and Year	N	GSH	GPx	SOD2	GLRX2	TXN2
Ma et al. (2005) (50)	C: 19 HC: 4	-	-	-	-	-
Srivastava et al. (2013) (51)	C: 20 HC: 20	C: 30.43 ± 2.90*** HC: 22.90 ± 1.10***	C: 22.99 ± 3.43*** HC: 15.16 ± 0.48***	-	-	-
Banerjee et al. (2020) (52)	C: 12 HC: -	C: 12.4 ± 0.432 mM** HC: 11.3 ± 0.716 mM**	C: 48.58 ± 0.46 (GPX4)** 25.28 ± 0.55 (GPX1)** HC: 95 ± 0.43 (GPX4)** 85 ± 0.32 (GPX1)**	C: 40.8 ± 0.44** HC: 85 ± 0.2**	C: 146.17 ± 0.43** HC: 90 ± 0.57**	C: 146.11 ± 0.87** HC: 102 ± 0.70**
Barros et al. (2022) (53)	C: 44 HC: 10	-	-	-	-	-

C: OLK cases; HC: healthy control; GSH: reduced glutathione;GPx: glutathione peroxidase;SOD2: Superoxide Dismutase 2;GLRX2: Glutaredoxin 2;TXN2: Thioredoxin 2*:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

10. DISCUSSION

This systematic review analyzed scientific evidence published over the past two decades regarding the potential involvement of oxidative stress in patients with OLK. To achieve this, studies were selected based on their focus on evaluating the presence or absence of oxidative stress and antioxidant biomarkers in both case and control groups. The primary biomarkers assessed across the included articles encompassed a wide range of oxidative and antioxidant indicators, such as GSH, GPx, 8-OHdG, MDA, SOD, CAT, vitamins C and E, lipid peroxidation/TBARS, UA, GR, tGSH, GSSG, the GSH/GSSG ratio, iNOS, 8-nitroguanine, E-SOD, SOD2, GLRX2, and TXN2. Several studies also accounted for confounding factors known to influence oxidative stress and antioxidant equilibrium, such as tobacco use, alcohol consumption and betel nut chewing. In addition, most case-control studies ensured matching based on age and sex, with participant ages ranging from 17 to 77 years, with a median age of 50.6 ± 10.1 for cases and 47.4 ± 10.9 for controls.

The overarching objective of this paper was to evaluate the presence of oxidative stress in OLK by analyzing variations, either increases or decreases, in the levels of specific oxidative and antioxidant biomarkers. Biological samples used for analysis varied across studies and included saliva, tissue, blood, serum, and plasma, thereby offering a broader perspective on oxidative stress status in different biological compartments. Differentiating between the different samples is essential, as oxidative stress presents differently depending on the biological material. For instance, lipid peroxidation is typically reduced in tissues but elevated in blood, both serving as markers of oxidative stress-induced damage (51,58). Consequently, values obtained from tissue samples requires a distinct interpretation, as dysplastic, and precancerous cells may actively suppress oxidative stress to support continued proliferation (66). Lipid peroxidation levels are typically lower in such cells, reflecting an inverse relationship between oxidative damage and cell growth. This contrasts with findings in serum, plasma and saliva samples, where elevated oxidative stress markers indicate systemic oxidative damage associated with OLK and carcinogenesis (43,51,58,67).

GSH, tGSH and GSH/GSSG ratio:

GSH stands out as the most consistently investigated biomarker across the studies included in this review, underscoring its pivotal role in reflecting oxidative stress and redox imbalance in OLK. The antioxidant exists in cells primarily in two forms: the reduced form (GSH) and the oxidized form (GSSG). Together, these make up total glutathione (tGSH), which reflects the overall availability of glutathione in the system. The GSH/GSSG ratio is commonly used to assess the cellular redox status, as it indicates the balance between antioxidant capacity and oxidative burden (68). These three measures are closely related and provide complementary information about the redox environment within cells (34,67,68).

GSH is the primary and most abundant antioxidant within cells and serves a vital function in protecting the organism from damage and disease, thus the most important scavenger against ROS. Therefore, it's an interesting biomarker to focus on when studying the body's antioxidant defense system. Hence, it was the biomarker mentioned the most throughout all the articles utilized in this systematic review. The antioxidant plays a key role in redox signaling and regulates cell proliferation, apoptosis, and immune function (63,68). The oxidant-antioxidant status alteration is more pronounced in the advanced clinical stages of OLK (51). An important role of GSH is detoxification of chemical carcinogens and protecting the cells against the cytotoxic ROS. GSH achieves this by neutralizing harmful H_2O_2, counteracting oxidative stress linked to increased ROS activity, and supplying reducing power for various biochemical processes. Carcinogens from tobacco smoke and quid are primarily detoxified by GSH dependent enzymes. Continuous exposure of the oral mucosa to carcinogenic agents results in their gradual accumulation within the surrounding tissues, which in turn enhances GSH expression in tumor sites (51). Evidently, it is important for prevention of oral cancer appearance, as GSH detoxifies carcinogens and lipid peroxidation products while supporting immune function (63).

Moreover, several studies (51,52,57,59,62,63) report similar increases in GSH in early and advanced stages of leukoplakia, in tumor tissue, which supports the hypothesis

of a compensatory adaptive antioxidant defense to reduce DNA damage. This pattern was consistently observed across the studies focusing on tissue samples. At the same time, lower levels of GSH in serum and saliva were observed in patients with OLK and OSCC, suggesting that GSH consumption exceeds synthesis during prolonged oxidative stress (51,57,62,63). This discrepancy between the different sample variants suggests a compartment specific regulation of oxidative stress. Which in turn may reflect a localized upregulation in antioxidant response to ROS within lesions, while systemic reserves are depleted due to chronic oxidative stress excess (51,63).

However, the central role of GSH maintaining redox homeostasis is not limited to oral tissues. In a recent study from 2024 by Lana et al. (2024) (69) they analyzed the antioxidant in relation to neurodegenerative disorders, such as Parkinson's and Alzheimer. Interestingly, they found a depletion of GSH levels in brain tissue and blood and further concluded that GSH serves as a crucial antioxidant for mitochondrial health. Although the conditions differ from OLK, this illustrated the broader relevance of GSH dysregulation in chronic disease processes involving oxidative stress (34,69). Another study from 2016 (70) by Asher et al., studied the relation between GSH and ear-nose-throat diseases, such as tonsilitis, rhinitis and sinusitis, and found similar increases of oxidative stress, as well as both local and systemic depletion of GSH. Together, these findings reinforce the hypothesis that GSH dysregulation is a common feature of various diseases, reflecting a compensatory response to the oxidative stress burden associated with disease progression (51,54,69,70).

GR and GPx:

The antioxidant enzyme GR is closely linked to the GSH system, as it catalyzes the NADPH-dependent reduction of GSSG back to its active reduced form, GSH (71). This process is essential for maintaining both the GSH/GSSG ratio and the tGSH, which are widely used indicators of redox balance and oxidative stress within the cell. Under oxidative stress, intracellular levels of GSSG increase as GSH is consumed by enzymes such as GPx in the detoxification of ROS (51,62,71). Without sufficient GR activity, GSH cannot be efficiently regenerated, and the redox balance shifts toward a more oxidized

state, which may impair cellular function or trigger apoptosis (62,71). GR therefore plays a central role in preserving redox homeostasis and supporting antioxidant defense. Although only limited data were available in the reviewed articles, one study reported higher GR levels in saliva from patients with potentially malignant lesions compared to healthy controls, though the difference was not statistically significant (62). This trend may reflect an adaptive upregulation of GR in response to increased oxidative load. Given its role in replenishing GSH, GR may be particularly important in maintaining antioxidant capacity under sustained oxidative pressure, such as in the development and progression of OLK (62,68,71). Supporting this, a study Lorestani et al. (2021) (72) found significantly lower GR activity in colon tissue compared to controls ($p=0.007$), suggesting that inadequate GR function may contribute to oxidative imbalance in chronic inflammatory or stress-related disorders. Although IBS and OLK are distinct conditions, the significant reduction in GR activity observed in IBS tissue offers an interesting parallel, suggesting that a similar insufficiency in enzymatic antioxidant defense may also contribute to redox imbalance in OLK (62,72). Further research is needed to clarify this potential mechanism.

GPx is a family of eight antioxidant enzymes, GPx1-GPx8, and in this systematic review it was measured in general as well as GPx1 and GPx4 specifically was measured (73). GPx levels showed variation across sample types: they were higher in OLK cases in tissue, but higher in controls in both saliva and blood. This variation may reflect compartment-specific responses or differences in local versus systemic antioxidant regulation. GPx1 and GPx4 are both key selenoprotein that uses GSH to neutralize lipid peroxides. While GPx1 is abundantly and widely expressed across tissues and primarily targets H_2O_2 and small peroxides, GPx4 is unique in its ability to directly reduce complex lipid hydroperoxides within membranes. This makes GPx4 essential for preventing ferroptosis, a form of regulated cell death driven by lipid peroxidation. Their dysfunction contributes to oxidative stress-related damage and is implicated in cancer, inflammation, and other pathologies (74). GPX1 shows dual roles in cancer biology, acting both as a tumor suppressor and promoter depending on context, and influences processes such as cell proliferation, apoptosis, and therapy resistance (75).

Interestingly, while only tissue GSH showed decreased levels, GPx concentrations were consistently lower in cases, both in serum and tissue samples. One possible explanation is that GPx, being an enzyme, may become functionally impaired or downregulated under prolonged ROS exposure. As GPx depends on both sufficient GSH availability and intact enzymatic activity, sustained oxidative stress may not only deplete its substrate but also compromise the enzyme itself. Additionally, the fact that GPx is a selenium-dependent enzyme suggests that systemic factors such as nutritional status may also influence its activity (76).

Lipid peroxidation, TBARS and MDA:

Lipid peroxidation refers to the oxidative degradation of lipids, initiated when ROS attack polyunsaturated fatty acids in cell membranes (77,78). This peroxidation cascade leads to structural damage of the cell membrane integrity and contributes to cellular dysfunction. This process generates various reactive compounds, among which MDA is one of the most abundant and widely studied (79,80). MDA can be measured directly, or indirectly through TBARS assay, in which it is the primary detectable product. Although TBARS is not fully specific to MDA, it is commonly used to estimate lipid peroxidation levels (77,78). Together, measurements of lipid peroxidation, TBARS, and MDA provide complementary insight into oxidative membrane damage and the extent of redox imbalance in tissue or body fluids (79,81).

According to the study by Kumar Chandan Srivastava et al. (2014) (51), who did their measurements in biopsied tissue samples, the results for TBARS were significantly lower in cases compared to controls (82). Further, the study concludes that there is a decreased in lipid peroxidation alongside increased levels of GSH and GPx in affected tissue. This may appear contradictory to what has been presented previously, given that oxidative stress markers are generally elevated and antioxidant levels decreased in systemic samples. However, at tissue level, antioxidant defenses may be locally upregulated in response to the increase of ROS, as seen for GSH in similar samples. Further, there could possibly be a local suppression of the lipid peroxidation within the lesion itself. In contrast, several studies report significantly elevated MDA levels in

systemic compartments, such as serum and saliva, in patients with established leukoplakia, as well as in patients with OSCC, when compared to healthy controls (46,61,63). An increase in MDA means increased lipid peroxidation and suggests an overall redox imbalance where ROS outweighs the antioxidant capacity systemically. Furthermore, the local suppression of lipid peroxidation and redox alteration may create a favorable microenvironment for tumor development and facilitation of malignant transformation (78,80,83). Paradoxically, suppressed lipid peroxidation may promote survival of dysplastic cells that otherwise undergo oxidative stress induced apoptosis. Thus, by limiting lipid peroxidation, key apoptotic and immune signaling pathways may be downregulated, thereby creating a microenvironment that not only permits malignant transformation but may also provoke uncontrolled cell proliferation (1–4).

Evidently, these shifts in the oxidant–antioxidant status appear more pronounced in advanced clinical stages of leukoplakia. Consequently, tissue levels of TBARS, together with GSH and GPx, may serve as valuable oxidative markers for identifying lesions at higher risk of progression (51). Another article stated that the role of TBARS, GSH and GPx were identified as particularly central markers in the development of oxidative stress (56). To gain a more profound understanding of the interaction between them, correlation and regression statistical tools were used. The results demonstrated a strongly statistically negative correlation between TBARS and the two antioxidant enzymes which underlines the antagonistic dynamic between lipid peroxidation levels and the body's antioxidant capacity, which is already well documented in literature (54).

Although some studies have reported non-significant differences between individual degrees of dysplasia or OSCC differentiation (63), the overall data shows a clear trend of increasing MDA levels with disease progression, from PML to established cancer. This is also supported by observations of higher levels of MDA directly in tumor tissue, suggesting that cancer cells themselves are the source of increased oxidative stress (46). Intervention studies with antioxidants, such as curcumin, have shown that MDA levels are reduced after treatment, in parallel with clinical improvement, further confirming MDA as a useful biomarker of disease activity and treatment efficacy in oral

premalignant conditions (64). Despite variations in methodology and population, the studies suggest that MDA, particularly in saliva and serum, may serve as a readily available, non-invasive indicator of disease status and progression risk in OLK and oral cancer (46,61,63).

Overall the evidence supports the role of MDA as a reliable indicator of lipid peroxidation and overall oxidative stress in OLK and OSCC (37,78,83). As a stable end-product, MDA reflects the cumulative effect of ROS damage to polyunsaturated fatty acids, and its levels correspond with disease severity. TBARS, a broader assay that includes MDA and related compounds, further underscores this connection. Thus, the assessment of lipid peroxidation via MDA and TBARS offers a valuable, non-invasive approach to monitor oxidative damage and may serve as a complementary marker for disease progression and therapeutic response (64,77).

SOD, SOD2, E-SOD, CAT, and UA:

SOD catalyzes the dismutation of superoxide anions ($O_2^{\bullet -}$) into H_2O_2, thereby converting reactive species into less harmful non-radical products, which is then further neutralized by CAT or GPx. Through this mechanism, SOD helps prevent $O_2^{\bullet -}$-induced lipid peroxidation and protects cellular components from oxidative damage (58). Several studies have shown that salivary and serum SOD levels are significantly reduced in patients with OLK and oral cancer compared to healthy controls, which may be due to consumption of the enzyme in response to increased free radical production (60,62,86). The decline in SOD levels reflects a weakened antioxidant defense, and its position as the first enzymatic barrier to ROS gives it potential as a biomarker for early detection and monitoring of OLK conditions.

The mitochondrial isoform of SOD, SOD2, plays a crucial role in controlling ROS levels in the mitochondria (87). It protects against apoptosis and regulates cell proliferation. In studies of oral premalignant conditions, it was observed that SOD2 levels were significantly reduced in OLK compared to controls, with lowest expression in leukoplakia (52). This suggests a possible link between reduced mitochondrial ROS

control and disease development in oral lesions. Another relevant biomarker is E-SOD, which is found in the extracellular space and helps to remove superoxide in the tissue environment.

One study, using serum, showed that E-SOD levels, were significantly reduced in patients with oral cancer and premalignant lesions compared to controls (55). This suggests a systemic impairment of antioxidant defenses and supports the role of E-SOD in protecting against DNA damage in the extracellular microenvironment. While SOD and SOD2 levels were consistently reduced in blood and tissue samples from OLK cases (51,52,54,55,58,60), an increase was observed in saliva samples (62), possibly reflecting a localized compensatory response to oxidative stress. The elevated SOD levels observed in saliva likely reflect a localized adaptive response to oxidative stimuli in the oral cavity, such as alcohol, tobacco, or chronic inflammation. Notably, the study itself reported significantly higher salivary SOD activity in individuals with moderate or heavy alcohol intake, suggesting that external oxidative exposures may induce local antioxidant upregulation. As the oral mucosa is directly exposed to such stressors, increased SOD secretion in saliva may serve to neutralize ROS locally, even in the context of reduced systemic antioxidant capacity (62).

Complementing the antioxidant activity of SOD, CAT is a heme-containing enzyme that detoxifies H_2O_2 (88). In addition, CAT interact with a wide range of oxidizing species, including OH•, • NO, and $ONOO^-$. Notably, many of these interactions inhibit CAT activity. Such inhibition may result in the local accumulation of H_2O_2, which can contribute to oxidative damage in surrounding tissues. However, in certain pathological contexts, such as the induction of apoptosis in cancer cells with membrane-associated CAT, this may surprisingly be beneficial to the host (89). Reduced CAT levels have been observed in serum from patients with OLK and OSCC, which can be explained by the enzyme being consumed in line with increased H_2O_2 load (51,52,58). The role of CAT consequently becomes central in the chain of enzymatic ROS handling, and its reduction reflects a weakening of the defense system against oxidative damage. Although CAT was not assessed in saliva in the included studies, this may be due to its predominantly

intracellular localization and low stability in oral fluids, which make it technically challenging to measure reliably in saliva-based assays (90).

The non-enzymatic antioxidant UA is primarily found in plasma, although it is the most abundant in saliva (91). UA has a dual role as an antioxidant and potential pro-oxidant. The antioxidant participates in redox reactions and has a protective function against oxidative processes. It accounts for approximately 60% of the body's ability to neutralize free radicals, underlining its important role in human antioxidant defense (56). In addition, UA forms a stable nitric oxide donor molecule through interaction with $ONOO^-$, which may contribute to increased vasodilation and reduced risk of oxidative damage caused by $ONOO^-$. However, UA levels are influenced by several external factors, including alcohol consumption and diet, and research has shown that both alcohol and tobacco can have an impact on salivary UA levels (92,93). In a study comparing serum levels of UA in patients with olk and healthy controls, slightly lower mean values were found in the patients to be lower compared to the control group. However, this difference was not statistically significant (56). In a study assessing UA (56), the authors noted that serum UA can be measured using a simple and cost-effective assay, however, the findings from this cross-sectional cohort did not support its clinical utility in evaluating patients with OLK or OSCC (56,92–94).

According to a study by Gherghina et al. (2022) (93), UA may also contribute to oxidative stress under pathological conditions such as cardiovascular disease, chronic kidney disease, and metabolic syndrome. In these contexts, elevated UA levels have been linked to increased DNA damage, inflammatory cytokine production, and cellular apoptosis. These findings suggest that while UA serves a protective antioxidant function under normal physiological conditions, it may shift toward a pro-oxidant and pro-inflammatory role when homeostasis is disrupted. This dual behavior underscores the importance of interpreting UA levels in OLK with caution, particularly in patients with comorbidities or systemic oxidative burden (93,94).

Vitamins A, C and E:

The vitamins C and E were studied specifically, whereas vitamin A was only mentioned briefly, mainly as a supportive antioxidant in combination treatments. Vitamin A and its precursor β-carotene were, although not studied in all the articles, associated with reduced disease progression when given as a supplement in combination with C and E. This suggests a possible preventive role, although the evidence is currently limited and more research is needed to confirm effects and mechanisms (59). The fat- and water-soluble vitamins act as non-enzymatic antioxidants that help protect cell membranes and DNA from ROS-induced damage. Vitamin C also acts in the regeneration of vitamin E, and both are consumed during oxidative stress (61). In patients with OLK and OSCC, significantly lower levels of vitamins C and E were reported in saliva compared to healthy controls, with further reduction in more advanced disease stages (59,61). This supports the theory that free radicals consume the body's antioxidant reserves in the progression of premalignant conditions. At the same time, one study showed that treatment with curcumin increased levels of vitamins C and E, as well as reducing lipid peroxidation and DNA damage, suggesting that levels can also be altered therapeutically (64,95,96).

8-nitroguanine, 8-OHdG and iNOS:

In addition to the enzymatic and thiol-based redox markers discussed previously, several studies have highlighted the role of oxidative DNA damage markers, specifically 8-OHdG and 8-nitroguanine, as indicators of genotoxic stress in OLK and its potential malignant transformation. These DNA lesions arise from oxidative and nitrative stress, respectively, and provide direct insight into ROS- and RNS-mediated mutagenesis (97,98).

There were two independent studies (50,53) using IR through immunohistochemical analysis as their way of measuring the biomarkers. In both studies, strong immunoreactivity for oxidative stress markers such as 8-OHdG and 8-nitroguanine was observed in leukoplakia tissue, while normal mucosa showed minimal or no such activity. One study (53) reported increasing cytoplasmic expression of 8-OHdG with increasing severity of epithelial dysplasia, with particular immunostaining in the intermediate cell layer. This suggests that damage linked to ROS is not only present but

also graded according to disease progression, and that the marker is localized predominantly in the cytoplasm rather than the nucleus. Another study (50) showed that both epithelial cells and inflammatory cells in OLK tissue had strong immunoreactivity for 8-nitroguanine and 8-oxodG, further supporting the hypothesis that both local inflammatory responses and epithelial changes play a role in the oxidative damage observed in OLK.

Consequently, elevated levels of both 8-OHdG and 8-nitroguanine have been observed in oral epithelial tissue from OLK patients, with particularly intense staining in dysplastic areas (50,53). Their colocalization, especially in the basal and suprabasal layers, and the parallel increase in iNOS expression suggest that nitrative stress plays a mechanistic role in early oral carcinogenesis, likely mediated by $ONOO^-$ generation and subsequent DNA base modification (58). Moreover, 8-nitroguanine formation has also been reported in other inflammation-driven cancers, supporting its role as a potential inflammation-linked biomarker of carcinogenesis (58).

8-OHdG, one of the most widely studied oxidative stress biomarkers, is consistently elevated in saliva and tissue samples from patients with OLK and OSCC compared to healthy controls (53,61,64). However, a few studies have reported lower levels in plasma, which may reflect impaired DNA repair mechanisms or differences in the source of measurement (65). In particular, cytoplasmic accumulation of 8-OHdG has been linked to mitochondrial DNA damage, which could play a role in early malignant transformation by altering cellular energy metabolism (53). The same study noted a correlation between dysplasia severity and cytoplasmic 8-OHdG expression, suggesting its relevance in disease progression (53).

Interestingly, intervention with curcumin resulted in a reduction of 8-OHdG levels in both saliva and serum, along with clinical improvement in patients with OLK (64). This indicates the potential therapeutic responsiveness of this biomarker, further supporting its value in monitoring disease activity. Taken together, 8-OHdG and 8-nitroguanine reflect different but complementary aspects of oxidative DNA damage. While 8-OHdG serves as a broad marker of ROS activity, 8-nitroguanine appears more specific to inflammation-

related nitrative stress. Their presence in tissue, and in the case of 8-OHdG, also in saliva or plasma, may help identify high-risk lesions and provide insight into the underlying mechanisms of oral carcinogenesis (50,53).

TAS/TAC, TXN2, GLRX2 and zinc:

TAS and TAC are two terms that are often used interchangeably in the literature, as both refer to the overall antioxidant capacity of the body. However, there may be slight differences in how they are measured and which biofluids or laboratory techniques are used (99). Both biomarkers provide an integrated measure of the interaction between known and unknown antioxidants in the body, reflecting the balance between oxidants and antioxidants. Additionally, one of the articles utilized showed significantly reduced levels of TAS in OLK patients compared to healthy controls, suggesting an impaired antioxidant defense mechanism in the pathogenesis of the condition (59). Although no statistically significant differences were found between the groups in TAC levels, the study showed higher median TAC values in patients with OLK and OSCC, which may indicate an adaptive response against persistent oxidative stress (59).

The mitochondrial protein GLRX2 contributes to redox activity and plays a role in cell proliferation and in preventing apoptosis by inhibiting the release of cytochrome c and activation of caspases (100,101). In the article by Banerjee et al. (2020) (52), increased expression of GLRX2 was shown in OLK compared to the control group. These findings suggest that GLRX2 may be involved in the proliferative properties of oral cancer precursors. The observed upregulation in cases of OLK may reflect a cellular adaptation to maintain mitochondrial integrity and avoid programmed cell death (52).

TXN2 is part of the thioredoxin system in the mitochondria and protects cells from oxidative stress by reducing oxidized proteins (102). Interestingly, one article found that the highest level of TXN2 was observed in the OLK group, while control, oral lichen planus and oral submucous fibrosis had similar and lower levels. This pattern suggests a specific activation of TXN2 in OLK, possibly as an homeostatic reaction to increased mitochondrial stress or to maintain DNA integrity in cells with high proliferation activity (52).

In addition, zinc is an essential trace element that is involved in many enzymatic processes, regulates immune response, and has both antioxidant and anti-inflammatory properties (103). It functions both by stabilizing sulphydryl groups and by inhibiting metal-catalyzed oxidative reactions. Findings from one of the included papers (59) reported significantly lower levels of plasma zinc were measured in patients with OLK compared to the control group. Since zinc is required for the function of the antioxidant enzymes GSH and CAT, such results suggest that zinc deficiency may contribute to oxidative stress and thus play a role in the development of OLK (59). On top of that, zinc induces the expression of metallothioneins, which are powerful free radical scavengers, and acts immunosuppressively by downregulating NF-κB activation.

Risk factors in relation to oxidative stress:

As mentioned previously GSH detoxifies chemical carcinogens and protects the cells against the cytotoxic ROS. Hence, carcinogens from tobacco smoke and quid are primarily detoxified by GSH dependent enzymes. Continuous exposure of the oral mucosa to carcinogenic agents results in their gradual accumulation within the surrounding tissues, which in turn enhances GSH expression in tumor sites (51).

Extensive research has demonstrated that tobacco use enhances the production of ROS and RNS. These species, either directly or via the activation of inflammatory pathways, are implicated as both initiators and promoters of carcinogenesis. While low concentrations of ROS are essential for maintaining cellular homeostasis and are normally neutralized by antioxidant defense mechanisms, an imbalance, either due to excessive ROS production or diminished antioxidant capacity, leads to oxidative stress. This state can trigger lipid peroxidation, ultimately resulting in DNA damage (51). Another article (54) reported that all participants had a history of tobacco use, with or without additives, reinforcing the well-documented link between tobacco consumption and the development of OLK. Thus, another article found that all control subjects without a history of tobacco use demonstrated significantly lower levels of peroxidation activity compared to their counterparts with tobacco-related habits (91). However, elevated levels of MDA in saliva and serum are influenced not only by tobacco consumption, but also by the overall intensity of oxidative stress. This reinforces the hypothesis that cancer cells

possess significantly altered ROS metabolism, leading to increased ROS generation compared to non-neoplastic cells, along with a suppression of the antioxidant systems, such as GSH, that are crucial for maintaining cellular defense (63). Tobacco use contributes to increased lipid peroxidation in saliva, which is partly due to the presence of carcinogenic compounds and oxidative degradation products in smokeless tobacco users. Nicotine and heavy metals found in tobacco exert a localized toxic effect on the oral mucosa, while nicotine is absorbed both through the mucosa and when swallowed. This leads to a persistent systemic exposure, even after the tobacco has been removed from the oral cavity, and allows harmful substances to spread and affect tissues at a distance from the site of exposure (63).

The continuous irritation caused by tobacco contact, combined with nicotine's genotoxic effects and accumulation in saliva, promotes the development of chronic inflammation and oxidative stress. This can lead to oxidative damage also in large and small salivary glands. In fact, degenerative changes have been detected in more than 40% of the minor salivary glands in people with long-term and intensive use of smokeless tobacco. The elevated MDA levels detected in patients with OLK and OSCC reflect the influence of multiple carcinogenic factors and confirm increased lipid peroxidation and oxidative stress in these conditions. In addition, reduced activity of the body's antioxidant defense systems, such as GSH, contributes to enhanced free radical damage, which may play a central role in carcinogenesis (63).

The study by Kumar Chandan Srivastava et al. (2016) (54) suggest that GSH plays a central regulatory role within the antioxidant defense system. The significant predictive relationship between GSH and other enzymatic antioxidants such as GPx, CAT, and SOD, as well as markers of oxidative damage like TBARS, highlights GSH as a potential upstream modulator in redox homeostasis. In particular, the regression model indicates that 53% of the variance in GPx activity can be attributed to fluctuations in GSH levels, underscoring a strong functional interdependence between these two components. This may reflect a compensatory mechanism where increased GSH availability enhances GPx-mediated detoxification of peroxides, thereby contributing to cellular protection against oxidative stress. In addition, alcohol and smoking habits also showed a correlation with

TAC values (59). The observed associations further support the hypothesis that alterations in GSH concentration may have downstream effects on both antioxidant enzyme activity and lipid peroxidation levels, which could be relevant in the pathophysiology of disorders characterized by redox imbalance (54).

The articles that investigated saliva samples consistently demonstrated a pattern characterized by elevated levels of oxidative stress biomarkers, such as MDA and 8-OHdG, alongside a noticeable reduction in antioxidant parameters, including GSH, SOD, and CAT. These findings collectively suggest a systemic imbalance between oxidative damage and antioxidant defense mechanisms in patients with OLK (63).

This systematic review underlines the theory that oxidative stress plays a central role in the pathogenesis and progression of OLK. Across diverse biological samples, including tissue, saliva, and serum, patients with OLK consistently exhibited elevated levels of oxidative stress markers and reduced antioxidant capacity. Furthermore, tobacco use, and other exogenous factors were repeatedly shown to exacerbate oxidative stress, reinforcing the need for preventive strategies targeting lifestyle modification. Despite the consistency in findings, limitations in study design, sample size, and heterogeneity of methods highlight the necessity for further large-scale, longitudinal research. A deeper understanding of the molecular mechanisms governing redox homeostasis in OLK could pave the way for improved risk stratification and novel therapeutic interventions.

10.1 Possible preventative measures and treatment

The exact etiological factors contributing to OLK remain unknown, although it is believed to be multifactorial. Throughout this review it has been proven that OLK patients exhibit an imbalance in redox homeostasis, characterized by reduced levels of antioxidants and elevated oxidative stress. These findings are consistent with the objectives outlined in this systematic review. The prevention of OLK should be approached from an integral perspective based on the three classical levels of prevention in public health: primary, secondary, and tertiary.

First, as previously mentioned, tobacco smoke, alcohol consumption, and betel nut chewing are all sources of oxidative stress and therefore increasing the risk of OLK appearance. Additionally, constant irritation of the oral mucosa from for example badly fitted prosthesis and a diet lacking sufficient antioxidants may also contribute to appearance of oxidative stress. The elimination of these risk factors may represents a possible method of primary prevention (104).

Secondarily, as with most other diseases, early diagnosis is crucial in slowing disease progression. This is particularly important in cases of precancerous lesions with malignant transformation potential, such as OLK, as these can develop rapidly, and the outcome can be severe. Early detection significantly reduces the risk of extensive damage and reduce the likelihood of extensive epithelial dysplasia or progression to OSCC. Routine screening in high-risk populations play a key role in secondary prevention.

Lastly, early therapeutic intervention in patients, especially in cases that have progressed to advanced stages, may play a role in limiting further deterioration and improving patient outcomes. However, the current lack of standardized treatment protocols and limited long-term data underline the importance of continued research. A more refined understanding of the disease process, including factors such as oxidative stress, may pave the way for improved preventive and therapeutic strategies in patients with OLK.

Furthermore, a study conducted by Giovani Ladi et al. (2016) (105) reviewed different treatments and preventative measures of OLK. What they concluded was that high-quality evidence on the treatment of OLK remains scarce. To this date, no randomized controlled trials have evaluated surgical interventions or smoking cessation in comparison to no treatment. Although agents such as vitamin A and beta-carotene may promote lesion regression, their use is frequently associated with relapse and adverse effects. Currently, no therapy has been proven to effectively prevent the progression to oral cancer. Robust, large-scale studies with extended follow-up are necessary to determine the efficacy of available interventions.

Another study (22) points out that despite OLK being one of the most common potentially malignant disorders, it remains clinically unpredictable. Moreover, lesions that appear similar may follow very different biological courses, with some progressing to malignancy. This diagnostic uncertainty complicates both prevention and treatment, particularly for large or high-risk lesions. These limitations highlight the importance of reducing modifiable risk factors and the need for stronger evidence to guide therapeutic decisions.

In conclusion, while prevention strategies focusing on modifiable risk factors such as tobacco and alcohol use are essential, the management of OLK remains a clinical challenge. The lack of reliable predictive markers and limited evidence for effective treatments, especially for high-risk lesions, highlight the need for continued research. A deeper understanding of the molecular mechanisms involved, particularly oxidative stress, may contribute to more targeted and individualized therapeutic approaches in the future (22,104,105).

10.2 Limitations of the study

One limitation of this systematic review is the heterogeneity of the included studies in terms of the specific oxidative stress biomarkers assessed. Thus, some of the biomarkers in example GSH is measured in eight articles, whereas for example uric acid is only measured in one. This could potentially lead to misinterpretations and errors in the results. However, the aim of this systematic review was to study the overall concept of oxidative stress and not necessarily any specific biomarker. Therefore, by studying and comparing various markers of oxidative damage such as MDA and antioxidant markers like GSH on the contrary, gives a good overview over the biochemical process. Furthermore, three different types of samples were used throughout the study which calls for careful interpretations of the results and further comparisons across the different sample types.

Nevertheless, some of the studies included did not score a full nine stars in the Newcastle-Ottawa scaling. Even though a score equal to or over six was considered reliable, it could be considered a limitation. Furthermore, the types of studies utilized are not all the same design, as there are case-controls, cross-sectional studies, interventional and cohort studies, which can on the other hand be a strength as it gives the study range and gives a good base for comparison. In addition, this review only included articles published in English and available in full text, which introduces a risk of publication bias and language bias. Relevant studies published in other languages or not indexed in the selected databases may have been overlooked.

Additionally, one non-randomized interventional study included in the review was assessed using the ROBINS-I tool, which is designed for evaluating risk of bias in non-randomized studies of interventions. This study was found to have a serious risk of bias, primarily due to lack of a control group and absence of adjustment for confounding variables. While it contributes exploratory insight, its findings should be interpreted with caution and considered a limitation in the overall evidence synthesis.

Future studies with standardized methodologies, larger and more diverse populations, and longitudinal designs would be valuable in confirming the role of oxidative stress in the pathogenesis and progression of OLK. It would also be beneficial to explore the clinical utility of specific biomarkers across different biological samples to better assess their diagnostic and prognostic potential. Such efforts may contribute to more precise risk stratification and the development of targeted preventive and therapeutic strategies.

11. CONCLUSION

General conclusions:

Patients with oral leukoplakia (OLK) show higher levels of oxidative stress than healthy controls.

Specific conclusions:

1. A significant increase in the levels of oxidative markers in saliva, blood and tissue samples has been detected in patients with OLK.

2. At the same time, a clear reduction in the antioxidant defense in the same sample types has been registered in this patient group.

3. The imbalance between oxidative markers and antioxidant defenses in OLK patients suggests that oxidative stress may play a key role in the pathogenesis and potential malignant transformation of this lesion.

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13. APPENDICES

Use of artificial intelligence (AI):

Tool used: Chat GPT 4o

Function: Artificial intelligence (ChatGPT, OpenAI) has been used as a support tool in the work on this thesis, mainly for help with language formulation, restructuring of text, summarization of information and suggestions for professional formulation. All content assessment, analysis and conclusion are done by me, and the AI tool has not been used to generate professional interpretations, systematic reviews or analyze primary data. The use of AI has been in line with ethically sound practice and served as an aid in the writing process.

Examples of prompts used with artificial intelligence (ChatGPT):

1. “What is a synonym for this word in an academic context?”
2. “What can I use instead of this word to avoid repetition?”
3. “Can you explain this text to me in a simple way so that I can summarize it in my own words?”
4. “Can you give me advice to improve the flow of this paragraph?”

Link: http://chatgpt.com

PRISMA GUIDE 2020

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Cover page
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	3
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	25-26
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	28
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	31
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	31-33
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	32-33
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	33
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	33-34
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	_
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	_
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	34
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	_

Section and Topic	Item #	Checklist item	Location where item is reported
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	_
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	_
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	_
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	_
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	_
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	34
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	_
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	_
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	36-37
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	37-38
Study characteristics	17	Cite each included study and present its characteristics.	41-44
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	45-46
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	48-62
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	_
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	_
	20c	Present results of all investigations of possible causes of	_

Section and Topic	Item #	Checklist item	Location where item is reported
		heterogeneity among study results.	
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	_
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	_
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	_
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	64-76
	23b	Discuss any limitations of the evidence included in the review.	78-79
	23c	Discuss any limitations of the review processes used.	_
	23d	Discuss implications of the results for practice, policy, and future research.	_
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	30
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	_
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	_
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	_
Competing interests	26	Declare any competing interests of review authors.	_
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	_

LOCAL AND SYSTEMIC OXIDATIVE STRESS IN ORAL LEUKOPLAKIA: A SYSTEMATIC REVIEW.

Running title: Oxidative stress in oral leukoplakia

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Abstract

Introduction: Oral leukoplakia (OLK) is one of the most common potentially malignant disorder of the oral mucosa. Oxidative stress is increasingly recognized as a key factor in the pathogenesis and malignant transformation of OLK.

Aim: To evaluate local and systemic oxidative stress in OLK by synthesizing evidence on biomarker levels in tissue, serum, plasma, blood, and saliva.

Materials and Methods: A systematic search was conducted in PubMed, Web of Science and Scopus, following PRISMA guidelines. Studies were included if they compared oxidative stress biomarkers between OLK patients and healthy controls. Data were qualitatively synthesized, and study quality was assessed using the Newcastle-Ottawa Scale, ROBIN-I (Cochraine) and AXIS.

Results: 15 studies were included. Oxidative damage markers such as malondialdehyde (MDA) and 8-OHdG were elevated in OLK patients across tissue, serum, plasma, blood, and saliva. Enzymatic antioxidants such as GPx, SOD, CAT were generally reduced, whereas tissue levels of GSH were elevated, suggesting a local compensatory response. Total antioxidant capacity (TAS/TAC) was decreased systemically. Uric acid (UA) and glutathione reductase (GR) showed inconsistent trends.

Conclusion: The evidence supports a consistent redox imbalance in OLK. Certain biomarkers, especially MDA and GSH, show potential as non-invasive indicators of oxidative stress and disease progression. Standardized protocols and longitudinal studies are needed to clarify their clinical value.

Key words:

Oral leukoplakia, oxidative stress, antioxidants, biomarkers, reactive oxygen species

Introduction

Oral leukoplakia (OLK) is the most common potentially malignant lesion of the oral mucosa, defined as a predominantly white patch that cannot be classified as any other condition (1). It carries a variable risk of malignant transformation (1–20%), depending on factors such as lesion size, location, and the presence of epithelial dysplasia (2,3). Understanding its molecular

mechanisms is key to preventing progression to oral squamous cell carcinoma (OSCC), an aggressive and prevalent head and neck malignancy (3).

Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and antioxidant defenses, has emerged as a relevant contributor to carcinogenesis (4–6). ROS can damage lipids, proteins, and DNA, promoting genomic instability and tumor development. The oral cavity is especially vulnerable to oxidative damage due to chronic exposure to tobacco, alcohol, inflammation, and microbial agents (3,7). In response, the body activates antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH), alongside markers of oxidative damage like malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) (8,9). These biomarkers are measurable in tissues, serum, plasma, saliva, or erythrocytes.

Although multiple studies have investigated oxidative stress in OLK (4,5), the findings remain partially contradictory, and no clinically validated biomarkers exist to predict malignant transformation. Diagnosis still relies on exclusion and clinical experience, often challenging for less experienced clinicians (1,4). The variability of biomarker expression between local and systemic compartments adds further complexity (6,8,9). Given the potential clinical value of identifying reliable redox markers, this systematic review aims to evaluate and compare oxidative stress biomarkers in patients with OLK versus healthy controls, across different biological samples, to identify the most consistently altered indicators and better understand their diagnostic and prognostic potential.

Materials and Methods

This systematic review was conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (10). The protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO) under registration number CRD42023438437.

Focus question:

Based on the objectives of this systematic review, the research question focuses on the following components:

- **P** (Population): Patients with OLK
- **I** (Intervention): Measurement of oxidative and antioxidant biomarkers

- **C** (Comparison): Healthy controls
- **O** (Outcome): Concentrations of oxidative stress and antioxidants biomarkers from patients with OLK and healthy controls

Eligibility criteria:

Inclusion criteria: Type of study: Observational studies (Cohort, Case-Control studies, and Cross-sectional studies), experimental studies (Randomized control trials), studies written in Spanish or English, articles published from 2000 to 2024. Type of patients: Patients with OLK and healthy patients, human adult patients. Type of intervention: Measurements of oxidative stress compared to antioxidant levels, biomarkers of oxidative stress present in presence of OLK lesion. Type of control: Healthy controls without OLK or other premalignant lesions. Type of result variables: Measurement of biomarkers, oxidants, and antioxidants, the association between oxidative stress and OLK, oxidative stress as an etiopathogenic factor in OLK.

Exclusion criteria: Type of studies: Clinical trials, systematic reviews, articles that does not distinguish between the oral precancerous lesions, preclinical studies, studies that are not done in humans, animal or in vitro studies, studies published before year 2000, studies done in other languages than English or Spanish, studies with less than 5 patients. Type of patients: Pediatric patients, patients without a confirmed diagnosis of OLK or those with other oral lesions unrelated to premalignant conditions, patients with underlying HIV diagnosis or other immunosuppressive diseases.

Information sources and data search:

A comprehensive literature search was performed in three electronic databases: PubMed/MEDLINE (Medical Literature Analysis and Retrieval System Online), Web of Science and Scopus. The Boolean operator "AND" was applied to ensure the inclusion of both oxidative stress and oral leukoplakia in the results, while "OR" was used to integrate synonyms and related terms, broadening the search scope. The following combination of Medical Subject Headings (MeSH) and keywords was used: “oral leukoplakia”, “OLK”, “potentially malignant disorders”, “oxidative stress”, “redox”, “reactive oxygen species”, “oxidative damage”,

“biomarkers” and “antioxidants”. The following search strategy was carried out for the following data bases. In PubMed the following search was done: ("leukoplakia, oral"[MeSH Terms] OR ("leukoplakia"[All Fields] AND "oral"[All Fields]) OR "oral leukoplakia"[All Fields] OR ("oral"[All Fields] AND "leukoplakia"[All Fields]) OR "OLK"[All Fields]) AND ((("oxidative stress"[MeSH Terms] OR ("oxidative"[All Fields] AND "stress"[All Fields]) OR "oxidative stress"[All Fields]) AND ("biomarker s"[All Fields] OR "biomarkers"[MeSH Terms] OR "biomarkers"[All Fields] OR "biomarker"[All Fields])) OR ("oxidability"[All Fields] OR "oxidable"[All Fields] OR "oxidant s"[All Fields] OR "oxidants"[Pharmacological Action] OR "oxidants"[MeSH Terms] OR "oxidants"[All Fields] OR "oxidant"[All Fields] OR "oxidate"[All Fields] OR "oxidated"[All Fields] OR "oxidates"[All Fields] OR "oxidating"[All Fields] OR "oxidation"[All Fields] OR "oxidations"[All Fields] OR "oxidative"[All Fields] OR "oxidatively"[All Fields] OR "oxidatives"[All Fields] OR "oxide s"[All Fields] OR "oxides"[MeSH Terms] OR "oxides"[All Fields] OR "oxide"[All Fields] OR "oxidic"[All Fields] OR "oxiding"[All Fields] OR "oxidisability"[All Fields] OR "oxidisable"[All Fields] OR "oxidisation"[All Fields] OR "oxidise"[All Fields] OR "oxidised"[All Fields] OR "oxidiser"[All Fields] OR "oxidisers"[All Fields] OR "oxidises"[All Fields] OR "oxidising"[All Fields] OR "oxidization"[All Fields] OR "oxidize"[All Fields] OR "oxidized"[All Fields] OR "oxidizer"[All Fields] OR "oxidizers"[All Fields] OR "oxidizes"[All Fields] OR "oxidizing"[All Fields]) OR ("react oxyg species apex"[Journal] OR "ros"[All Fields]) OR ("reactive oxygen species"[MeSH Terms] OR ("reactive"[All Fields] AND "oxygen"[All Fields] AND "species"[All Fields]) OR "reactive oxygen species"[All Fields]) OR (("antioxidant s"[All Fields] OR "antioxidants"[Pharmacological Action] OR "antioxidants"[MeSH Terms] OR "antioxidants"[All Fields] OR "antioxidant"[All Fields] OR "antioxidating"[All Fields] OR "antioxidation"[All Fields] OR "antioxidative"[All Fields] OR "antioxidatively"[All Fields] OR "antioxidatives"[All Fields] OR "antioxidizing"[All Fields]) AND "status"[All Fields]) OR ("ieee trans affect comput"[Journal] OR "tac"[All Fields]))

Search strategy:

The articles to be studied in this systematic review were selected through a three- stage process. The selection of studies was carried out by two reviewers (UPCU, CEN). The first stage involved selecting articles based on their titles to exclude any publications unrelated to the research. In

the second stage, studies were filtered by reviewing abstracts and selected based on study type, patient characteristics (type and number), oxidative marker measurements, intervention type, sample types assessed, and outcome variables. For the third stage, we selected the eligible articles for our review by reading them in full and conducted data extraction using a pre-established collection form to confirm study eligibility. The degree of agreement regarding the inclusion of potential studies was calculated by k-statistics (11). There were no disagreements among the reviewers at any stage of the process.

Data Extraction:

After removal of duplicates, titles and abstracts were screened independently by two reviewers. Full-text articles were then assessed for eligibility based on the inclusion criteria. For each included study, the following data were extracted and organized in tables: Author and year, type of sample analyzed (tissue, serum, plasma, saliva, blood, and erythrocyte lysate), biomarkers assessed, analytical methods and main findings regarding oxidative stress markers in OLK vs. healthy controls.

Quality and risk of bias assessment:

Methodological quality was assessed using the Newcastle-Ottawa Scale (NOS) for case-control and cohort studies, the AXIS tool for cross-sectional studies, and ROBINS-I for non-randomized interventional studies. Each tool evaluates bias through specific domains, allowing classification into high, moderate, or low quality based on standardized criteria.

Results

Study Selection:

During the initial search in total 288 articles were obtained. Medline-PubMed (n= 155), Web of Science (n=70), SCOPUS (n= 63) and manual search (n=2). Out of these, 95 were duplicated. Furthermore, 153 articles were excluded based on title and 26 were excluded based on abstract. Then the full text of the remaining 22 articles was obtained and evaluated for its eligibility. Out of

these seven were excluded, four of them for not dividing between the different premalignant lesions (PML), one because it was an in vitro study which does not align with the inclusion criteria. Furthermore, one article was excluded because it only measured the effect of the treatment and lastly one was excluded because it didn't include comparison to healthy controls. In total 15 articles met the inclusion criteria and were included in the review (Fig. 1). Agreement between reviewers concerning study inclusion yielded k-values of 0.92 for titles and abstracts, and 1.0 for full-text articles, indicating "substantial" and "perfect" agreement, respectively, based on the criteria by Landis and Koch (11).

Study Characteristics:

Across the different sample types, of the 15 articles included, the oxidative stress biomarker GSH was the most frequently assessed biomarker, measured in eight articles (12-19) four of them were using serum samples (15-17), two of them tissue (12,13), one saliva (18) and one used both serum and saliva (19). 8-OHdG was evaluated in six articles (20-25), while GPx (13,14,16,18,26,27) and MDA were each measured in six. SOD and CAT were assessed in four articles, as were vitamins C and E. Lipid peroxidation was reported in three articles, and uric acid in two. 8-nitroguanine was measured in one article (21). Several other biomarkers were also measured in only one article, including TXN2, GLRX2, SOD2, E-SOD, GSH/GSSG ratio, tGSH, iNOS and GR. Six of the studies also took into consideration and noted the habits of the patients such as cigarette smoking and alcohol ingestion (17, 18, 20, 25, 27, 28). Nevertheless, some articles also divided leukoplakia into stages, where the oxidative damage increased as the disease progressed as in the study by Kumar Chandan Srivastava et al. (2013) (12) where they divide the OLK patients into stages I-IV. In that specific study they used tissue samples and concluded that a significant decrease in thiobarbituric acid reactive substances (TBARS) levels ($P < 0.05$) was observed only in stage IV leukoplakia patients. Among the antioxidant enzymes, GSH and GPx were the only ones to show a significant reduction ($P < 0.001$) across the different disease stages (Table 1)

Risk of bias:

Risk of bias was assessed using the Newcastle-Ottawa Scale for case-control and cohort studies, evaluating selection, comparability, and exposure or outcome. For cross-sectional studies, the AXIS tool was applied, while ROBINS-I was used to assess risk of bias in non-randomized intervention studies (Tables 2,3, 4 and 5)

Synthesis of results:

Out of the 15 articles included, there were studies using serum, plasma, blood, and erythrocyte lysate, as well as saliva and tissue samples.

Serum, plasma, erythrocyte lysate and blood biomarkers:

Nine studies assessed biomarkers in serum, plasma, or blood samples. GSH was measured in five studies and consistently found at lower levels in OLK patients compared to controls, e.g., 1.04 ± 0.22 vs. 1.88 ± 0.36 mg/L (15). SOD was also reduced in OLK cases, with one study reporting 2.09 ± 0.08 vs. 4.70 ± 1.26 U/ml (14). Similarly, lower levels of GPx and CAT were observed, such as GPx 19.09 ± 0.56 vs. 25.07 ± 1.55 U/g Hb and CAT 1.37 ± 0.08 vs. 3.46 ± 0.85 (14). Three studies found significantly higher MDA levels in OLK cases, e.g., 5.68 ± 0.32 vs. 1.96 ± 0.15 nmol/ml (16). TBARS levels were also elevated in OLK and increased with clinical stage (14). Vitamin C and E levels were consistently lower in OLK patients, as were zinc and E-SOD. One study reported decreased 8-OHdG in OLK cases (24). All values are shown in table 6 and 7.

Salivary biomarkers:

Four studies analyzed saliva samples. MDA was elevated in OLK patients, with values such as 0.33 ± 0.07 vs. 0.08 ± 0.07 µmol/L (23). 8-OHdG was also higher in all studies, ranging from 0.34 to 11.54 ng/mL in cases and 0.07 to 8.58 ng/mL in controls. Vitamins C and E were lower in OLK saliva, e.g., vitamin C 0.55 ± 0.13 vs. 1.2 ± 0.6 µmol/L (23). One study measured several additional markers. TAC, SOD, GR and UA were higher in OLK cases, while GPx and GSH were reduced. GSH was reported at 0.01 vs. 0.02 µmol/ml (18). Strong correlations were found between markers, particularly a positive correlation between 8-OHdG and MDA ($R = 0.79$, $p < 0.001$) and negative

correlations between oxidative markers and antioxidant vitamins. All values are shown in table 8 and 9.

Tissue biomarkers:

Four studies analyzed oxidative stress biomarkers in biopsy tissue samples from OLK patients (*16,24,25,29*). Two studies assessed 8-OHdG and 8-nitroguanine, showing strong immunoreactivity (IR) in epithelial and inflammatory cells of OLK tissue, whereas normal mucosa showed no or weak IR (21,22). One study also found elevated expression of inducible nitric oxide synthase (iNOS) in inflammatory cells of OLK tissue (21). One article measured lipid peroxidation (TBARS) and found lower values in OLK tissue (91.99 ± 2.97) compared to controls (127.93 ± 2.97) (*29*). This was accompanied by reduced SOD and CAT levels in OLK cases (SOD: 14.48 ± 1.05 vs. 18.54 ± 0.54; CAT: 6.36 ± 1.10 vs. 10.46 ± 0.79) (26), with similar CAT reduction confirmed in a second study (13). GSH levels were slightly higher in OLK tissue than in controls in two studies, while GPx activity showed inconsistent findings, either elevated or reduced depending on the isoform measured. Notably, GPX1 and GPX4 were significantly reduced in OLK tissue, while GLRX2 and TXN2 were elevated (13). SOD2, in contrast, was markedly decreased in OLK cases (40.8 ± 0.44) compared to controls (85 ± 0.20) (13). These results reflect a complex antioxidant response, with some compensatory upregulation and other markers significantly depleted in OLK tissue. All values are shown in table 10 and 11.

Discussion

This systematic review analyzed the role of oxidative stress in oral leukoplakia (OLK), focusing on the levels of oxidative and antioxidant biomarkers in tissue, serum, plasma, and saliva. The findings revealed a consistent pattern of increased oxidative stress markers, particularly MDA and 8-OHdG and reduced antioxidant defenses such as GSH, SOD, CAT, and vitamins C and E in OLK patients across sample types (14,16,19,23,26).

Glutathione (GSH) was the most frequently assessed biomarker, reflecting its key role in redox balance. While elevated levels were observed in OLK tissue, suggesting a local adaptive

antioxidant response, serum and saliva samples consistently showed reduced GSH, indicating systemic depletion under prolonged oxidative stress (15,18,19,26). The GSH/GSSG ratio and total GSH (tGSH) further supported these findings, with compartment-specific differences suggesting a compensatory response in lesions and systemic oxidative burden (19,36,29,30). The enzyme glutathione reductase (GR), responsible for regenerating GSH, was found to be slightly elevated in OLK saliva but generally under-researched. One study in a different disease context showed reduced GR activity associated with chronic inflammation, supporting the potential importance of GR in OLK as well (18,31). GPx activity, including isoforms GPx1 and GPx4, was inconsistently altered across studies, but generally lower in OLK patients. This may reflect impaired enzymatic function or substrate depletion due to sustained ROS exposure (13,26,32). Lipid peroxidation, measured via MDA and TBARS, showed contrasting patterns: while tissue levels were lower in OLK lesions, likely due to local ROS suppression or adaptation, serum and saliva samples revealed significantly elevated MDA, correlating with disease severity and progression (6,19,23,26). This paradox suggests that while tissue may suppress lipid peroxidation to permit cell survival, systemic compartments reflect ongoing oxidative damage. Furthermore, strong inverse correlations between MDA and antioxidant enzymes reinforce its relevance as a marker of redox imbalance (17,19,27). SOD and CAT, both central to enzymatic antioxidant defense, were generally reduced in serum and tissue samples of OLK patients, indicating impaired ROS detoxification (16,19,26,31). However, salivary SOD was found to be increased in one study, possibly due to local upregulation in response to external oxidative stimuli like tobacco or alcohol (19). SOD2, the mitochondrial isoform, was significantly downregulated in OLK tissue, suggesting compromised control of mitochondrial ROS (13). Uric acid (UA), a non-enzymatic antioxidant, showed slightly reduced levels in OLK patients, but the findings were inconsistent and not statistically significant in serum (33). Its dual antioxidant/pro-oxidant role and sensitivity to dietary and lifestyle factors make it less reliable as a clinical marker in this context (33).

Vitamins C and E were consistently lower in OLK patients across saliva and serum studies, supporting the hypothesis that antioxidant reserves are consumed during oxidative stress. Curcumin treatment increased these vitamin levels while reducing oxidative damage, suggesting therapeutic responsiveness (17,23,24). DNA damage markers such as 8-OHdG and 8-nitroguanine

were elevated in tissue and saliva, with increased staining in dysplastic areas and strong immunoreactivity observed in OLK lesions (21,22). These markers indicate oxidative and nitrative DNA damage and may serve as indicators of malignant transformation risk (16,23,24). Other mitochondrial-related markers, including TXN2 and GLRX2, were also elevated in OLK tissue, possibly reflecting cellular attempts to preserve redox balance and avoid apoptosis (13). TAS/TAC measurements varied across studies but suggest that antioxidant capacity is compromised in OLK (17). Tobacco and alcohol use emerged as strong contributors to oxidative stress in OLK, with studies linking them to increased MDA and reduced antioxidant levels (14,19,26,33). The chronic exposure to carcinogens induces persistent ROS generation, which not only damages cellular structures but also depletes antioxidant systems like GSH and CAT, creating an environment conducive to malignant transformation (12,19). The evidence indicates that oxidative stress contributes significantly to OLK pathogenesis. Altered redox markers across sample types suggest systemic imbalance influenced by modifiable risks. Still, methodological differences and limited long-term data highlight the need for further research on biomarkers in diagnosis and treatment.

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Figure 1: *PRISMA flowchart of searching and selection process of titles during systematic review.*

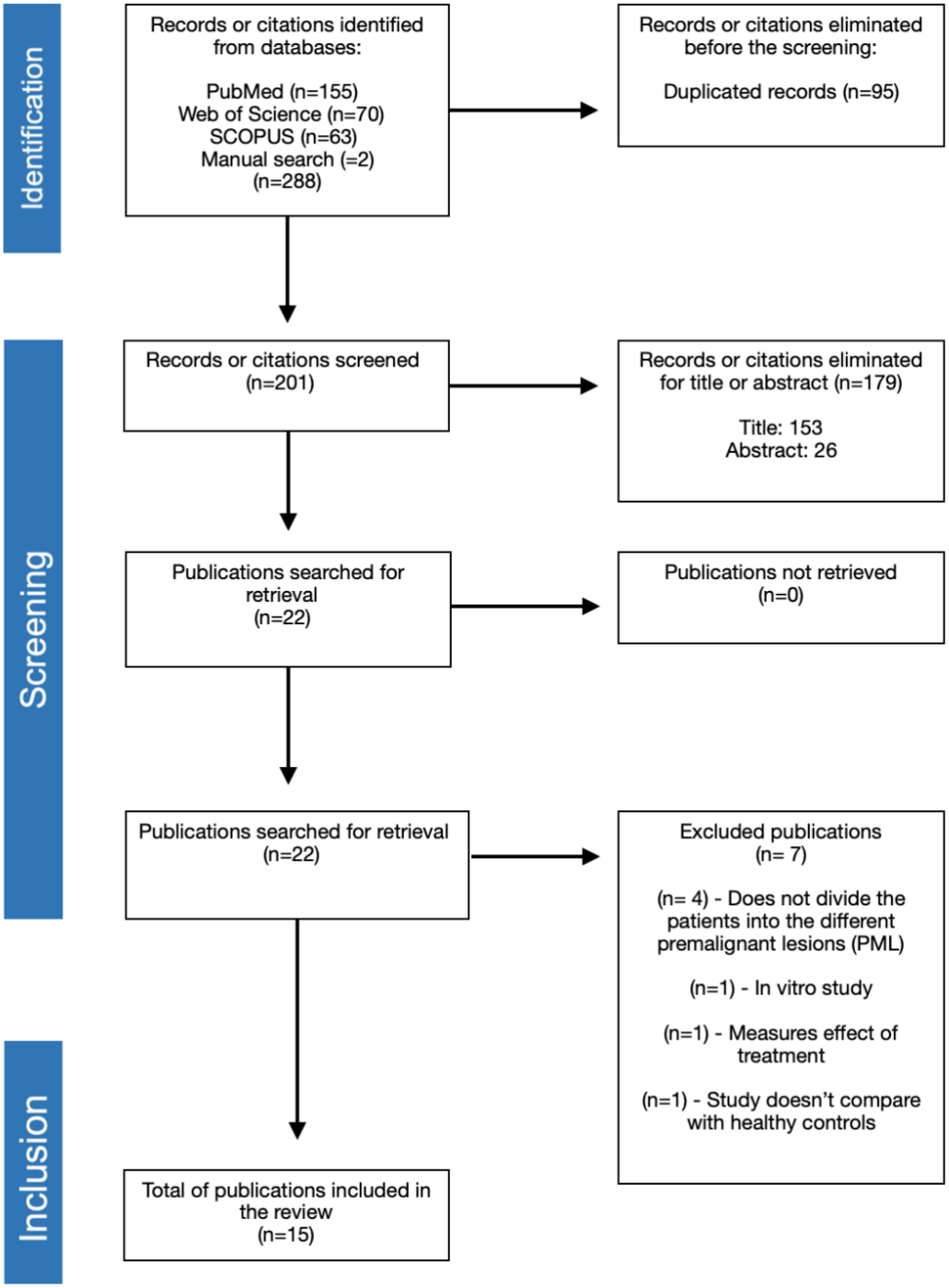

Table 1: *Characteristics of the included studies*

Author and year	Sample type	Type of study	Age	Biomarkers measured	Measuring method
(Ma et al., 2005) (21)	Tissue	Case-control study	Cases: (63.9 +- 9.6 years) Controls: (67.7 +- 10.1 years)	8-nitroguanine 8-oxodG	8-Nitroguanine and 8-oxodG were detected in oral epithelium using double immunofluorescence labelling.
(Srivastava et al., 2013) (12)	Tissue	Case-control study	Age 46,20 ± 11,08 years	Lipid peroxidation, SOD, CAT, GSH and GPx	Lipid peroxidation (TBARS) and antioxidant enzymes (SOD, CAT, GSH, GPx) were analyzed using spectrophotometry.
(Srivastava et al., 2016) (14)	Plasma	Case-control study	Cases: 46.20±11.08 years Positive controls: 39.55±9.22 years Negative controls: 37±7.56 years	TBARS, GSH, SOD, CAT and GPx	TBARS, GSH, SOD, CAT, and GPx were measured using standard colorimetric assays based on spectrophotometric absorbance at specific wavelengths.
(Metgud et al., 2014) (19)	Saliva and serum	Case-control study	Cases: 51.7 years Controls: 48.3 years	Lipid peroxidation, MDA and GSH	MDA (as TBARS) and GSH were measured using standard colorimetric methods, with absorbance read at 532 nm and 412 nm, respectively.
(Kaur et al., 2015) (23)	Saliva	Cross-sectional study	Age 49 ± 5.9 years	8-OHdG, MDA, Vit C and Vit E	MDA, 8-OHdG, and vitamins C/E were analyzed by TBARS, ELISA, and HPLC, respectively. ROC analysis defined thresholds; histopathology was the gold standard.

(Gurudath et al., 2012) (27)	Whole blood, serum, and erythrocyte lysate	Case-control study	Cases: 40.73±9.65 years Controls: age/sex matched	E-SOD and GPx	E-SOD and GPx activities were measured spectrophotometrically using commercial kits.
(Yadav et al., 2019) (32)	Serum	Cross-sectional study	Cases: 42.8 years Controls: 37 years	UA	UA was measured using the uricase method.
(Shetty et al., 2013) (15)	Serum	Case-control study	Age 20-65 years	GSH	Serum GSH was measured colorimetrically using the Beutler method with DTNB at 412 nm. Data were analyzed using one-way ANOVA
(**Babiuch et al., 2018)** (18)	Saliva	Case-control study (pilot study)	The median age was 59 years for cases and 51 years for controls.	TAC, SOD, GPx, GR, tGSH, GSH, GSSG, GSH/GSSG ratio, 8-OHdG and MDA	TAC: Measured by FRAP assay based on reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. Absorbance was read at 593 nm. SOD: Assayed via inhibition of epinephrine autooxidation at pH 10.2. Absorbance measured at 480 nm. GPx:Measured indirectly by GSH oxidation and NADPH consumption at 340 nm. GR: Activity assessed by monitoring NADPH oxidation to $NADP^+$ at 340 nm. tGSH: Quantified using a colorimetric kit measuring both GSH and GSSG at 405 nm. GSH: Measured by DTNB reaction producing TNB, read at 412 nm. GSSG: Calculated by subtracting GSH from tGSH. UA: Determined via enzymatic oxidation with uricase and colorimetric detection at 546 nm.

					8-OHdG: Measured using sandwich ELISA with monoclonal antibodies and colorimetric detection at 450 nm. MDA: Determined by competitive ELISA using monoclonal anti-MDA antibody, read at 450 nm.
(Sachdev et al., 2022) (16)	Plasma, serum, and erythrocyte lysate	Cross-sectional study	Age 20-60 years	Lipid hydroperoxide, MDA, SOD, GPx, CAT, GSH, Vit C and Vit E	The levels of lipid peroxidation products, antioxidants, and NO products were determined by colorimetric methods.
(Bose et al., 2011) (17)	Plasma	Cross-sectional study	Age 28-40 years	Zinc, TAS, Vit A, Vit C, Vit E and GSH	Plasma levels of β-carotene, vitamins C and E, GSH, TAS, and zinc were measured using standard colorimetric methods. Absorbance was read at specific wavelengths, and group comparisons were analyzed using Student's t-test.
(Rai et al., 2010) (24)	Saliva and serum	Pre-post interventional study	Age 17-50 years	MDA, 8-OHdG, Vit. E and Vit. C	MDA was measured by the TBARS method, vitamins C and E by liquid chromatography, and 8-OHdG by competitive ELISA. Data were analyzed using ANOVA and Spearman correlation ($p < 0.05$).
(Kuthoor et al., 2023) (28)	Plasma	Case-control study	Cases: 50.6 ± 10.1 years Controls: 47.4 ± 10.9 years	SOD	SOD levels were measured and analyzed using one-way ANOVA, Student's t-test, and Pearson's Chi-square test, as appropriate.
(Banerjee et al., 2020) (13)	Tissue	Cross-sectional study	Cases: 52.69±5.35 years Controls: age/sex matched	SOD2, CAT, GLRX2, GSH, GPx and TXN2	Mitochondria were isolated from precancerous oral tissues by differential centrifugation and validated by immunoblotting with specific cellular and mitochondrial markers. Control tissue was obtained via vestibuloplasty.

(Barros et al., 2022) (22)	Tissue	Prospective longitudinal study	Cases: 60 ± 13,3 years Controls: -	8-OHdG	8-OHdG expression in oral mucosa was assessed by immunohistochemistry.

GSH: glutathione; GPx: glutathione peroxidase; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; Vit C: vitamin C (ascorbic acid); Vit E: vitamin E (α-tocopherol); TBARS: thiobarbituric acid reactive substances; UA: uric acid; GR: glutathione reductase; tGSH: total glutathione; GSSG: oxidized glutathione; iNOS: inducible nitric oxide synthase; 8-nitroguanine: a nitrative DNA lesion; E-SOD: extracellular superoxide dismutase; SOD2: mitochondrial superoxide dismutase (Mn-SOD); GLRX2: glutaredoxin 2; TXN2: thioredoxin

Table 2: Risk of bias assessment with Newcastle-Ottawa scale in case-control studies

Articles	Case definition	Representativeness	Selection of controls	Control definition	Comparability (most important factor)	Comparability (any other variable)	Ascertainment of exposure	Same method for both groups	Dropout rate	Total
Ma et al. (2005)	★	★	★	–	–	–	★	★	★	6
Srivastava et al. (2013)	★	★	★	★	–	★	★	★	★	8
Srivastava et al. (2016)	★	★	★	★	★	★	★	★	★	9
Metgud et al. (2014)	★	★	★	★	★	–	★	★	★	8
Gurudath et al. (2012)	★	★	–	★	★	–	★	★	★	7
Shetty et al. (2013)	★	★	★	★	★	–	★	★	★	8
Babiuch et al. (2018)	★	★	★	★	★	★	★	★	★	9
Kuthoor et al. (2023)	★	★	★	★	★	–	★	★	★	8

Table 3: Risk of bias assessment with Newcastle-Ottawa scale in cohort studies.

Article	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that the outcome of interest was not present at start	Comparability (main factor)	Comparability (additional factors)	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	Total
Barros et al. (2022)	★	★	★	★	★	_	★	★	_	7

Table 4: *Bias assessment of cross-sectional studies with AXIS.*

Article	Criteria clearly defined	Sample description	Valid and reliable exposure measurement	Use of objective criteria to measure condition	Identification of confounding factors	Strategies to deal with confounders	Appropriate statistical analysis
Kaur et al., (2015)	yes	yes	no	yes	?	no	no
Yadav et al., (2019)	yes	yes	no	yes	yes	no	no
Sachdev et al., (2022)	yes	yes	no	yes	yes	no	?
Bose et al., (2011)	yes	yes	?	yes	yes	no	no
Banerjee et al., (2020)	yes	yes	no	yes	yes	no	no

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Table 5: *Bias assessment of interventional studies with ROBIN-I tool (by Cochrane).*

Rai et al. (2010) **Domain**	**Risk Level**	**Justification**
Bias due to confounding	Serious	No adjustment for smoking, age, sex, or other potential confounders.
Bias in selection of participants into the study	Moderate	Selection criteria described but unclear representativeness.
Bias in classification of interventions	Low	All patients received curcumin; no misclassification possible.
Bias due to deviations from intended interventions	Low	No deviations from planned intervention reported.
Bias due to missing data	Low	No relevant missing outcome data reported.
Bias in measurement of outcomes	Moderate	No mention of blinding; outcome measures could be influenced by knowledge.
Bias in selection of the reported result	Moderate	Full results reported, but no protocol registration noted.

Table 6: *Analysis results of serum/plasma/erythrocyte lysate and blood samples and their associated oxidative stress markers.*

Author and year	N	GSH	SOD	CAT	GPx	E-SOD	UA	MDA
(Srivastava et al., 2016) (14)	C: 20 HC: 20	C: 40.15±3.09** Negative control: 51.10±2.09** Positive control: 48.93±0.86**	C: .09±0.08* Negative control: 4.70±1.26* Positive control: 2.28±0.30*	C: 1.37±0.08** Negative control: 3.46±0.85** Positive control: 1.95±0.48**	C: 19.09±0.56** Negative control: 25.07±1.55** Positive control: 21.68±1.18**	–	–	–
(Metgud et al., 2014) (19)	C: 20 HC: 30	C: 21.47 ± 3.35** HC: 32.18 ± 5.53**	–	–	–	–	–	C: 3.31 ± 0.41* HC: 2.93 ± 0.79*
Gurudath et al. (2012) (27)	C: 25 HC: 25	–	C: 91.52 (U/ml) *** HC: 199.35 (U/ml)***	–	C: 21.55(U/g Hb)*** HC: 60.46* (U/g Hb)***	C: 91.52 ±19.45 (U/ml)*** HC: 164-240 (U/ml)***	–	–
Yadav et al. (2019) (32)	C: 25 HC: 30	–	–	–	–	–	C: 3.79±1.23 HC: 5.16± 0.98	
Shetty et al. (2013) (15)	C: 25 HC: 25	C: 01.04 ± 0.22** HC: 1.88 ± 0.36**	–	–	–	–	–	–

(Sachdev et al., 2022) (16)	C: 70 HC: 70	C: 2.02±0.322** HC: 13.24±0.94**	C: 188.45±8.54 (units/100 mg protein)*** HC: 233.64±11.89 (units/100 mg protein)***	C: 13.51±2.32** HC: 35.3±3.11**	C: 2.67±1.34** HC: 15.23±2.68**	–	–	C: 5.68±0.322*** HC: 1.96±0.145***
(Bose et al., 2011) (17)	C: 23 HC: 23	C: 6.09±0.67* mg/L HC: 10.09±0.89 *mg/L	–	–	–	–	–	–
(Rai et al., 2010) (24)	C: 25 HC: 25	–	–	–	–	–	–	C: 1.23 (0.56)** HC: 0.98 (0.86)**
Kuthoor et al. (2023) (28)	C: 29 HC: 25	–	C: 0.052±0.012 (U/ml)*** HC: 0.074±0.014 (U/ml)***	–	–	–	–	–

C: OLK cases; HC: healthy control; GSH: glutathione; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; E-SOD: extracellular superoxide dismutase; UA: uric acid; MDA: malondialdehyde; lipid hydroperoxide: lipid hydroperoxide; *:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

Table 7: *Analysis results of serum/plasma/erythrocyte lysate and blood samples and their associated oxidative stress markers.*

Author and year	N	Lipid peroxidation / TBARS	8-OHdG (ng/ml)	TAS	Vitamins	Zinc
(Srivastava et al., 2016) (14)	C: 20 HC: 20	C: 2.20±0.44** Negative control: 1.30±0.40 Positive control: 2.050±0.94*	–	–	–	–
(Metgud et al., 2014) (19)	C: 20 HC: 30	–	–	–	–	–
Gurudath et al. (2012) (27)	C: 25 HC: 25	–	–	–	–	–
Yadav et al. (2019) (32)	C: 25 HC: 30	–	–	–	–	–
Shetty et al. (2013) (15)	C: 25 HC: 25	–	–	–	–	–
(Sachdev et al., 2022) (16)	C: 70 HC: 70	C: 467.65±17.43*** HC: 276.46±17.66***	–	–	**<u>Vit E:</u>** C: 0.73±0.211 (mg/dL)*** HC: 11.74±0.566 (mg/dL)*** **<u>Vit C:</u>** C: 0.41±0.162 (mg/dL)**	–

					HC: 2.78±0.31 (mg/dL)**	
(Bose et al., 2011) (17)	C: 23 HC: 23	–	–	C: 1.23±0.45*** HC: 2.47±0.43***	**Vit E:** C: 5.99±0.82 (mg/dL)** HC: 10.54±1.1 (mg/dL)*** **Vit C:** C: 0.57±0.16 (mg/dL)** HC: 1.08±0.16 (mg/dL)***	C: 59.9±6.91*** HC: 91.2±11.8***
(Rai et al., 2010) (24)	C: 25 HC: 25	–	C: 2.13 (1.12)*** HC: 2.17 (1.45)***	–	**Vit E:** C: 8.01 (1.23) (µmol/l)*** HC: 8.97 (2.34) (µmol/l)*** **Vit C:** C: 8.78 (3.12) (µmol/l)*** HC: 9.05 (2.21) (µmol/l)***	–
Kuthoor et al. (2023) (28)	C: 29 HC: 25	–	–	–	–	–

C: OLK cases; HC: healthy control; TNO-2: total nitrite/nitrate; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; 8-ISO: 8-isoprostane; TAS: total antioxidant status; Vit A: vitamin A; Vit E: vitamin E; Vit C: vitamin C; antioxidant mineral zinc: zinc; GSH^{+}: total glutathione; *:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

Table 8: *Analysis results of salivary samples and their associated oxidative stress markers.*

Author and Year	N	8-OHdG (ng/ml)	MDA (µmol/l)	Vitamins (µmol/l)	TAC (mmol/l)	SOD (U/ml)
Kaur et al. (2015) (23)	C: 40 HC: 40	C: 0.36 (0.07)** HC: 0.07 (0.07)**	C: 0.33 (0.07)** HC: 0.08 (0.07)**	**Vit C:** C: 0.55 (0.13)** HC: 1.2 (0.6)** **Vit E:** C: 0.57 (0.16)** HC: 1.4 (0.6)**	-	-
Metgud et al., 2014) (19)	C: 20 HC: 30	-	C: 20.87 ± 1.23* HC: 19.98 ± 0.81*	-	-	-
Babiuch et al. (2018) (18)	C: 20 HC: 20	C: 11.54 (8.22) HC: 8.58 (4.59)	C: 8.30 (14.22) HC: 2.32 (5.36)	-	C: 0.74 (0.44) HC: 0.51 (0.34)	C: 3.40 (3.92)** HC: 2.36 (2.42)**
Rai et al. (2010) (24)	C: 25 HC: 25	C: 0.34 (0.24)*** HC: 0.11 (0.12)***	C: 0.36 (0.17)*** HC: 0.11 (0.13)***	**Vit C:** C: 1.08 (0.98)*** HC: 1.46 (0.86)*** **Vit E:** C: 0.65 (0.31)*** HC: 0.91 (0.43)***	-	-

C: OLK cases; HC: healthy control; 8-OHdG: 8-hydroxy-2-deoxyguanosine; MDA: malondialdehyde;Vit C: vitamin C; Vit E: vitamin E;TAC: total antioxidant capacity; SOD: superoxide dismutase; *:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

Table 9: *Analysis results of salivary samples and their associated oxidative stress marker.*

Author and Year	N	GPx [U/l]	GR [U/l]	GSSG / GSH [µmol/l]	GSH/GSSG ratio	UA [µmol/l]
Kaur et al. (2015) (23)	C: 40 HC: 40	-	-	-	-	-
Metgud et al., 2014) (19)	C: 20 HC: 30	-	-	**GSH** C: 8.67 ± 1.20*** HC: 9.74 ± 0.53***	-	-
Babiuch et al. (2018) (18)	C: 20 HC: 20	C: 81.34 (22.56), HC: 90.60 (18.65)	C: 17.7 (27.48), HC: 7.68 (6.47)	**GSH:** C: 0.01 (0.02)*** HC: 0.02 (0.01)*** **GSSG:** C: 0.26 (0.25) HC: 0.23 (0.22) **tGSH:** C: 0.27 (0.26) HC: 0.25 (0.23)	C: 0.21 (0.64)** HC: 0.27 (0.43)**	C: 386.36 (235.96), HC: 256.79 (185.20)
Rai et al. (2010) (24)	C: 25 HC: 25	-	-	-	-	-

C: OLK cases; HC: healthy control; GPx: glutathione peroxidase; GR: glutathione reductase; tGSH: total glutathione; GSH: reduced glutathione;GSSG: oxidized glutathione;UA: uric acid; *:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

Table 10: *Analysis results of tissue samples and their associated oxidative stress markers.*

Author and Year	N	8-nitroguanine (nitric stress)	8-OHdG	Lipid peroxidation / TBARS	SOD	CAT	iNOS (nitric stress)
(Ma et al., 2005) (21)	C: 19 HC: 4	C: Strongly positive (IR)** HC: Negative (IR)**	C: Strongly positive (IR)** HC: Negative (IR)**	-	-	-	C: Strongly and weakly positive (IR)*** HC: Negative (IR)***
(Srivastava et al., 2013) (12)	C: 20 HC: 20	-	-	C: 91.99 ± 2.97 HC: 127.93 ± 2.97	C: 14.48 ± 1.05 HC: 18.54 ± 0.54	C: 6.36 ± 1.10 HC: 10.46 ± 0.79	-
(Banerjee et al., 2020) (13)	C: 12 HC: -	-	-	-	-	C: 75.35 ± 0.56 HC: 98 ± 0.32	-
(Barros et al., 2022) (22)	C: 44 HC: 10	-	C: Strongly positive (IR in cytoplasm)* HC: Negative (IR in cytoplasm)*	-	-	-	-

C: OLK cases; HC: healthy control; IR: immunoreactivity:TBARS:thiobarbituric acid reactive substances;SOD: superoxide dismutase;CAT:catalase;iNOS: inducible nitric oxide synthase*:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.

Table 11: *Analysis results of tissue samples and their associated oxidative stress markers.*

Author and Year	N	GSH	GPx	SOD2	GLRX2	TXN2
(Ma et al., 2005) (21)	C: 19 HC: 4	-	-	-	-	-
(Srivastava et al., 2013) (12)	C: 20 HC: 20	C: 30.43 ± 2.90*** HC: 22.90 ± 1.10***	C: 22.99 ± 3.43*** HC: 15.16 ± 0.48***	-	-	-
(Banerjee et al., 2020) (13)	C: 12 HC: -	C: 12.4 ± 0.432 mM** HC: 11.3 ± 0.716 mM**	C: 48.58 ± 0.46 (GPX4)** 25.28 ± 0.55 (GPX1)** HC: 95 ± 0.43 (GPX4)** 85 ± 0.32 (GPX1)**	C: 40.8 ± 0.44** HC: 85 ± 0.2**	C: 146.17 ± 0.43** HC: 90 ± 0.57**	C: 146.11 ± 0.87** HC: 102 ± 0.70**
(Barros et al., 2022) (22)	C: 44 HC: 10	-	-	-	-	-

C: OLK cases; HC: healthy control; GSH: reduced glutathione;GPx: glutathione peroxidase;SOD2: Superoxide Dismutase 2;GLRX2: Glutaredoxin 2;TXN2: Thioredoxin 2*:p value <0.05 = statistically significant: **:p value <0.001= very highly significant:***:p value <0.0001= extremely significant.