

# Saliva-Based Biomarkers in Oral Squamous Cell Carcinoma Using OMICS Technologies: A Systematic Review

Fariba Esperouz <sup>1,\*</sup> , Domenico Ciavarella <sup>1</sup>, Andrea Santarelli <sup>2</sup> , Mauro Lorusso <sup>1</sup> , Lorenzo Lo Muzio <sup>1</sup> , Luigi Laino <sup>3</sup>  and Lucio Lo Russo <sup>1</sup> 

<sup>1</sup> Department of Clinical and Experimental Sciences, University of Foggia, 71122 Foggia, Italy

<sup>2</sup> Department of Clinic Specialistic and Odontostomatological Sciences, Polytechnic University of Marche, 60122 Ancona, Italy

<sup>3</sup> Multidisciplinary Department of Medical-Surgical and Odontostomatological Specialties, University of Campania “Luigi Vanvitelli”, 81100 Naples, Italy

\* Correspondence: fariba.esperouz@unifg.it; Tel.: +0881-588082

**Abstract:** (1) Background: Head and neck cancer (HNC) is a major public health challenge worldwide, with oral squamous cell carcinoma (OSCC) being the predominant form. Despite advances in treatment, OSCC remains a major cause of morbidity and mortality due to delayed diagnosis and limited therapeutic efficacy. This study reviews omics technologies to assess new salivary biomarkers for the early detection of OSCC. (2) Methods: A comprehensive literature search in the last 20 years identified four relevant studies focusing on salivary biomarkers in OSCC. (3) Results: Proteomic and genomic analyses revealed significant changes in salivary composition between OSCC patients and healthy controls, suggesting promising diagnostic and prognostic biomarkers. However, studies showed varying degrees of bias, indicating the need for further research and improved standardization. (4) Conclusions: Saliva, with its advantages of ease of collection, minimal invasiveness, and potential for large-scale screening, is an emerging promising substrate for non-invasive biomarker research. Nonetheless, there is a need for improved biomarker sensitivity and specificity; currently, histological examination remains the golden standard.

**Keywords:** saliva; biomarkers; OSCC; proteomics; genomics; liquid biopsy



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## 1. Introduction

Head and neck cancer (HNC) ranks as the sixth most prevalent cancer globally, affecting over 757,509 individuals worldwide [1]. HNC includes upper aerodigestive tract mucosal tumors (the mucosal epithelium of the oral cavity, pharynx, and larynx) [2], and more than 90% of HNCs are attributed to squamous cell carcinoma (OSCC), originating from the mucosal surface of the mouth.

Among HNCs, oral cancer is of particular interest and a significant health issue due to its high incidence, recurrence, and mortality rates (440,000 new deaths annually globally) due to limited symptoms, leading to delayed diagnoses, and rapid progression from early to advanced stages [2].

In advanced tumor stages, distant metastases are more common and require aggressive and clinically difficult multi-disciplinary treatments (surgery, chemotherapy, and radiotherapy).

These treatments have unsatisfactory success rates and are burdened by considerable complications, with a high negative impact on survival and quality of life (postoperative difficulty in chewing and swallowing, dysarthria, and loss of facial aesthetics) [3].

Researchers aim to create less invasive early-stage diagnostic methods to revert these figures.

Attempts have been made to apply various techniques and technologies, such as spectroscopy, brush biopsy, autofluorescence, blue toluidine staining, and “OMICS” methodologies (transcriptomic, genomic, metabolomic, and proteomic), to identify molecular and tissue signatures, along with potential biomarkers in cells, tissues, and biological matrices like saliva. Now, the golden standard still remains biopsy, which may be responsible, at least in part, for the current, negative epidemiological figures of head and neck malignancies and the associated health and social burden [4,5].

In recent years, saliva liquid biopsy has emerged as a promising minimally invasive alternative [6], to reduce biopsy-associated complications.

Saliva’s simplicity in collection, its non-invasiveness, and its potential for identifying various analytes [7] make it suitable for diagnostic tool development.

Several studies [8–13] in the past have investigated the possibility of detecting RNA biomarkers, particularly the expression of miRNA in saliva as potential biomarkers in head and neck cancer using genomics techniques [14]; salivary miRNA-21, miRNA-145, and miRNA-184 are examples of miRNAs that may be used as quick, non-invasive diagnostic indicators for oral malignant transformation. However, for the time being, given their degree of sensitivity and specificity, they could act as a confirmatory or follow-up tool in addition to biopsy, which is still the golden standard [12].

However, as the tumor is in direct contact with saliva, it contains a range of other analytes including proteins, microorganisms and metabolites, peptides, electrolytes, and organic and inorganic salts secreted by the salivary glands, which, through the use of various ‘omics’ technologies, can be identified and used as biomarkers for prevention, monitoring, diagnosis, and prognosis [6].

Proteomics is defined as “the study of the interactions, function, composition, and structures of proteins and their cellular activities” [15], and among the omics technologies currently used, proteomic profiling now has the potential to become standard practice in the clinical laboratory thanks to innovations in proteomic technology [16]; proteomics can analyze the expression of a protein at different levels, allowing the assessment of specific quantitative and qualitative cellular responses related to that protein [17], and currently, the most promising application of proteomics is in the screening of protein biomarkers for certain diseases, including oral cancer.

Three forms of proteomics exist: structural proteomics, functional proteomics, and expression proteomics [18]. The latter is particularly useful in the detection of head and neck cancer because it may identify proteins unique to a certain illness [19]. Moreover, proteomics can be used to diagnose cancer and discover novel therapies [20].

Despite being a high-coverage, sensitive, and quick proteome technology [21], proteomics does not yet have any approved biomarkers for the diagnosis of OSCC.

Furthermore, the potential use of protein-based salivary indicators in the identification of oral potentially malignant disorders (OPMDs) has been assessed in recent research [22]; OPMDs are defined as “a heterogeneous group of clinically defined conditions associated with a variable risk of progression to oral squamous carcinoma. Most produce clinically visible lesions” [23]. Nowadays, a biopsy is required to determine whether or not dysplasia is present, even in cases with OPMDs [24].

This systematic review aims to determine the current state of research in the identification of potential salivary biomarkers identified using omics technologies (proteomics and genomics) for the early diagnosis of OSCC, and whether or not the biomarkers actually identified are effective for diagnosis.

## 2. Materials and Methods

### 2.1. Protocol and Search Strategy

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement and the Patient or Population, Intervention, Control or Comparison and Outcome (PICO) methodology were adhered to during the review process.

The formulated PICO question was as follows: “Is it possible to use saliva biomarkers, detected by OMICS technologies, for the early diagnosis in patients with OSCC”?

Utilizing the following databases, PUBMED, SCOPUS, and Cochrane Database of Systematic Reviews, an electronic search of the English-language literature was conducted up to February 2024. The following search strategies were used: “Saliva, oral fluid, oral cancer, head and neck cancer, markers, biomarkers, diagnosis, prognosis, and proteomics”.

Additionally, the gray literature was retrieved via a manual research. No restrictions were applied on the year of publication.

## 2.2. Eligibility Criteria, Studies Selection, and Data Collection Process

Studies fulfilling the following eligibility criteria were considered eligible for inclusion in this review:

- (a) Published English-language studies focusing on the potential role of saliva for biomarker identification;
- (b) Studies conducted on human saliva;
- (c) Studies that exploited omics technologies.

Failure to meet these criteria resulted in the exclusion of the study.

The following study types were also excluded from this review: biographies, editorials, lectures, reviews, systematic reviews, letters to editors, book chapters, animal samples, and studies for which English translations were unavailable.

During the initial phase, the reviewers screened the titles and abstracts of the papers obtained from the search data sources. Studies that did not center around the identification of salivary biomarkers were excluded at this stage. At the conclusion of the second round of the selection process, papers that met all the inclusion criteria were chosen for the extraction of data. For each included study, the following data items were systematically recorded: author names, year of publication, method of sample collection, technologies used for analysis, and outcome data (Table 1).

**Table 1.** Characteristic of included study.

Author	Year	Type of Study	Cases of OSCC or OPMD	Control	Salivary Biomarkers	Type	Detection Method	Principal Conclusion
Lo Russo et al.	2012	Proteomics	45	30	8041 and 6239 <i>m/z</i>	Proteins	SELDI-TOF-MS and Protein Chip	The peptide with 8041 Da mass was 22-fold more expressed in OSCC, thus being a suitable potential biomarker
Hsu et al.	2014	Proteomics	96	47	DKK1c, VEGFCc, dATP1A1, LCP1, NT5Ed, LOXL2, LUM, RAP1Bc, COL5A1, UFD1Ld, CFB, DNAJB11d, HLA-C, GOLM1, THBS2, FN1c, C1S, SFRS3, F3c	Proteins	SDS-PAGE LC-MS/MS	THBS2, UFD1L, and DNAJB11 were found to be elevated in OSCC tissues
Gallo et al.	2016	Proteomics	45	30	74 peaks	Proteins	SELDI-TOF/MS	74 mass peaks whose intensities were significantly different between controls and OSCC
Chu et al.	2019	Genomics	233	115	A1BG, AFM, ANXA2, APOA1, APOA2, APOA4, APOB, APOH, ITIH1, KNG1, PLG, SERPINA1, SERPIND1, VTN, C3, CA2, CFB, CFH, FGA, FGB, FN1, HP, HPX, HRG	Gene	LC-MS/MS; LC-MRM-MS; iTRAQ; Sandwich ELISA	CFH, FGA, and SERPINA1 were demonstrated to have the potentials as biomarker candidates for early detection and/or prognosis of OSCC.

### 2.3. Risk of Bias Assessment

Reviewers adapted the techniques used in a related systematic review to assess the risk of bias in each included paper [25].

The quality assessment of the included studies was carried out using the “Scottish Intercollegiate Guidelines Network” (SIGN) methodology checklist for case–control studies. The SIGN checklist consisted of 11 statements for evaluating the risk of bias of the internal validity across 7 domains: group comparability, differentiation, assessor blinding, outcome measures, confounding, statistical analysis, and overall assessment.

The scores for each statement were as follows: “yes” (low risk) or “no” (high risk). In case of a lack of details, the score “can’t say” (moderate risk) was given.

After the piloted test of the checklist was conducted to confirm its compatibility with this review, the reviewer decided that the grouped domains, i.e., “groups comparability and differentiation”, would obtain one of the following risks:

- (a) “low risk” in case all responses to the statements were “yes”;
- (b) “high risk” in case of the presence of a negative response (no);
- (c) “can’t say” for the presence of two or more “no” responses;
- (d) “moderate” was given in the case of the presence of one response of “can’t say” to one of the statements [26].

## 3. Results

### 3.1. Study Selection

A flowchart of the selection process is detailed in Figure 1. In total, seven records were retrieved from the database and screened by title and abstract; of these, only four studies met the eligibility criteria and were included in the study (Table 1).

### 3.2. Study Features

The included studies were published between 2012 and 2019 and analyzed the potential role of saliva in the research on new possible biomarkers for the diagnosis and prognosis of oral squamous cell carcinoma (OSCC) using omics technologies. The analyses in the studies by Hsu et al. [27] and Chu et al. [28] were performed on a cohort of Taiwanese patients. Participants included patients with early and advanced OSCC.

The studies by Gallo et al. [4] and Lo Russo et al. [29] involved 45 patients with OSCC and 30 healthy controls from the Department of Head and Neck Pathology at the Second University of Naples.

Most of the studies focused their attention on searching for biomarkers for the early and non-invasive diagnosis of OSCC, rather than on the search for markers for the early diagnosis of oral potentially malignant disorders (OPMDs), for which only one study set up an analysis.

In addition, three out of four studies used proteomics techniques to detect and analyze possible biomarkers in the saliva of OSCC patients, and only one study used a genomics technique.

All studies set the standards for their research by evaluating the differences in concentrations of possible salivary biomarkers between cancer and control subjects.

The two main methods used to carry out the analyses were surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (Seldi Tof-Ms), a mass spectrometry technique used to analyze biomolecules, particularly proteins and peptides, and liquid chromatography with tandem mass spectrometry (LC-MS/MS), this being used for both protein and gene detection.

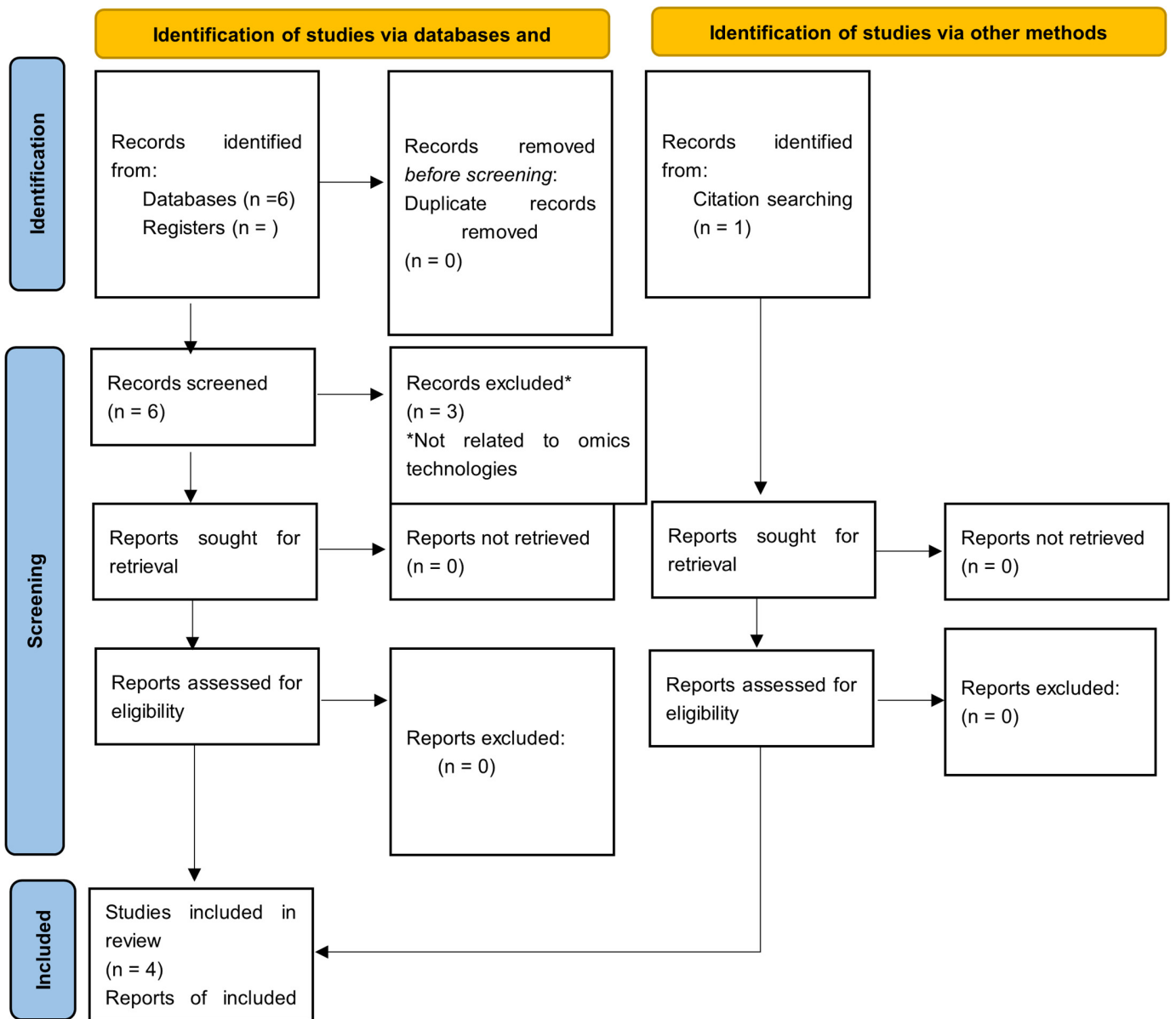


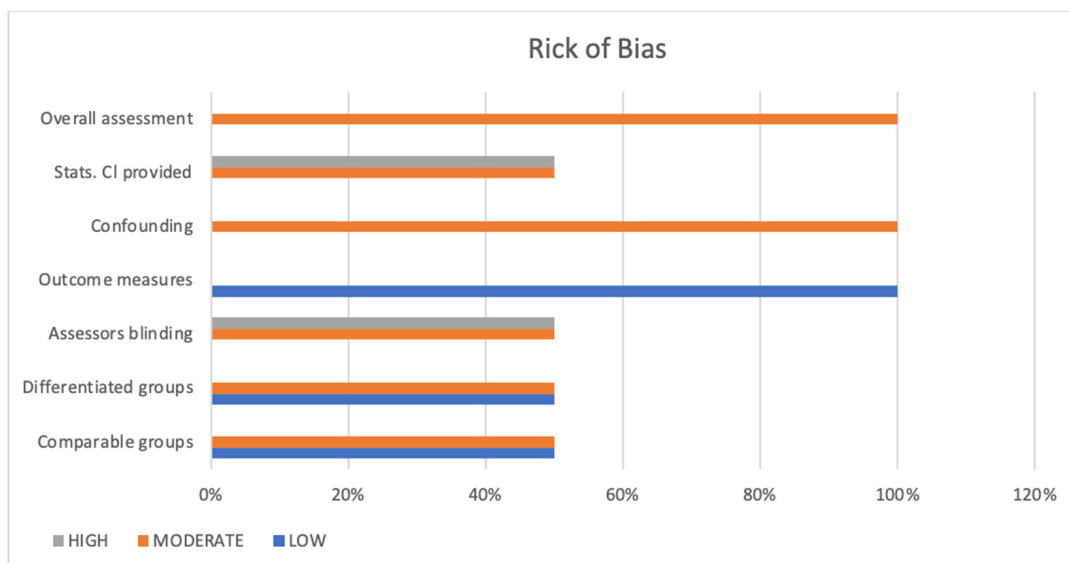
Figure 1. Flowchart of study selection process.

### 3.3. Risk of Bias Assessment

None of the studies analyzed reported a low BIAS level following the SIGN checklist. In detail, the risk of bias is summarized in Table 2 and in Figure 2.

Table 2. Risk of bias assessment scores of the included studies with the SIGN methodology checklist for case-control studies.

Study	Comparable Groups	Differentiated Groups	Assessors Blinding	Outcome Measures	Confounding	Stats. CI Provided	Overall Assessment
Lo Russo et al.	Low Risk	Low Risk	Moderate Risk	Low risk	Moderate Risk	High Risk	Moderate risk
Hsu et al	Moderate Risk	Low Risk	High Risk	Low risk	Moderate Risk	High Risk	Moderate risk
Gallo et al	Moderate Risk	Moderate Risk	High Risk	Low risk	Moderate Risk	Moderate risk	Moderate risk
Chu et al	Low Risk	Moderate Risk	Moderate Risk	Low risk	Moderate Risk	Moderate risk	Moderate risk



**Figure 2.** The proportion of the studies of this review with a low, moderate, or high risk of bias across the different considered domains of the SIGN methodology checklist.

### 3.4. Synthesis of Results

In all the studies conducted, the omics analysis methods showed that there were differences in the levels of both proteins and genes in saliva samples from cancer patients and saliva samples from healthy people (Table 3).

**Table 3.** Methods used in the studies and results of analysis.

Study	Samples	Store	Methods	Analysis	Results
Chu et al.	Unstimulated saliva	−80°	Centrifugation at 3000 × g for 15 min at 4 °C.	(MRM)-MS	↑ CFH ↑ FGA ↑ SERPINA 1
Hsu et al.	Unstimulated saliva	−80°	Centrifugation at 3000 × g for 15 min at 4 °C.	Label-free quantification	↑ THBS2, ↑ UFD1L, ↑ DNAJB11
Lo Russo et al.	Unstimulated saliva	−80°	Centrifugation 13,000 × g per minute at 4 °C for 10 min	SELDI-TOF	19 mass peaks
Gallo et al.	Unstimulated saliva	−80°	Centrifugation 13,000 × g per minute at 4 °C for 10 min	SELDI-TOF	74 mass peaks

The salivary proteomes of patients with OSCC, of individuals with OPMDs, and of healthy volunteers were analyzed using isobaric tags for relative and absolute quantitation (iTRAQ)-based mass spectrometry (MS) in Chu et al.’s study to find potential biomarkers for OSCC [27].

To obtain unstimulated saliva for the study, patients were instructed to refrain from eating, drinking, or smoking for at least two hours beforehand. An amount of 4 mL of saliva was treated right away with protease inhibitor mixtures in order to stop the activity of natural salivary enzymes. The supernatants were collected and kept at 80 °C until needed following centrifugation at 3000 × g for 15 min at 4 °C.

In the OSCC group, salivary levels of 67 proteins were found to be raised, while the non-cancer group (OPMD and healthy groups) showed that 18 proteins were present in decreased amounts. Multiple reaction monitoring (MRM)-MS was used to further narrow the candidate biomarkers, and immunoassays were used to validate them. Advanced

OSCC stages were associated with elevated salivary levels of three proteins: complement factor H (CFH), fibrinogen alpha chain (FGA), and alpha-1-antitrypsin (SERPINA1).

These results were confirmed by conducting statistical analyses, reporting an AUC for CFH of 0.661, and 0.740 for SERPIN1 and FGA.

In the study conducted by Hsu [28], unstimulated whole saliva was taken during oral mucosal examination, and volunteers refrained from eating, drinking, smoking, and using oral hygiene products for at least one hour before sample collection. The gathered specimens underwent 15 min,  $3000 \times g$  centrifugation at 4 °C. A protease inhibitor mixture (2 µL/mL; Sigma-Aldrich, St. Louis, MO, USA) was applied right away to the supernatants. After that, they were aliquoted into smaller quantities, kept at −80 °C, and analyzed via spectral counting based on label-free quantification; it was found that the salivary concentration of 64 proteins was significantly higher in patients with OSCC, compared with that of healthy subjects, using LC-MS/MS as the sample analysis method. The final results reported that the levels of THBS2, UFD1L, and DNAJB11 were significantly higher in patients with OSCC; in particular, THBS2 reports an AUC of 0.756, suggesting that THBS2 is a fairly good biomarker for the detection of OSCC, with a sensitivity and specificity of 55.1% and 89.4%, respectively.

In the work of Lo Russo et al. [29], collected saliva samples were immediately frozen in dry ice for transport to the laboratory, and once there, protease inhibitors were added in a 10/100 (*v/v*) ratio; then, the samples were divided into 1 mL aliquots, stored at −80 °C until use, and analyzed using the SELDI-TOF method. Eleven mass peaks were found to be excreted in higher amounts in the saliva of patients with OSCC, while eight were found to be excreted in lower amounts. In particular, 2 (8041 and 6239 *m/z*) of the 19 mass peaks showed the strongest association with OSCC (22- and 17-fold increases, respectively); indeed, 8041 and 6239 reported high ROC/AUC values (close to 1), indicating that the identified biomarkers have excellent diagnostic capabilities, making them valuable for the early diagnosis of OSCC.

The same results were obtained by Gallo et al., who identified 74 mass peaks with significantly different intensities in the saliva of OSCC, compared with that of controls, via SELDI-TOF analysis [4]; the results were confirmed via the use of the radial basis function, which correctly classified 80% of the controls and 100% of the OSCC cases, resulting in an overall performance of 91.89%.

#### 4. Discussion

Much work has been conducted recently to enhance OSCC prognosis, therapy, and diagnosis. On the other hand, GLOBOCAN states that OSCC is one of the main causes of morbidity and death globally [1]. To provide more accurate and customized methods for early diagnosis, prognosis, and the development of effective treatments, the most recent research has concentrated on understanding the molecular underpinnings of OSCC.

Saliva is a newly developed specimen for diagnosing illnesses because it can be obtained easily and non-invasively; for these reasons, liquid biopsy has been proposed in numerous studies [25] as a possible alternative to traditional biopsy performed on a histological sample.

The primary objective of this systematic review is to answer the first question: “Is it possible to use saliva biomarkers, detected by OMICS technologies, for the early diagnosis in patients with OSCC?” Among the results obtained from proteomic and genomic analyses performed on saliva samples collected from OSCC patients, the salivary proteomes of healthy controls, individuals with OPMD, and patients with OSCC, quantitatively profiled using isobaric tags for relative and absolute quantification and non-targeted mass spectrometry, showed CFH, FGA, and SERPIN1 as potential candidates for the early diagnosis and/or prognosis of OSCC [27].

Proteomic studies, conducted using the SELDI-TOF method, a mass spectrometry technique capable of analyzing complex biological samples with high sensitivity, have shown that alterations in low-molecular-weight proteins are more common in the early

phase of neoplastic disease, while alterations in high-molecular-weight proteins are more common in the metastatic phase [4]. The same results were obtained in a previous study, in which, again, when applying SELDI-TOF and Protein Chip<sup>®</sup> technology, the latter enabling the separation, detection, and identification of proteins and peptides present in complex biological samples, such as serum, plasma, tissue, and cell samples, it was found that the salivary proteomes of oral cancer patients differed from those of healthy controls and that these variations could follow the progression of oral cancer.

Another method, liquid chromatography with tandem mass spectrometry, proved to be a promising technique for the search for biomarkers, and it was found that in individuals with OSCC, there is a higher level of Thrombospondin 2 (TBH2), an adhesive matricellular glycoprotein that participates in the modulation of cell–matrix interaction, and in the modulation of UFD1L and DNAJB1, two protein folding-related molecules, in contrast to the case of normal epithelia.

The result for TBH2 is very encouraging as this gene produces a protein of the thrombospondin family, which is responsible for mediating interactions between cells and in the cell matrix; the glycoprotein is a disulphide-linked homotrimeric glycoprotein [30]. Epithelial–mesenchymal transition (EMT) is the process underlying tumor growth. It involves the remodeling of cell–cell and cell–extracellular matrix junctions and the detachment of epithelial cells from the basement membrane and from each other [31]. EMT increases the potential for tumor growth and metastasis, as well as the resistance of tumor cells to elimination by various treatments; therefore, the identification of a potential biomarker present in the early stages of OSCC bodes well for the possibility of using such molecules in the early diagnosis of this tumor, with the possibility of reducing the still high mortality associated with this neoplasm.

Limitations of this study include the presence of bias and other limitations, such as the small number of samples in the studies reviewed. Thus, further research is needed to validate these findings.

## 5. Conclusions

Based on the results of this systematic review, it is possible to draw the conclusion that saliva might be used as a substrate for biomarker research by utilizing omics technologies; indeed, recent developments in nanotechnology applied to proteomics and genomics have revealed a plethora of potential biomarkers for OSCC.

However, detailed studies with a high number of samples must be conducted to verify their specificity; as a result, while there is significant potential, histological testing remains the gold standard for oral cancer diagnosis [32].

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