UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN MEDICINA MOLECOLARE

CICLO XXXVII

Identification and validation of salivary biomarkers for systemic and oral diseases

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Anni Accademici 2021/2022 – 2023/2024

The present Thesis reports the activities carried out during the PhD course in Molecular Medicine - XXXVII cycle, under the supervision of Professor Marco Meleti at the University Center for Dentistry (Department of Medicine and Surgery, University of Parma), in collaboration with the Laboratory of Biochemistry and Metabolomics (Professor Thelma Pertinhez, Department of Medicine and Surgery, University of Parma), with the Breast Unit (Professor Antonino Musolino, Parma University Hospital), and with the Laboratory of Industrial Toxicology and Advanced Analytical Methods (Professor Paola Mozzoni, Department of Medicine and Surgery, University of Parma).

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SUMMARY

An established target of medicine and modern healthcare is *early diagnosis* of diseases and the evaluation of *patient risk profile*. Detect a disease at an early stage and modulate the risk factors may improve the success of treatment, prevent complications and enhance prognosis and quality of life of patients.

The possibility to obtain an accurate diagnosis and effective screenings through reliable and non-invasive tools, seems to be a very attractive.

Within such background, the concept of "*precision medicine"* is rapidly developing. It usually employs molecular profiles to adapt a therapeutic strategy to a patient, with specific stage of disease and/or to determine the predisposition for. "Precision medicine" is based on biomolecular disciplines usually indicated with the suffix "omics". It encompasses genomic, epigenomic, transcriptomic, proteomic, metabolomic, and others.

The combination of the above-mentioned concepts is leading to the development of novel technologies, based on easy and non-invasive methods to collect diagnostic human specimens, possibly with a high specificity and sensitivity and customized for specific patient. One of the human fluids which has gained importance within this context is saliva.

The aim of the present thesis is to present translational data about salivary biomarkers and their potential application in clinical setting.

An update of the current knowledge about salivary diagnostics is provided in Chapter 1. Latest literature confirms the fluid high potential as a matrix for multipurpose analysis with several different technological platforms; the number of recently published studies confirms the increasing interest in saliva. However, as routinely reliable diagnostic tools, salivary diagnostics has still little spreading.

Chapter 2 reports preliminary data of a pilot study of nutriepigenomic, which aims to highlight the role of nutrition in epigenetic variations (induced and inducible), as cause of cancer risk increasing or decreasing. The study evaluated the effect of a nutritional and lifestyle program [Nutritional and Lifestyle Intervention (NLI)] on salivary miRNAs expression in women carriers of BRCA1/2 germ mutations. Preliminary statistically results seem to demonstrate that 5 salivary miRNAs have a statistical difference between their expression before nutritional intervention and after 18 months.

The progression from normal mucosa to different grades of dysplasia is explored in relation to salivary metabolome in an experimental case-control study (Chapter 3). Metabolomics was performed on saliva samples using high resolution ^{1}H-NMR (Nuclear Magnetic Resonance). A cohort of voluntary patients with histologically confirmed oral leukoplakia and oral lichen planus was enrolled and salivary specimens were collected before surgical procedures (incisional/excisional biopsies). Preliminary results are focused on the differences between metabolomes of patients with dysplastic and non-dysplastic leukoplakia as well as healthy controls.

Chapter 4 reports data about the possible relationships between the salivary and serum metabolomes to gain a comprehensive view of the metabolic phenotype under physiological conditions. Using ^{1}H-NMR spectroscopy, we obtained the serum metabolite profiles of 20 healthy young individuals and compared them with the metabolomes of parotid, submandibular/sublingual, and whole saliva samples collected concurrently from the same individuals. The salivary gland-specific metabolic composition and the effective saliva collection protocol is reported in the Annex n° 1. During the samples collection, the Full-Mouth Bleeding Score (FMBS) was employed as a classification parameter. We identified a panel of metabolites differentially expressed in healthy subjects with high but physiological FMBS scores compared to those with lower score, and this set of metabolites may be associated with early stages of gingival inflammation. This correlation between salivary metabolites and gingival bleeding score is described in Chapter 5. The salivary fluid has been widely studied and genomic, transcriptomic, and proteomic profiles have been reported. Conversely, its metabolic composition is still mainly unknown: metabolites in submandibular/sublingual saliva have never been analyzed systematically. These results could provide basis to a further identification of salivary biomarkers in oral and systemic diseases.

Results emerging from 2 systematic reviews are presented in chapter 6 and 7.

The first was developed to answer the question: “Are salivary biomarkers reliable for diagnosis of dental caries?” (Chapter 6), while the second aims to identify the salivary metabolites for the diagnosis of oral cancer (Chapter 7).

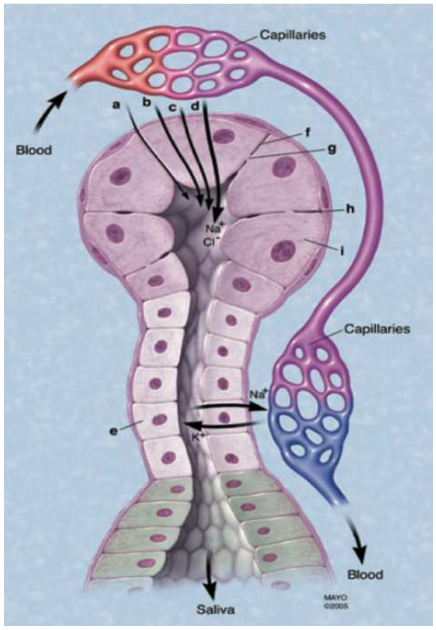
For both reviews, results and quality assessment of selected studies are presented, highlighting the potential risks of bias in current literature. Moreover, a summary of statistical data and reported evidence are given.

Conclusions are provided in chapter 8.

CHAPTER 1

Salivary diagnostics – un update

Saliva is a complex biological fluid produced by secretions of major and minor salivary glands. In normal conditions, salivary glands produce about 1-1.5 litres of serous and mucinous saliva daily, by combining water, salts, salivary proteins (locally produced by the glands) and a subset of very different molecules derived from the blood. The quantity and composition of saliva depends on several factors, such as flow rate, circadian rhythm, type and size of glands, duration and type of salivary stimulus, diet, drugs, age, gender and psychological status [1] [2].



Such a fluid may provide a picture of the health status of a person, being an ultra-filtrate of the blood and presenting its specific molecules (Figure 1.1). As matter of facts, saliva has been defined as the "mirror of the body" or "window on health status" [3].

For its intrinsic characteristic, like complexity of composition, abundance of molecules, similarity with blood composition, good production quantity, combined with non-invasive sample, easy collection, transport and storage, saliva represents an excellent and innovative substrate for the research of biological molecules, defined as "biomarkers".

Figure 1.1 Mechanisms of transport of proteins and ions from serum into salivary gland ducts.

Salivary biomarkers

Biomarker, short term for "biological marker", is an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions [FDA-NIH definition].

Biomarkers are used in several branches of medicine. Through the analysis of alterations or concentration of biomarkers it is possible to detect or confirm presence of a disease or condition of interest or identify individuals with subtype of the disease [4].

Biomarkers exist in a variety of different forms; in a diagnostic contest they can be classified into 5 groups:

a) Genomic biomarkers, which are based on the analysis of DNA. Detecting genomic alteration may possibly indicate a pathology, based on the hypothesis that the accumulation of specific genetic aberrations can lead to the development of illness. Saliva contains approximately 24 μg of DNA, ranging from 0.2 to 52 μg, sufficient to genotyping procedures (it is sufficient about 5 ng/mL of DNA to work effectively) [5].
b) Transcriptomic biomarkers, which are referred to RNA transcript of a cell. The information contained in the DNA are transmitted with transcription, through RNA-molecules type: mRNA and non-coding RNA; the letter has several functions and can be further subdivided into a number of categories including microRNA and small interfering RNA. All these molecules could be used as target of analysis. A consistent portion of RNA into the saliva is the microbial-RNA, because of abundant presence of oral microbiota. Saliva sample should previously be centrifuged in order to obtain cell-free whole saliva, which is the richest in genuine human RNA (range from 0.108 ± 0.023 μg/mL to 6.6 ± 3.6 μg/mL) [5]. Cell-free whole saliva contains more than 3000 species of mRNA, derived from several sources, beside microorganisms. Such sources are the secretion of salivary glands (originated by circulation or directly produced into the acini), the gingival crevicular fluid, desquamated oral cells and blood coming from microwounds in the oral mucosa [6].
c) Proteomic biomarkers, defined as specific protein target, particularly involving in alteration of their structure, function and quantity (increase or decrease). Proteomic biomarkers may have a strong diagnostic potential because they are more diversified than genome: they consist of an estimated 500000 protein isoforms derived from about 20000 protein-coding genes. Such an increase in protein diversity is largely due to alternative splicing and post-translational modification of proteins. Furthermore, genomic technologies can only provide a static snapshot, as not all gene-encoded proteins are expressed at all the time and in all cell types [7].
d) Metabolomic biomarkers, which are based on metabolites, intermediate and final products of metabolism. The metabolome represents the collection of all metabolites in cells, tissues, organs or organisms (which are the final products of genic expression). It includes molecules, like nucleic acids, lipids, amino acids, peptides, vitamins, organic acids, thiols and carbohydrates. The different concentration of endogenous

metabolites, respect a healthy status, may be the expression of a pathological process in act, and thus represent a possible target for the molecular analysis [8].

e) Microbiota, that is an ecological community of commensal, symbiotic and pathogenic microorganisms found into organisms. Oral microbiota is abundant, composed by more than 300 species. There are several approaches for characterizing the salivary microbiome, two of these are the analysis of their RNA or their metabolites [8].
   Modification of oral microbiota may suggest correlation with some tumors, as found in pancreatic [9] and stomach cancer [10].

State of salivary biomarkers in diagnostic

The composition of the saliva virtually reflects the entire state of health and disease in a body The composition of the saliva may virtually reflect the entire state of health and disease in a body and it has the potential of being a diagnostic medium for a broad range of diseases, such as in the detection of caries risk, periodontal diseases, oral cancer, non oral cancer, neurological disorders, inflammatory, cardiovascular and metabolic diseases and much more (Figure 1.2).

High-impact human diseases are challenging to diagnose without supplementing clinical evaluation with laboratory testing. Even with the current laboratory tools, definitive diagnosis often remains elusive due to clinical or public health factors.

Salivary sample as a diagnostic tool has many advantages and could exceed the limits of other diagnostic fluid, as serum. Saliva is easy to collect, store and ship. For patients, the non-invasive collecting techniques reduce anxiety and discomfort and simplify procurement of repeated samples [11].

Caries risk

Dental caries represents a complex disease that, if diagnosed and treated early, can be stabilized and, in some cases, reversed with the remineralization of the tooth surface [12].

Innovative methods for the early diagnosis of carious lesions are currently available [13]. Some of such new approaches could lead to a decrease in invasive treatments and costs for patients and health care systems.

Dental caries is in direct contact with saliva, and some of its components react to the acidic environment induced by bacterial metabolism, contrasting their biofilm's development and

adhesion. Indeed, the detection and measurement of salivary caries biomarkers may represent an attractive alternative for the early diagnosis of caries. However, using a single biomarker predictive of disease occurrence appears unsuitable given the multifactorial aetiology of caries.

According to the results of our published systematic review (showed in Chapter 7), most of the salivary molecules analysed might potentially play an important diagnostic or predictive role[14]. Based on the "good" quality studies, salivary mucins, glycoproteins (sCD14), interleukins (IL-2RA, 4-13), urease, carbonic anhydrase VI, and urea appear to exhibit significant different levels in healthy and active-caries subjects [15–21]. These salivary molecules should be the target of clinical research to validate or exclude their relevance as biomarkers for dental caries.

Periodontal diseases

Early diagnosis can significantly improve the evolution and prognosis of periodontal diseases. Approaches based on periodic clinical screening of periodontal tissues are the most used, even if their efficacy has recently been debated.

The possibility to detect specific salivary signatures of gingival inflammation can be of paramount importance to identify subjects that are potentially predisposed to develop periodontal diseases and that cannot be intercepted by current clinical parameters (such as Bleeding on Probing).

Papers since 2010 have shown that a wide variety of classes of biomarkers found in saliva including proteins of host and bacterial origin (IL-1β and TNFα, MMP-8) [22] [23],DNA and mRNA of host, bacterial, and viral origin, ions and steroid hormones [24–26] are informative in the detection of gingival inflammation and periodontal disease activity [27].

As end-products of many physiological and pathological processes, also salivary metabolites can be regarded as markers of specific pathways, including those resulting from host-microbial interactions (e.g. gingival inflammation) [28].

Particularly, metabolites originating from gingival tissues and bacteria are possibly released within the crevicular fluid and eventually in the whole saliva[29].

There are currently two salivary diagnostic tests available for the detection of periodontal diseases. One test identifies the type and concentration of specific periodontal pathogenic microorganisms in patients' saliva samples. Another salivary test claims to detect genetic

susceptibility to periodontitis in individuals, analysing the variation in the production of the inflammatory cytokines, interleukin-1 a and b [30].

A novel Salivary Occult Blood Test (SOBT) is currently available in Japan. This method has been reported to detect individuals with poor periodontal health (bleeding on probing in ≥20% of teeth or the presence of probing pocket depth ≥6 mm plus bleeding on probing in ≥1 teeth), using a paper strip containing gold-labelled anti-human haemoglobin monoclonal antibody that is dipped into the saliva sample [30].

Oral cancer

Oral squamous cell carcinoma (OSCC) is among the most frequent cancers worldwide, with an estimated new 354,864 cases (2% of all sites) and 177,384 related deaths (1.9% of all sites) occurring in 2018 [31]. Despite treatment advances, the 5-year survival rate remains below 50%. Stage at diagnosis remains an important indicator for predicting patient survival, with survival rates for early-stage disease being approximately 84%, but decreasing to approximately 40% for advanced-stage disease [32, 33].

To avoid diagnostic delay and possibly detect subclinical forms, tools platforms and biomarkers for malignancy identification than histology on a biopsy, which remains the current gold standard, have been proposed [34].

For its strict contact with salivary fluid, oral cancer is one of the most investigated diseases for salivary biomarkers identification.

Genetic and epigenetic alterations were found in salivary circulating tumour DNA from OSCC, as well as deletions, loss of heterozygosity, gene mutations and methylation; such alterations were comparable to those identified in tissue specimens for histology. Two genes, HOXA9 and NID2, emerged as valuable in early detection and follow-up of patients with OSCC [35]. Moreover, somatic mutations of TP53 in saliva identified OSCC in a whole of oral cancer patients[36].

The study of salivary microRNAs concentration seems to be reliable both for OSCC identification and follow-up. Significant decreased levels of miR-125a and miR-200a and elevated miR-31 were16 reported in saliva of OSCC, with evidence of decrease of the latter after disease treatment[37, 38]. In addition, a pilot study reports a comparison between OSCC patients and HC which returned diminished concentrations of miR-139–5p in cancer group [39].

Several authors investigated the alteration of metabolic pathways and their consequent metabolites expression between OSCC and HC through targeted and untargeted metabolomic procedures [40–42]. The majority of the metabolomic study on salivary samples from OSCC cases report about the role of sialic acid, cortisol, pyruvic acid, neopterine, 8-hydroxy-2-deoxyguanosine and malondialdehyde, endogenous porphyrin. Overall, the presence and/or concentration of more than 100 salivary metabolites was investigated in association with histologically confirmed diagnosis of OSCC [43] (Chapter 6).

Non oral cancer

Several studies have investigated the potential of human salivary components for cancers diagnosis, therapy efficacy evaluation and follow-up.

Most of the studies found in literature investigated the correlation between salivary biomarkers and breast carcinoma. Among these, some authors reported a correlation between increase of c-erbB2 [44] [45] [46], CA15-3 and cancer[47], cathepsine D,p53 and cancer[48–51]; Brooks al. have detected a significant increase of vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and carcinoembryonic antigen (CEA) in patients with breast cancer compared to non-cancer patients [52]; other studies, show statistically significant association between the tumor and the concentration of lung resistance protein (LRP) [53].

Delmonico L et al. (2015) investigated the methylation pattern of cyclin-dependent kinase Inhibitor 2A (CDKN2A) and ATM gene promoter, into the saliva of women with benign and malignant breast lesion. The genes present a good percentage of methylation, but there was not significant statistical difference between the two groups [54].

Tsutsui H et al. (2013) and Takayama T et al. (2016) evaluated several metabolites, as polyamines (ornithine (ORN), putrescine (PUT), cadaverine (CAD), spermine (SPM), N1N8-diacetyl-spermidine (DAc-SPD), N1N12-diacetyl-spermine (DAc-SPD), diaminopropane (DAP), 1,6-diaminohexane (DAH), N8-acetylspermidine (Ac-SPD), N1-acetyl-putrescine (Ac-PUT), N1-acetyl-spermine (Ac-SPM) and spermidine (SPD))[55, 56] . Takayama T et al. used a larger group of patients and one more molecule was identified (N1-acetylspermidine). This study showed strong correlation between salivary biomarkers and cancer. As a matter of fact, the test was positive in more than 80% of the patients with breast cancer [57].

Other authors reported the association between salivary metabolites and breast cancer (BC) and showed a significant difference between cancer group and healthy control group (HC).

Zhong et al. (2016) evaluated 18 metabolites, that have a difference of salivary expression between BC and HC groups. In particular, 3 of these (two form of lysophosphatidylcholine and monoacylglycerol) are more significant (Area Under the Curve (AUC) of 0.920) [58].

The second most studied malignant tumour is lung pancreatic carcinoma. Particularly, molecules as EGFR gene, 5 mRNA (CCNI, EGFR, FGF 19, FRS 2, GREB1) and proteins such as the calprotectin and AZGP1 HP showed a good association with diagnosis of lung carcinoma at several stages [59–61].

Also, for pancreatic carcinoma, it is been demonstrated a significant association between the salivary molecules and the diseases. Molecules reported in literature derived from transcriptomics (4mRNA (KRAS, MBD3L2, ACRV1, DPM1), and 2 miRNAs (miR-3679-5p and miR-940) [62].

Pancreatic carcinoma is an aggressive tumour and usually diagnosed at a late stage, with not peculiar symptoms. When diagnosed, distant metastases are present, and the prognosis is therefore very poor[63]. Based on that, the possibility of early diagnosis, not based on possible subjective radiographical images interpretation, could prevent most of the deaths related to this cancer.

Other malignant tumour studied are gastric, prostatic and ovarian cancer [64–68].

Particularly, in the field of oncology, an easy, non-invasive and fast repeatable method based on salivary diagnostic, may prevent a late diagnose and hypothetically avoid and invasive treatment, and sometimes the death.

Neurological disorders

Studies on neurological disorders are mainly focused on Parkinson's and Alzheimer disease. About Alzheimer's disease, Aβ40-42 is considered a valid biomarker, with a decrease under pathological conditions. Instead, *p*-Tau and *p*-tau/t-tau ratio show increased salivary concentration in presence of Alzheimer's disease[69]. Other biomarkers have been evaluated for Alzheimer's disease such as lactoferrin, decreased in the saliva of affected patients compared to the control group and subjects with fronto-temporal dementia, and acetylcholinesterase, that decreases in affected patients [70–72].

For Parkinson's disease, salivary biomarkers most analysed are alpha-synuclein, whose correlation with progression of the pathology is debates, and DJ-1, increased in the saliva of patients [73–75].

Inflammatory, cardiovascular and metabolic diseases

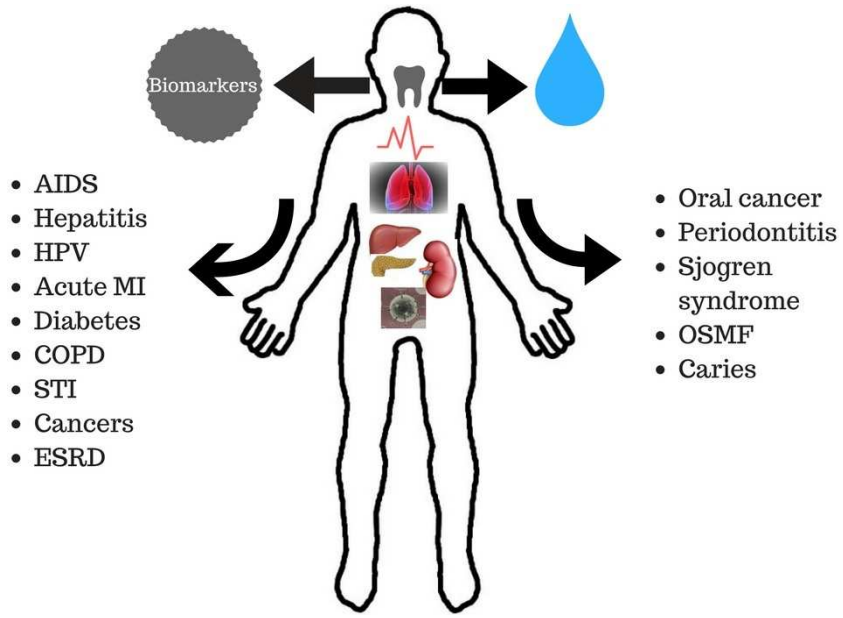
Studies on inflammatory, cardiovascular and metabolic diseases evaluated several pathologies. Most studies are focused on acute myocardial infarction (AMI) and rheumatoid arthritis.

Recent literature reports that the identification of troponins, C-reactive protein and creatine phosphokinases may serve as salivary tests able to promptly discriminate patients with AMI [76].

The study on rheumatoid arthritis patients highlighted the role of GRP78/BiP, a protein significantly correlated to the diseases [77].

Rheumatoid arthritis is difficult to diagnosis, mostly in the early stage, because of the variability of the symptoms and the absence of specific markers. Early diagnosis of the disease improves the success of the treatment, reducing the quantity of drugs administered.

Figure 1.2 Depiction of the detection of various oral and systemic diseases through salivary biomarkers [78].



CHAPTER 2

Identification of salivary miRNAs in women with mutations of BRCA 1 and 2 genes: a pilot study of Nutriepigenomic

Introduction

Breast cancer (BC) is the most common malignancy in women worldwide and ovarian cancer (OC) is the most lethal gynaecological malignancy. Approximately, 20%–25% of the patients with OC and 5%–10% of the patients with BC carry an inherited predisposition to their pathologic condition, and the most involved mutated genes are BRCA 1 and 2 [79]. Tumorigenesis in women with germline BRCA mutations requires somatic inactivation of the remaining wild-type allele, suggesting that the BRCA genes are tumour suppressors [80].

In BRCA1 and BRCA2 mutation carriers, the cumulative risk of breast cancer at 70 years has been estimated to 65 and 45%, respectively, and the risk of ovarian cancer to 39 and 10%, respectively [81]. The identification of a BRCA1/BRCA2 mutation is crucial for the medical follow-up, as mutation carriers should be offered annual Magnetic resonance Imaging (MRI) or, alternatively, prophylactic mastectomy and prophylactic salpingo-oophorectomy. Furthermore, in a breast cancer patient, the detection of a germline BRCA1 or BRCA2 mutation may have important therapeutic consequences: complete mastectomy instead of partial mastectomy and, in the future, the prescription of specific targeted therapies [79].

Although the risk of breast and ovarian cancer increases considerably, not all women with BRCA1 and BRCA2 mutations develop cancer, thanks to the different expression of the carcinogenic potential of genes, defined as "penetrance".

Penetrance rates of BRCA1/2 genes vary because of endogenous factors, such as gene polymorphisms, as well as exogenous factors, such as the number of pregnancies, year of birth, and physical activity during youth [82]. It is interesting to note that the risk for breast cancer is lower if genotype carriers were born before 1940, suggesting the role of lifestyle in the expression of the carcinogenic potential of genes. Nutrition also influences the risk of breast

cancer. Obesity and weight increase the risk of breast cancer in both pre- and postmenopausal subjects [83].

A weight gain of more than 20 kg after the age of 18 doubles the risk of breast cancer. Furthermore, women with a body mass index (BMI) of >30 kg/m^2 have a greater risk of developing distant metastases and early mortality indicating a calorie and fat-reduced nutrition program led to a significant reduction in recurrence rates [84, 85].

The branch of medicine that studies the mechanisms of gene regulation by exogenous factors is called "Epigenetics".

Epigenetics encompasses DNA methylation, chromatin variation, and noncoding RNAs, particularly microRNAs (miRNAs).

MicroRNAs are 18–25 nucleotides noncoding RNAs that post-transcriptionally regulate gene expression. These molecules are involved in RNA interference (RNAi) machinery by binding to the untranslated regions (UTRs) of mRNA to suppress protein translation or decay mRNA [86].

Moreover, the expression of miRNAs is also regulated by epigenetic machinery, including DNA methylation, RNA modification and histone modification. The reciprocity relationship between miRNAs and epigenetic regulation forms the miRNA-epigenetic feedback loop (Figure 2.1). The modulation of miRNA-epigenetic feedback loop and its cellular function has emerged as a novel mechanism of regulating cell process, including cell proliferation, apoptosis, and differentiation [87].

Due to their cell cycle interference, miRNAs are involved either as oncogenes or as oncosuppressors in the pathogenesis of a huge variety of human cancers such as lung cancer, prostate cancer, colorectal cancer, leukaemia, gliomas and medulloblastoma, diffuse large B-cell lymphoma, hepatocellular carcinoma (HCC), gastric cancer, osteosarcoma, renal cell carcinoma, BC, and OC[88]. Particularly, four significantly altered and specifically regulated miRNAs (miR-21, miR-125b, miR-451, and miR-155) were identified in BC patients as compared to healthy controls, and some of them, such as mir-155, are more expressed in women carrying mutations of the BRCA1 and BRCA2 genes [89].

Moreover, several studies indicate that some miRNAs are related to certain lifestyles or individual modifiable or voluptuous risk factors, as nutrition [90].

Some gene panels, which evaluate several miRNAs at the same time, are able to identify different miRNAs expression profiles between healthy women, women with sporadic breast

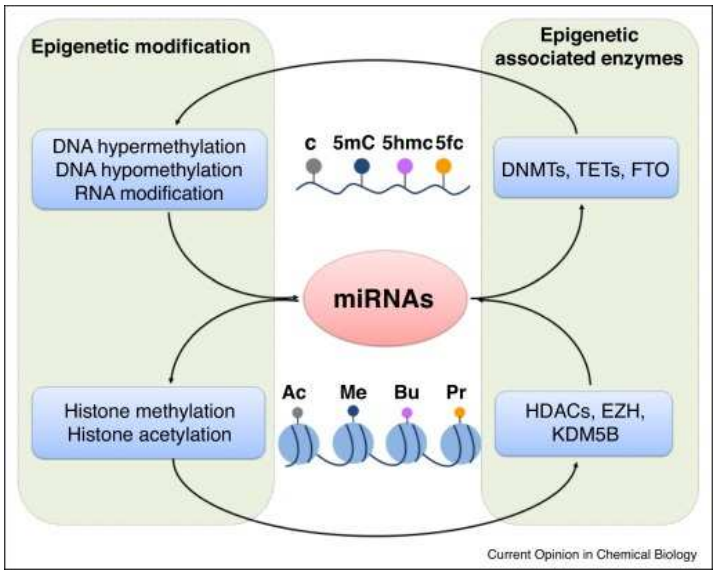
cancer and women with BRCA-mutated breast cancer [91]. miRNAs are easily available from blood, saliva or urine samples, making their analysis manageable and repeated [91].

There are many aspects that would identify salivary testing as an excellent alternative of screening method. Several studies have already identified more than 100 biomarkers present in saliva that could act as indicators of an ongoing malignant process such as cytokines, interleukins, enzymes, genes [92, 93]. Saliva is a non-invasive, quick, safe, easily repeatable diagnostic tool, therefore well accepted by patients; usable thanks to the presence of "salivary biomarkers" [94].

Nutriepigenomic is a new area of investigation that aims to highlight the role of nutrition in modifying epigenetic variations (induced and inducible), cause of cancer risk. Recent studies have shown how the expression of miRNAs can be influenced by nutrition. Some easily introduced foods in the diet, e.g. curcumin contained in curry or epigallocatechin-3-gallateil (EGCG) contained in green tea, may reduce the expression of oncogenic miRNAs and promote the increase of miRNAs tumour suppressors [95]. An adequate intake of nutrients, vitamins and antioxidants with a diet rich in fruits and vegetables (e.g., Mediterranean diet), increases the expression of miRNAs oncosuppressors, easily searched with a salivary sample [96].

Further understanding of the dysregulation of miRNA-epigenetic feedback loop and its mechanism during the development of different diseases has great potential to lead to the discovery of novel therapeutic targets of diagnostic strategies for cancer [97].

Figure 2.1 Schematic illustration of the miRNA-epigenetic feedback loop. MicroRNAs are regulated by epigenetic regulators, including DNA methylation, RNA modification and histone modification. Epigenetics-associated enzymes are also under the control of miRNA regulation.



Rational and aim of the study

A nutritional and life-style strategy can regulate the expression of epigenetic variables (miRNAs) associated with cancer development and modulate penetration and phenotypic manifestations (age of onset and type of tumor event) of BRCA1 and/or 2 germinal mutations.

Primary Aim

Monitor the expression of a salivary and serum miRNAs panel, which may affect the risk of cancer and penetration of BRCA1/2 germline mutations. The study evaluates the effect of a nutritional and lifestyle program [Nutritional and Lifestyle Intervention (NLI)] on miRNAs expression. Study population include unaffected women, involved in the enhanced breast/ovarian cancer screening program as carriers of BRCA1/2 germ mutations.

Secondary Aim

1. Evaluate the improvements of NIL program in terms of Health-Related quality-of-life improvement (HR-qol), dietary habits, habitual levels of physical activity (AF), Body Mass Index (BMI), metabolic syndrome parameters, demoralization levels and anxiety.
2. Describe the relationship between miRNAs measurements from blood and saliva samples.

Ethical Committee and Funding

The present pilot study was approved by the Ethical Committee of the “Area Vasta Emilia Nord” (AVEN) - Ethical Committee Approval number: 1036/2018/SPER/AOUPR NUTRIEPIGENOMICA. It was conducted in association with the Breast Unit of the Academic Hospital of Parma (coordinator: Prof. Antonino Musolino) and “Centro Universitario di Odontoiatria” of the University of Parma (coordinator: Prof. Marco Meleti)

It obtained a funding by “Lega Italiana per la Lotta contro I Tumori” (LILT) with a Call for Health Research 2017.

Materials and methods

Study design

Prospective observational non-pharmacological interventional study, aimed to collect data of women not affected but involved in the intensified screening program for breast/ovarian cancer, active at the Hospital-University of Parma, as carriers of BRCA1/2 germ cell mutations. The assignment to a specific NLI program was determined during clinical practice, and decisions were made by practitioners involved in the study (doctor dietician, nutritionist biologist and oncologist). Even if NLI programs were customized, the following guidelines - recommendations of the World Cancer Research Fund (WCRF) and of the American Institute for Cancer Research (AICR) – were the common elements in defining the programs.

As shown in Figure 2.2, the study design was characterized by the following 3 periods:

1. pre-exposure period: 6 months prior to the start of NLI program, relevant historical data of patients were recorded. These data were used to obtain standardized profiles of participants, as socio-demographic, genetic and nutritional/behavioural aspects.
2. Transition period: based on the data obtained in the pre-exposure period, the patient met professionals, including doctor dietician, biologist nutritionist, oncologist and dentist, in order to develop a personalized NLI program. Although the program has begun, the month of transition period is necessary to be considered as active and stable in the individual's life.
3. Post-exposure period: adherence to the NLI program was monitored with regular visits for 23 months starting from the day following the transition month.

In order to:

- not to force the participants into strict timelines and deadlines,
- ensure the care feasibility,

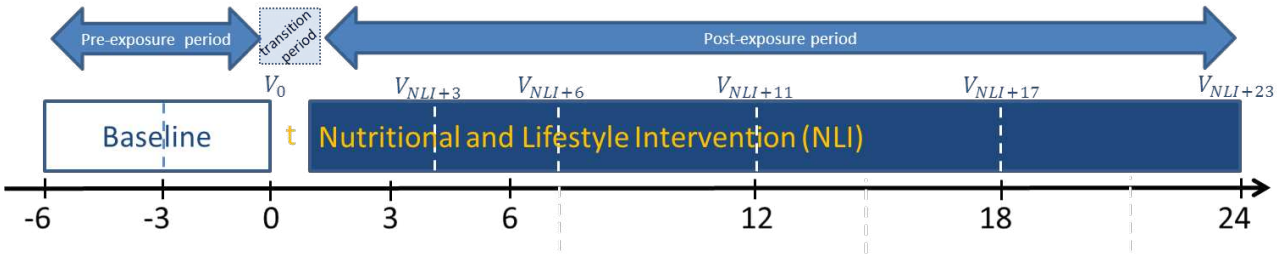
for each periodic visit we considered tolerable, in proportion to the time since the last survey, the following deviations (in terms of days) from the next estimated date of recognition:

- 1 month + [0-7] days
- 3 months + [0-14] days
- 6 months + [0-21] days
- 12 months + [0-28] days
- 18 months + [0-28] days

– 24 months + [0-28] days

This design provides to assess, for each subject, the changes induced by the intervention and at the same time avoid the burden of a control group, that no interest would have been received for participation and it would be unethical, as the benefits of NLI's intervention are already proven [98].

Figure 2.2 Timeline of the experimental intervention



Study population

Young women, not affected, carriers of germline mutations of BRCA1 and BRCA2 genes, submitted to the Emilia-Romagna Region's Breast and/or Ovarian Cancer Hereditary Risk Assistance Program at the University Hospital of Parma (http://salute.regione.emilia-romagna.it). were included in the study.

Written consent was obtained from all volunteers who participated in this study, according to the Declaration of Helsinki.

Inclusion Criteria

– Evidence of germ cell mutation of BRCA1 and/or BRCA2 genes
– Age 18 - 40 years
– Participation in the assistance program for the hereditary risk of breast cancer and/or ovarian cancer of the Emilia-Romagna Region at the University Hospital of Parma
– Performance status (Eastern Cooperative Oncology Group) ≤ 1
– Informed written consent

Exclusion Criteria

– Previous diagnosis of breast and/or ovarian cancer
– Previous intervention of mastectomy and/or prophylactic oophorectomy
– Clinical conditions in active or uncontrolled phase that would limit compliance with the study requirements

- Pregnancy or breastfeeding

Experimental intervention

- Individual visit to start the study:
  - Collection of family and personal anamnesis (physiological and pathological)
  - Lifestyle and voluptuous habits assessment (alcohol, smoking, eating habits)
  - Weight, height, body mass index (BMI), waist circumference, blood pressure and heart rate measurement
  - Assessment of habitual levels of physical activity through the International Physical Activity Questionnaire (IPAQ) [99]
  - Baseline blood chemical tests (routine blood chemistry, total cholesterol, triglycerides, HDL cholesterol, basal blood glucose and insulin levels for the calculation of the Homeostasis Model Assessment - HOMA index)
  - Blood and saliva sampling for miRNAs analysis
  - Filling out quality of life questionnaire (HRQOL) through European Organization for Research and Treatment-QOL questionnaire (EORTC QLQ-C30/B23) [100, 101]
  - Nutritional counselling and production of a personalized lifestyle program.
- Monthly telephone interviews (1 time per month):
  - Collection of patient-reported outcomes (PRO) through standardized questionnaires [102]
  - Assessment of adherence to the NLI program
- Educational and group training events on nutrition and lifestyle every 2 months
- Individual periodic visits (+ 3/+6/+12/+18/+24 months):
  - Collection of family and personal anamnesis (physiological and pathological)
  - Lifestyle and voluptuous habits assessment (alcohol, smoking, eating habits)
  - Weight, height, body mass index (BMI), waist circumference, blood pressure and heart rate measurement
  - Assessment of habitual levels of physical activity through the International Physical Activity Questionnaire (IPAQ)[103]
  - Baseline blood chemical tests (routine blood chemistry, total cholesterol, triglycerides, HDL cholesterol, basal blood glucose and insulin levels for the calculation of the Homeostasis Model Assessment - HOMA index)

- Blood and saliva sampling for miRNAs analysis
- Filling out quality of life questionnaire (HRQOL) through European Organization for Research and Treatment-QOL questionnaire (EORTC QLQ-C30/B23)

Nutritional and Life-stye Intervention (NLI)

The main objective of the nutrition intervention was to provide a food education program, that was able to help subjects achieve long-term changes in their eating habits and to improve or change previous habits to get as close as possible to the Mediterranean Diet model (DM). The assumption is that the NLI program can regulate the expression of epigenetic variables (miRNAs) associated with cancer development and modulate penetration of BRCA1 and/or 2 germinal mutations.

Eating habits were recorded using the EPIC (European Prospective Investigation into Cancer and Nutrition Food Frequency Questionnaire - EPIC FFQ) [104]. This was administered to all the 'timepoints' of the study.

Dental examination of the study participants

Eligible subjects underwent a comprehensive oral exam and a sialometric test at the Centro Universitario di Odontoiatria of the University of Parma. Oral inspection included a careful examination of teeth, periodontal tissues, and oral mucosa (alveolar, labial, buccal mucosa, and mucosa covering tongue, palate, and attached gingiva). Assessments did not include invasive procedures and only employed a mirror and dental/periodontal probes.

Dental status was assessed by the Decayed, Missing, and Filled Teeth (DFMT) index. Periodontal health status was assessed by the Periodontal Screening and Recording (PSR) index [25] and by plaque and bleeding indexes (Full Mouth Plaque Score - and Full Mouth Bleeding Score -)[26]. Subjects achieving a FMPS and FMBS ≥ 10% were subjected to a professional oral hygiene

The salivary flow rate was assessed by the modified Saxon Test to exclude subjects suffering from hyposalivation (whole saliva flow <0.5 mL/5 min) [105].

Saliva collection

Saliva collection for miRNA analysis was carried out at the Centro Universitario di Odontoiatria of the University of Parma (baseline, + 3/+6/+11/+23 months).

Patients who did not require the preliminary procedure of "professional oral hygiene" were submitted to a saliva sample on the same day as the dental visit.

In patients who were subjected to "professional oral hygiene" the collection of saliva was performed with a distance of 14 days.

Participants were asked to refrain from eating, smoking, and performing intense physical activity for at least 12 h before salivary sampling and to drink only water. Furthermore, it was requested not to carry out oral hygiene (tooth brushing and flossing) in the 45 min before saliva collection. The procedure took place between 8:00 a.m. and 10:00 a.m. to minimize the influence of the circadian rhythm on salivary composition. Immediately before collection, patients rinsed their mouth with water for 1 min.

Unstimulated whole saliva was collected by the passive drooling method (Figure 2.3)

During collection, salivary samples were transferred to Eppendorf tubes and kept on ice until a volume of 3 mL and then frozen at −80 °C.

Figure 2.3 Passive drooling method for saliva collection

Blood collection

Blood collection for miRNA analysis was carried out at the Day Hospital for Oncology of the University Hospital of Parma (baseline, + 3/+6/+11/+23 months). The sample was about 10 cc of venous blood. The biological material obtained was appropriately processed and stored at the molecular biology laboratory of the Day Hospital for Oncology of the University Hospital of Parma.

Laboratory analysis (miRNA)

Blood and saliva samples were taken from each study participant at baseline and at the time points: + 3/+6/+11/+23 months. Each sample was tested with a panel of 41 miRNAs selected

from the literature and whose expression is related to breast cancer and BRCA1/2 mutations [106, 107] (Table 2.1).

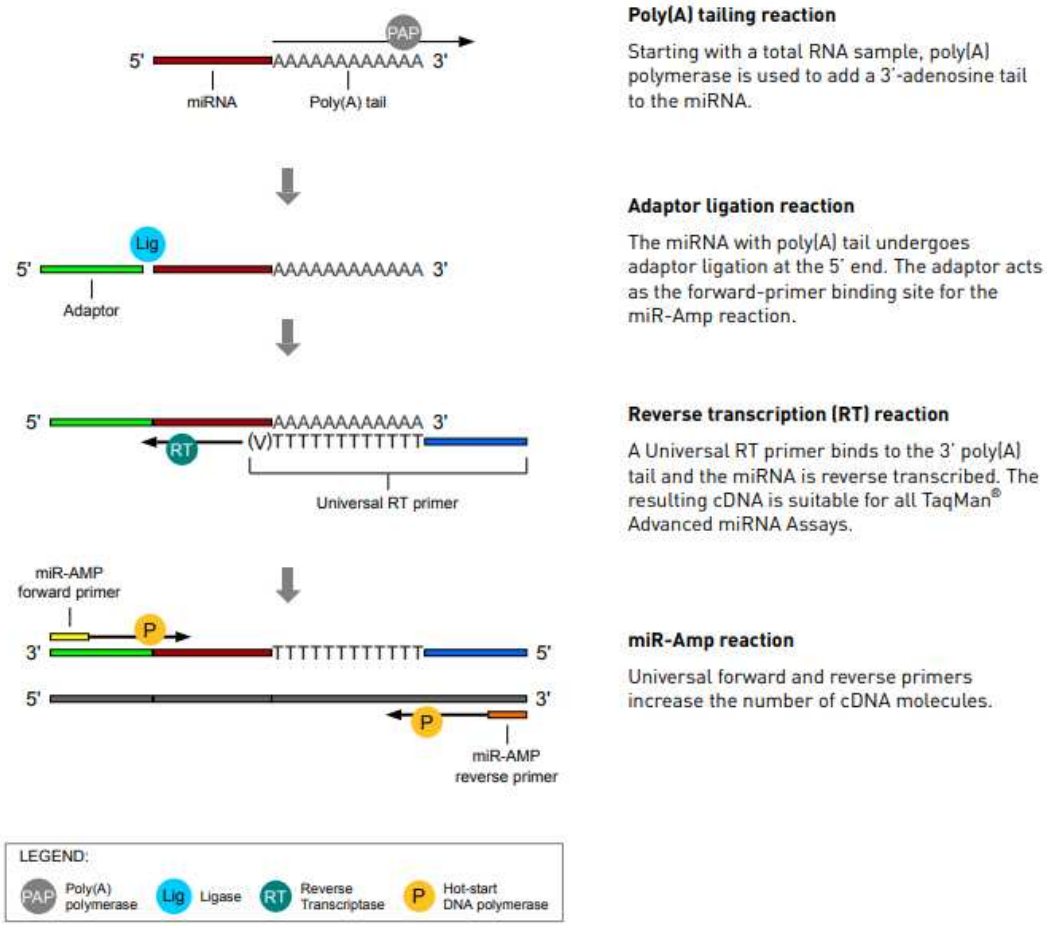
Table 2.1 miRNAs selected by literature and analysed by RT-PCR

miRNAs analysed				
145b-5p	181b-5p	146a-5p	191-5p	133b
31-5p	378a-3p	222-3p	221-3p	27a-3p
17-5p	181a-5p	155-5p	106b-5p	24-3p
16-5p	122-5p	663a	21-3p	15a-5p
132-3p	489-5p	335-5p	125b-5p	182-5p
200c-3p	10a-5p	663b	34-5p	19b-3p
503-3p	138-5p	214-3p	542-3p	210-3p
143-3p	206	429	141-3p	532-5p
22-3p				

The laboratory analyses were carried out at CoreLab and the Medical Genetics laboratory of the University Hospital of Parma.

RNA was isolated from saliva and serum samples with the dedicated commercial kits, RNeasy Protect Saliva kit and miRNeasy Serum/Plasma Advanced Kit respectively (QIAGEN, https://www.qiagen.com/us/). The miRNAs was converted into cDNA through a reverse transcription reaction and then amplified as provided by the miScript PCR System (Figure 2.4). The test involved the use of a plate (miScript miRNA PCR Array) with a defined number of wells, that allow the quantification of the relevant miRNAs and the verification of certain quality controls by a real-time PCR using the miScript SYBR Green PCR Kit. The raw data analysis was performed using the miScript miRNA PCR Array Data Analysis Tool according to the manufacturer's instructions. All the described steps will be conducted following the protocol and guidelines provided by the manufacturer.

Figure 2.4 Reverse transcription reaction



Statistical analysis

Generalized linear mixed models for continuous variable were used for the simultaneous comparison of differences between patients and timepoints. The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to investigate the normality of the data. *p*-values < 0.05 were regarded as statistically significant.

Results

Clinical data of the study participants

The whole population of 25 subjects was enrolled.

Study participants underwent an interview and a complete dental exam to collect demographic data, medical history, and oral health status. Their aggregate data are presented in Table 2.2.

Table 2.2 Demographics, clinical data, and social habits of the study participants

	Patient (n=25)
	Mean values ±SD
Age (years)	28.1 ± 6.2
BMI (kg/m^2)	22.0 ± 9.8
Salivary flow[a] (mL/5min)	2.8 ± 1.5
% FMPS	29.5 ± 8.3
% FMBS	3.3 ± 2.0
DMFT	2.7 ± 2.7
PSR	0.9 ± 0.3
	No. of subjects
Drugs:	
Under medication	*8*[b, c]
No treatment	17
Smoke:	
Cigarette smokers[d]	4
Non-smokers	21
Alcohol:	
drinkers[e]	0
Non-drinkers	25

[a]determined by modified Saxon Test; [b]Eutorix 50-100 mg (2 subjects); [c]contraceptive therapy (6 subjects); [d]less than to 7 cigarettes/day; [e]more than 2 drinks/week.

All the participants had a normal salivary function with salivary flow ranging from 0.6 mL to 5 mL/5 min. The FMBS index ranged from 0 to 44.8% (mean value of 3.3 ± 2.0), but only six subjects had an FMBS > 10% and < 30% (13.3, 12.6, 12.5, 16.8, 10.2, 13.2) and, therefore, a state of localized gingivitis [108]. One subject had a FMBS > 30%, with a state of generalized gingivitis. All 7 subjects with FMBS>10% had a PSR value = 1 and, therefore, they didn't have any disease in periodontal tissues. Anyway, they underwent to a dental hygiene session before the salivary sample, to remove the gingival inflammation.

Salivary miRNA analysis

One hundred and five (105) salivary samples of 25 enrolled woman were analysed. During the 2 years of study conduct, 5 patients have formally withdrawn from the project (NE07, NE08, NE09, NE23, NE24) (Table 2.3).

Table 2.3. salivary samples taken up to 24 months

	T0	T1 (3)	T2 (6)	T3 (12)	T4 (18)	T5 (24)
NE01	X	X	X	X	X	X
NE02	X	X	X	X	X	
NE03	X	X	X	X	X	
NE04	X	X	X	X	X	X
NE05	X	X	X	X	X	X
NE06	X	X	X	X	X	X
NE07	X	X	X	X	X	
NE08	X	X				
NE09						
NE10	X	X	X	X	X	X
NE11	X	X	X	X		
NE12	X	X	X	X	X	X
NE13	X	X	X	X	X	
NE14	X	X	X	X	X	
NE15	X	X	X	X	X	X
NE16	X	X	X	X	X	X
NE17	X	X	X	X		
NE18	X	X	X	X		
NE19	X	X	X			
NE20	X	X	X			
NE 21	X	X	X	X		
NE22	X	X	X			
NE23						
NE24	X					
NE25	X	X	X	X		

Twenty miRNAs did not amplify in any sample. Instead, the remaining 21 were expressed in the salivary samples. In table 2.4 are reported the values, obtained by the following formula:

$$\frac{2^{-C_t(GOI)}}{2^{-C_t(HKG)}} = 2^{(-C_t(GOI)-C_t(HKG))} = 2^{-\Delta C_t}$$

Table 2.4 Values obtained by analysis of miRNAs

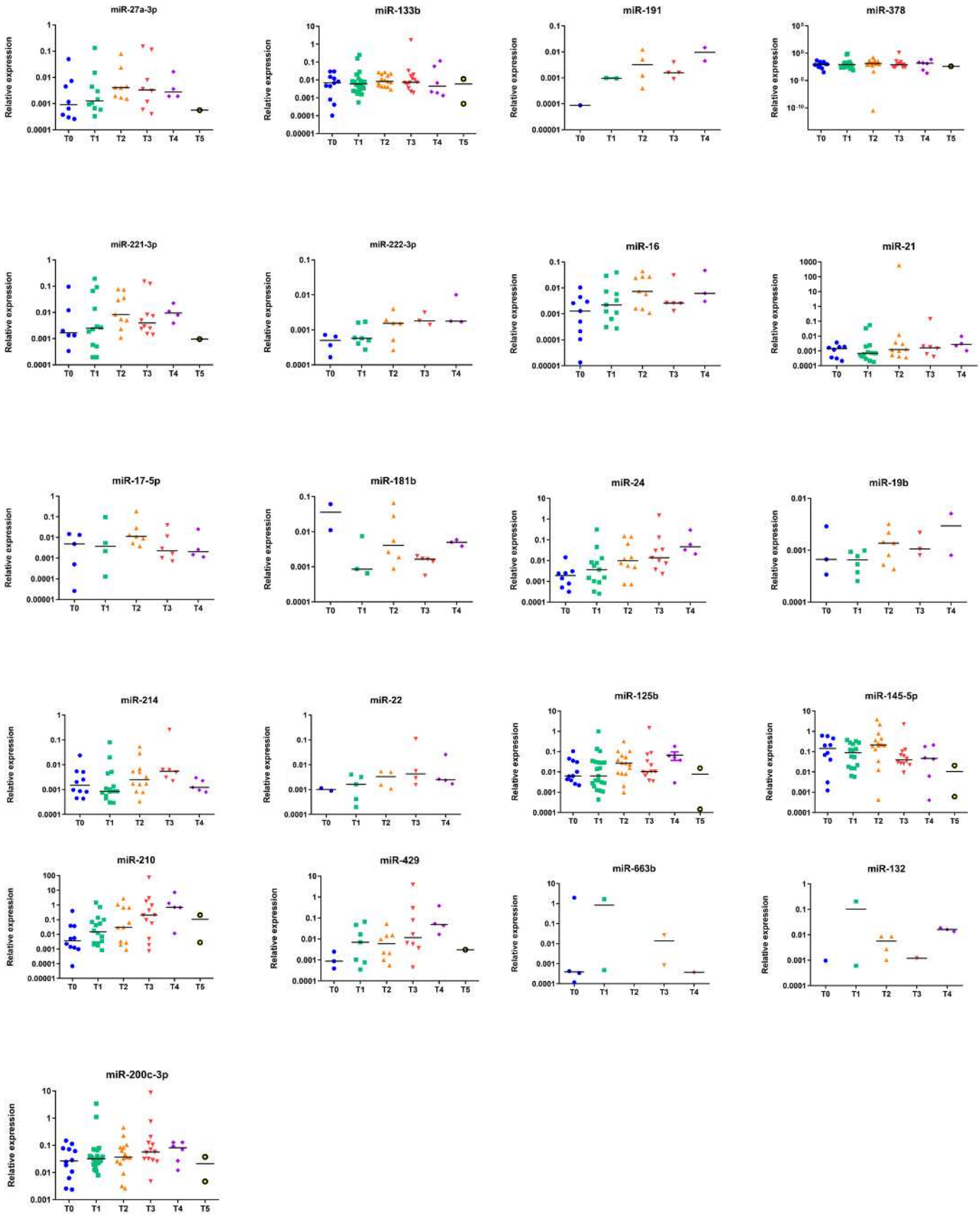
Campioni	27a	133b	221	222	17-5p	181b	191	378	16	21-3p	24	19 b	214	22	210	429	663b	132-3p	125b	145-5p	200c-3p
NE01 T0	0,007313	0	0	0	0,014726	0	0	0,048995	0,002604	0,001678	0,000794	0,002889	0,023847	0	0,005025	0,002511	0	0,000966	0,035908	0,040567	0,147979
NE01 T1	0	0,011632	0	0	0	0	0	0,010911	0	0	0	0,000769	0,005245	0	0	0	0	0	0,000435	0,134309	0,031876
NE01 T2	0,00416	0,011232	0,035972	0,001933	0,028779	0,065686	0,001249	0,053692	0,043484	0	0,01201	0,003227	0,028966	0	0,030102	0,015298	0	0,00273	0,063791	0,791687	0,10345
NE 01T3	0	0,002225	0	0	0,000712	0,001391	0,000903	0,00317	0	0,000621	0,003722	0	0	0	0,196082	0	0	0	0,009968	0,033754	0,028441
NE02 T0	0,0003	0,008109	0	0	0	0	0	0,007553	0,002945	0,00031	0,002408	0,000341	0,000864	0	0,005471	0,000393	0,000345	0	0,030758	0,453787	0,018699
NE02 T1	0,000333	0,028765	0,066165	0	0	0	0	0,009518	0	0,000539	0,000319	0,000378	0,00044	0	0,003452	0	0	0	0,029139	0,277122	0,080053
NE03 T0	0,00453	0,006855	0,012076	0,000364	0,013412	0,011115	0	0,002813	0,010443	0,000363	0,003074	0,000667	0,00045	0,000899	0,036269	0	0,000429	0	0,010205	0,213291	0,002347
NE03 T1	0	0,002313	0,000194	0	0	0	0	0,004622	0,000307	0,000621	0	0	0,000855	0	0,000826	0	0,000481	0	0,001984	0,057484	0,037145
NE03 T2	0	0,021141	0	0	0	0	0	0,034717	0	0	0	0,001454	0,005448	0	0	0,001017	0	0	0,008572	0,205875	0,226305
NE04 T0	0,000375	0,014919	0,00197	0,000721	0,000502	0,06101	0	0,00768	0,001283	0,00187	0,0014	0	0,002016	0	0,001399	0	0	0	0,045402	0,614892	0,077638
NE04 T1	0,000946	0,005444	0,013808	0,000572	0	0	0	0,003012	0,00124	0	0,000255	0,000255	0,000293	0	0,002425	0	0	0	0,003828	0,122523	0,027616
NE04 T2	0,023656	0,004162	0,029465	0,00052	0,011309	0,028266	0	0,026023	0,005754	0	0,007687	0	0,006901	0,005068	0,062762	0,002269	0	0,001034	0,054505	0,222371	0,026007
NE 04T3	0	0,0019	0,002467	0	0	0	0	0,002599	0	0	0,007266	0	0	0	0,427569	0,006552	0	0	0,021158	0,026736	0,032092
NE05 T0	0,000259	0,000415	0,000338	0	2,59E-05	0	0	0,001707	0,000505	0	0,000509	0	0	0	0,002269	0	0,000116	0	0,002213	0	0,006235
NE05 T1	0	0,004531	0	0	0	0	0	0,012547	0	0	0	0	0	0	0,002083	0,007058	0	0	0,035354	0	0,007911
NE05 T2	0	0,027441	0	0	0	0	0	0,014751	0	0	0	0	0	0	0,001967	0	0	0	0,016114	0,434221	0
NE05 T3	0,114272	0,005603	0,122895	0	0	0	0	0,006932	0	0	0	0	0,004922	0	0,004873	0	0,026369	0	0,010406	0	0,748461
NE06 T0	0	0,00453	0	0	0	0	0	0,015099	0	0	0	0	0,005446	0	0,000977	0	0	0	0,006078	0,202412	0,071638
NE06 T1	0,015035	0,002757	0,191788	0,001611	0,005374	0	0	0,003547	0,003339	0,000295	0,008331	0	0,000554	0,003213	0,041349	0	0	0	0,029513	0,329306	0,038141
NE07 T0	0	0,011982	0	0	0	0	0	0,025862	0	0,001802	0	0	0,000974	0	0	0	0	0	0,003877	0	0,113401
NE07 T1	0	0,020624	0,000577	0	0	0	0	0,005446	0	0	0	0	0	0	0	0	0	0	0,003162	0	0,011343
NE07 T2	0	0,008292	0	0	0	0	0	0,00044	0	0	0	0	0	0	0	0	0	0	0	0,012749	0,002592
NE07 T3	0	0,019991	0	0	0	0	0	0,002677	0	0	0	0	0	0	0	0	0	0	0	0,061704	0,004745
NE 07T4	0	0,00192	0	0	0	0	0	0,00023	0	0	0	0	0	0	0,011538	0	0	0	0,002912	0,006208	0,026956
NE08 T0	0,049639	0,004759	0,001339	0	0	0	0	0,003196	0,000214	0,000215	0,000319	0	0,000823	0	0,001244	0	0	0	0,00438	0,069266	0,02466
NE08 T1	0,000665	0,001699	0,00228	0,000273	0	0	0	0,008464	0,007248	0	0,005677	0	0,000848	0,000418	0,012162	0	0	0	0,031763	0,236187	0,028242
NE08 T2	0,001686	0,006617	0,077589	0	0,013429	0	0	0,043813	0,027007	0,00129	0,004894	0	0,005071	0	0,029127	0,002127	0	0	0,104748	2,199232	0,021733
NE09 T0	0,001162	0,030081	0,095988	0,000643	0,004873	0	0	0,025042	0,004455	0,003662	0,014408	0	0	0,001141	0,038447	0,000879	0	0	0,105587	0,583175	0,061426
NE09 T1	0	0,005939	0,0005	0,0005	0	0,007518	0	0,019853	0,001117	0,000424	0	0,000991	0	0	0,001769	0	0	0	0,006317	0	0,069879
NE09 T2	0	0,002865	0	0	0	0,001895	0	0,005866	0	0,000364	0	0	0	0	0	0	0	0	0,027195	0	0,03674
NE 09T3	0,003066	0,010023	0,004942	0,001811	0,00165	0,001641	0	0,009258	0,002572	0,001458	0,029589	0	0,002204	0,002945	1,742891	0,078519	0	0	0,071551	0,022603	0,104745
NE10 T1	0,001446	0,000559	0	0,000406	0,000127	0	0	0,003112	0,000637	0,000214	0,001552	0	0,001098	0	0	0,000352	1,669019	0	0,00119	0,118134	0,012713
NE10 T2	0	0,00443	0,002325	0,000265	0	0	0	0,007916	0	0,000533	0	0,000526	0,000334	0	0,003379	0	0	0,008555	0,000973	0,00043	0,063678
NE 10T3	0,00786	0,016479	0,008159	0,003148	0,011244	0,001693	0,003969	0,034663	0,030693	0	0,130709	0,002169	0,006127	0,005599	2,838949	0,294094	0	0	0,08673	0,125002	0,203324
NE11 T1	0,004412	0,008472	0,09141	0	0	0,000653	0,000953	0,018396	0,039596	0,000728	0,01308	0	0,001005	0	0,017229	0,000778	0	0	0,014067	0,278935	0,040992
NE11 T2	0,003992	0,004058	0,001092	0	0,005294	0	0,000395	0,015555	0,001627	0,001191	0,005731	0,000432	0,003282	0	0,002735	0,000557	0	0	0,008108	0,130761	0,035612
NE11 T3	0	0,006827	0	0	0	0	0	0,024072	0	0,001772	0	0	0,00308	0	0,001863	0	0	0	0,003761	0,046763	0,032195
NE 11T4	0,016356	0,006858	0,022535	0,010101	0,025338	0,005016	0,014623	0,07262	0,04716	0,009679	0,297972	0,005089	0,003003	0,025895	7,167742	0,384952	0	0,01613	0,183724	0,179412	0,129102
NE 11T5	0	0,000465	0	0	0	0	0	0	0	0	0	0	0	0	0,002786	0	0	0	0,000147	0,000607	0,004678
NE12 T1	0,001096	0,002446	0,000194	0,000601	0	0	0	0,002416	0,000275	0,000179	0,000904	0	0,000304	0,000201	0,002501	0	0	0	0,003931	0,006481	0,021937
NE12 T2	0	0,005019	0	0	0	0	0	0,009398	0	0,000423	0	0	0,000799	0	0,000891	0	0	0	0,002061	0	0,033331
NE12 T3	0	0,007404	0,00239	0	0	0	0	0,012448	0	0,000403	0	0,000803	0	0	0,000721	0	0	0	0,003497	0,025613	0,025471
NE 12T4	0,003606	0,002183	0,010982	0	0,001469	0,003883	0	0,013288	0	0,002246	0,020937	0	0,001233	0,002363	1,301629	0,016621	0	0	0,038048	0,049878	0,070473
NE13 T0	0	0,029698	0	0	0	0	0	0,011218	0	0	0	0	0,002535	0	0	0	1,966323	0	0	0,003065	0,028429
NE13 T2	0	0,010177	0	0	0	0	0	0,009996	0	602,5762	0,000738	0,000815	0,001732	0	0	0	0	0	0,009059	0,262611	0,009204
NE 13T4	0	0,001328	0	0	0	0	0	0,000842	0	0	0	0	0,002276	0	0	0	0	0	0	0,000408	0,012042
NE 13T5	0,000565	0,011433	0,000961	0	0	0	0	0,004145	0	0	0,004834	0	0	0	0,204465	0,00309	0	0	0,015511	0,020763	0,037687
NE14 T1	0	0,007853	0,002656	0	0	0	0	0,023309	0,002209	0,002418	0,003705	0	0,004468	0	2,21E+08	0,00103	0	0	0,028252	0,379192	0,071545
NE 14T2	0,004537	0,022767	0,008329	0,003967	0,008952	0,005571	0,012279	0,016765	0,027202	0,003727	0,143933	0,002202	0,001643	0,005443	2,704367	0,053246	0	0,008703	0,105174	0,199572	0,082045
NE 14T3	0,003622	0,003398	0,007223	0,001389	0,002902	0	0,001582	0,007934	0,002648	0	0,033274	0	0	0,001549	0,904936	0,005745	0	0,001186	0,033869	0,103572	0,069818
NE 14T4	0,001918	0,115852	0,007927	0,00177	0,002632	0,00591	0,004514	0,020908	0,006178	0,003143	0,059666	0,000801	0,00096	0,002634	0,657454	0,054046	0,000375	0,013468	0,061956	0,211594	0,093242
NE15 T0	0	0,000102	0	0,000165	0	0	8,77E-05	0,000333	0,000105	0	0	0	0,000431	0	6,87E-05	0	0	0	0,002599	0,00123	0,002557
NE15 T2	0,078715	0,026942	0,073659	0	0,185052	0	0	0,173379	0,024298	0,012023	0,152513	0	0,053475	0	1,070289	0	0	0	0,322529	3,891968	0,448755
NE 15T3	0,152134	1,683346	0,151364	0	0,039231	0	0	1,280471	0	0,14384	1,488559	0	0,257427	0,110851	73,07787	3,881748	0	0	1,468204	2,287258	8,660592
NE 15T4	0,001906	0,05869	0,003867	0,001706	0,001152	0	0	0,016804	0,003029	0,001044	0,033327	0	0,000792	0,00171	0,694351	0,044285	0	0,018677	0,052154	0,043097	0,127849
NE16 T1	0	0,159707	0	0	0,096924	0	0	0,787854	0,029188	0,055707	0,314798	0	0,080325	0	0,054015	0,047104	0	0,204051	0,999307	0	3,465347
NE16 T2	0	0	0	0	0	0	0	3,25E-11	0,001082	0	0,000726	0	0	0	0	0	0	0	0,02356	0,358365	0,003177
NE 16T3	0,0004	0,011929	0,001504	0	0	0,000557	0	0,003808	0	0	0,002283	0	0	0	0,055824	0,003667	0	0	0,00657	0,009397	0,054063
NE17 T1	0,132243	0,251129	0	0	0	0	0	0,576743	0	0,033146	0	0	0,020081	0	0	0	0	0	0,138264	0	1,110724
NE 17T3	0,000591	0,007252	0,00293	0	0,001013	0,002012	0,001575	0,002556	0,001296	0	0,009813	0	0	0	0,093269	0,000442	0	0	0,01033	0,068343	0,056879
NE 18T1	0	0,004071	0	0	0	0	0	0,002441	0	0,000822	0,001471	0,00093	0,001355	0	0,13392	0	0	0	0,002788	0,021656	0,023484
NE 18T2	0,001964	0,018053	0,004983	0,001537	0,003759	0,002654	0,00516	0,008173	0,007352	0,002982	0,066684	0,001374	0,00173	0,001564	0,650966	0,009807	0	0	0,028367	0,191575	0,046463
NE 18T3	0,001167	0,031904	0,001407	0	0	0	0	0,012157	0,0025	0,002025	0,013799	0,001057	0,006276	0	0,206015	0,0167	0,000831	0	0,009083	0,02968	0,123218
NE 19T1	0,000592	0,007895	0,001876	0	0	0	0	0,009281	0	0,000699	0,007771	0	0,000819	0,001625	0,710701	0,0167	0	0,000606	0,015294	0,018162	0,040799
NE 19T2	0,001533	0,007341	0,005506	0,001556	0	0,000895	0	0,012371	0,001542	0,001022	0,014758	0	0,000841	0,001099	0,665194	0,014189	0	0	0,03957	0,034623	0,084127
NE20T1	0	0,009176	0	0	0	0	0	0,002377	0	0	0	0	0	0	0,099977	0	0	0	0,001301	0,005793	0,019886
NE 21T1	0,002969	0,020463	0,002905	0,001707	0,002208	0,000859	0,000985	0,012927	0,005764	0,001998	0,045858	0,000536	0,000749	0,004049	1,436169	0,067136	0	0	0,106478	0,054371	0,065756
NE 23T0	0,00065	0,000812	0,001336	0	0	0	0	0,009747	1,34E-05	0,001203	0,002481	0	0,005142	0	0,386126	0	0	0	0,006341	0,087428	0,010858
NE 23T1	0	0,001577	0	0	0	0	0	0,001398	0	0	0	0	0	0	0,069033	0	0	0	0,002875	0,014917	0,030433
NE 24T1	0	0,003372	0	0	0	0	0	0,00077	0	0	0,00102	0	0	0	0,007149	0	0	0	0,001072	0,015508	0,015733

By relating the expression of each miRNAs to the expression of reference miRNA (cel-39), it is obtained the relative expression value of the 21 miRNAs.

Using the relative expression, graphs were created to assess the change during the experimental intervention (T0, T1, T2, T3, T4) by © 2024 GraphPad Software.

Every miRNAs, except miR-133b, miR-378 and miR-21 had a modify in their relative expression, starting from time T0. (Figure 2.5).

Figure 2.5 Graphs of the 21 miRNA relative expressions up to time T5 (24 months)



Statistical analysis

The generalized linear mixed model analysis for groups and timepoints revealed significant differences at timepoint 2 (after 12 months) for miRNA 24-3p and 191-5p (Table 2.5,2.6 and Figure 2.6,2.7), and at timepoint 3 (after 18 months) for miRNA 214-3p, 222-3p and 429 (Table 2.7-2.9 and Figure 2.8-2.10). All other comparisons between groups and timepoints within groups were not statistically significant. Statistical results were made up to timepoint 3, because there was not enough data collected at timepoint 4 (after 24 months)

Table 2.5 Statistical data for miRNA 24-3p

Model Informations		
Model Type	Mixed Model	Linear Mixed model for continuous y
Model	lmer	Log24 ~ 1 + tempo + (1 | ID)
Distribution	Gaussian	Normal distribution of residuals
Direction	y	Dependend variable scores
Optimizer	bobyqa	
DF method	Satterthwaite	
Sample size	40	
Converged	yes	
Y transform	none	
C.I. method	Wald	

Names	Effect	Estimate	SE	Lower	Upper	df	t	p
(Intercept)	(Intercept)	-2.188	0.128	-2.4484	-1.93	36.0	-17.05	< .001
tempo1	T1 - T0	0.387	0.359	-0.3416	1.12	36.0	1.08	0.287
tempo2	T2 - T0	0.857	0.379	0.0878	1.63	36.0	2.26	0.030
tempo3	T3 - T0	1.154	0.388	0.3657	1.94	36.0	2.97	0.005

Test for Normality of residuals	Statistics	p
Kolmogorov-Smirnov	0.123	0.582
Shapiro-Wilk	0.957	0.130

Figure 2.6 Statistical plot for miRNA 24-3p

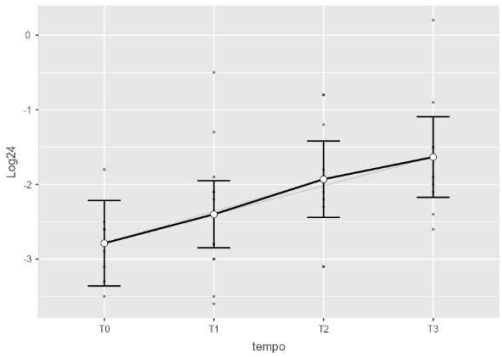


Table 2.6 Statistical data for miRNA 191-5p

Model Info

Info		
Model Type	Mixed Model	Linear Mixed model for continuous y
Model	lmer	Log191 ~ 1 + tempo + (1 | ID)
Distribution	Gaussian	Normal distribution of residuals
Direction	y	Dependend variable scores
Optimizer	bobyqa	
DF method	Satterthwaite	
Sample size	11	
Converged	yes	
Y transform	none	
C.I. method	Wald	

				95% Confidence Intervals				
Names	**Effect**	**Estimate**	**SE**	**Lower**	**Upper**	**df**	**t**	**p**
(Intercept)	(Intercept)	-3.11	0.165	-3.5368	-2.69	6.39	-18.91	< .001
tempo1	T1 - T0	1.12	0.566	-0.3320	2.58	7.00	1.98	0.088
tempo2	T2 - T0	1.48	0.517	0.1506	2.81	7.00	2.86	0.024
tempo3	T3 - T0	1.34	0.517	0.0142	2.67	7.00	2.60	0.036

Test for Normality of residuals	Statistics	p
Kolmogorov-Smirnov	0.23	0.684
Shapiro-Wilk	0.99	0.629

Figure 2.7 Statistical plot for miRNA 191-5p

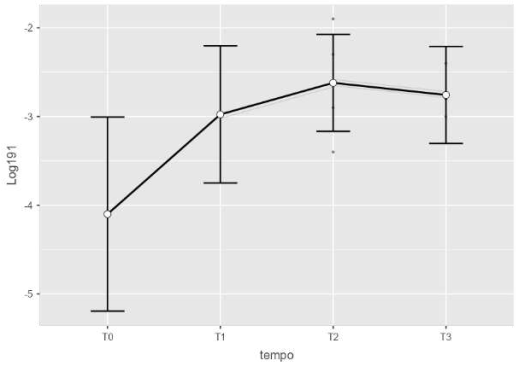


Table 2.7 Statistical data for miRNA 214-3p

Model Info

Info		
Model Type	Mixed Model	Linear Mixed model for continuous y
Model	lmer	Log214 ~ 1 + tempo + (1 | ID)
Distribution	Gaussian	Normal distribution of residuals
Direction	y	Dependend variable scores
Optimizer	bobyqa	
DF method	Satterthwaite	
Sample size	43	
Converged	yes	
Y transform	none	
C.I. method	Wald	

				95% Confidence Intervals				
Names	**Effect**	**Estimate**	**SE**	**Lower**	**Upper**	**df**	**t**	**p**
(Intercept)	(Intercept)	-2.5251	0.124	-2.776	-2.274	16.8	-20.411	< .001
tempo1	T1 - T0	-0.0275	0.239	-0.512	0.457	26.3	-0.115	0.909
tempo2	T2 - T0	0.3058	0.248	-0.197	0.809	24.5	1.232	0.230
tempo3	T3 - T0	0.7829	0.317	0.140	1.426	33.1	2.469	0.019

Test for Normality of residuals	Statistics	p
Kolmogorov-Smirnov	0.0983	0.764
Shapiro-Wilk	0.9723	0.379

Figure 2.8 Statistical plot for miRNA 214-3p

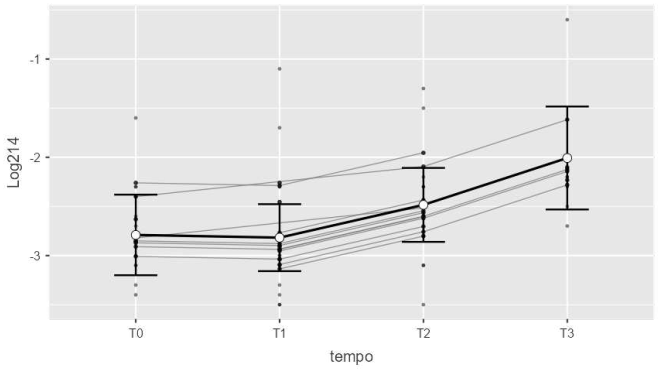


Table 2.8 Statistical data for miRNA 222-3p

Model Info

Info		
Model Type	Mixed Model	Linear Mixed model for continuous y
Model	lmer	Log222 ~ 1 + tempo + (1 | ID)
Distribution	Gaussian	Normal distribution of residuals
Direction	y	Dependend variable scores
Optimizer	bobyqa	
DF method	Satterthwaite	
Sample size	20	
Converged	yes	
Y transform	none	
C.I. method	Wald	

				95% Confidence Intervals				
Names	**Effect**	**Estimate**	**SE**	**Lower**	**Upper**	**df**	**t**	**p**
(Intercept)	(Intercept)	-3.049	0.0803	-3.2208	-2.876	16.0	-37.958	< .001
tempo1	T1 - T0	0.189	0.2131	-0.2678	0.646	16.0	0.888	0.388
tempo2	T2 - T0	0.442	0.2195	-0.0290	0.912	16.0	2.013	0.061
tempo3	T3 - T0	0.675	0.2597	0.1181	1.232	16.0	2.599	0.019

Test for Normality of residuals	Statistics	p
Kolmogorov-Smirnov	0.118	0.943
Shapiro-Wilk	0.975	0.862

Figure 2.9 Statistical plot for miRNA 222-3p

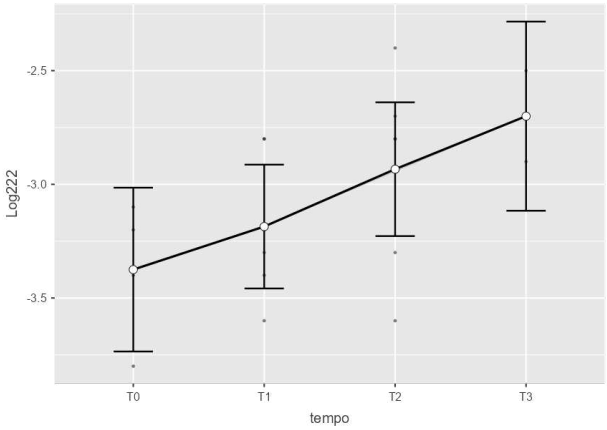


Table 2.9 Statistical data for miRNA 429

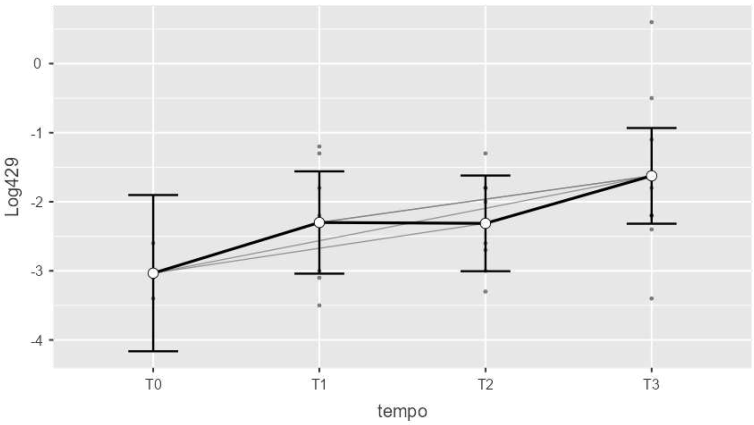
Model Info

Info		
Model Type	Mixed Model	Linear Mixed model for continuous y
Model	lmer	Log429 ~ 1 + tempo + (1 | ID)
Distribution	Gaussian	Normal distribution of residuals
Direction	y	Dependend variable scores
Optimizer	bobyqa	
DF method	Satterthwaite	
Sample size	26	
Converged	yes	
Y transform	none	
C.I. method	Wald	

				95% Confidence Intervals				
Names	**Effect**	**Estimate**	**SE**	**Lower**	**Upper**	**df**	**t**	**p**
(Intercept)	(Intercept)	-2.318	0.201	-2.7375	-1.90	22.0	-11.52	< .001
tempo1	T1 - T0	0.733	0.652	-0.6264	2.09	22.0	1.13	0.273
tempo2	T2 - T0	0.721	0.640	-0.6132	2.05	22.0	1.13	0.272
tempo3	T3 - T0	1.408	0.640	0.0743	2.74	22.0	2.20	0.038

Test for Normality of residuals	Statistics	p
Kolmogorov-Smirnov	0.0887	0.987
Shapiro-Wilk	0.9749	0.751

Figure 2.10 Statistical data for miRNA 214-3p



Discussion

Nutriepigenomics is the study of nutrients and their effects on human health through epigenetic modifications (DNA methylation, histone modification, e.g.). Numerous studies have suggested that body mass index (BMI), components of food and lifestyle may interfere with miRNA expression, thus affecting tumors' initiation and progression [109].

The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) described the evidence for associations between diet, nutrition and physical activity and cancer survival from intervention and observational studies [110].

Some studies suggested that the dietary pattern of Mediterranean diet (MD) could reduce BC risk and improve BC survival via anti-inflammatory effects, antioxidant properties and hormone–receptor interactions [111, 112].

Similarly, dietary elements, as polyphenols, curcumin and flavonoids play an onco-suppressor role and, especially, affect the epigenetic regulation of gens involved in BC progression and drug-resistance [109]

This pilot study aims to monitor the salivary and serum miRNAs expression of 25 unaffected women involved in the breast/ovarian cancer screening program as carriers of BRCA1/2 germline mutations, after undergoing personalised nutritional and lifestyle program based on literature data for 24 months (T4).

At the moment, we have the results about the salivary samples analysis.

The relationship between 21 salivary miRNAs relative expression and timing (T0-T4) shows an increase of miR-27a-3p, miR-191, miR-221-3p, miR-222-3p, miR-16, miR-24, miR-19b, miR-22,

miR-125b, miR-210, miR-429, miR-200c-3p relative expressions, and a decrease of miR-17-5p, miR-181b, miR-214, miR-145-5b. Instead, the relative expression of miR-133b, miR-378, and miR-21 are unchanged. The value of miR-132 and miR-663b cannot be considered because few salivary samples have been analysed (Figure 2.5).

Statistical analysis highlights the significance of 5 salivary miRNAs (miRNA-24-3p, miRNA-191-5p, miRNA-214-3p, miRNA-222-ep, miRNA-429) that have a statistical difference between their expression before nutritional intervention (T0) and after 18 months (T3). In particular, miRNA-24 and miRNA-191 show a statistical significance from time T2 (after 12 months).

The statistical data are processed up to time t3 (18 months), because there was not enough data at time T 4. Anyway, these preliminary expression modulations seem probably confirm the influence of nutrition and lifestyle on miRNAs.

About the 5 statistically significance miRNAs, Khodadadi-Jamayran et al (2018) suggests the prognostic role of elevated mir-24 in breast cancer and its association with the metastatic process [113]. MiRNA-24 promotes cell proliferation and inhibits apoptosis in human breast cancer by targeting p27Kip1 [114]. Jang JY et al. (2020) confirm this hypothesis, reporting that miRNA-24 has a sensitivity of 98%, a specificity of 96% and an accuracy of 97% for breast cancer detection, as well as miRNA-1246, miRNA-206 and miRNA-373 [115].

Ju-Yeon Kim et al. (2023) reports the role of miRNA-221 and 222 levels in breast cancer cell lines and cancer tissues. MiR-221/222 are considered to be important modulators of cancer progression that are involved in several aspects related to malignant tumors, such as cell invasion, metastasis, angiogenesis, apoptosis and drug resistance [116, 117]. In breast cancer cell lines, it has also been reported that miR-221/222 regulates epithelial-to-mesenchymal transition by targeting trichorhinophalangeal or adiponectin receptor. Moreover, they have also been reported to promote cell cycle progression, migration and invasion by targeting cyclin-dependent kinase inhibitor 1B [117].

Plantamura I et al. (2018) reports that oncogenic miRNAs target tumor suppressor genes miR-221/222 are usually upregulated in tumor [118].

MiR-214 is a microRNA that plays a significant role in BC biology, influencing processes as cell proliferation, migration, invasion and apoptosis. Schwarzenbach H et al. (2012) report that the concentrations of miR-214 were significantly higher in breast cancer patients than in serum of patients with benign disease and healthy women ($p = 0.0001$), and thus, could discriminate between benign and malignant disease [119]. Kalniete D *et al*. (2015) also report that a high

expression level of tissue miR-214 is associated with a significantly worse disease-specific survival than patients with low expression of miR-214 [120]. It is not clear how BRCA1 dysfunction can influence the level of miR-214 in breast tumors as yet. It is known that miR-214 targets the PTEN gene; by targeting PTEN Akt pathway is activated thus resulting in the cell survival [121].

MiR-429 plays several complex roles in the occurrence and development of cancer. Zhang L et al. (2020) analyzed cell proliferation and migration in patients with BC and found that the overexpression of miR-429 could significantly promote the proliferation and migration of MDA-MB-468 and T47D cells [122]. Some studies found that the re-expression of one or more miR-200 family members may reduce cell proliferation and migration [123, 124]. This discrepancy may be related to the differences in the cell lines and cancer subtypes analyzed.

MiR-191 plays in a multitude of diseases including cancer and also in normal development and differentiation processes. In BC, studies suggest that miR-191 can play a dual role, acting both as an oncogene and as a tumor suppressor, depending on the context and specific environment [125]. It can promote BC progression by targeting tumour suppressor genes and pathways that inhibit cell growth. Conversely, lower levels of miR-191 have been linked to more aggressive tumour behavior. This indicates that its expression must be finely tuned for normal cellular function and that dysregulation can contribute to malignancy. [126]MiR-191 was observed with other 8 miRNAs (miR-16, miR-21, miR-27a, miR-150, miR-200c, miR-210, miR-451 and miR-145) to be deregulated in both plasma and tumor tissues from BC patients [127]. Among statistically significant salivary miRNAs of our pilot study, it's interesting to highlight that in literature the expression levels of miR-191_5p have been inversely associated with BMI [109]. Levels of miR-191-5p significantly increased during a six-month weight-loss intervention (Lifestyle, Exercise, and Nutrition; LEAN trial) in 100 BC survivors [128].

It's important underling that all studies reported in literature about the analyzed miRNAs were performed on tissue and serum samples and that only one of the miRNAs with a statistically significant role in our study has a positive response in literature about the nutriepigenomics relationship (miR-191). Therefore, much remains to be discovered regarding dietary modulation of salivary miRNA expression, including cell- or tissue-specific responses.

The next step will be to compare the salivary samples with serum miRNAs of enrolled women, and evaluate the significance of any differences, correlating with their personalized diet.

CHAPTER 3

Progression from normal oral mucosa to epithelial dysplastic lesions: a metabolic profiling study on saliva

Introduction

Oral dysplasia

Oral dysplasia is defined as an abnormal growth of the epithelium, characterized by cellular atypia and loss of normal maturation and architecture. The World Health Organization refers to dysplasia using the term “epithelial precursor lesions” and defines it as “altered epithelium with an increased likelihood for progression to squamous cell carcinoma (2005)”[129]. The progressive accumulation of genetic and epigenetic alterations in the oral mucosa is manifested by a series of well-defined clinical features and histological changes depicting dysplasia [130]. Criteria used for diagnosing oral epithelial dysplasia are listed in Table 3.1.

Table 3.1 Criteria used for diagnosing dysplasia

Architecture	Cytology
Irregular epithelial stratification	Abnormal variation in nuclear size (anisonucleosis)
Loss of polarity of basal cells	Abnormal variation in nuclear shape (nuclear pleomorphism)
Basal cell hyperplasia	Abnormal variation in cell size (anisocytosis)
Drop-shaped rete ridges	Abnormal variation in cell shape (cellular pleomorphism)
Increased number of mitotic figures	Increased nuclear-cytoplasmic ratio
Abnormally superficial mitoses	Increased nuclear size
Pre-mature keratinization in single cells (dyskeratosis)	Atypical mitotic figures
Keratin pearls within rete ridges	Increased number and size of nucleoli
	Hyperchromasia

The severity of dysplasia depends on the evidence and/or numerous features present in the biopsy specimen. Such alterations can affect cell layers from the lowest one-third (basal/parabasal) to the middle one-third until the upper one-third. Typically, the extension of involvement is related to the grade definition: mild, moderate, and severe [130].

- Mild dysplasia: it appears as an architectural alteration limited to the lower third of the epithelium, within which minimal cytological atypia is noticeable.
- Moderate dysplasia: the architectural alteration extends into the middle third of the epithelium. However, it is essential to consider the degree of cellular atypia. Lesions with mildly atypical features extending into the middle third of the epithelium may merit being graded as mild dysplasia. Conversely, marked atypia that do not reach the upper third may classified as severe dysplasia (Figure 3.1).
- Severe dysplasia: architectural alteration and cell atypia has reached the upper third of the epithelium. However, as already mentioned, the architectural disturbance extending into the middle third of the epithelium with sufficient cytological atypia can be described as severe dysplasia. A full-thickness change in the epithelium can also be referred to as carcinoma in situ (CIS) (Figure 3.2).

Recently, it was demonstrated that the architectural pattern of dysplasia, termed “architectural dysplasia (AD)” (or differentiated dysplasia) is a strong predictor of malignant progression of oral leukoplakia in Oral Squamous Cell Carcinoma (OSCC) (Figure 3.3) [131].

Oral leukoplakia is one of the Oral Potentially Malignant Disorders (PMDs), that are a heterogeneous group of epithelial-connective pathologies with a higher risk of malignant transformation, compared to healthy oral mucosa. Potential malignant disorders include, among others, oral leukoplakia, erythroplakia, oral lichen planus, oral submucous fibrosis, and oral lichenoid lesion. The risk of malignant transformation depends on numerous variables such as the type of PMDs, the characteristics of disorder such as colour, location, size, duration, the presence and grade of dysplasia [132].

Figure 3.1 A) Oral leukoplakia with mild grade of dysplasia in a 61 y/o female, localized on upper vestibular gingival mucosa B) Histological slides from OLK biopsy. Presence of moderate grade dysplasia at Hematoxylin & Eosin (H&E) – 50X.

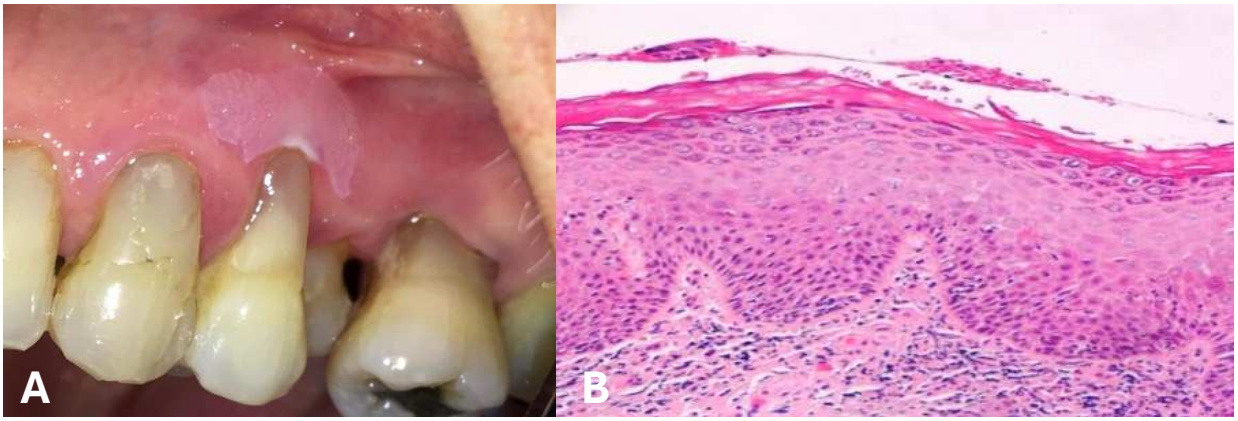


Figure 3.2 A biopsy highlighted the presence of severe dysplasia at H&E in a chronic ulcerative lesion of the tongue in 74 y/o

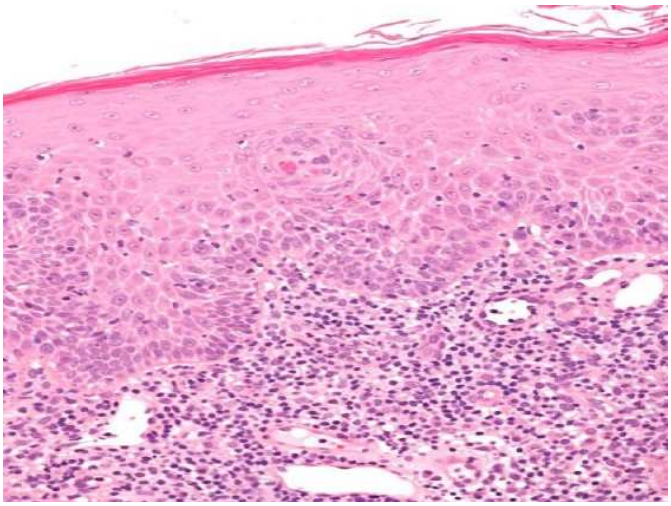
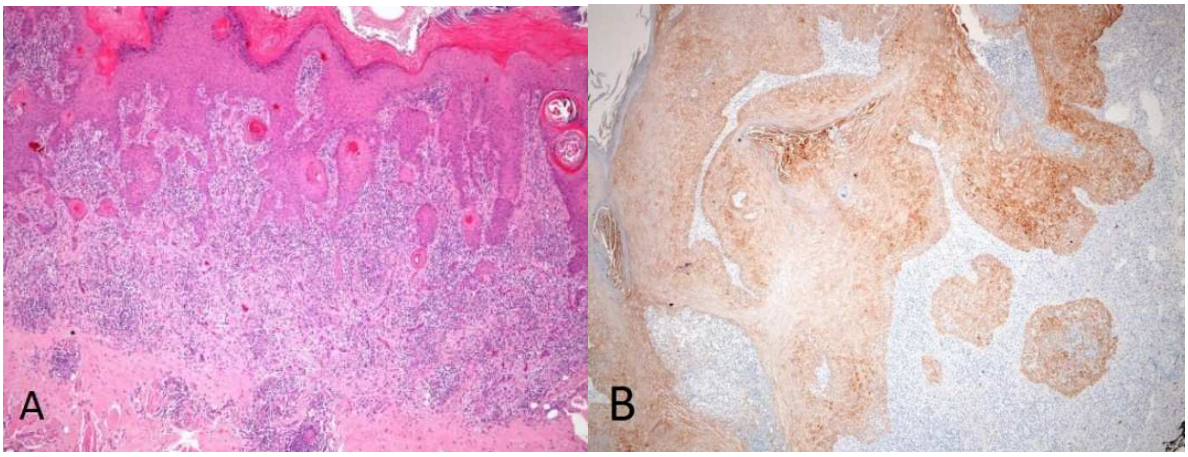


Figure 3.3 A) Infiltrative low-differentiated OSCC, H&E – 25X. Keratin pearls and inflammatory infiltrate are present in the stroma. B) Stromal infiltration of neoplastic cells marked with MNF116, a broad-spectrum cytokeratin immunostaining which react with high and low molecular weight cytokeratins (e.g., CK 5, 6, 8, 17 and 19) – 25X.



Oral leukoplakia

Oral leukoplakia (OLK) is defined by the World Health Organization (WHO) as a "white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" [133]. The worldwide prevalence of OLK is 2.6% [134]. Oral leukoplakia is a clinical term, and an appropriate diagnosis should be confirmed only after a histopathologic evaluation and the exclusion of other types of clinically similar diseases.

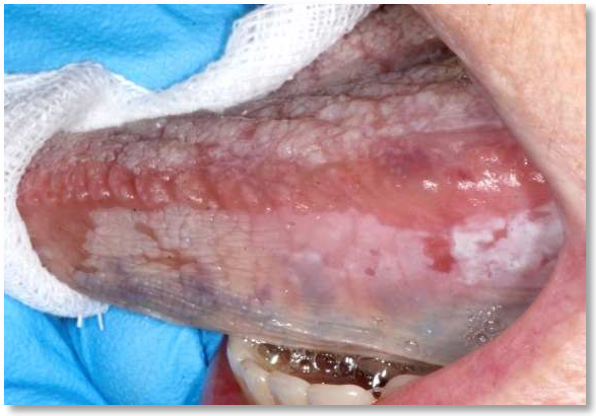
Traditionally, OLK is clinically subdivided in a *homogeneous variant* (uniform white lesion with flat or slightly wrinkled surface), *non-homogeneous variant* (white areas often accompanied by erythema, also possibly associated to nodulous and warty areas) and *erythroleukoplakia*, lesion characterized by a mix of white and red plaques (Figure 3.4) [135]. There is also a variety called *proliferative verrucous leukoplakia* (PVL), non-homogenous lesions which frequently involving gingiva, buccal mucosa, and tongue [136].

A systematic review on 24 studies reported that malignant transformation rate of OLK varies from 0.13 to 34% with an average rate of 3.5%. The rate of annual transformation was between 0.3% and 6.9% (mean 3.8% yearly) with a follow-up period varying from 2.4 to 11 years [137]. There are specific determinants that increase OLK's malignant transformation potential, such as grade of dysplasia, advanced age, female sex, OLK extension exceeding 200 mm^2 and non-homogeneous type (such as erythroleukoplakia and PVLs), but also oral sites associated with a higher risk of transformation (e.g., lingual margin and ventral surface, floor of the mouth and soft palate). Interestingly, a recurrent OLK (lesion appearing at the same site after complete excision) has been suggested to have a high risk of malignant transformations independent of other clinicopathological factors [137].

Oral leukoplakia with dysplasia exhibits histological and cytological features of keratinizing dysplasia, such as hyperkeratosis and parakeratosis, epithelial atrophy or epithelial hyperplasia, hyperplasia of basal cells with large and hyperchromatic nuclei, altered nucleus / cytoplasm ratio, atypical mitosis, dyskeratosis and loss of cells cohesion [138]. On the other hand, OLK without dysplasia exhibits hyperkeratosis, without histological features of a frictional/reactive process, such as hyper-granulosis, verrucous architecture and epithelial atrophy or hyperplasia [139].

Several chemopreventive and surgical strategies have been employed to treat OLK, with surgical interventions being the mainstay of treatment.

Figure 3.4 Non-homogeneous OLK in a 75 y/o female, extensively involving the tongue



Oral Lichen Planus

Oral lichen planus (OLP) is a chronic inflammatory immune-mediated disease, affecting the oral mucosa with characteristic relapses and remissions [140].

The global prevalence of OLP has rates range from 0.5% and 2,6%; the disease has a female predilection (1.4-2:1) and is more commonly observed in middle-aged adults, with an increasing prevalence after the fourth decade [141].

It has a very low transformation rate (0.28-1.14%) and is classified as a potentially malignant disorder (PMD) [142].

The modified WHO criteria for OLP diagnosis require a pathognomonic bilateral and relatively symmetric reticular clinical appearance involving oral mucosa (Figure 3.5,3.6). These forms could be accompanied by atrophic and/or erosive patterns, describing a more complex and symptomatic mucosal involvement [143].

Moreover, histological evidence of degeneration of the basal epithelial cell layer accompanied by liquefaction, a well-defined band-like zone of lymphocytic infiltration confined to the superficial chorion and the absence of epithelial dysplasia must be observed. In addition, a peculiar deposition of fibrinogen band at the basement membrane zone could be observed through direct immunofluorescence [144].

A well-established pathogenetic event is the auto-activation of T CD8+ that can induce basal membrane keratinocytes apoptosis and cytokine releasing (e.g TNF-α), promoting the attraction of many more lymphocytes in the lesional area. Many etiological factors have been considered as disease triggers such as genetic, self-peptides or altered self-peptides, immunity, reaction to viral infections and response to drugs or topical agents; however, OLP cause is not yet clearly understood [145].

Spontaneous healing of OLP lesions is described but in most cases are long lasting. All the lesions commonly share a see-sawing behavior, with recurring remission and relapse events. Nonetheless, the prognosis is favorable.

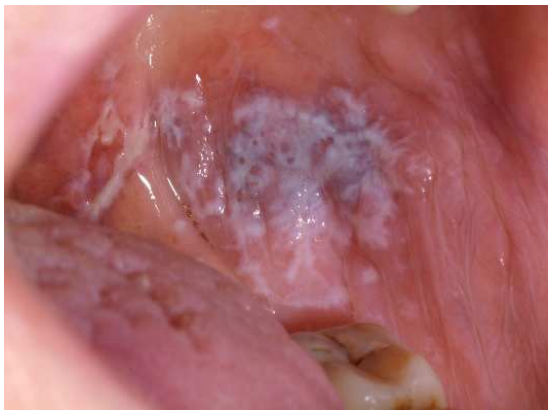


Figure 3.5. Oral lichen planus in a 45 y/o male extended to the entire right buccal mucosa

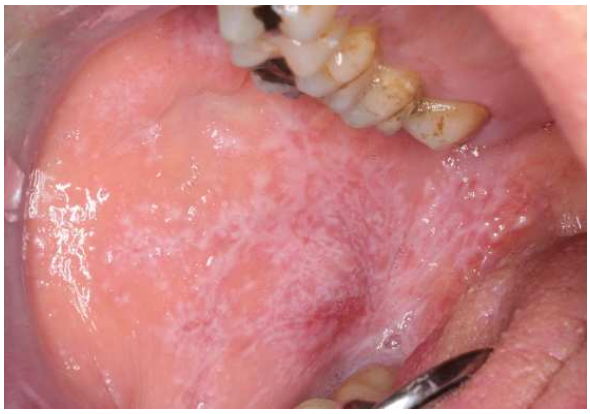


Figure 3.6. Oral liken planus in a 68 y/o female, on the right buccal mucosa

Diagnosis of Potentially Malignant Disorders

Diagnosis of oral cancer is currently based on clinical examination and histological analysis of suspicious areas. Most common clinical signs and symptoms of cancer of the oral mucosa: persistent mouth sores, alterations in the appearance and consistency of the oral mucosa, white and/or red raised patch or plaque in the oral mucosa, localized bleeding, pain. Diagnosis is histopathological, obtainable by multiple incisional biopsies or in any case through a reasonably large biopsy sampling [146].

Similarly, PMDs, such as OLP and OLK, also require a diagnosis based on clinical examination and histopathological analysis. Small homogeneous lesions can be immediately removed by excisional biopsy, while PVL and large non-homogeneous lesions require multiple biopsies for "field mapping" the involved area, to intercept the presence of different degrees of dysplasia or oral cancer, a situation that requires a radical surgical approach [146].

In case of oral squamous cell carcinoma (OSCC) or PMDs, the problems relating to the invasiveness and post-operative morbidity of this intervention are well known. There are also alternative non-invasive screening techniques such as including brush biopsy (cytological test based on the collection of cells from the deep layers of the lesion collected by a brush), supravital straining (staining of the lesion with Toluidine blue), light-based detection systems and optical diagnostic technologies [147]. However, they present several pitfalls, and we shouldn’t consider these substitutes for biopsy, especially in cases where we suspect the

presence of malignant lesion, but they should only be used as additional test [148]. Furthermore, the use of toluidine blue tests is debated due to its sensitivity (between 72.5% and 84%), specificity (between 61.4% and 70%) and being extremely dependent on the operator [149].

There are still many limitations in these diagnostic systems relating to the invasiveness of the biopsy, the timing of analysis or due to reduced sensitivity and specificity of the tests. To overcome these problems, a valid alternative, which today is seen in an increasingly promising way, is represented by the field of salivary metabolomics, and in particular by identification of salivary biomarkers associated with the presence of PMDs or dysplasia, that may signal the onset of OSCC. Metabolites serve as direct signatures of biochemical activity,and may become an effective methodology that has been largely implemented for clinical diagnostics [150].

Unlike non-oral diseases, oral PMDs are in direct contact with saliva. For its proximity to the anatomical sites of lesions development, the oral fluid may be regarded as the ideal carrier of information on the possible presence of neoplastic alterations in the oral cavity [151]. The possible detection and measurement of specific molecules released from oral malignant and potentially malignant lesions in saliva seems, therefore, to be an attractive possible alternative or precursor to tissue biopsies [152]

Aim of the study

The aim of this study is:

- to characterize the salivary metabolic profile of oral lichen planus, oral leukoplakia with and without dysplasia by ^{1}H-NMR, compared to salivary healthy control profiles

The findings of this study could be implemented in screening programs for early diagnosis and therapy planning.

Materials and methods

The present pilot study was approved by the Ethics Committee of the "Area Vasta Emilia Nord" (AVEN) (protocol number: 38/2017/TESS/AUOMO-509/2019/TESS/UNIPR). According to the Declaration of Helsinki, written consent was obtained from all volunteers who participated in this study.

Study population selection

The patients (n=60) and the health control (n=27) were enrolled in 3 centers, one in Parma (University Dentistry Center of the University of Parma) and two based in Modena (Dermatology and Dentistry Unit, Modena University Hospital).

They undergo routine diagnostic or surgical procedures as part of outpatient oral surgery (e.g., third molar extraction, clinically benign oral lesions excision), from September 2019 to April 2022. No samples were taken in 2020 and 2021 due to Coronavirus SARS-CoV-2 restrictions. Enrolment was voluntary, following adequate information on the aim and methods of the research, after the acceptance and signature of informed consent. Cases were subdivided into groups for data acquisition and comparison with HC: OLP, non-dysplastic OLK, dysplastic OLK.

Patients were interviewed for personal data, medical and pharmacology history both remote and recent, exposure to any risk factors (alcohol, tobacco, HPV infection, diabetes). Enrolled subjects underwent a careful dental examination, following a protocol previously published and showed in Annex n° 1 [153]. We evaluated their general oral health (Decay Missing Filled Teeth index-DMFT and Full Mouth Plaque Score-FMPS), periodontal health status (Periodontal Screening and Recording,PSR), presence or absence of infectious diseases and other potential pathological conditions. For cohorts of patients with OLP and OLK, lesions were photographed, described clinically and removed surgically, after a salivary sample. Total collection time of saliva, amount of saliva and type of surgical instrument used were recorded.

Criteria of enrolled subjects are summarized in Table 3.2.

Table 3.2 Inclusion and exclusion criteria for patient enrolled

Groups	Inclusion	Exclusion
HC	Tobacco smoking: yes/no Alcohol consumption: yes/no	History of PMDs or OSCC Anaemic conditions Uncontrolled diabetes Onco-haematological diseases in the last 12 month
PMDs	Tobacco smoking: yes/no Alcohol consumption: yes/no Clinical and histological diagnosis for PMDs	Clinical and histological diagnosis for OSCC Anaemic conditions Uncontrolled diabetes Onco-haematological diseases in the last 12 mm

Saliva Collection

The protocol for saliva collection was described by Meleti et al. (2020) (Annex n° 1) [153]. For each participant, a sample of whole saliva (WS) was collected in the absence of stimulation. The procedure took place between 9:00 a.m. and 11:00 a.m. to minimize the influence of the circadian rhythm on salivary composition. Immediately before collection, patients rinsed their mouths with water for 1 min. During collection (5-15 min), salivary samples were transferred to a tube containing NaN_3 (0.5% final concentration) and kept on ice until a volume of approximately 2 mL was obtained and then frozen at −80 °C.

Sample preparation and ^{1}H-NMR spectra acquisition

Each frozen saliva sample was thawed on ice and centrifuged at 15,000 × g for 10 min at 4 °C to remove eukaryotic and prokaryotic cells, cellular debris, and mucins, according to Quartieri et al. [154]. Saliva supernatants were separated from pellets and stored at −80 °C until metabolomic analysis.

For NMR sample preparation, saliva supernatants were thawed on ice. One millilitre of each saliva supernatant was ultra-filtered using Amicon Ultra-4 Centrifugal filters (3000 MWCO, Merck Millipore) at 4,000 x g at 10 °C for 60 min to deplete proteins that may interfere with metabolite quantification by NMR [155]. Then, ultra-filtered supernatants were lyophilized and

resuspended in 600 µL of 25 mM phosphate buffer pH 7.4, containing 1.45 mM 3-trimethylsilyl propanoic acid (TSP), used as a quantitative standard, and 5 % D_2O for the solvent signal lock. One-dimensional ^{1}H-NMR spectra of serum samples were acquired at 25 °C with a JEOL 600 MHz ECZ600R spectrometer (JEOL Inc., Tokyo, Japan). The spectra were processed and analysed with the Chenomx NMR suite 9.0 software (Chenomx Inc., Edmonton, AB, Canada), zero-filling to 256 K points, using a line broadening of 0.5 Hz.

Metabolomics statistical data analysis

Statistical analysis was conducted using Metaboanalyst 6.0 (https://www.metaboanalyst.ca). The metabolite concentration data of each sample were normalized by the median and auto scaled. Multivariate statistical analysis was performed using the Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). Results were visualized as 2D score plots, and metabolites with high PLS-DA VIP scores were identified. The heatmap used the Euclidean distance measure and the Ward clustering method. The volcano plot with 1.5 and 0.05 as fold change and p-value thresholds were performed for univariate analysis.

Results

A whole population of 87 patients was enrolled, but 54 were included for the analysis; subgroups were composed by 13 OLP, 13 with OLK with dysplasia, 13 with OLK without dysplasia, 15 HC. Thirty-three subjects were excluded from the study for incorrect sample collection and insufficient material to be analysed.

Table 3.3 reports the demographic and clinical features of study participants.

We have profiled the saliva samples of all the study participants, identifying 68 metabolites by comparation between OLP patients and HC and 72 compounds by comparation between OLK patients and HC.

Table 3.3 Characteristics of the cohort of saliva donors

Variables		Subject			
		OLK with dysplasia (n=13)	OLK without dysplasia (n=13)	OLP (n=13)	HC (n=15)
Sex, age (min-max, media ± SD)					
	Female	6 22-73 (46.5 ± 21)	6 54-77 (63.0 ± 9.59)	9 43-79 (61.3 ±11.6)	10 42-71 (59.2 ± 10.8)
	Man	7 64-80 (64.8.0 ± 9.08)	7 53-76 (66.9 ± 7.34)	4 45-66 (56.0 ± 10.1)	5 44-60(51.8 ± 6.5)
Smoking					
	Never / former	8	8	10	11
	Current	5	5	3	4
Location of lesion					
	Alveolar mucosa	2	4	-	-
	Buccal mucosa	2	3	0	-
	Lip	-	1	12	-
	Oral floor	-	-	-	-
	Palate	-	1	-	-
	Retromolar trigone	2	2	-	-
	Tongue	7	2	1	-
Histopathology					
	Non-dysplastic	-	13	13	-
	Low-medium grade dysplasia	13	-	-	-
	High grade dysplasia	-	-	-	-

Oral leukoplakia – Healthy controls

The heat map shows apparent differences between oral leukoplakia (with and without dysplasia) and healthy controls. This is confirmed by PLS-DA, where principal components identify variables driving a separation between the 3 sets of samples (Figure 3.7, 3.8).

Figure 3.7 Heatmap visualization of all metabolic features. Each line in the heat map represents a metabolite. Colors change from red to blue, red and blue meaning higher and lower metabolite concentrations in the saliva sample of donors.

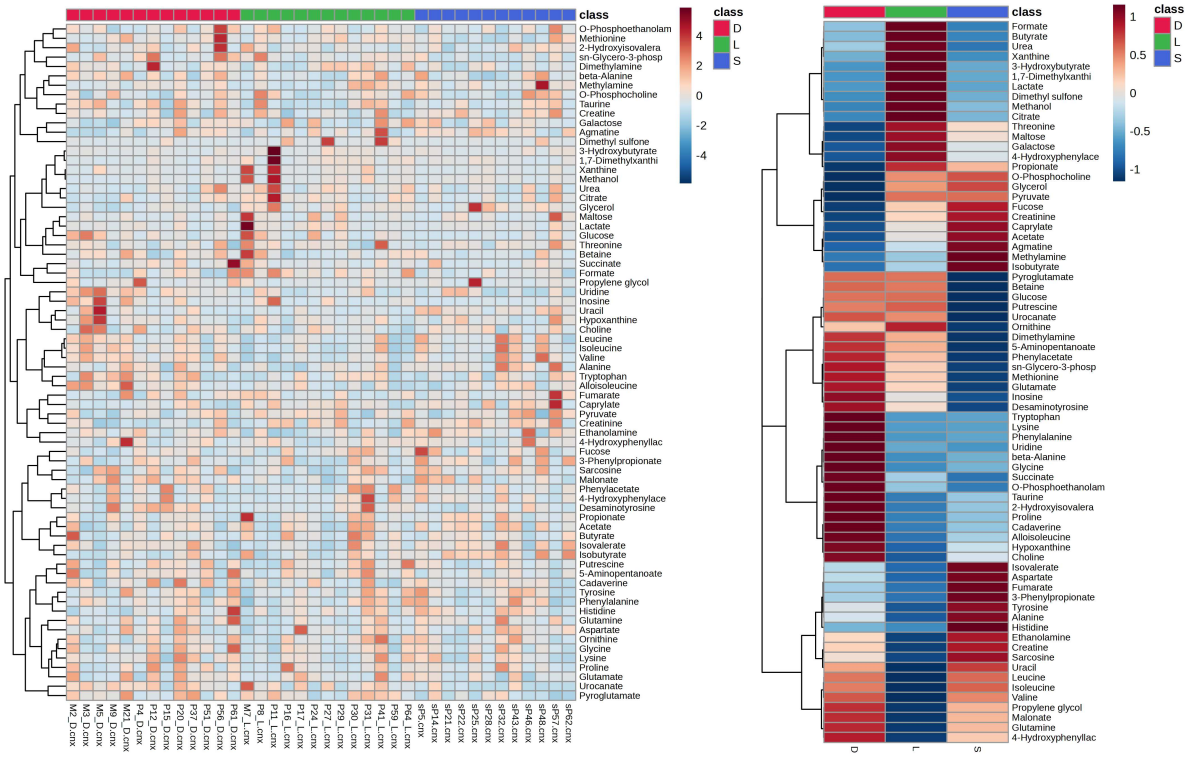
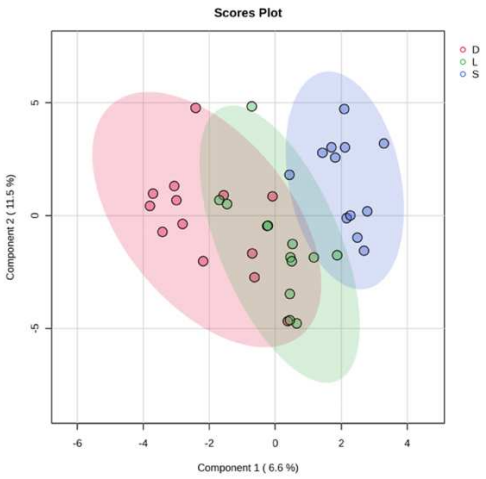


Figure 3.8 A PLS-DA Scores Plot of HC (S), OLK with dysplasia (D) and OLK without dysplasia (L)



Particularly, PLS-DA revealed separate clustering of OLK with dysplasia and healthy controls, with components 1 and 2 explaining 9 and 8.7% of the total variance between the two groups. (Figure 3.9). Compounds driving separation in the PLS-DA model are presented in Figure 3.10. The most discriminant compounds, exhibiting a VIP score > 2, are urocanate (most concentrated in OLK with dysplasia), isobutyrate, and creatinine (most concentrated in HC). We set the fold change threshold at 1.5 with a p-value > 0.05. Urocanate, isobutyrate and creatinine were confirmed as discriminant metabolites and, in addition, inosine, glycerol, uridine, pyroglutamate, sn-glycero-3-phosphocoline, desaminotyrosine and glucose (Figure 11). Vulcano Plot and Box Plots showed that urocanate is the most discriminant metabolite between sets of samples (Figure 3.11, 3.12).

Figure 3.9 PLS-DA Scores Plot of HC (S), OLK with dysplasia (D).

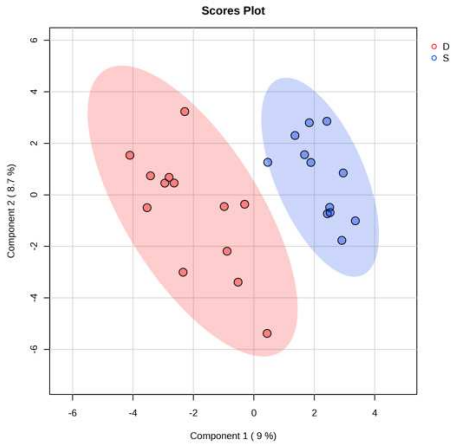


Figure 3.10 PLS-DA variable importance projection (VIP) scores depict the 15 most significant metabolites contributing to the separation of OLK with dysplasia and healthy donors. Red or blue on the right indicates the low or high average metabolite concentration by comparing the concentration of each metabolite in OLK with dysplaisa vs. healthy donors' samples. D: OLK with dysplasia; S: healthy controls.

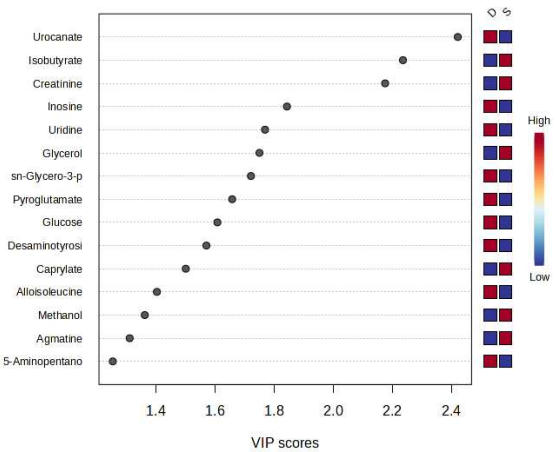


Figure 3.11 Volcano plot analysis of the differential salivary metabolites of OLK with dysplasia vs. healthy controls. Volcano plot of the quantified metabolites illustrating a comparison between the two groups of donors. Each point on the volcano plot is based on *p* and fold-change (FC) values. The points which satisfy the condition $p < 0.05$ and $|FC| > 1.5$ were considered significant and appear in red or blue if the concentration of the corresponding metabolite is higher or lower in lichen than in healthy donors.

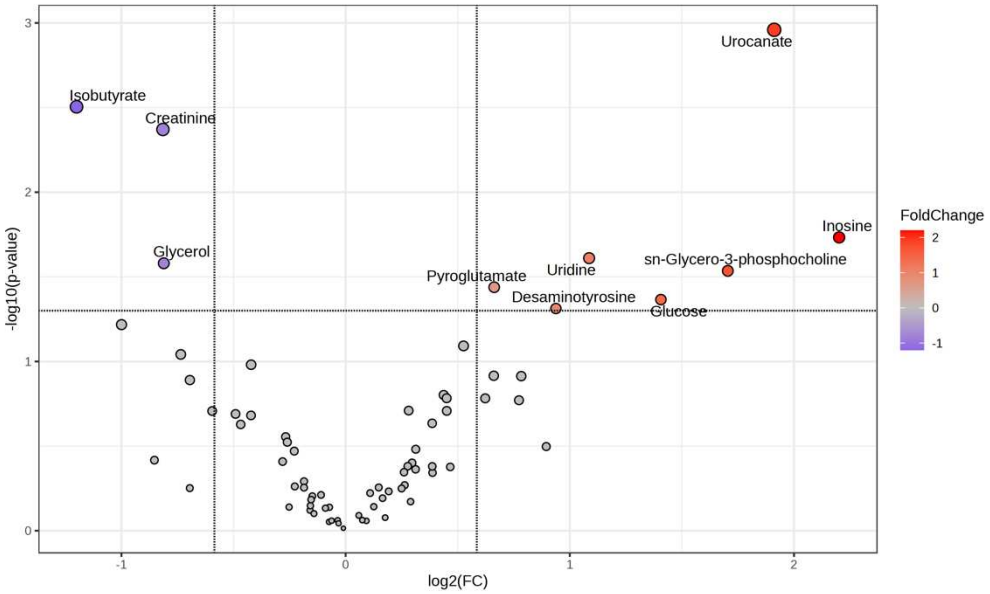
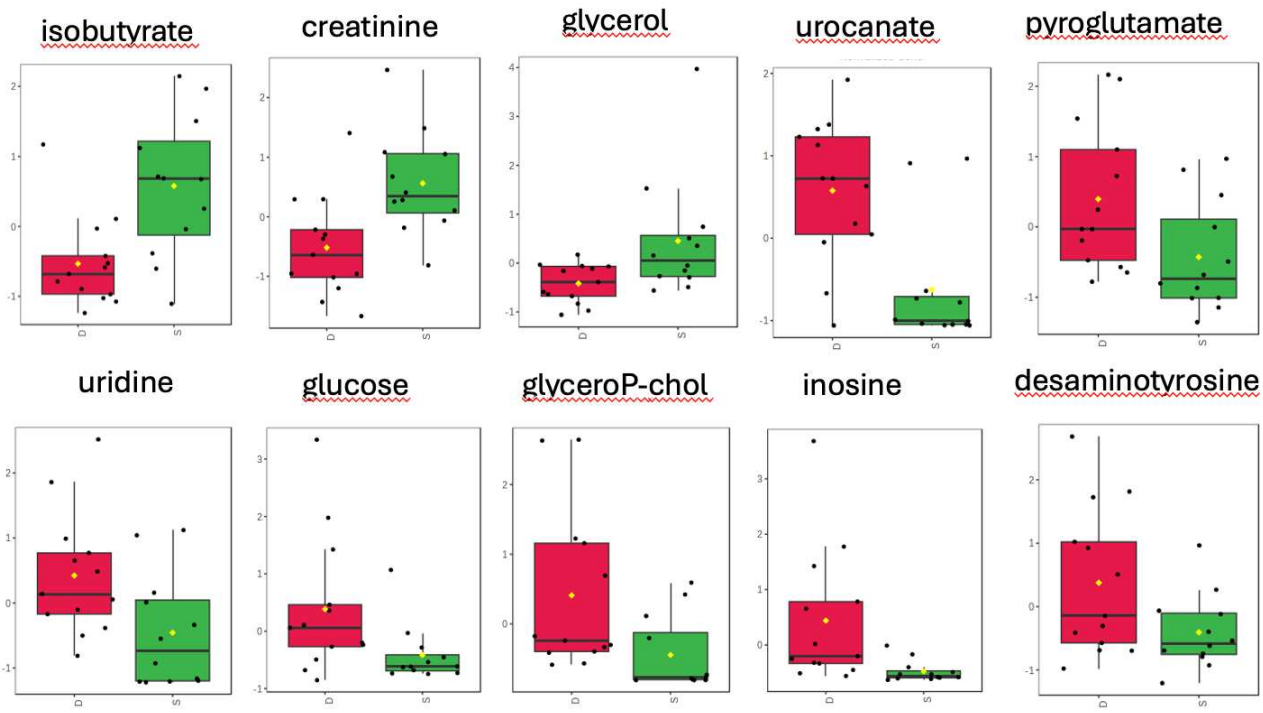


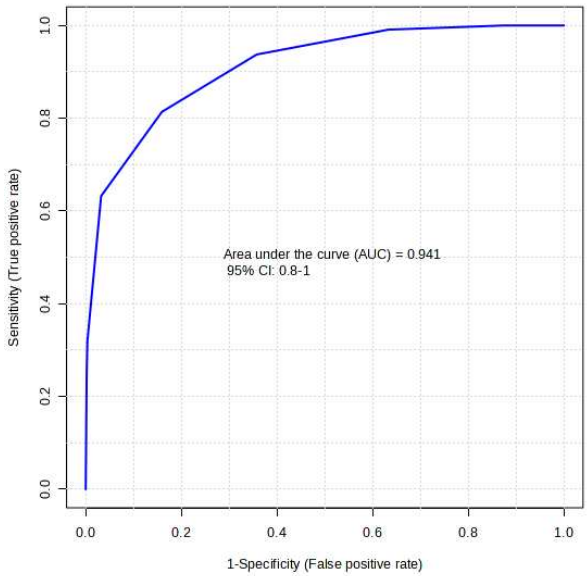
Figure 3.12 Box plots of the normalized concentrations of the significant metabolites identified in Vulcano Plot for both sets of samples; red is used for depicting OLK with dysplasia group and green for the healthy controls.



Using the relevant metabolites found with Vulcano Plot (inosine, creatinine, urocanate, isobutyrate, glycerol, sn-glycero-3-phosphocholine, univariate), it was realizing a ROC curve

with Metaboanalyst 6.0 software. Area under curve (AUC) was of 0.941, indicating a good experimental model, with a probability of 94% to consider the discriminating values of metabolites as reliable (3 3.13).

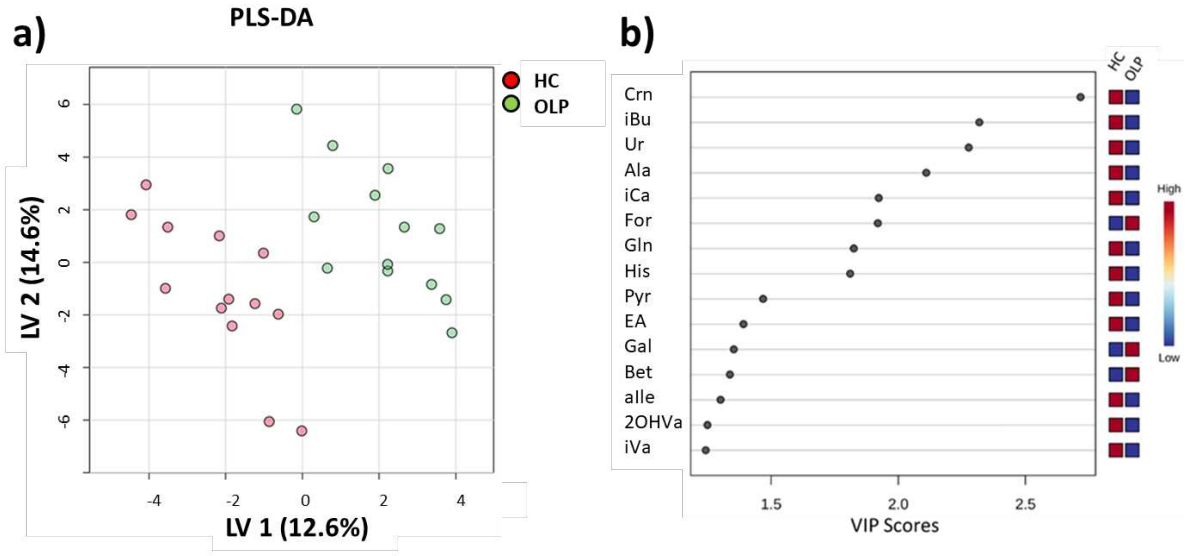
Figure 3.13. ROC curve dysplasia x healthy (model built with inosine, creatinine, urocanate, isobutyrate, glycerol, Sn-glycero-3-phosphocholine with a AUC > 0.78)



Oral Lichen Planus patients – Healthy controls

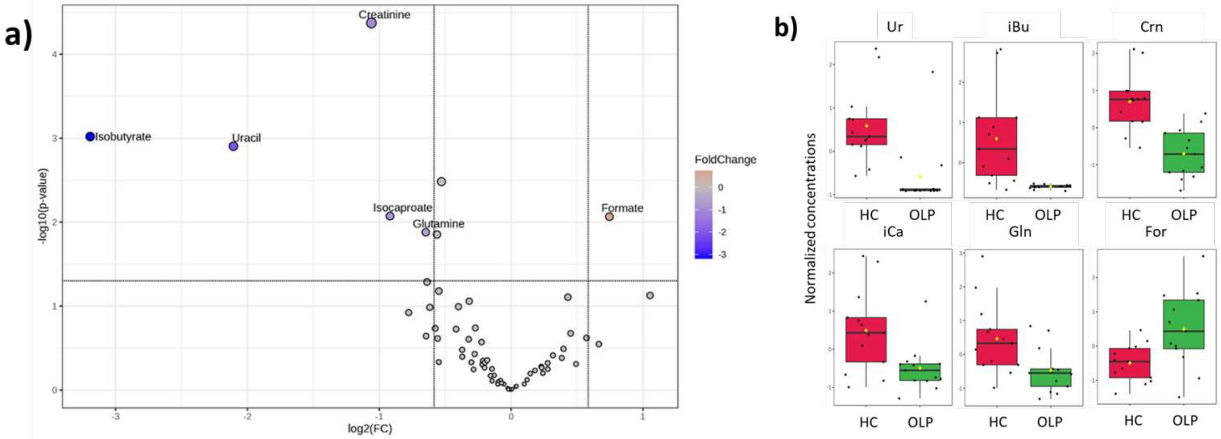
Partial Least Squares Discriminant Analysis (PLS-DA) revealed separate clustering of OLP patients and HCs, with LV1 and LV2 explaining 12.6 and 12.4% of the total variance between the two groups (Figure 3.14a). The compounds driving the separation in the PLS-DA model are presented in Figure 3.14b. The most discriminant compounds exhibiting a VIP score >2 are creatinine (Crn), isobutyrate (iBu), uracil (Ur), and Alanine (Ala).

Figure 3.14. Partial Least Squares Discriminant Analysis (PLS-DA) of salivary metabolic profiles of OLP vs. HC. PLS-DA 2D score plot (**a**). PLS-DA variable importance projection (VIP) scores (**b**) depict the 15 most significant metabolites contributing to the OLP and HC separation. The red and blue boxes on the right side of the VIP score plot indicate the relative metabolite abundance in each cluster of the PLS-DA.



The univariate volcano plot analysis was employed as a means of further validating the statistically significant compounds. By setting the fold change threshold at 1.5 and a p-value <0.05 (Figure 3.15a), Crn, iBu, Ur, iCa, and Gln appear as more concentrated in healthy controls. In contrast, formate is present in higher amounts in OLP patients. The box plots in Figure 3.15b show the distribution of the normalized concentrations of the six most significant metabolites which are responsible for discriminating between HC and OLP samples.

Figure 3.15 (a) Volcano plot analysis of the differential salivary metabolites of OLP vs. HC. Each point on the volcano plot is based on specific p and fold-change (FC) values. The points that satisfy the condition p <0.05 and |FC| > 1.5 were considered significant. (b) Box plots of the normalized concentrations of the significant metabolites identified in (a) for both sets of samples; red is for OLP patients, and green is for HC.



Discussion

Literature data about salivary untargeted metabolomics for exploration of Oral Potentially Malignant Disorders (OPMDs) are particularly limited in literature. Research on multiple databases (Medline, Scopus and World of Science) returned 9 original articles published in English language in the last 20 years [156–164]. Most studies have explored salivary metabolome of leukoplakia or epithelial dysplastic lesions as a group of comparison with OSCC and HC, and only 2 about the salivary metabolomics in OLP.

The preliminary data from exploration of salivary metabolome in leukoplakia through ^{1}H-NMR, returned a panel of metabolites capable to aggregate well distinguished clusters of cases and controls (Figure 3.8). Following the importance attributed by VIP scores output, the most representative are discussed below.

Urocanic acid is a breakdown (deamination) product of histidine. In the liver, urocanic acid is an intermediate in the conversion of histidine to glutamic acid, whereas, in the epidermis, it accumulates and may be both a UV protectant and an immunoregulator. The problem is that it provides glutamic acid to the cancer cell, which may enhance the glutaminergic pathway. In fact, by our preliminary results urocanate seem increased in patients with dysplasia [165]. Jauhonen et al. demonstrated the productive immunosuppressive activity of cis-urocanate by reducing mast cell degranulation in both IgE mediated as well as IgE independent models. In the same study, the authors also reported the efficacy of this molecule as anti-inflammatory effect [166].

Isobutyric acid is a carboxylic or short chain fatty acid, and there is little evidence about the influence of isobutyrate on the biological characteristics of cancer cells. Chen H et al. (2023), explored the tumor suppressor role of isobutyrate in lung cancer (LC), performing cell proliferation, migration, and invasion assays on cancer cells after treatment with isobutyric acid. Compared with the control group, they noticed that cell activity was remarkably decreased in the isobutyric acid-treated group [167]. In fact, our results show a decrease of this metabolite in the saliva of patients with oral dysplasia. Probably, this short chain fatty acid modulates the tumorigenesis activating G protein-coupled receptors and inhibiting histone deacetylases [168].

Creatinine, the final product of arginine and glycine degradation, has been investigated in different types of cancer, sustaining its prognostic character in several types of epithelial cancers (e.g., vulvar, colorectal and ovarian) [169].

It is a nitrogen-containing organic acid that abundantly exists in human body with 120–140 g for a 70-kg adult. About 2–4 g of creatine is obtained daily from creatine-enriched diet such as fish, and poultry and red meat. Moreover, creatine is phosphorylated by creatine kinase to form phosphocreatine, an important metabolite for efficient energy buffering in organism [170].

There are controversial results from literature about the role of creatinine in cancer patients.

In hepatocellular carcinoma patients, breast cancer and glioblastoma the creatine level is significantly increased in serum and urine [171, 172]. Otherwise, a retrospective cohort study evaluated serum creatine and creatinine levels in 170 patients with invasive vulvar cancer, finding that elevated serum creatine and creatinine levels were significantly associated with both and overall survival [173]. Creatine levels were also found lowly existing in some cancer tissue, while creatine treatment increased cancer creatine content along with suppressive cancer cell growth, as sarcoma[174]. Thus, the correlation between creatine levels in cancer patients and cancer progression is less clear.

Inosine is an essential metabolite for purine biosynthesis and degradation; it also acts as a bioactive molecule that regulates RNA editing, metabolic enzyme activity, and signaling pathways. As a result, inosine is emerging as a highly versatile bioactive compound and second messenger of signal transduction in cells with diverse functional abilities in different pathological states [175].

Multiple studies have suggested that inosine functions as a crucial biomarker metabolite associated with cancer metastasis, drug resistance and/or treatment, and tumor progression [176–178]. A recent study showed that inosine can predict the metastatic potential of lung squamous cell carcinoma [177]. Inosine has also been shown to be associated with acute myeloid leukemia [176], esophageal squamous cell carcinoma [178], colorectal cancer [179], and head and neck squamous cell carcinoma [180]. Inosine is a crucial serum metabolite that can differentiate between low- and high-grade bladder cancer patients [181]. These data, together with our preliminary results, suggest that inosine has a promising role in the diagnosis and grading of tumors as a signature metabolite.

Presence of abundant salivary glycerol characterized the HC group. Glycerol is a precursor for synthesis of triacylglycerols and of phospholipids, both representing cellular membrane constituents. It is also released in bloodstream when stored fat goes to breakdown for energy production purposes.

The increase of glycero-phosphocoline in patients with dysplasia is confirmed by its role described in literature. In fact, activated choline metabolism is a hallmark of carcinogenesis and tumor progression, which leads to elevated levels of phosphocholine and glycerophosphocholine in all types of cancer tested so far [182].

Regarding the PLS-DA output, a sharp group clustering is observable. Such aggregation allows to clearly distinguish healthy controls from non-dysplastic and dysplastic leukoplakia respectively. Moreover, HC and LK_1 (dysplastic lesion) clusters do not have point of contact, suggesting a true distinction in metabolic profiles between groups. This result is comparable to other authors data output. Wei et al. for example, demonstrated a good separation of clusters resulting from UPLC-QTOF MS analysis, emerging 27 discriminant salivary metabolites capable of distinguish OLK group from HC [183].

Regarding OLP data, statistical analysis of salivary metabolite concentrations revealed significant changes in some metabolites of bacterial origin, such as isobutyrate, isocaproate, and formate, suggesting that OLP lesions are possibly associated with an imbalance in microbiota composition.

Uracil, together with isobutyrate and isocaproate, is found only in whole saliva [184] and reflects the metabolic interaction between the salivary glands' secretions, gingival crevicular fluid, suspended bacteria, desquamated cells, and food debris. Instead, blood-derived molecules, such as creatinine, Glutamine, and formate [184] can also enter the saliva via the highly vascularized salivary glands and may be associated with systemic diseases that require special attention from health professionals [185].

In particular, glutamine seems to be an important regulator of oxidative stress [186] that is associated with inflammation correlated with the onset and progression of OLP [187].

Therefore, our results showed metabolic differences between the 2 cohorts studied (HC and OLP): not sufficiently consistent to define a clear difference between the metabolic profiles but sufficient to highlight a possible alteration in the oral ecosystem. The differences found in saliva are derived from alterations in the oral mucosa of patients affected by oral lichen planus. Within the limitations of this study, salivary metabolic footprint seems to be capable to distinguish patients with oral lichen planus, Oral leukoplakia from the healthy condition and to discriminate the presence of dysplastic alteration. Further studies are needed to select reliable biomarkers; such molecules could be associated to clinical examination or lesion biopsy to successfully early detect potentially malignant disorders and cancer.

Additionally, the comparisons through ^{1}H-NMR analysis with the lesional, perilesional and healthy tissues from the same subjects allowed us to define a complex metabolic signature of these pathologies.

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CHAPTER 4

Human Serum and Salivary Metabolomes:

Diversity and Closeness

*Ferrari E, Gallo M, Spisni A, **Antonelli R**, Meleti M, Pertinhez TA. Human Serum and Salivary Metabolomes: Diversity and Closeness. Int J Mol Sci. 2023 Nov 22;24(23):16603. doi: 10.3390/ijms242316603. PMID: 38068926; PMCID: PMC10706786.*

Published on International Journal of Molecular Sciences
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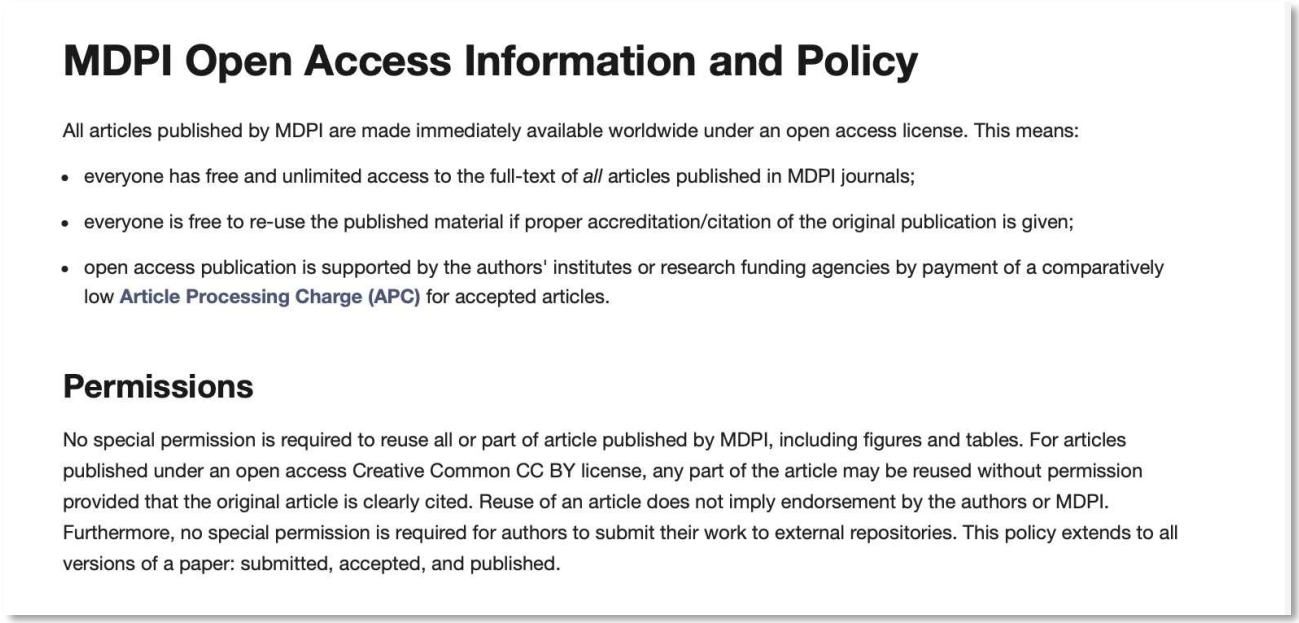

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International Journal of
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Human Serum and Salivary Metabolomes: Diversity and Closeness

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Abstract: Saliva, which contains molecular information that may reflect an individual's health status, has become a valuable tool for discovering biomarkers of oral and general diseases. Due to the high vascularization of the salivary glands, there is a molecular exchange between blood and saliva. However, the composition of saliva is complex and influenced by multiple factors. This study aimed to investigate the possible relationships between the salivary and serum metabolomes to gain a comprehensive view of the metabolic phenotype under physiological conditions. Using ^{1}H-NMR spectroscopy, we obtained the serum metabolite profiles of 20 healthy young individuals and compared them with the metabolomes of parotid, submandibular/sublingual, and whole-saliva samples collected concurrently from the same individuals using multivariate and univariate statistical analysis. Our results show that serum is more concentrated and less variable for most of the shared metabolites than the three saliva types. While we found moderate to strong correlations between serum and saliva concentrations of specific metabolites, saliva is not simply an ultrafiltrate of blood. The intense oral metabolism prevents very strong correlations between serum and salivary concentrations. This study contributes to a better understanding of salivary metabolic composition, which is crucial for utilizing saliva in laboratory diagnostics.

Keywords: salivary metabolites; serum metabolites; metabolite profile; parotid gland; submandibular gland; sublingual glands; whole saliva

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Citation: Ferrari, E.; Gallo, M.; Spisni, A.; Antonelli, R.; Meleti, M.; Pertinhez, T.A. Human Serum and Salivary Metabolomes: Diversity and Closeness. *Int. J. Mol. Sci.* **2023**, *24*, 16603. https://doi.org/10.3390/ijms242316603

Academic Editor: Anne Vejux

Received: 28 October 2023
Revised: 15 November 2023
Accepted: 20 November 2023
Published: 22 November 2023

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1. Introduction

Whole saliva (WS) contains molecular information that may reflect the health status of an individual. It is a complex water mixture composed of organic and inorganic secretions of major and minor salivary glands, gingival crevicular fluid, suspended bacteria, desquamated cells, and food debris [1–3]. Its production is significantly affected by circadian rhythm, age, hydration status, physical exercise, oral hygiene, and food consumption [1,4]. In addition, blood-derived molecules may enter the highly vascularized salivary glands via transcellular and paracellular routes, affecting the biochemical composition of saliva [2,5,6]. In the oral cavity, the metabolite composition of whole saliva is further modified by the metabolism of the resident oral microflora, desquamated host cells, and immune cells [7]. As a result, oral fluids contain molecular information that may reflect the health status of an individual.

1.1. Salivary Glands

The major glands that bilaterally secrete saliva are the parotid, submandibular, and sublingual glands (Figure 1A). Under the control of the autonomic nervous system, these major salivary glands create about 90% of the saliva, with the remaining saliva, which is

produced by 600 to 1000 minor salivary glands, found mainly on the lips, buccal mucosa, palate, and tongue [8].

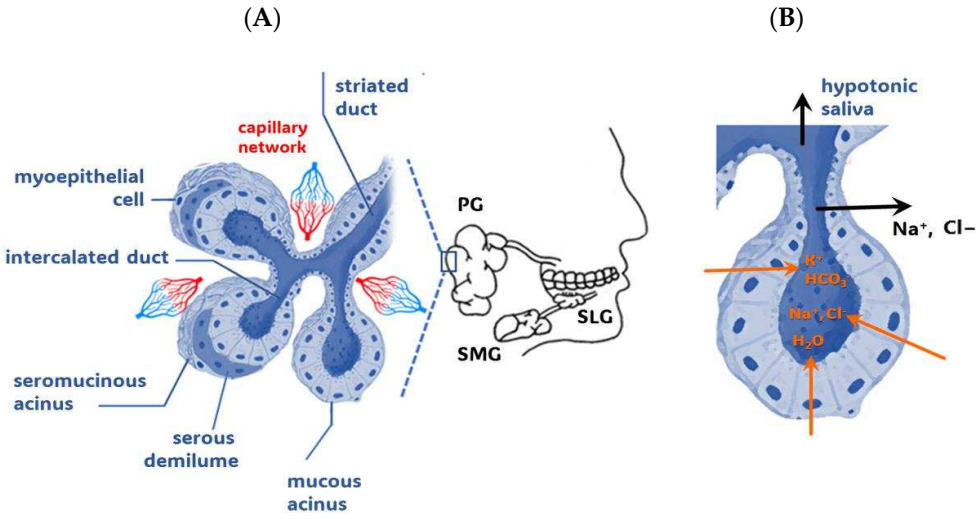


Figure 1. Salivary gland components. (**A**) Detail of a mixed salivary gland showing one mucous acinus and two seromucinous acini, the ductal system, and the capillary network. Contractile myoepithelial cells wrap around the acinar cells and their ducts to promote gland secretion. In mixed seromucinous acini, the serous cells form a demilune around the mucous acinus. Each acinar portion merges into an intercalated duct, which continues as a striated duct. (**B**) An acinus with its intercalated duct. According to the current model of saliva secretion, in the first step, the acinar cells secrete a relevant number of Na^+ and Cl^- ions into the acinar lumen. This ionic flux drives water efflux transcellularly (through aquaporin channels) and paracellularly, producing an isotonic fluid that enters the ductal lumen. In the second step, the ductal cells reabsorb most of the Na^+ and Cl^- and secrete K^+ and HCO_3^- ions, resulting in hypotonic saliva reaching the final secretory duct [4,9,10]. PG, parotid gland; SMG, submandibular gland; SLG, sublingual gland.

Salivary glands are composed of specialized epithelial cells that form acini, the glandular end portions that initiate the secretion of saliva (Figure 1A), and branched ducts through which saliva enters the oral cavity. The acini can be serous, mucous, or a combination of both, determining the secretion type they produce. While a serous acinus cell secretes a watery salivary fluid rich in proteins, a mucous one produces a viscous secretion rich in mucins [2].

1.2. From Capillary Blood to Whole Saliva

A dense network of capillaries surrounds the ductal system, while a less intense vascularization supplies the terminal acini. When stimulated by parasympathetic vasodilator fibers, blood flow increases up to 20-fold and produces the flux of a solution, containing inorganic and small organic substances (Figure 1B), toward the gland lumen via (a) ultrafiltration through the narrow junctions between acinar cells, (b) intracellular passive diffusion according to a concentration gradient, or (c) energy-dependent transport against a concentration gradient [4,5,11–13].

1.3. Salivary Metabolomics

Over the last two decades, knowledge about saliva and its homeostasis has increased to such an extent that a new term, salivaomics, has been coined to describe the reservoir of information present in WS, referring to the subject's genome, epigenome, transcriptome, proteome, metabolome, and microbiome [14,15].

Among the organic macro-components, salivary proteins have been extensively investigated, revealing their glandular (e.g., α-amylase, mucins, histatins, cystatins, proline-rich

proteins, statherins, lactoferrin, and lysozyme) and plasma (e.g., albumin, secretory immunoglobulin A, and transferrin) origin [1]. The presence of plasma proteins in saliva represents a source of new markers of diseases, offering a diagnostic alternative to specific blood tests [1,16–18].

The comprehensive analysis of metabolites in a salivary specimen has been employed (a) as a source of biomarkers for oral and general disorders, (b) to study changes in saliva composition with exercise performance, and (c) to correlate the salivary metabolome with oral microbiome [3,19–21]. Our recent investigations of the salivary metabolome of whole, parotid, and submandibular/sublingual saliva in healthy and young individuals [22,23] prompted us to explore the relationships between the salivary and serum metabolomes to provide a comprehensive view of the metabolic phenotype in physiological conditions. Here, we present (a) the serum metabolite composition of young and healthy subjects obtained using ^{1}H-NMR spectroscopy and (b) a comparison of their serum and salivary metabolite profiles.

2. Results

2.1. Sample Collection and Metabolite Profiling

Serum, parotid saliva (PS), submandibular/sublingual saliva (SM/SLS), and WS samples were collected from each subject according to a previously established protocol [22].

Metabolomic analysis based on ^{1}H-NMR spectroscopy allowed the identification of 43 metabolites in the serum of all study participants. Table S1 (Supplementary Materials) reports their mean concentrations and standard deviations.

Each participant released the three salivary samples (5.4 mL) at different rates (Figure 2, Table S2). The mean sample collection rates of PS, SM/SLS, and WS were consistent with the salivary flow rates presented in the literature [24] and corresponded to an approximate ratio of 1:2:4.5, suggesting a comparable correlation between their secretion rates.

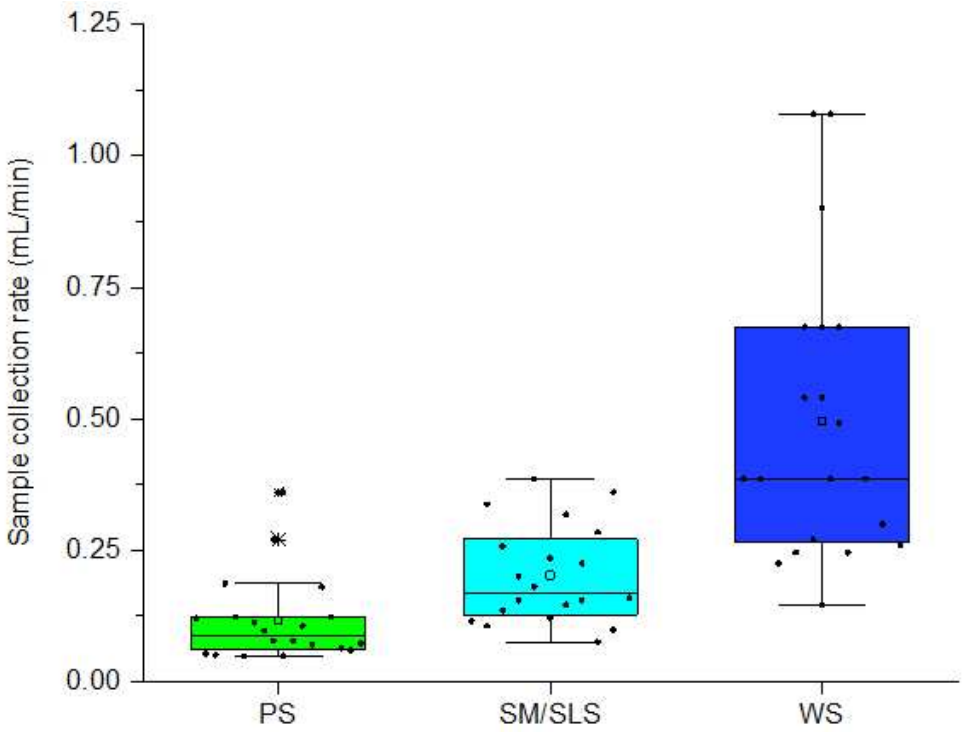


Figure 2. Collection rates of the salivary samples. The boxes are determined by the 25th and 75th percentiles; the horizontal line within each box is the median of the group, while the empty square inside the box corresponds to the mean value of the group; the asterisks identify two outliers. Black dots represent individual sample collection rates.

A list of the metabolites' mean concentrations in the three types of saliva and an analysis of the resulting salivary profiles have already been published [22,23]. The obtained salivary profiles, rearranged according to chemical category, are presented in Table S1.

2.2. Comparison of Serum and Salivary Metabolite Profiles

The number of metabolites detected in all sample types was computed and plotted in a Venn diagram (Figure 3). In this representation, the area covered by the three circles highlights the core of 31 shared metabolites identified in all matrices (Table S1). This number drops to 27 when separating the glandular saliva profile (green circle) into two distinct profiles, i.e., PS and SM/SLS (27 = 31 − 4 metabolites common to PS and SM/SLS). The overlapping section of each pair of circles shows the number of shared metabolites. Metabolites that are unique to each sample type are listed in Figure 3.

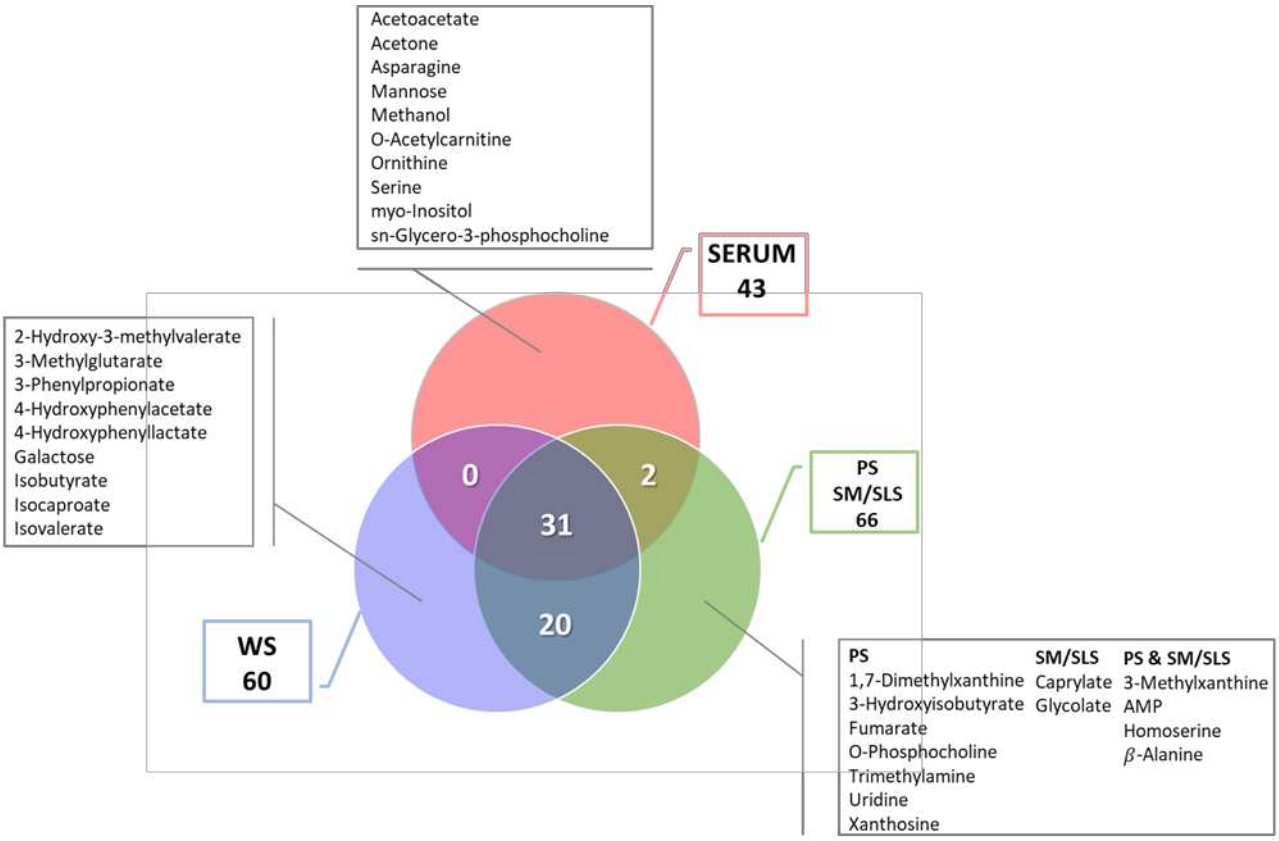


Figure 3. Venn diagram of the number of metabolites detected in each sample type. The red, blue, and green circles refer to the metabolite profile of serum, whole saliva (WS), and saliva produced by parotid and submandibular/sublingual glands (PS and SM/SLS), respectively. Overlapping regions reveal the number of shared metabolites. Unique metabolites of each sample are framed in gray squares. For PS and SM/SLS samples, exclusive metabolites are reported as present only in PS, only in SM/SLS, and both in PS and SM/SLS. For each metabolite profile, the total number of metabolites can be calculated by adding the number of shared and unique metabolites.

We performed a partial least-squares–discriminant analysis (PLS-DA) using the NMR-derived concentrations of the metabolites shared by all sample matrices (n = 27). The PLS-DA score plot presented in Figure 4A evidences a net clustering for serum, PS, and SM/SLS samples, whereas WS samples exhibit a notable dispersion. The model denotes a good separation by component 1 between serum and all salivary samples; metabolite concentration differences do not appreciably distinguish the three salivary samples. Figure 4B reports the Variable Importance in Projection (VIP) scores of the top 15 discriminant metabo-

lites in order of importance in the PLS-DA model, according to the variance explained by component 1. Differences in Acetate, Glucose, and Lactate concentrations, with VIP scores >2, are the most important in generating cluster separation.

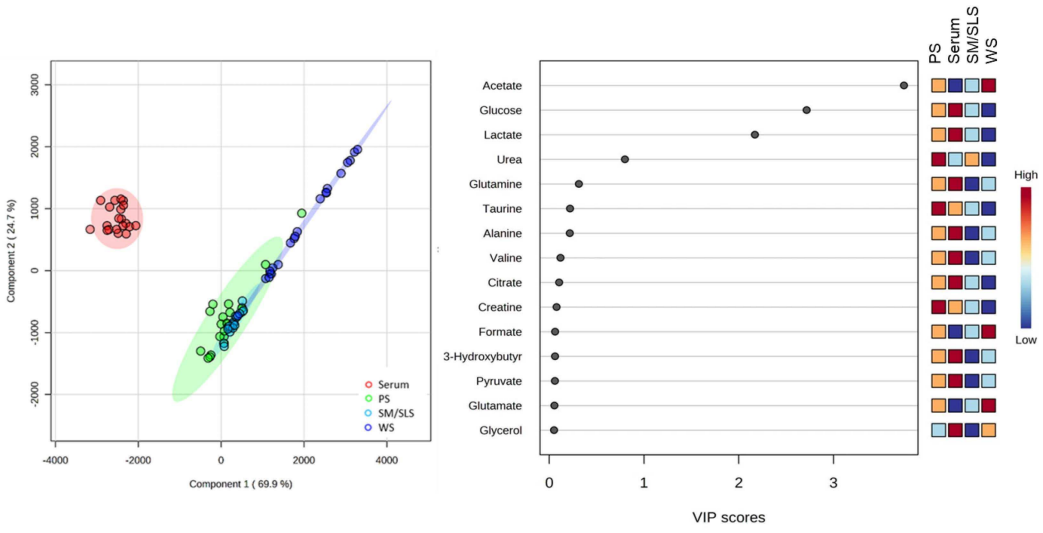


Figure 4. Partial least-squares–discriminant analysis (PLS-DA) based on the metabolites common to serum, WS, and PS plus SM/SLS profiles. (**A**) PLS-DA 2D scores plot. (**B**) Metabolite ranking (top 15 metabolites) according to the Variable Importance in Projection (VIP) scores, resulting from the separation by component 1 in the PLS-DA score plot. A higher metabolite VIP score denotes a more significant contribution to sample separation. The colored boxes on the right indicate the relative metabolite abundance in each sample type.

Upon excluding the most discriminant metabolites, Acetate, Glucose, and Lactate, the PLS-DA model still allowed for some separation between serum and the salivary samples by component 2. In this case, the most discriminant metabolites (1 < VIP score < 4) were Urea, Glutamine, Alanine, and Taurine. Serum exhibited higher levels of Glutamine and Alanine than the salivary samples. The role of these amino acids as gluconeogenic precursors, being mainly released by muscle tissue during fasting, may explain these findings. However, Taurine's prevalence in PS suggests a role of this metabolite in salivary gland function.

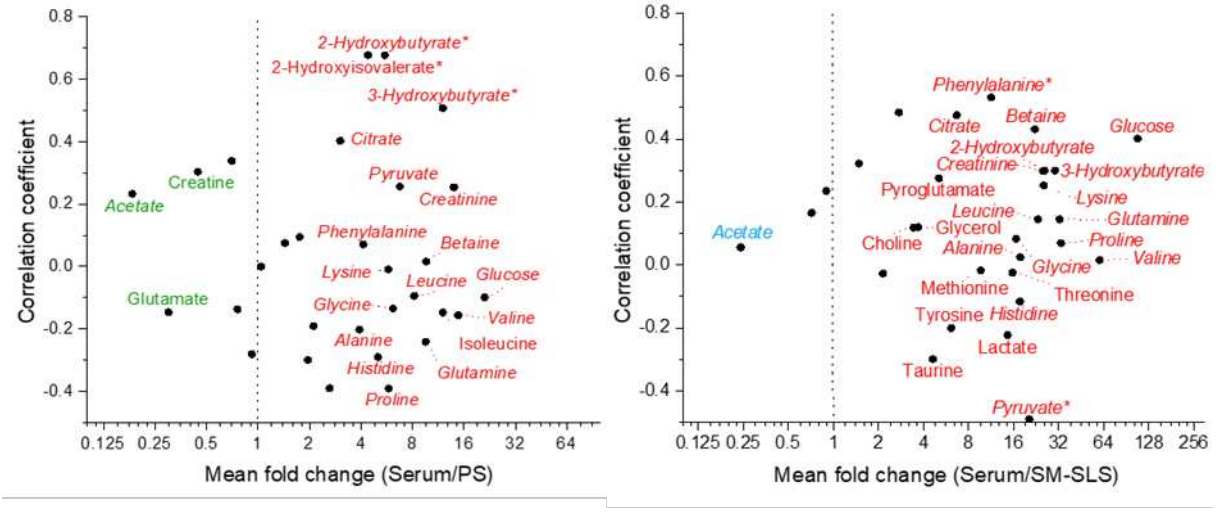
2.3. Comparison of Salivary and Serum Metabolite Concentrations

2.3.1. Metabolites Common to Serum and Saliva Collected from Parotid or Submandibular/Sublingual Glands

We used the Mann–Whitney test to compare the metabolite composition of serum and PS or SM/SLS. The concentration distribution significantly differed for all metabolites, except for Choline in serum and PS and Creatine, Glutamate, and Urea in serum and SM/SLS.

We performed a correlation analysis for metabolite concentrations. For most of them, the correlation was negligible when comparing serum to PS or SM/SLS. Based on Pearson analysis, the highest degree of correlation was obtained with 2-Hydroxyisovalerate, 2-Hydroxybutyrate, and 3-Hydroxybutirate ($0.5 \leq r < 0.7$) when comparing serum to PS and with Phenylalanine ($r = 0.5$) and Pyruvate ($r = -0.5$) when comparing serum to SM/SLS. In serum vs. PS, Spearman correlation analysis confirmed all the strong/moderate correlations found in Pearson analysis and assigned a moderate correlation ($|r| \approx 0.5$) to Creatine and Lactate. In serum vs. SM/SLS, Spearman correlation analysis confirmed the moderate negative correlation of Pyruvate found in Pearson analysis and assigned a moderately positive correlation ($r \approx 0.5$) to 3-Hydroxybutyrate, Creatine, and Creatinine.

We calculated the concentration ratios of "serum/saliva" for each metabolite present in serum and saliva. These ratios were averaged across the 20 study participants and are referred to as "mean fold change", a parameter that expresses the metabolite concentration difference between serum and saliva samples. Figure 5 depicts those metabolites' mean fold change as a function of their correlation coefficients. In both graphs, we have labeled the metabolites with mean fold changes <0.5 or >3, likely reflecting significant differences: for values lower than 0.5, we expected the metabolite salivary concentration to be higher than in serum; for values higher than 3, we expected the metabolite salivary concentration to be lower than in serum.



(**A**) (**B**)

Figure 5. Mean fold change in the metabolites shared by serum and parotid saliva (**A**) or submandibular/sublingual saliva (**B**) expressed as a function of their Pearson's correlation coefficients. For each metabolite, the mean fold change was obtained by averaging the ratios obtained by dividing the concentration in serum by the concentration in the PS or SM/SLS of the 20 study subjects. Line x = 1 marks the boundary between two graph subsections: on the left, the salivary concentrations are expected to be higher than the serum concentration; on the right, the salivary concentrations are expected to be lower than the serum. Metabolites with a mean fold change lower than 0.5 and higher than 3 are labeled. They appear in green or cyan when all their original metabolite concentrations are higher in PS or SM/SLS than in serum, respectively, and in red when their original metabolite concentrations are higher in serum than in PS or SM/SLS. Metabolites in *italics* appear in the same subsection of the two graphs. The asterisks mark the metabolites with a correlation coefficient $\geq \pm 0.5$. X axis is in $\log_2$ scale.

In the comparison of serum vs. PS (Figure 5A), among the metabolites which exhibited a mean fold change >3 or <0.5, only Proline and Pyruvate resulted in a limited number of cases where the concentration ratio was reversed. In fact, their serum concentrations were lower than the corresponding salivary concentrations in some subjects (10% and 25%, respectively), despite the resulting mean fold change being >3. When comparing serum to SM/SLS (Figure 5B), among the metabolites exhibiting a mean fold change higher than 3 or lower than 0.5, Taurine (mean fold change >3) exhibited a reversed concentration ratio in 35% of the subjects. In both comparisons, the concentration ratios of all other metabolites remained consistent (> or <1) for all subjects.

Metabolites with a mean fold change between 0.5 and 3 are listed in Table 1. The closer a mean fold change is to 1, the more likely its concentration ratios will vary around 1. For example, in the comparison of serum vs. SM/SLS, the mean fold change in Creatine was 1.5 (Table 1), but its concentration in serum was higher than in SM/SLS only in 12 out of 20 subjects.

Table 1. Metabolites with a mean fold change between 0.5 and 3.

Metabolite	SERUM vs. PS Mean Fold Change	Metabolite	SERUM vs. SM/SLS Mean Fold Change	Metabolite	SERUM vs. WS Mean Fold Change
Aspartate	0.7	Formate	0.7	Aspartate	0.7
Formate	0.8	Glutamate *	0.9	Choline *	1.3
Taurine	0.9	Creatine *	1.5	Glycerol	1.6
Choline *	1.0	Urea *	2.1	Tyrosine *	1.9
Urea	1.4	Aspartate	2.7	Creatine	2.1
Glycerol	1.7			Lysine [a]	2.5
Tyrosine	1.9			Phenylalanine	2.6
Arginine	2.1				
Lactate	2.6				

* Serum and salivary distributions of the observed concentrations did not significantly differ at $p = 0.05$. [a] Metabolite with a correlation coefficient of 0.52.

With a mean fold change <0.5 threshold, Acetate, Glutamate, and Creatine were significantly more concentrated in PS than in serum, while only Acetate was significantly more concentrated in SM/SLS than in serum (Figure 5A,B).

Finally, the data indicate that the metabolites shared by the two major salivary gland samples were more concentrated in PS than SM/SLS (Figure S1).

2.3.2. Metabolites Common to Serum and WS

Despite the complex origin of the WS, metabolite levels were compared using the same approach as for PS and SM/SLS. Statistical analysis showed that serum and WS concentrations differed significantly except for Choline and Tyrosine.

Based on Pearson analysis, 3-Hydroxybutyrate, Creatinine, Glutamate, and Lysine concentrations correlated with the corresponding serum concentrations with a coefficient >0.5 (Figure 6 and Table 1).

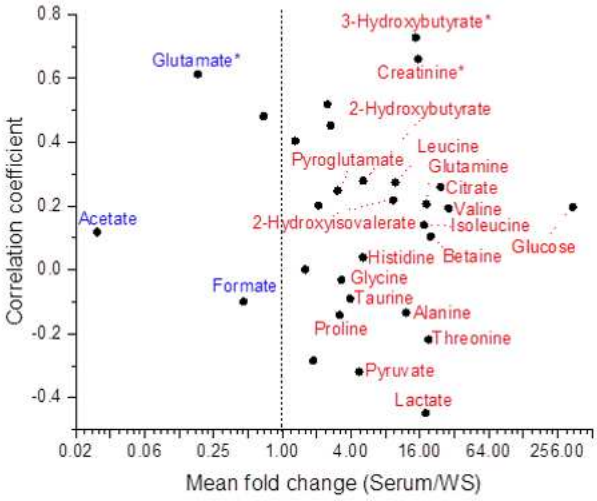


Figure 6. Mean fold changes in the metabolites shared by serum and WS are expressed as a function of their correlation coefficients. The line x = 1 marks the boundary between two graph subsections: on the left, the salivary concentrations are expected to be higher than the serum concentrations; on the right, the serum concentrations are expected to be higher than the salivary concentrations. Metabolites with mean fold changes <0.5 and >3 are labeled. They appear in blue when all their original metabolite concentrations are higher in WS than in serum and red when they are higher in serum than in WS. Urea was omitted as it was not found in several WS samples. The asterisks mark the metabolites with a correlation coefficient >0.5. X axis is in $\log_2$ scale.

Spearman correlation analysis confirmed the strong correlation of 3-Hydroxybutyrate and Creatinine found in Pearson analysis and assigned a moderate correlation ($|r| \approx 0.5$) to Lactate and Lysine.

Most of the mean fold changes (74%) fell between 1 and 20, with a few close to 1 (Figure 6 and Table 1). Among those metabolites with a mean fold change >3 or <0.5 (Figure 6), only Glycine, Proline, Pyroglutamate, Taurine, and Formate showed a few cases (10–30%) of a reversed concentration ratio.

Acetate and Glutamate were present in WS at a higher concentration than in serum for all study subjects. This is consistent with the result obtained when comparing serum and PS and partially in line with the comparison between serum and SM/SLS (Figure 5), where the amount of Glutamate was higher in SM/SLS than in serum in only 13 out of 20 subjects (0.9 mean fold change, Table 1).

3. Discussion

Recent metabolomics studies have been performed to compare the diagnostic performance of serum and saliva or investigate their relationships in various pathologies [15,25–28]. Establishing concentrations and conditions for every analytical feature in healthy and pathological states is crucial for utilizing saliva in laboratory diagnostics. To our knowledge, this is the first metabolomics study to compare serum and salivary metabolite profiles obtained under physiological conditions.

We have already devised a standardized procedure for saliva collection, optimized the preparation of samples for ^{1}H-NMR-based metabolomics, and generated the PS, SM/SLS, and WS metabolite profiles of young and healthy subjects [22,23]. Integrating these data with the corresponding serum metabolite profile allowed for new comparisons and more reliable clinical assessments.

The mean metabolite concentrations obtained from the sera of the cohort of young and healthy subjects of this study were coherent with the data reported in The Human Metabolome Database (normal conditions, both sexes and age >18, https://hmdb.ca, accessed on 30 September 2023) [29], except for Glycerol and Urea. However, our saliva collection protocol requires a fasting period starting from midnight, which likely induces lipolysis. This process could cause an elevation in our serum Glycerol concentrations. As for the discrepancy in Urea content, we ascribe this fact to our experimental data acquisition conditions. The ^{1}H-NMR Urea signal may have been affected by the exchange with water, leading to an underestimation of Urea serum concentration.

Based on the total signal area distribution of the NMR spectra (n = 20), serum samples revealed a more compact distribution when compared with WS and PS samples [23], suggesting a minor individual variability in serum metabolite content compared with the salivary samples.

In agreement with Tzimas and Pappa [30], we verified that human saliva and serum metabolomes are comparable in chemical composition but present significant differences in the concentrations of the common metabolites. In fact, in the pairwise comparisons of serum with PS, SM/SLS, or WS, the concentration distributions significantly differed for most metabolite features, with a few exceptions.

By applying PLS-DA to the datasets, we found that VIP scores for component 1 effectively distinguished serum from saliva samples, while the three saliva types remained grouped, indicating quite comparable metabolomes in the salivary samples. Glucose and Lactate, whose concentrations were markedly higher in serum (Table S1), and Acetate, which is predominantly derived from oral microbiota, are the principal compounds responsible for the observed separation.

The metabolite concentrations measured in serum and saliva samples revealed a low incidence of correlations in agreement with a previous study [26]. In serum vs. PS, 2-Hydroxyisovalerate, 2-Hydroxybutyrate, and 3-Hydroxybutyrate showed the highest correlation coefficients, and their presence in PS appeared to be supported by a favorable concentration gradient from serum to PS (Figure 5A, Table S1). 2-Hydroxyisovalerate is

derived from ketogenesis and branched-chain amino acid metabolism. Mammalian tissues (principally hepatic) that catabolize threonine (a ketogenic and glucogenic amino acid) produce 2-Hydroxybutyrate. 3-Hydroxybutyrate blood levels reflect fatty acid β-oxidation and ketogenic amino acid catabolism. The observed moderate to strong correlations relating to amino acid metabolism may arise from a transfer of 2-Hydroxyisovalerate, 2-Hydroxybutyrate, and 3-Hydroxybutyrate from serum to saliva.

When comparing the serum and SM/SLS concentration datasets, Spearman's correlation analysis assigned a moderately positive correlation ($r \approx 0.5$) to 3-Hydroxybutyrate, in line with the serum vs. PS correlation analysis results. As this metabolite is more concentrated in serum than saliva, the parotid and submandibular/sublingual glands likely use the same molecular mechanism to transfer it from blood to saliva. Based on Pearson and Spearman analyses, 3-Hydroxybutyrate, Glutamate, Creatinine, and Lysine showed a positive correlation when comparing serum vs. WS. As for 3-Hydroxybutyrate, our data agree with the study of Miyazaki et al. [31]. We found a comparable correlation between serum and PS, suggesting that the parotid gland may be an entry point for that metabolite. Its increased concentration in the WS of patients with liver cirrhosis evidences its potential as a biomarker associated with the upregulation of the catabolic pathways of fatty acids/ketogenic amino acids [31]. However, we cannot exclude the contribution of oral microbiota in WS. In line with our findings, Jasim et al. found a correlation between Glutamate concentrations in stimulated WS and blood [32]. However, in that study, the concentration of Glutamate in WS was lower than that in serum, in contrast to our findings. Though we cannot exclude a contribution of oral microbiota metabolism, this apparent discrepancy may be due to the fact that glutamate salivary concentration declines with age [33], which may explain the higher salivary amounts measured in our young participants. The correlation between serum and WS Creatinine concentrations agrees with the literature [34], supporting the use of salivary Creatinine concentration as a non-invasive diagnostic tool for chronic kidney disease [35,36]. The literature does not support the correlation between serum and WS Lysine (essential amino acid) levels.

Overall, for most of the metabolites shared by serum and saliva, we observed a prevalence of serum concentrations over the salivary ones (mean fold change >1, Figures 5 and 6) without any significant correlation. In these instances, it might be inappropriate to attribute the levels of PS and SM/SLS metabolites exclusively to a transport mechanism from serum to saliva since the gland environment may have a role in producing and/or consuming these metabolites.

The highest mean fold change values were obtained with serum vs. SM/SLS, indicating that saliva secreted from those glands is much more diluted than serum; the only exceptions were the Citrate, Creatine, Lactate, and Glucose mean fold changes obtained with serum vs. WS (Figure S2). When comparing the mean fold changes in serum vs. PS and serum vs. WS, the highest mean fold changes derived from the serum vs. WS, except for 2-Hydroxybutyrate, Glycerol, Glycine, Lysine, Phenylalanine, Proline, Pyruvate, and Tyrosine (Figure S2). The mean fold changes in the 12 amino acids in Figure S2 indicate that the saliva secreted by SM/SLS has a lower aminoacidic content than PS and WS, and that PS has a lower Glycine, Lysine, Phenylalanine, Proline, and Tyrosine content than WS. These results suggest a potential use of amino acids for synthesizing secretory proteins in the parotid and submandibular/sublingual glands, whereas the proteolytic activity of the oral microbiota has a major influence on the amino acid content of WS. Nonetheless, it is plausible that differences in saliva flow and/or collection rate between the salivary types may have affected their metabolite content. Therefore, the prevalence of PS metabolite concentrations over SM/SLS (Figure S1B) could be attributed to the lower salivary collection rate measured for PS (0.11 ± 0.08 vs. 0.20 ± 0.09 mL/min). We expect that the longer the saliva spends in the secretory acini of the gland, the more concentrated it may become. We calculated the fractional abundance of all amino acids in serum and saliva samples (Figure S3). Due to the different roles and origins of amino acids in blood and saliva, ratios of the same magnitude were observed only in limited cases (Histidine, Phenylalanine,

and Leucine). Serum is remarkably rich in the gluconeogenic precursors Glutamine and Alanine, while Alanine, Glycine, Glutamine, and Glutamate are the most abundant amino acids in PS and SM/SLS. In WS, instead, the most abundant amino acids are Glycine, Glutamate, Lysine, and Proline. Notably, whilst amino acids undergo a complex homeostatic regulation resulting in stable plasma levels, salivary amino acid content is influenced by the proteolytic activity of oral microflora, which degrades salivary proteins and produces organic acids from amino acid fermentation. From this perspective, the high level of WS Proline can be explained by the degradation of salivary proline-rich proteins.

Notably, the mean concentration of Glucose in PS is significantly higher than in the other salivary samples (Table S1), supporting the idea that parotid acini are the main route of entry for this metabolite [27]. However, the reduced collection rate of PS samples compared with SM/SLS and WS could have facilitated the accumulation of Glucose in PS. The same interpretation applies to PS Lactate, although this metabolite is also a product of the oral microbiota metabolism. We speculate that the Glucose concentration in WS is lower than that in PS due to its utilization as a microflora substrate [22].

However, our saliva-collecting protocol does not entirely exclude a certain degree of WS cross-contamination. Acetate, Glutamate, and Formate showed a mean fold change <1 in all saliva sample types (Figures 5 and 6, Table 1 and Table S1). The abundance of Acetate and Formate in WS is mainly due to the metabolism of oral microflora, and diffusion from WS could explain the presence of these metabolites in PS and SM/SLS. Notably, the mean concentrations of Acetate and Formate in saliva are associated with high standard deviation values (Table S1), reflecting the variability in the composition of saliva microbiota. Glutamate is an important component of saliva because it contributes to taste [33], and it is the most abundant excitatory neurotransmitter in the central nervous system of vertebrates related to pain; its salivary level increases in patients with chronic migraine, validating its use as a clinical biomarker [37].

Finally, our evaluation revealed that some metabolites are unique to each sample type (Figure 3). We hypothesize that they represent the following: (a) in serum, metabolites characterized by a poor permeation of the hemato-salivary barrier; (b) in WS, intermediates of oral microbiota metabolism, shared by a cohort of subjects without any sign or symptom of oral/periodontal disease; and (c) in PS and/or SM/SLS, a group of endogenous metabolites produced by acinar and/or ductal cells of the major salivary glands. Further investigations in a larger population are necessary to confirm these hypotheses.

4. Materials and Methods

According to the ethical principles of the Declaration of Helsinki, written informed consent was obtained from all the study participants.

As described in reference [22], the cohort of participants consisted of twenty healthy volunteers aged between 20 and 25 years (Table S2). Subjects were eligible for saliva collection after completing an oral clinical examination and a sialometry test to exclude dental/periodontal disease or hyposalivation. Health status was checked with a medical history interview. The metabolite profiling of WS, PS, and SM/SLS samples was the topic of a previous publication wherein the study population, the saliva collection procedure, the saliva sample preparation, and the ^{1}H-NMR spectra collection and analysis are described [22]. Briefly, saliva and blood collection took place from 8 to 10 a.m. The participants were instructed to refrain from eating, smoking, and performing intense physical activity for at least 12 h before sampling. They were also asked to refrain from oral hygiene practices for 45 min before saliva collection. Immediately before saliva collection, patients rinsed their mouths with water for 60 s. PS, SM/SLS, and WS were collected separately under unstimulated conditions. To collect PS and SM/SLS, a sterile sponge was positioned over the duct outlets of the glands to absorb the secreted saliva. The sponge was squeezed with a syringe to collect saliva in a vial at regular intervals. WS was collected using the passive drooling method. The serum metabolite profile derived from the subjects and its comparison with saliva profiles from the same subjects is the topic of this manuscript.

4.1. Blood Collection, Serum Sample Preparation, and Processing

After saliva collection and under fasting conditions, 9 mL of cubital venous blood was collected from all participants. Blood samples were kept at 37 °C for 30 min in tubes without anticoagulant and then centrifuged at 4000× *g* for 10 min at 25 °C to separate serum. The procedure resulted in 20 serum samples ranging in size from 2.0 to 3.8 mL. Serum samples were stored at −80 °C until metabolomic analysis.

For NMR sample preparation, sera were thawed at room temperature and, since the presence of plasma proteins may interfere with metabolite quantification via NMR [38], they were protein-depleted via ultrafiltration using centrifugal filters (3000 MWCO, Amicon Ultra-4 Centrifugal filters, Merck Millipore, Darmstadt, Germany) at 4000× *g* for 120 min at 10 °C. Each serum ultrafiltrate was used to prepare a 600 µL sample, including the addition of 10 µL of 1 M phosphate buffer (pH 7.4) and 15 µL of 1% 3-trimethylsilyl propanoic acid (TSP) in 2.5% D_2O (as a quantitative internal standard).

One-dimensional ^{1}H-NMR spectra of serum samples were acquired at 25 °C with a JEOL 600 MHz ECZ600R spectrometer (JEOL Inc., Tokyo, Japan) as described in [39]. The spectra were processed and analyzed with the Chenomx NMR suite 9.0 software (Chenomx Inc., Edmonton, AB, Canada), zero-filling to 256 K points and using a line broadening of 0.5 Hz.

4.2. Metabolomics Data Analysis

Multivariate statistical analysis was conducted on target metabolites using Metaboanalyst 5.0 (https://www.metaboanalyst.ca, accessed on 30 September 2023) [40]. Partial least-squares–discriminant analysis (PLS-DA) was used to compare the original metabolite composition of all sample matrices. The PLS-DA results were visualized as 2D score plots and VIP scores [41]. VIP scores ≥2 were considered relevant for the PLS-DA analysis.

4.3. Statistical Analysis

Data were analyzed using Origin 2019 software. Descriptive statistics for each sample type and variable are presented as mean ± SD or median and interquartile range. The mean fold change was obtained for each metabolite by averaging the ratios calculated by dividing the serum concentration by the PS, SM/SLS, or WS concentration of all study subjects (n = 20).

Differences between concentration distributions of two sample types were evaluated using the Mann–Whitney test, a non-parametric statistical test used to compare two samples or groups.

The Pearson and Spearman correlation tests were performed on the observed concentrations of shared metabolites to explore the relationships between serum and saliva composition. The correlation coefficient (r) ranges from +1 (perfect positive correlation) to –1 (perfect negative correlation). For absolute values of r, the correlation is considered strong if $0.6 < r < 0.8$, moderate if $0.4 < r < 0.6$, and weak if $0.2 < r < 0.4$.

For all analyses, the significance level was set at $p < 0.05$.

Metabolites with concentrations below the quantification limit [23] in ≥20% of participants were excluded from all analyses.

5. Conclusions

Serum and salivary metabolite profiles derived from samples collected concurrently from healthy subjects under physiological conditions were compared to assess the relationships between their compositions. Many researchers have focused exclusively on WS, especially when searching for biomarkers, because it can be collected using non-invasive procedures and is rich in many analytes of interest for diagnosis or screening. Instead, we intended to study the three types of saliva separately to provide a comprehensive analysis of their metabolomes in relation to serum composition under physiological conditions. Our results highlight that none of the saliva types are a mere ultrafiltrate of blood, as saliva composition is influenced by several factors, such as intrinsic glandular metabolism and

the enzymatic activities of the oral microbiota. The proximity of the capillary bed and acinar/ductal structures suggests that metabolites may diffuse or be transported from serum to saliva because of a favorable concentration gradient and/or via specific transport systems. Based on our analyses, however, an intense oral metabolism is likely to alter the concentrations of most excreted metabolites, both at the gland level and in other oral niches, thus preventing very strong correlations between serum and salivary concentrations. In some cases, the salivary metabolite was not detected in the serum, or its concentration exceeded the serum level, indicating intense endogenous production.

We did find some moderate to strong correlations between the serum and saliva concentrations of specific metabolites, supporting the idea that these metabolites may have been transferred from serum to saliva to an extent dependent on their concentrations. Our results align with the literature focused on those metabolites.

In terms of future applications, the metabolomic profiles of serum, WS, PS, and SM/SLS generated using standard operating procedures and the resulting assessments may contribute to a valuable knowledge base for salivary metabolomics aimed at identifying biological markers of oral and systemic health disorders [7,30].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms242316603/s1.

Author Contributions: Conceptualization, E.F. and T.A.P.; methodology, M.G.; software, M.G. and T.A.P.; formal analysis, E.F.; investigation, R.A., E.F. and M.G., resources, M.M. and T.A.P.; data curation, T.A.P.; writing—original draft preparation, E.F.; writing—review and editing, E.F., T.A.P. and A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This cross-sectional study was approved by the Ethical Committee of the "Area Vasta Emilia Nord" (AVEN) (protocol number: 808/2018/SPER/UNIPR METASAL3) and conducted in 2019 (March–May).

Informed Consent Statement: According to the ethical principles of the Declaration of Helsinki, written informed consent was obtained from all the study participants.

Data Availability Statement: Data is contained within the article or supplementary material.

Acknowledgments: Centro Interdipartimentale Misure "Giuseppe Casnati" of the University of Parma is thanked for permitting use of its facilities.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 5

The Association between Salivary Metabolites and Gingival Bleeding Score in Healthy Subjects: A Pilot Study

Antonelli R, *Ferrari E, Gallo M, Ciociola T, Calciolari E, Spisni A, Meleti M, Pertinhez TA.*
Int J Mol Sci. 2024 May 17;25(10):5448. doi: 10.3390/ijms25105448. PMID: 38791486; PMCID: PMC11122368.

Published on International Journal of Molecular Sciences
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The Association between Salivary Metabolites and Gingival Bleeding Score in Healthy Subjects: A Pilot Study

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Abstract: Periodontal diseases, including gingivitis and periodontitis, are among the most prevalent diseases in humans. Gingivitis is the mildest form of periodontal disease, characterized by inflammation of the gingiva caused by the accumulation of dental plaque. Salivary diagnostics are becoming increasingly popular due to the variation in saliva composition in response to pathological processes. We used a metabolomics approach to investigate whether a specific saliva metabolic composition could indicate preclinical stage of gingivitis. ^{1}H-NMR spectroscopy was used to obtain the salivary metabolite profiles of 20 healthy subjects. Univariate/multivariate statistical analysis evaluated the whole saliva metabolite composition, and the Full-Mouth Bleeding Score (FMBS) was employed as a classification parameter. Identifying a signature of specific salivary metabolites could distinguish the subjects with high FMBS scores but still within the normal range. This set of metabolites may be due to the enzymatic activities of oral bacteria and be associated with the early stages of gingival inflammation. Although this analysis is to be considered exploratory, it seems feasible to establish an FMBS threshold that distinguishes between the absence and presence of early inflammatory alterations at the salivary level.

Keywords: saliva; Full-Mouth Bleeding Score (FMBS); gingivitis; salivary metabolomics; salivary diagnostics

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Citation: Antonelli, R.; Ferrari, E.; Gallo, M.; Ciociola, T.; Calciolari, E.; Spisni, A.; Meleti, M.; Pertinhez, T.A. The Association between Salivary Metabolites and Gingival Bleeding Score in Healthy Subjects: A Pilot Study. *Int. J. Mol. Sci.* **2024**, *25*, 5448. https://doi.org/10.3390/ijms25105448

Academic Editor: Maria Gazouli

Received: 13 April 2024
Revised: 6 May 2024
Accepted: 15 May 2024
Published: 17 May 2024

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1. Introduction

Periodontal diseases affect most of the adult population [1]. Particularly, severe periodontitis, a major irreversible cause of tooth loss, has a prevalence of 11% and is the sixth most prevalent disease worldwide [2–4]. Remarkably, periodontitis has been correlated with several inflammatory-based systemic diseases, such as diabetes, cardiovascular diseases, rheumatoid arthritis, Alzheimer's, and pulmonary infections, as well as adverse pregnancy outcomes [4–9]. Moreover, it affects vulnerable segments of the population, negatively impacts quality of life, and is a consequence of social inequality [10].

Plaque-induced gingivitis is a site-specific inflammatory condition caused by microbial plaque accumulation in the gingival sulcus [11–13]. Such inflammation remains confined to the gingiva and is reversible by reducing plaque with professional dental cleaning and practicing good oral hygiene (brushing and flossing) [14]. In addition to pathogenetic microorganisms and the host immune response, genetic and environmental factors (e.g.,

tobacco use and plaque retentive factors) contribute to its development [15]. Clinically, gingivitis affects the marginal gingiva, causing erythema, edema, bleeding on probing, and sometimes increasing gingival volume [11]. If not treated, gingivitis can eventually lead to periodontitis in susceptible subjects [16]. Yet, early diagnosis of gingivitis and periodontitis can be challenging due to their slow progression.

Overall, the presence, extent, or severity of bleeding is the most widely accepted indicator of the prevalence of gingivitis, and according to the current classification of periodontal and peri-implant diseases, bleeding on probing is the primary sign for the diagnosis of gingivitis [12]. The Full-Mouth Bleeding Score (FMBS), which is the percentage of sites in the entire dentition that bleed on probing, can provide an overall assessment of gingival inflammation; a patient with an intact periodontium is diagnosed as a case of gingivitis according to an FMBS $\geq$10%. In turn, an FMBS < 10% excludes gingivitis and is generally consistent with the patient's perception of healthy gums [13].

The diagnostic approach to gingivitis and periodontal disease could be revolutionized by detecting salivary components associated with the early development of gingival changes, even at a subclinical level, before bleeding on probing manifests.

Whole saliva, a mixture of fluids produced by the major and minor salivary glands and crevicular fluid, includes endogenous molecules, components derived from the oral microflora, and exogenous substances [17]. Its composition, the result of a dynamic exchange between microflora and mucosa cells, as well as components derived from capillary blood, holds significant clinical relevance. For this reason, saliva is an excellent tool for the identification of clinically relevant biomarkers [18–21]. Reliable biomarkers specific to a disease can provide useful information regarding the type, molecular etiology, and stage of the disease, driving the development of personalized therapeutic interventions [22,23]. The advantages of using saliva for disease diagnosis include ease of access, non-invasive sample collection, increased patient acceptance, and reduced risk of infectious disease transmission.

Biofluid-based metabolomics has generated vast knowledge over the past decades. In the case of saliva, research studies have focused on the characterization of the salivary metabolome in relation to oral function, oral microbiome, and the identification of disease biomarkers [19,24,25]. Recent literature reviews have reported using salivary metabolomics to diagnose systemic diseases, systemic cancers, and mental illnesses [25,26]. Regarding the oral cavity, many publications on salivary metabolomics based on whole saliva have focused on the discovery of diagnostic biomarkers for oral cancer [27,28] and periodontitis [29,30]. NMR-based metabolomics provides detailed, qualitative, and quantitative information that is valuable for discovering specific biomarkers. Its routine use for screening or diagnostic purposes is limited due to the hardware and maintenance costs. Nevertheless, once a biomarker (or a pattern of biomarkers) has been identified, it can be employed to develop novel, reliable, and non-invasive devices for early diagnosis.

Interestingly, in an experimental gingivitis model, asymptomatic and suboptimal gum health was associated with a shift in plaque microbiome structure, plaque metabolome, and host immune response during gingivitis onset and progression [30].

This metabolomics study was conducted on whole saliva from a healthy young population. Using the FMBS as a classification parameter, we investigated whether a particular saliva composition could reflect the early stages of gingival inflammation (preclinical stage of gingivitis). We speculated that subjects with high FMBS scores but still within the normal range might have a different composition of salivary metabolites than subjects with low FMBS scores. According to our metabolomics analysis, detecting a signature of specific salivary metabolites could help identify individuals who may be more susceptible to gingival inflammation based on their FMBS scores.

2. Results

2.1. Clinical Data of the Study Participants

The enrolled subjects' demographic data, dental/periodontal indexes, and whole saliva flow are shown in Tables S1 and S2. All the participants had a normal salivary

function, with salivary flow ranging from 0.9 to 5 mL/5 min. Their FMBS scores ranged from 0 to 11.4%, and their Full-Mouth Plaque Score (FMPS) ranged from 2.8 to 24.8% (Table S2). Only one of the twenty participants had an FMBS score higher than 10% (11.4%), which is the highest value compatible with a gingivitis-free state [13]; this finding resulted in the exclusion of this subject from subsequent evaluations. The observed FMPS scores indicated adequate plaque control, and Periodontal Screening and Recording (PSR) indices excluded the presence of active periodontal disease. Aggregate data of the selected subjects (n = 19) are presented in Table 1.

Table 1. Demographics, oral health status, and social habits of the selected subjects.

	MALE (n = 10)	**FEMALE (n = 9)**
	Mean ± SD	Mean ± SD
Age (years)	23.7 ± 1.3	23.6 ± 2.1
BMI (kg/m^2)	23.2 ± 1.4	20.9 ± 1.3
Salivary flow [a] (mL/5 min)	2.3 ± 1.2	2.2 ± 1.5
% FMPS	12.8 ± 7.1	14.1 ± 7.5
% FMBS	2.6 ± 1.9	4.6 ± 3.4
DMFT	1.7 ± 1.3	1.0 ± 1.3
PSR	0.9 ± 0.3	1 ± 0.0
	No. of subjects	No. of subjects
Drugs:		
- In the last 12 h	-	3 [b]
- No drugs	10	6
Smoke:		
- Cigarette smokers [c]	2	3
- Non-smokers	8	6
Alcohol:		
- Moderate drinkers [d]	4	4
- Non-drinkers	6	5

[a] Determined by modified Saxon test; [b] contraceptive (2 subjects) or antihistamine therapy (1 subject); [c] up to 5 cigarettes/day; [d] less than 7 alcohol units/week.

2.2. Eukaryotic and Prokaryotic Cell Counts in Saliva

Procaryotic and eukaryotic cell counts performed in whole saliva samples showed a relevant variability compared to their mean values (Table S3). No significant correlations were found between the FMBS scores and the number of prokaryotic or eukaryotic cells suspended in whole saliva (R = 0.43 with *p*-value = 0.07 and R = 0.40 with *p*-value = 0.09, respectively) and between the FMPS scores and the number of prokaryotic or eukaryotic cells (R = 0.19 with *p*-value = 0.43 and R = 0.003 with *p*-value = 0.99, respectively).

2.3. Bleeding Score and Salivary Metabolomes Analysis

We selected an FMBS of 3.75%, the midpoint of the observed FMBS range (0–7.5%), as a suitable threshold for investigating the metabolite composition of saliva samples to search for metabolite alterations associated with the preclinical stage of gingivitis. According to our classification based on FMBS score, 12 subjects were allocated to the group with FMBS < 3.75% (DOWN) and 7 to the group with FMBS > 3.75% (UP) (Figure 1).

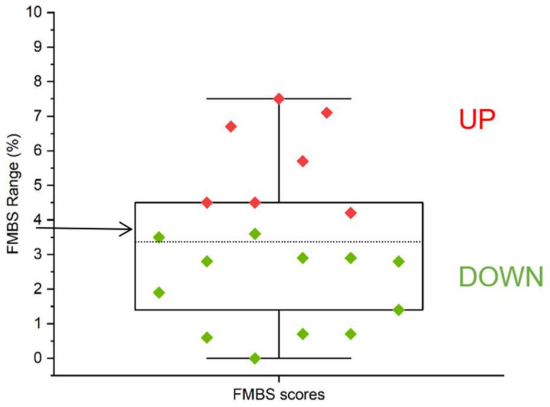

Figure 1. FMBS score distribution. The arrow at y = 3.75% corresponds to the FMBS threshold selected for the enrolled subjects' arbitrary UP and DOWN categorization (see Section 4). Green diamonds have FMBS values < 3.75% (DOWN group), and red diamonds have FMBS values > 3.75% (UP group). The boxes are determined by the 25th and 75th percentiles; the dotted line corresponds to the mean (3.4%).

We applied Partial Least Squares–Discriminant Analysis (PLS-DA) to the concentrations of the sixty metabolites identified in the whole saliva samples of the two groups of subjects. The obtained PLS-DA model was effective in separating the metabolomes of the UP and DOWN groups (Figure 2A), and the Variable's Importance in Projection (VIP) score plot suggested the metabolites with the highest contribution to the separation by component 1 (Figure 2B).

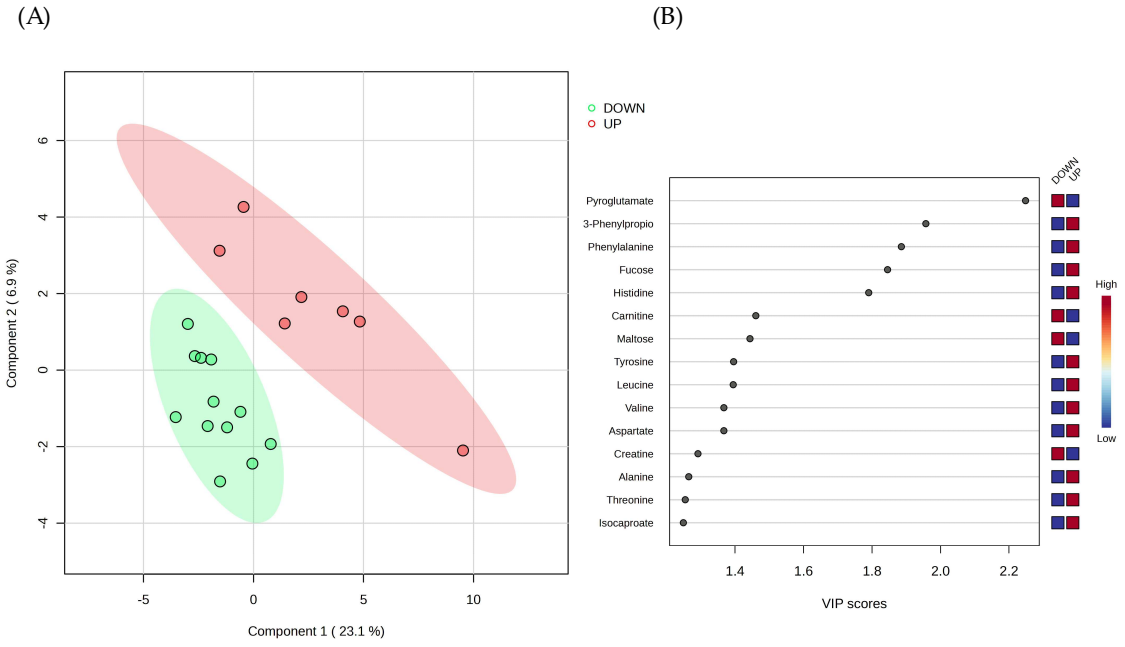

Figure 2. Supervised multivariate analysis of the salivary metabolite datasets. (**A**) Partial Least Squares–Discriminant Analysis (PLS-DA) scores plot of whole saliva metabolomes. The classification model separates the DOWN (FMBS < 3.75%) from the UP (FMBS > 3.75%) salivary metabolomes. Components 1 and 2 account for 23.1% and 6.9% of the variance, respectively, and colored ellipses represent each cluster's 95% confidence region. (**B**) Metabolite ranking (top 15 metabolites) according to the Variable's Importance in Projection (VIP) scores, resulting from PLS-DA component 1. The higher the VIP score of a variable, the better its ability to discriminate between groups. Variables with a VIP score close to or greater than 1 are considered relevant [31]. The blue and red boxes on the right denote the relative metabolite abundance in the two clusters.

Among the top 15 metabolite features identified by the VIP score plot, we arbitrarily selected a VIP score $\geq$ 1.8 to extract the variables that contribute most to class discrimination in the PLS-DA model. These variables are Pyroglutamate, 3-Phenylpropionate, Phenylalanine, Fucose, and Histidine (Figure 2B).

The volcano plot in Figure 3 highlights the significantly different metabolite concentrations observed in the UP and DOWN groups.

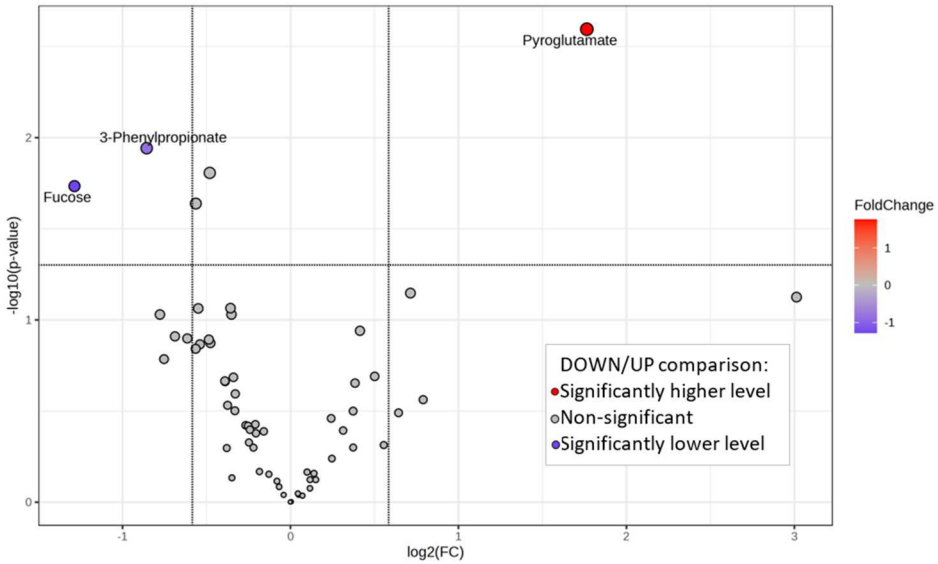

Figure 3. Volcano plot of whole saliva metabolomes shows the statistically significant metabolites. Fold change (FC) threshold of 1.5 and a p-value < 0.05 were considered for significance. Significantly different metabolite concentrations are highlighted.

When comparing the DOWN vs. UP group, Pyroglutamate shows a higher concentration, while 3-Phenylpropionate and Fucose show a lower concentration. A graphical summary of their concentration distributions is displayed in Figure 4.

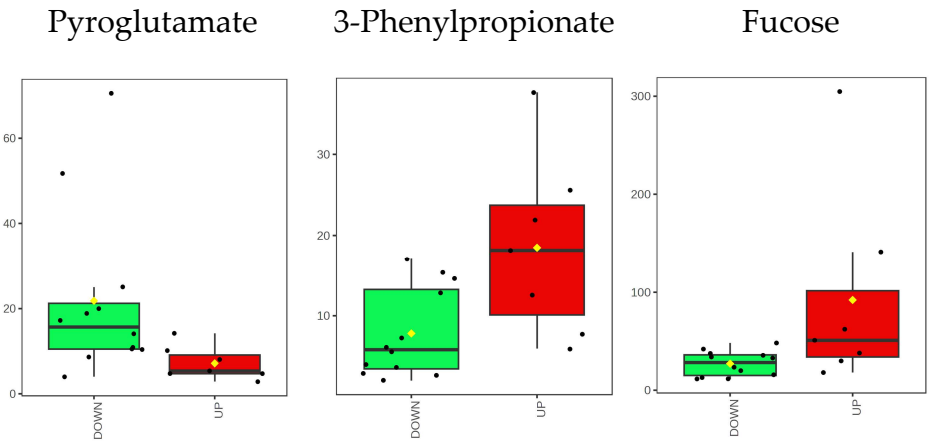

Figure 4. Box plots of the distributions of the concentration (μM) of the discriminant metabolites. Black dots represent the metabolite concentrations. The black line inside each boxplot is the median, and the yellow diamond is the mean.

Figure S1 provides the DOWN vs. UP comparison of all metabolite concentrations to complement the statistical analysis above.

2.4. Biomarker Evaluation by Receiver Operating Characteristic (ROC) Curve Analysis

Classical univariate ROC curve analysis was conducted using all variables as potential classification elements to identify the metabolites that perform better in predicting UP or DOWN group membership. The analysis identified six metabolites with area under the ROC curve (AUC) scores greater than 0.82 and statistical significance for the *t*-test with a *p*-value < 0.05 (Table 2, Figure S2). This response indicates that the single concentrations of Pyroglutamate, Maltose, Histidine, Fucose, Phenylalanine, and 3-Phenylpropionate have reasonable accuracy in discriminating between the UP and DOWN groups. However, their AUC values primarily indicate the biomarker potential of the single variables rather than their predictive performance.

Table 2. Significant metabolites in univariate ROC curve analysis.

Metabolite	AUC §	Optimal Cutoff *	*p*-Value
Pyroglutamate	0.99	−0.66	0.0025
Maltose	0.90	−0.45	0.0749
Histidine	0.88	−0.24	0.0229
Fucose	0.88	−0.14	0.0184
Phenylalanine	0.83	−0.23	0.0156
3-Phenylpropionate	0.82	0.30	0.0114

§ AUC, area under the Receiver Operating Characteristic (ROC) curve; * optimal cutoff is the metabolite normalized concentration with the best performance in discriminating the DOWN and UP groups.

In multivariate analysis, the group-discriminating ability of selected salivary metabolites was used to develop a predictive model. We chose the combination of the three metabolites Pyroglutamate, Fucose, and 3-Phenylpropionate differentially expressed in the DOWN and UP groups (Figure 3) to generate an ROC curve-based model (Figure 5). With an AUC of 0.93, the analysis attributed a good discriminatory power to the metabolite ensemble. The average accuracy based on cross-validations was 0.74. However, the predicted group probability of each sample, resulting from 100 cross-validations, classified three of the DOWN samples in the wrong group, indicating that they might be outliers.

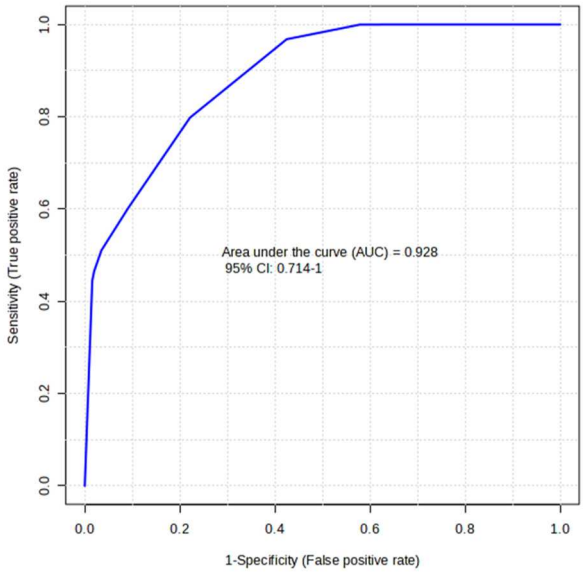

Figure 5. Multivariate ROC curve as a predictive model based on selected salivary metabolites. The ROC curve was constructed using Pyroglutamate, Fucose, and 3-Phenylpropionate normalized concentrations.

In a subsequent analysis aimed at predicting the category (DOWN vs. UP) of new samples, the obtained model was tested on the whole saliva metabolome of the subject excluded from our study because his FMBS was higher than 10%. The metabolite profile of this subject fitted into the DOWN group, albeit with an FMBS of 11.4%.

3. Discussion

The current gold standard for diagnosing periodontal diseases is based on clinical examination with a periodontal probe, which may be combined with a radiographic examination. However, clinical and radiographic assessments provide a picture of what has happened to the patient in the past. Still, they cannot predict how the disease will progress. Instead, the identification of biomarkers has the potential to detect the disease at an early stage, before clinical signs appear, and forecast disease progression or response to treatment.

As end-products of many physiological and pathological processes, metabolites are markers of specific pathways that also result from host-microbial interactions, such as gingival inflammation [32]. Metabolites originating from gingival tissues and plaque bacteria are likely released in the crevicular fluid and eventually in saliva [33]. The ability to detect specific salivary signatures of early gingival inflammation, even at a preclinical level, may be paramount to identifying individuals liable to developing periodontal disease not intercepted by current clinical parameters.

We previously detailed the metabolic composition of whole, parotid, and submandibular/sublingual saliva of the healthy subjects involved in this study, finding a certain degree of individual variability in the metabolite composition of salivary samples [19,24]. In the present study, we arbitrarily selected the FMBS score of 3.75% as a threshold to classify the study participants based on their tendency to gingival bleeding. This threshold has enabled the selection of subjects with FMBS scores near or above the 75th percentile of the distribution (UP group, Figure 1) to compare their salivary metabolomes with those with a minor tendency for gingival bleeding (DOWN group). We expect the UP group to be more prone to gingival inflammation. PLS-DA analysis separated the saliva samples into two clusters according to the bleeding score threshold (Figure 2A). Further statistical analysis revealed that Pyroglutamate, 3-Phenylpropionate, and Fucose concentrations differ significantly between participants with FMBS> and <3.75% (Figure 3). As expected, they are on the list of metabolites with the highest contribution to cluster separation in the PLS-DA model (Figure 2B).

Pyroglutamate is found in whole saliva due to the cyclization reaction of Glutamine or Glutamic acid. This reaction occurs when these residues are at the N-terminus of several human salivary proteins, such as in salivary α-amylase [34]. According to our analysis, high levels of pyroglutamate characterize the salivary composition of subjects with low FMBS scores in the DOWN group (Figures 2B, 3 and 4).

3-Phenylpropionate is a metabolic product of aromatic amino acid fermentation by anaerobic bacteria of the subgingival plaque [35]. We detected 3-Phenylpropionate only in whole saliva, reflecting its microbial metabolic origin [19]. Subjects with FMBS scores > 3.75% exhibited higher levels of 3-Phenylpropionate (Figures 2B, 3 and 4), suggesting the contribution of oral microflora to this alteration. They also had higher levels of Phenylalanine, Histidine, and other amino acids (UP group in Figure 2B). Salivary Phenylalanine concentration has been reported to correlate with the proteolytic bacterial load, indicating that oral bacteria can generate that metabolite [36]. Interestingly, bacterial or endogenous proteases' degradation of salivary proteins is considered the primary source of salivary free amino acids also in plaque-induced gingivitis [37].

Fucose has a higher salivary concentration among subjects of the UP group, Figures 2B, 3 and 4. Such a finding is supported by the study of Shetty and Pattabiraman [38], in which they demonstrated that Fucose, determined as a protein-bound fraction, is higher in patients with gingivitis and periodontitis than in healthy subjects. Wsoo and Ahmed showed that salivary total Fucose and fucose-related parameters were significantly increased in patients

with advanced and moderate periodontitis compared to healthy subjects [39]. Roopa et al. observed an increase in salivary Fucose levels in periodontitis, probably caused by a rise in fucosidase activity associated with the breakdown of plasma and tissue glycoproteins caused by inflammation [40]. We hypothesize that the higher concentration of salivary Fucose in subjects with FMBS > 3.75% might reflect an increase in oral bacteria fucosidase activity, functional to the production of a free form of the carbohydrate [24].

Overall, our analysis has demonstrated that (a) the UP group (FMBS score > 3.75) is associated with higher salivary levels of specific amino acids (Phenylalanine, Histidine, Tyrosine, Leucine, Valine, Aspartate, Alanine, and Threonine, Figure 2B) and Fucose and lower levels of Pyroglutamate, and (b) the alterations in salivary metabolites that can distinguish between the groups with FMBS> or <3.75% may be attributed to oral bacteria metabolism. Remarkably, oral microflora perturbances have been recognized as involved in major clinical conditions, such as gingivitis and periodontitis [41].

It is worth noting that neither the FMBS nor FMPS scores of the study participants were correlated with eukaryotic or prokaryotic cell counts obtained from whole saliva samples. This could be because we only counted planktonic cells. It is important to acknowledge that oral microorganisms are organized with specificity in different oral niches, such as saliva, tooth, and soft tissue surfaces, and those that contribute most to gingival inflammation and bleeding are expected to adhere to teeth and gingiva as plaque biofilm [42].

Univariate ROC analysis was used to explain the performance of individual metabolites in predicting UP or DOWN group membership. Pyroglutamate, Maltose, Histidine, Fucose, Phenylalanine, and 3-Phenylpropionate were found to produce ROC curves with an AUC ranging from 0.99 to 0.82, thereby demonstrating a reasonable ability to discriminate between the two groups (Table 2, Figure S2). This suggests that the salivary levels of these metabolites could potentially serve as indicators of group membership.

By selecting the combination of Pyroglutamate, 3-Phenylpropionate, and Fucose variables, of which the concentrations were significantly different between the subjects with FMBS> or <3.75%, multivariate ROC analysis generated a model with an AUC of 0.93, indicating an acceptable predictive accuracy. When this ROC-based model was tested on the metabolite dataset of a subject with an FMBS score of 11.4%, the sample was assigned to the DOWN group despite the FMBS score. A possible explanation for this result is that the sample was obtained from a subject with localized gingivitis. This condition may have caused metabolite alterations that differ from those of healthy subjects with an FMBS score > 3.75% but still within the normal range.

We acknowledge that an FMBS within the range of 3.75–10% does not indicate the presence of gingivitis or any periodontal alteration and that our results are conditioned by the original study design and sample size, which were not specifically intended for the current purpose. As the study has a cross-sectional design, it did not provide information on any developments over time in subjects with higher FMBS scores. A longitudinal approach is needed to gain information on the possible development of gingivitis in at-risk subjects.

To confirm the validity of our biomarker model, a more comprehensive study with a larger cohort of subjects is required. The inclusion of two additional groups of patients with localized and generalized gingivitis (FMBS 10–30% and FMBS > 30%, respectively) will contribute to the validation of our metabolomics approach.

The complex pathogenesis of periodontal disease suggests that the golden key to diagnosing the onset of gingivitis should be based on a combination of factors rather than metabolites alone. Our biomarker model should be integrated with the analysis of molecules involved in innate and adaptive oral immunity, such as cytokines. These could not be targeted in the current analysis because the NMR samples, according to our sample preparation procedure [43], have been protein-depleted.

In clinical practice, the diagnosis of periodontal diseases can only assess the advanced stage of the disease, not its onset or evolution. These pathologies are not linear and are characterized by periods of progression and remission [44,45]. A salivary metabolite signature could allow early-stage periodontal diagnosis, offering an easy, safe, and non-

invasive approach to planning appropriate treatments. Transferring scientific findings to clinical practice is also relevant for developing rapid, low-cost, and accurate point-of-care technologies, thus improving the personalized approach to precision medicine [46].

4. Materials and Methods

4.1. Ethics Statement

The protocol of this pilot study was approved by the Ethics Committee of "Area Vasta Emilia Nord" (AVEN) (protocol number: 808/2018/SPER/UNIPR METASAL3). The study was conducted according to the criteria set by the Declaration of Helsinki. Written informed consent was obtained from all the eligible subjects before enrolment in the study.

4.2. Clinical Assessment of the Study Participants

The cohort of volunteers enrolled in this study was previously included in the project aimed at metabolite profiling of different saliva subtypes (whole, parotid, and submandibular/sublingual saliva) and serum [19,24].

Participant selection and enrolment were conducted at the Centro Universitario di Odontoiatria of the University of Parma, Italy, and lasted three months (March–May 2019). Inclusion and exclusion criteria for participant selection have been previously reported [24]. Twenty healthy subjects (10 males and 10 females) aged between 20 and 25 years were consecutively recruited. During selection, eligible subjects underwent a comprehensive oral exam and whole sialometry (by modified Saxon Test) [47]. Subjects with hyposalivation (whole saliva flow < 1 mL/5 min), systemic or oral diseases affecting dental or periodontal tissues, medication-induced salivary dysfunction, and pregnant or lactating women were excluded from the study. Any history of successfully treated periodontitis was an exclusion criterion.

A single trained dental specialist performed a thorough oral examination with the help of a mirror and periodontal probe (UNC15, University of North Carolina), including teeth, periodontal tissues, and oral mucosa (alveolar, labial, buccal mucosa, and mucosa covering tongue, palate, and attached gingiva). In doubtful cases, intraoral radiography and/or orthopantomography were performed to detect dental lesions that visual examination could not identify.

Dental status was assessed using the Decayed, Missing and Filled Teeth (DFMT) index. Periodontal health was assessed using the Periodontal Screening and Recording (PSR) index, Full-Mouth Plaque Score (FMPS), and Full-Mouth Bleeding Score (FMBS) [48]. FMPS and FMBS scores were assessed at six sites per tooth.

We used restrictive/precautionary measures for smoke, drugs, and alcohol confounders. For the subjects identified as 'light smokers' in Table S1, we required at least a 12 h smoking restriction before collecting saliva. We also verified that salivation was unaffected in the three drug-using participants listed in Table S1. Eight subjects were identified as "moderate drinkers", consuming less than 7 alcohol units per week (Table S1). Selected subjects were asked to refrain from eating and strenuous exercise for at least 12 h before the saliva sample was taken and to drink only water. They were also asked to avoid oral hygiene (brushing and flossing) for 45 min before saliva collection. Immediately before saliva collection, patients rinsed their mouths with water for 1 min. Unstimulated whole saliva collection was performed using the passive drooling method between 8:00 am and 10:00 am to minimize the influence of the circadian rhythm on salivary composition [24].

4.3. Cell Counting in Whole Saliva

Eukaryotic and prokaryotic cells were counted in whole saliva samples, according to Gardner et al. [49]. Eukaryotic cells (mainly oral epithelial cells and leucocytes) were counted on the same day of saliva collection. Briefly, 20 µL of each untreated salivary sample were mixed with 20 µL of 0.4% Trypan blue (Sigma-Aldrich, Poole, Dorset, UK), placed in a hemocytometer counting chamber, and counted with a light microscope Nikon eclipse TS100 (Nikon, Tokio, Japan) (100× magnification).

For prokaryotic cells, 2 µL of thawed saliva samples were heat-fixed to a glass slide and Gram-stained [50]. Cells were counted with a Nikon Eclipse 80i microscope using at least four random fields at 100× magnification. Each count was performed by two independent observers using at least four random fields. In all cases, variability was less than 10%.

4.4. NMR Metabolomics

NMR sample preparation was performed according to an optimized protocol for NMR-based metabolomics [43]. Briefly, saliva samples were assembled as follows: 10 µL of 1 M potassium phosphate buffer (pH 7.4) and 15 µL of 1% 3-trimethylsilylpropionic acid (TSP) in D_2O were added to 575 µL of the filtered saliva (achieving final concentrations of 1.45 mM TSP and 2.5% D_2O in 16 mM phosphate buffer). TSP was used as a reference for chemical shift (0.00 ppm) and quantitative internal standard.

One-dimensional ^{1}H-NMR spectra were acquired at 25 °C with a JEOL 600 MHz ECZ600R spectrometer using the first increment of the 1DNOESY pulse sequence, 128 scans, a sweep window of 20 ppm, 128 k points, and a relaxation delay of 5 s. The spectra were processed by zero-filling to 256 k points and line broadening at 0.5 Hz and analyzed. Metabolite identification and quantification were performed using Chenomx NMR suite 8.3 software (Chenomx Inc., Edmonton, AB, Canada) [51]. The chemometric analysis allowed the identification and quantification of sixty metabolites in all saliva samples, resulting in a whole saliva metabolic profile described in detail elsewhere [19].

Statistical analysis: Descriptive statistics summarized the subjects' data using mean ± SD (Origin 2019 software). Spearman correlation coefficient was computed to evaluate the relationship between FMBS or FMPS and the number of cells (procaryotic or eucaryotic) suspended in whole saliva samples; the significance level was set at $p < 0.05$.

Metabolomics data analysis: We preliminary classified the population into two groups based on the observed FMBS scores. The midpoint of the observed FMBS range (3.75%, [0–7.5%]), which approximately marks the boundary between the scores above and below the 75th percentile, was chosen as the threshold for assigning subjects to the UP (>3.75%) or DOWN (<3.75%) group (Figure 1).

Statistical analysis on metabolite datasets was carried out using the MetaboAnalyst 6.0 platform (www.metaboanalyst.ca, accessed on 1 March 2024). Metabolite concentration data were uploaded according to the group membership (UP or DOWN), normalized by the median value to adjust for systematic differences between samples, and auto-scaled (mean-centered and divided by the standard deviation of each metabolite concentration) to adjust for fold differences between variables. We applied supervised Partial Least Squares Discriminant Analysis (PLS-DA) and obtained the Variable's Importance in Projection (VIP) score for the variables that contributed most to group separation. We generated a volcano plot by combining the results from FC Analysis and t-test (FC threshold of 1.5 and a p-value < 0.05 for significance).

Potential biomarkers for classification in the UP or DOWN group were predicted by classical univariate Receiver Operating Characteristic (ROC) curve analysis. The area under the curve (AUC) was used to compare the performance of different variables. Multivariate ROC curve analysis was applied based on selected variables to generate a predictive model for group classification. The multivariate ROC curves were based on the cross-validation performance of the Support Vector Machine (SVM) classification method. To produce a smooth ROC curve, 100 cross-validations were performed, and the results were averaged to generate the final curve plot.

5. Conclusions

To the best of our knowledge, our pilot study is the first to assess the metabolite composition of whole saliva from healthy subjects according to their FMBS scores. We identified a panel of metabolites differentially expressed in healthy subjects with high but physiological FMBS scores compared to those with lower scores. Due to the study's

limited sample size, our results must be considered exploratory. However, as this set of metabolites may be associated with enzymatic activities of oral bacteria, it may help to identify individuals more susceptible to gingival inflammation. By expanding the study population to include patients with different degrees of gingival bleeding, we are confident that salivary metabolomics will help identify suboptimal conditions of gingival health and contribute to future point-of-care diagnostics.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijms25105448/s1.

Author Contributions: Conceptualization, T.A.P. and E.F.; validation, R.A., M.G. and T.C.; formal analysis, E.F.; investigation, R.A. and T.A.P.; data curation, E.F.; writing—original draft preparation, E.F.; writing—review and editing, A.S., E.C. and M.M.; supervision, T.A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The protocol of this pilot study was approved by the Ethics Committee of "Area Vasta Emilia Nord" (AVEN) (protocol number: 808/2018/SPER/UNIPR METASAL3). The study was conducted according to the criteria set by the Declaration of Helsinki. Written informed consent was obtained from all the eligible subjects before enrolment in the study.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 6

Salivary metabolomics in oral cancer: A systematic review

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Oral Oncology Reports,Volume 11, 2024,ISSN 2772-9060, https://doi.org/10.1016/j.oor.2024.100657

Published on Oral Oncology Reports
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Oral Oncology Reports 11 (2024) 100657

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Review Article

Salivary metabolomics in oral cancer: A systematic review

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ARTICLE INFO

Keywords:
Salivary metabolomics
Salivary biomarkers
Oral squamous cell carcinoma
Diagnosis

ABSTRACT

Diagnosis of oral squamous cell carcinoma (OSCC) is frequently delayed, with most tumors being detected in advanced stages.

Saliva may represent the ideal fluid for early detection of dysplastic and malignant oral lesions. Among available salivary analytical approaches (e.g., genomic, transcriptomic, proteomic), metabolomics appears promising in identifying the presence of small, specific molecules directly released from malignant cells that could potentially serve as diagnostic biomarkers.

This systematic review evaluates the scientific evidence that supports the role of salivary metabolites in the early diagnosis of OSCC.

Medline, Scopus and Web of Science databases were searched until February 2024. Two independent reviewers assessed the quality of the studies using a checklist proposed by the National Institutes of Health. They also assessed the level of evidence using the Oxford Center for Evidence-Based Medicine classification.

Twenty-eight out of 9623 papers fulfilled the inclusion and exclusion criteria. The quality of the included papers ranged from "good" (n = 2), "intermediate" (n = 22), and "poor" (n = 4).

Ten out of 25 studies evaluated one specific metabolite (sialic acid, cortisol, pyruvic acid, neopterine, 8-hydroxy-2-deoxyguanosine and malondialdehyde, endogenous porphyrin, among others), with the remainder analyzing a combination of 2 or more molecules. Overall, the presence and/or concentration of more than 100 salivary metabolites was investigated in association with histologically confirmed diagnosis of OSCC.

All studies showed a statistically significant correlation between one or more metabolites and OSCC.

These results suggest that metabolic analysis of saliva is a promising field for early diagnosis of OSCC, even though more well-conducted studies are needed.

1. Introduction

Oral Squamous Cell Carcinoma (OSCC) ranks among the most common cancers globally, with an estimated 354,864 new cases (2 % of all sites) and 177,384 related deaths (1.9 % of all sites) in 2018 [1]. Despite advancements in treatment, the 5-year survival rate remains below 50 %. The stage at diagnosis is crucial for predicting patient survival, with early-stage disease having survival rates around 84 %, which drop to approximately 40 % for advanced-stage disease [2,3].

Major risk factors for OSCC include tobacco and alcohol, responsible for about 75 % of cases [4]. Cigarette smoke contains over 60 carcinogenic elements, such as nitrosamines, benzopyrenes, and aromatic amines [5]. In the Indian subcontinent, Southeast Asia, and Taiwan, the use of betel quid containing areca nut and lime significantly increases oral cancer risk, often leading to oral submucosal fibrosis [6]. Chewing betel quid releases reactive oxygen species (ROS) that induce genotoxicity and genetic mutations or attack salivary proteins, causing structural mutations in the oral mucosa that facilitate the penetration of other environmental toxins [7].

Alcohol consumption is also highly correlated with oral cavity

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https://doi.org/10.1016/j.oor.2024.100657
Received 30 June 2024; Accepted 15 September 2024
Available online 16 September 2024
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tumors due to carcinogenic impurities like nitrosamine and mycotoxin. Alcohol abuse enhances the effects of other carcinogens like tobacco, exponentially increasing the risk of upper aero-digestive tract cancer in individuals who both smoke and drink. Ethanol induces cytochrome p450 (CYP2E1), a principal enzyme involved in activating N-nitrosamines associated with tobacco [7].

Additionally, a correlation between oral cancer and viruses, particularly human papillomavirus (HPV), herpes group virus, adenovirus, and hepatitis C virus, has been demonstrated [8]. Human Papillomavirus, especially genotypes HPV-16 and HPV-18, is the most frequently associated virus with human oral cancer, with an attributable risk of 2.2 % [9]. HPVs are epitheliotropic DNA viruses that can induce hyperplastic, papillomatous, and verrucous squamous cells in stratified squamous epithelia of the skin and mucous membranes [7].

Other suspected risk factors for oral cancer include chronic irritation, poor oral hygiene, occupational exposure, malnutrition, and genetic factors [4,10]. Studies have shown the significance of heredity in oral carcinogenesis, with several genes implicated in genetic predisposition. Gene polymorphisms involved in the metabolism of xenobiotic factors, such as cytochrome P450 1A1 (CYPIA 1) and glutathione S-transferase mu 1 (GSTM1), increase the relative danger in carriers. Individuals with the alcohol dehydrogenase 3 genotype are prone to developing oropharyngeal cancers [11–15].

Oral squamous cell carcinoma can develop de novo from non-aberrant keratinocytes chronically exposed to risk factors following a progression from epithelial hyperplasia, dysplasia, and carcinoma in situ (CIS), to invasive carcinoma. Sometimes, OSCC arises from pre-existing mucosal abnormalities called Oral Potentially Malignant Disorders (OPMDs), which include oral leukoplakia (OL), oral erythroplakia (OE), oral submucosal fibrosis (OSMF), and oral lichen planus (OLP) [14,15]. At diagnosis, most OPMDS patients are asymptomatic, though some may exhibit erythema, pain, or ulceration [16].

The gold standard for OSCC diagnosis is biopsy and histological examination. Early diagnosis techniques include micro-biopsy exfoliative cytology, vital staining, light-based detection systems, and optical diagnostic technologies [17,18]. Unlike other cancers, OSCC is in direct contact with saliva, making saliva testing for specific diagnostic and/or prognostic molecules highly feasible [19].

"Salivomics" refers to the comprehensive –omics approaches (genomics, transcriptomics, proteomics, microbiomics, and metabolomics) in salivary analysis. Current research focuses on the metabolome, as its changes reflect disturbances in metabolic pathways caused by pathophysiological processes.

This systematic review was designed to answer the question, “Are there significant metabolic alterations of the salivary profile in patients with histological diagnosis of OSCC?”, formulated according to the “Population or problem”, “Intervention or exposure”, “Comparison”, “Outcome” (PICO) worksheet [20].

2. Material and methods

This systematic review followed the Preferred Reporting Item for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [21].

2.1. Search strategy

The review involved a comprehensive search across multiple databases (Medline, Scopus, Web of Science) conducted until February 2024. The search terms used included.

- “Salivary diagnostic AND oral squamous cell carcinoma”,
- “Salivary metabolomic AND oral potentially malignant disorders”,
- “Salivary metabolomic AND oral squamous cell carcinoma”,
- “Salivary metabolomic AND oral cancer”.

Only English-language literature published after 2000 was considered. Duplicate records identified during the database searches were removed using End Note X9© software (Clarivate Analytics). Initial screening of titles and abstracts was conducted by two independent investigators. Any discrepancies were resolved through the evaluation of full-text articles.

Studies were selected based on their focus on salivary sampling and analysis for basic, translational, or diagnostic research related to OSCC. Included studies had to be performed on humans and provide detailed diagnostic information.

Excluded were case reports, conference proceedings, personal communications, letters to editors, reviews, and studies focusing on systemic diseases, systemic oral manifestations, microbial infections, hormone or drug dosage, and studies on animals or in vitro models. Papers discussing biochemical methods, technological aspects, or devices for saliva evaluation or molecule detection were also excluded (Table 1).

After applying the inclusion and exclusion criteria, reference lists of eligible papers were reviewed to identify additional relevant studies. To avoid data redundancy, overlapping patient series based on recruitment centers and study periods were consolidated, with preference given to the most recent or complete data sets.

2.2. Data extraction

Data from the included studies were compiled into Excel tables (Microsoft Office Suite©), capturing details such as authors, publication year, study type, population characteristics (gender, pathology, OSCC staging/grading), saliva type and collection methods, metabolomics techniques, and statistical significance. Studies were categorized as either “targeted” or “untargeted” metabolomics based on the nature of the metabolite analysis.

2.3. Quality assessment

Two independent investigators completed the “Study Quality Assessment Tool form Case-Control Studies” from the National Institutes of Health (NIH). Questions 9 and 10, which are related to exposure/risk confirmation and measure consistency, were excluded due to the multifactorial etiology of OSCC. Studies were scored from 0 % to 100 % based on the percentage of “yes” answers and categorized as “good” (80–100 %), “fair” (50–70 %), or “poor” (<50 %). Disagreements were resolved through discussion.

The level of evidence was assessed using the Oxford Center for Evidence-Based Medicine (CEMB) classification for diagnostic studies [22].

3. Results

As summarized in Fig. 1, the search process involved a detailed examination of numerous studies to identify the most relevant and high-quality data concerning salivary biomarkers for oral squamous cell

Table 1
Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
English literature, published between 2000 and 2024 **Studies considering salivary metabolomics for OSCC or PMD identification** **Studies using metabolites as diagnostic biomarkers to distinguish cases from healthy control** **Human case-control studies**	Publication type: case reports, conference proceedings, personal communications, letters to the editor, reviews Studies on animals Studies on the diagnostic use of salivary metabolomics in patients with: • neoplastic disease other than OSCC and its OPMDs • oral manifestations of systemic disease • oral microbial infections Studies exploring salivary transcriptome, lipidome, proteome and genome for diagnostic purposes

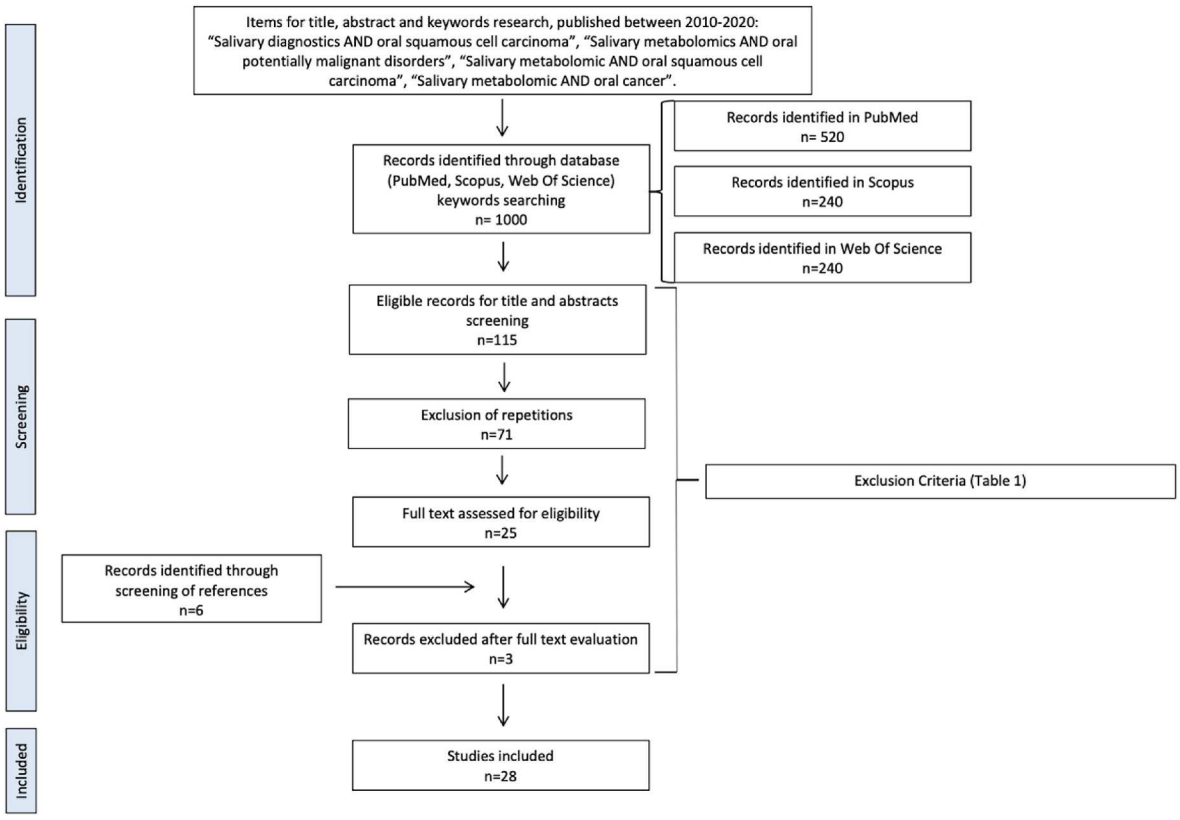

Fig. 1. Flowchart diagram for the selection of the 28 papers included in the review.

carcinoma (OSCC). From the initial pool of 9623 papers, a thorough evaluation led to the selection of 28 papers that met the inclusion and exclusion criteria. This meticulous selection process included resolving overlaps in data from studies by Ishikawa et al. (2016 and 2017) [23], [24] and Wang Q. et al. (2014) [25], [26], [27]. The most comprehensive records were chosen for inclusion to provide a robust data set [23, 25]. A summary of the features of the included studies is provided in Table 2.

3.1. Critical appraisal

The critical appraisal of these studies, detailed in Fig. 2, identified several methodological strengths and weaknesses. Reviewer disagreement was notable in two studies: Pink et al. [41] and Wang et al. [25]. The disagreements centered on the validity and consistency of their inclusion/exclusion criteria and the clarity in defining cases and controls. These issues were specifically related to the questionnaires' points on whether the selection processes and definitions were implemented consistently and reliably across all participants. After further discussion, both studies were rated as "intermediate" quality by consensus.

Several common risks of bias (ROBs) were identified.

- lack of concurrent controls (96.4 %): most studies did not employ concurrent controls, which could introduce temporal bias, affecting the reliability of the outcomes due to potential changes over time;
- no sample size justification (93 %): the absence of justification for the sample size undermines the statistical power and reliability of the findings, making it challenging to assess the true effect size;
- absence of randomization (82 %): without randomization, the studies are susceptible to selection bias, which can significantly affect the validity of the results;
- non-comparable recruitment timelines (50 %): differences in the recruitment timelines of study participants can introduce variability that complicates the interpretation and generalization of the results.
- lack of blinding procedures: almost all studies failed to adequately report blinding procedures, increasing the risk of subjective bias in the interpretation of results. Blinding is critical, especially in biomarker studies, to prevent evaluators' knowledge of participants' statuses from influencing their assessments.

Despite these biases, the overall quality of the studies varied, with the majority being rated as "intermediate" (79 %) [23,25,28–30,33–36, 38–44,46,48–51,53], followed by "good" (7 %) [37,47] and "poor" (14 %) [31,32,45,54].

3.2. Level of evidence

Applying the CEMB criteria, all included studies were classified as having a low level of evidence (level-4), primarily due to their case-control design. Even studies initially labeled as cross-sectional by their authors were reclassified as case-control due to insufficient information proving their cross-sectional nature. This low evidence level underscores the need for further research with more robust designs to confirm these preliminary findings.

3.3. Saliva collection and analytical techniques

Table 3 summarizes the methodologies used for saliva collection and analysis. All studies utilized unstimulated whole saliva, which is considered advantageous because it minimizes external influences compared to stimulated saliva. Unstimulated saliva provides a more stable baseline for metabolomic analysis.

Eleven studies described maintaining the collection tubes on ice during the procedure before storage [23,32–36,38,40,50,51,53], crucial

Table 2
General characteristics of the included 28 studies.

Ref.	Year	Type of study	Main features of subjects
[28]	2017	Case-control	30 OSCC 30 OPMDs: 10 SF; 10 OL; 7 OLP 30 CG
[29]	2012	Case-control	34 OSCC: 6 stage I; 7 stage II; 8 stage III; 13 stage IV 17 OL 27 smokers and/or drinkers 25 CG
[30]	2016	Case-control	25 OSCC (M:F = 18:7) 25 CG (M:F = 13:12)
[31]	2016	Case-control	30 OSCC (3 stage I; 3 stage II; 13 stage III; 11 stage IV) 30 OPMDs: 28 OL (8 stage I, 10 stage II, 2 stage III, 8 stage IV) 2 tobacco pouch keratosis 30 CG
[32]	2014	Case-control	30 OC 25 OPMDs 30 CG
[33]	2021	Case-control	27 OSCC (M:F = 19:8): 15 % stage I, 15 % stage II, 22 % stage III, 48 % stage IV 41 CG (M:F = 20:21)
[34]	2013	Case-control	20 HC 20 OL 20 OSCC
[35]	2020	Case-control	34 OSCC (M:F = 20:14):14 stage I; 9 stage II; 2 stage III; 9 stage IV 26 OLP (M:F = 5:21)
[36]	2019	Case-control	6 OSCC 10 OED 32 PSOML
[23]	2016	Case-control	24 OC (M:F = 14:10): 21 OSCC, 2 MM, 1 unknown 44 CG (M:F = 16:28)
[37]	2016	Case-control	40 OSCC (M:F = 20:20) 40 OLP (M:F = 20:20) 40 OL (M:F = 20:20) 40 OSF (M:F = 20:20) 40 CG (M:F = 20:20)
[38]	2018	Case-control	FIRST SET 79 OSCC: 45 N +; 34N -; 40 T1/T2; 39 T3/T4 20 CG SECOND SET 22 OSCC 58 OPC 20 CG
[39]	2018	Case-control	45 HNSCC 30 CG
[40]	2017	Case-control	22 OSCC (M:F = 13:9) 21 CG (M:F = 8:13)
[41]	2016	Case-control	15 OSCC 1 SCC of oropharynx 15 CG
[42]	2017	Case-control	21 OSCC: 12 well; 7 moderately; 2 poorly differentiated OSCC 10 CG
[43]	2012	Case-control	16 OSCC (8 well differentiated, 8 moderately differentiated) 8 HC
[44]	2019	Case-control	12 OSCC (M:F = 5:7): 5 stage I; 6 stage II; 0 stage III; 1 stage IV 8 CG (M:F = 7:1)
[45]	2011	Case-control	70 OPMDs, OSCC (moderately and well-differentiated) 50 CG
[46]	2020	Case-control	125 OSCC 124 OPMDs 124 CG
[47]	2019	Case-control	22 OSCC 21 OL 18 CG
[48]	2010	Case-control	69 OSCC 30 breast cancer 18 pancreatic cancer 11 periodontal disease 87 CG
[49]	2022	Case-control	30 OSCC 30 OLK 30 CG
[50]	2013	Case-control	100 HC 50 OPMDs: 39 oral submucous fibrosis, 11 homogenous leukoplakia 100 OSCC
[51]	2023	Case-control	1st phase (untargeted) 20 SCCT (M:F = 15:5): 30 % stage I, 20 % stage II, 35 % stage III, 15 % stage IV 40 % N_0, 35 % N_1, 25 % N_2 10 CG (M:F = 7:3) 2nd phase (targeted) 100 SCCT (M:F = 76:24): 18 % stage I, 52 % stage II, 13 % stage III, 17 % stage IV; 55 % N_0, 21 % N_1, 18 % N_2, 6 % N_3 30 CG (M:F = 21:9)
[52]	2014	Case-control	30 OSCC (M:F = 25:5): 4 stage I; 9 stage II; 3 stage III; 14 stage IV 30 CG (M:F = 25:5)
[53]	2011	Case-control	37 OSCC (M:F = 26:11): 9 stage I; 12 stage II; 6 stage III; 10 stage IV 32 OL (M:F = 13:19) 34 CG (M:F = 13:21)
[54]	2014	Case-control	63 OSCC 60 CG

Legend. SCCT: squamous cell carcinoma of the tongue; OSCC: oral squamous cell carcinoma; OC: oral cancer; OLP: oral lichen planus; OL: oral leukoplakia; OSF: oral submucosal fibrosis; MM: malignant melanoma; OPC: oropharyngeal cancer; CG: control group; OPMDs: oral potentially malignant disorders; "N+": presence of neck-nodes metastasis; "N-": absence of node metastasis; "T": tumor size according to the TNM staging system; OED: oral epithelial dysplasia; PSOML: persistent suspicious oral mucosal lesions; HNSCC: head and neck squamous cell carcinoma.

for preserving the samples' metabolic state. Centrifugation practices were generally consistent, with 2500-3000×*g* for 10–15 min at 4 °C. In some cases, a second centrifugation cycle was performed to ensure the removal of any remaining cellular debris, providing cleaner samples for analysis.

The identification of salivary metabolites was achieved through various techniques, reflecting the complexity and diverse nature of the salivary metabolome. Mass spectrometry and its derivates were the predominant methods due to their high sensitivity and ability to detect a wide array of metabolites. Techniques like ultraperformance liquid chromatography (UPLC-MS), triple quadrupole liquid chromatography (TPLC), and high-performance liquid chromatography (HPLC-MC) were frequently used, each providing unique advantages in separating and identifying compounds. Other methods included spectrophotometry, nuclear magnetic resonance (NMR), enzyme-linked immunosorbent assays (ELISA), autofluorescence, diphenylamine method (DPA) and thiobarbituric acid method (TBA), each contributing to a comprehensive understanding of the salivary metabolome in OSCC.

3.4. Target metabolomic

Twelve studies discriminate the concentration of known target compounds between cases and controls [28–32,34,37,41,42,45,50,54] (Table 4).

Seven studies focused on the detection of sialic acid and its concentrations [28,31,32,34,42,45,50]. Sialic acid extensively binds to glycoproteins; in such conjugated form, it is a major component of the cell membrane. With the development of neoplastic disease, cell membrane re-arrangement facilitates the accumulation of sialic acid on the cell surface; with membrane shredding, sialic acid becomes detectable in the bloodstream and probably also in other biological fluids, as saliva [55].

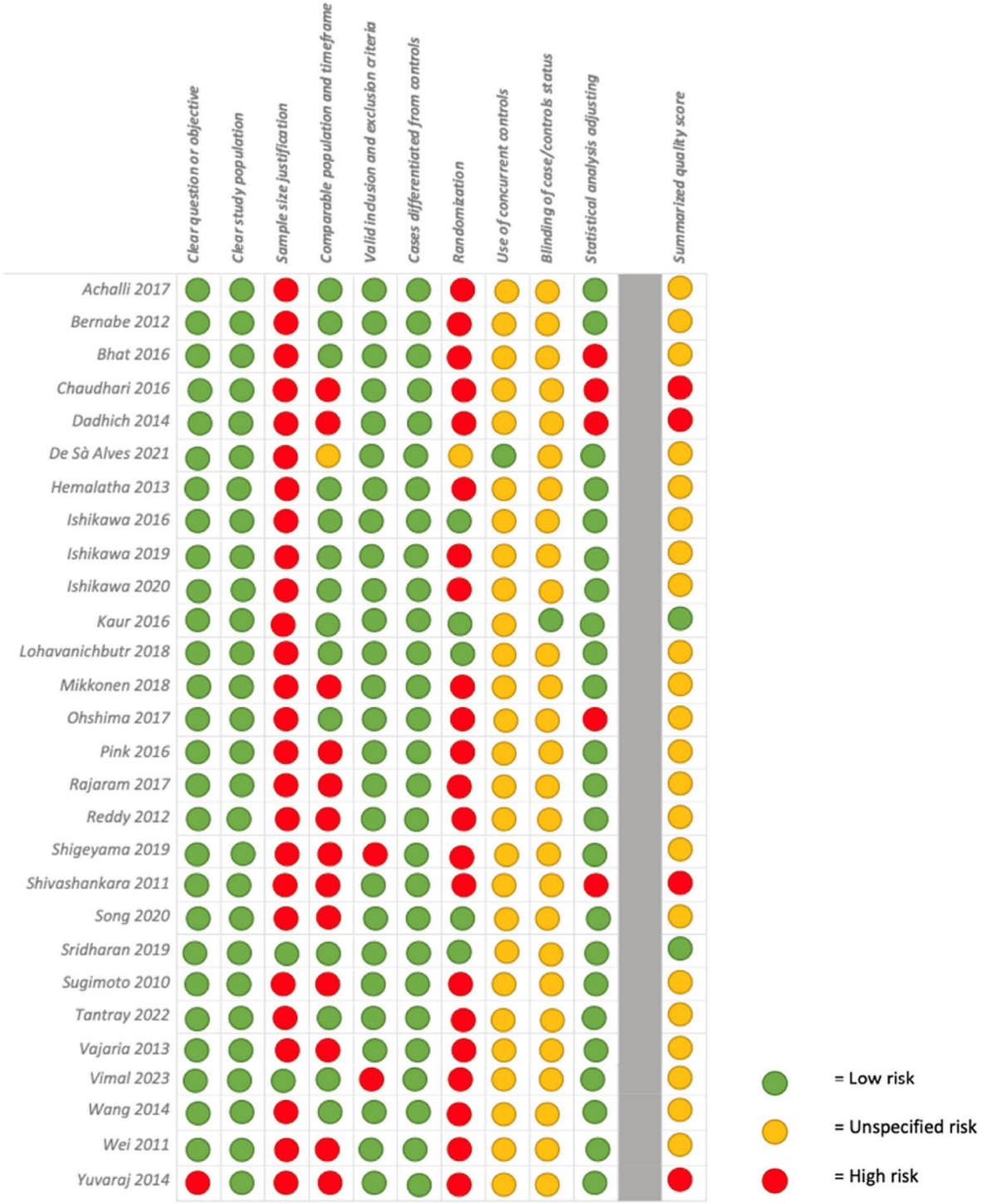

Fig. 2. Critical appraisal, including main potential risk of bias and quality score.

All studies report statistically significant differences in salivary concentration of sialic acid, with a higher value in patients with OSCC and OPMDs than HC. With such a present level of evidence, discrimination between OSCC stages seems not reliable.

Achalli et al. make multiple comparisons between HC, OPMDs and OSCC patients, always reporting statistically significant differences in salivary concentration ($P < 0.001$) [28]. Similarly, Rajaram et al. compared HC to well, moderately and poorly differentiated OSCC; significant differences emerged only in the comparison between saliva of well vs. moderately/poorly OSCC groups [42]. Chaudhari et al. identified higher concentrations of sialic acid in OSCC cases than OPMDs and HCs, with a highly significant difference at a 95 % confidence interval [31]. Such molecule expression was at the highest level in histological grade III OSCC (poorly differentiated), following Bryne et al. classification [56]. Moreover, a statistically significant correlation was observed in leucoplakia OPMDs classified following van der Waal *et* al., with the highest addressed to severe dysplasia [57]. Likewise, Dadhich et al. report that salivary sialic acid concentration is higher in OPMDs and OSCC groups than HC with significance ($P < 0.0005$) [32]. Specifically, the difference in concentration was found to be highly significant between the HC and OPMDs group ($P = 0.005$) and very highly significant between the HC and OSCC group and between the OPMDs and

Table 3
Biochemical techniques for identification and quantification of salivary metabolites.

Ref.	Type of Saliva and methods of collection	Setting of collection	Pre-analytical procedures	Method of analysis
[28]	Whole Unstimulated Spitting method	2 h after consumption of food	Storing: 2 °C Handling: NR Centrifugation: 3000 rpm for 10 min	DPA method spectrophotometry
[29]	Whole Unstimulated Spitting method	Collection 8–10am	Storing: 80 °C Handling: NR Centrifugation: 2000 rpm at 4 °C for 5 min	ELISA
[30]	Whole Unstimulated in the sublingual region. 3 mL of collected saliva was allowed to drool into sterile plastic tubes	NR	Storing: NR Handling: 1 mL of dinitrophenyl hydrazine was added to each solution and kept at 37 °C for 10 min; 10 mL of freshly prepared 0.4 M sodium hydroxide was added Centrifugation: NR	540 nm spectrophotometry.
[31]	Whole Unstimulated Draining method	Collection 10am-12pm, 2 h after breakfast; each subject rinsed their mouth with water	Storing: NR Handling: saliva was treated with ethanol to precipitate proteins Centrifugation: 1000 rpm for 15 min	Acidic ninhydrin method with 470 nm spectrophotometry (Yao *et* al. 1989)
[32]	Whole Unstimulated Spitting method	Collection during the morning hours; each subject rinsed their mouth with distilled water; collection in a wide-mouthed sterile plastic container on ice	Storing: supernatant at −80 °C Handling: NR Centrifugation: 3000 rpm for 15 min	Acidic ninhydrin method with 470 nm spectrophotometry
[33]	Whole Unstimulated	Patients were instructed not to ingest pasty or hardened foods for 1 h before collection and not to consume alcoholic beverages for at least 12 h before saliva collection. They could only swallow water and had to brush their teeth at least 2 h before the collection	Storing: 80 °C Handling: 3 mL collected in the plastic tubes, immersed in ice and transported within 1 h to the storage location. Metabolites were extracted by adding 300 μL of methanol containing methionine sulphonate as internal standard and stirred for 2 min, and the supernatant was dried in a vacuum centrifuge. After extraction, derivatization was performed by adding 100 μL of a solution with proportions (1:1) of N-methyl-N-(trimethylsilyl) trifluoroacetamide and a solvent solution: acetonitrile/dichloromethane/cyclohexane (5:4:1) and 5 % trimethylamine. The samples were stirred for 30 s and then kept in a thermal bath at 60 °C for 1 h. Centrifugation: 12,044×*g* for 2 min	GC-MS
[34]	Whole Unstimulated Spitting method	Collection during the morning hours; each subject rinsed their mouth with distilled water; collection in a sterile plastic container; transferred on ice to laboratory	Storing: NR Handling: NR Centrifugation: 3000 rpm for 15 min	Acidic ninhydrin method with 470 nm spectrophotometry
[35]	Whole Unstimulated	Dental plaque and calculus deposits were removed using a toothbrush and ultrasonic scaling at least 3 h before saliva collection; eating and drinking were not permitted for at least 1.5 h prior to saliva collection; each subject rinsed their mouth with water; collection in a 50 mL Falcon tube on ice	Storing: 80 °C Handling: 45 μL of each sample was added to a 1.5 mL Eppendorf tube, with 2 mM of methionine sulfone, 2-[N-morpholino]-ethanesulfonic acid (MES), D-Camphol-10-sulfonic acid, sodium salt, 3-aminopyrrolidine and trimesate. Frozen saliva was thawed at 4 °C for approximately 1.5 h and subsequently dissolved using a Voltex mixer at room temperature Centrifugation: through a 5-kDa cutoff filter at 9100g for at least 2.5 h at 4 °C	CE-TOF MS
[36]	Whole Unstimulated	Eating and drinking were not permitted for at least 1.5 h prior to saliva collection; each subject rinsed their mouth with water; collection in a 50 mL Falcon tube on ice	Storing: 80 °C Handling: 45 μL of each sample was added to a 1.5 mL Eppendorf tube, with 2 mM of methionine sulfone, 2-[N-morpholino]-ethanesulfonic acid (MES), D-Camphol-10-sulfonic acid, sodium salt, 3-aminopyrrolidine and trimesate. Frozen saliva was thawed at 4 °C for approximately 1.5 h and subsequently dissolved using a Voltex mixer at room temperature Centrifugation: through a 5-kDa cutoff filter at 9100g for at least 2.5 h at 4 °C	CE-TOF MS
[23]	Whole Unstimulated	Collection 8am-12pm eating and drinking were not permitted for at least 1.5 h prior to saliva collection; each subject rinsed their mouth with water; collection in a 50 mL Falcon tube on ice	Storing: 80 °C Handling: 45 μL of each sample was added to a 1.5 mL Eppendorf tube, with 2 mM of methionine sulfone, 2-[N-morpholino]-ethanesulfonic acid (MES), D-Camphol-10-sulfonic acid, sodium salt, 3-aminopyrrolidine and trimesate. Frozen saliva was thawed at 4 °C for approximately 1.5 h and subsequently dissolved using a Voltex mixer at room temperature Centrifugation: through a 5-kDa cutoff filter at 9100g for at least 2.5 h at 4 °C	CE-TOF MS

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Table 3 (*continued*)

Ref.	Type of Saliva and methods of collection	Setting of collection	Pre-analytical procedures	Method of analysis
[37]	Whole Unstimulated	NR	Storing: 20 °C Handling: NR Centrifugation: 3500g for 25 min	MDA was analyzed by TBA reaction; 8-OHdG was determined using ELISA; Vitamin C and vitamin E were estimated by HPLC
[38]	Whole NR	No eating or drinking anything, except water, within an hour before saliva collection; saliva was collected using a 50 mL sterile conical centrifuge tube and transferred on ice to the laboratory within 2 h of collection	Storing: 80 °C Handling: NR Centrifugation: 1300g for 10 min at 4 °C	NMR Three types of LC-MS: 1. targeted LC-MS for aqueous metabolite profiling; 2. global aqueous and lipidomics LC-MS experiments; 3. lipidomics LC-Q-TOF experiments
[39]	Whole Unstimulated	The collection of saliva samples was performed after dental treatment prior to radiotherapy; collection between 9 and 11am	Storing: 20 °C Handling: addition of 50 μL of NMR-buffer (1.5 M KH_2PO_4, 2 mM NaN_3, 5.8 mM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4, D_2O, pH 7.4) at each 450 μL of saliva sample Centrifugation: initially at 14,000 rpm for 6 min, after at 10,000 g for 5 min at 4 °C	^{1}H- NMR
[40]	Whole Unstimulated, Spitting method	Collection at 8am under fasting conditions after sufficient gargling and other oral hygiene steps	Storing: NR Handling: each saliva sample (45 μL) was added to 5 μL Milli-Q water containing internal standards and 20 mM each of methionine sulfone, D-camphor-10-sulfonic acid, 2-(n-morpholino) ethanesulfonic acid, 3-aminopyrrolidine and trimesate Centrifugation: 2600g for 15 min at 4 °C	CE-MS
[41]	Whole Unstimulated	Collection after morning oral hygiene, 30 min after drinking of 200 mL of water	Storing: 25 °C Handling: NR Centrifugation: NR	ELISA
[42]	Whole Unstimulated Spitting method	Collection 9–11am	Storing: NR Handling: NR Centrifugation: 3000 rpm for 15 min	Acidic ninhydrin Method
[43]	Whole Unstimulated Spitting method	Collection 7–8 m, 10 min after MilliQ water mouthwash	Storing: 4 °C Handling: addition of 0.3 mL of acid citrate dextrose (ACD) Centrifugation: 2500 rpm for 10 min. Supernatant was separated at −80 °C. For each 1 mL of supernatant, 0.1 mL of trichloroacetic acid was added (TCA) to precipitate proteins, after which the samples were once again centrifuged for 10 min at 2500 rpm	HPLC-MS
[44]	Whole Unstimulated Saliva collection in a 10 mL glass bottle over a period of 5–10 min	HC: saliva was collected continually for at least a period of 5 days 7–10am while the subjects fasted; OSCC: saliva was collected for at least 1.5 h after meals for multiple (1–3) time periods up to surgery after hospitalization; the subjects rinsed their mouths with water immediately prior to sample collection; smoking and using oral hygiene products were not permitted for at least 1 h before saliva collection	Storing: 80 °C Handling: 2 mL of saliva was diluted with 3 mL deionized water; VOCs in saliva were extracted by shaking the sample for 3 h and then the extraction bottle was gently rinsed with pure water and dried with nitrogen. The VOCs extracted onto the ZSM-5/PDMS film were condensed using 100 μL methanol Centrifugation: NR	GC-MS
[45]	Whole Unstimulated Spitting method	Collection during the morning hours; each subject rinsed their mouth with distilled water; after 10 min, collection in a sterile plastic container	Storing: NR Handling: NR Centrifugation: 3000 rpm for 10 min	Total protein: Lowry method (750 nm spectrophotometry). Total sialic acid: Acidic ninhydrin method with 470 nm spectrophotometry Malondialdehyde: Thiobarbituric acid method (470 nm spectrophotometry) GSH: Beutler method
[46]	Whole Unstimulated	Mouth rinsing with ultrapure water was required before saliva collection. Oral hygiene products (e.g., toothpaste) were also not allowed for use 1 h before sample collection	Storing: 80 °C Handling: NR Centrifugation: 5000 rpm for 3 min	CPSI-MS + ML
[47]	Whole Unstimulated under aseptic conditions by drooling method in a collecting jar	NR	Storing: at −80 °C before analysis, then at −20 °C Handling: 100 μL of the supernatant was mixed with chilled methanol in 1:4 ratio Centrifugation: 4000 rpm for 15 min (whole sample, immediately); 4.000 rpm for 10 min (100	LC-Q-TOF-MS

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Table 3 (*continued*)

Ref.	Type of Saliva and methods of collection	Setting of collection	Pre-analytical procedures	Method of analysis
			μL of the supernatant mixed with chilled methanol)	
[48]	Whole Unstimulated Spitting method	Mouth rinse with water and, 5 min later, spit into 50 mL Falcon tubes, kept on ice. Saliva collection was performed in a private room	Storing: frozen (temperature NR). Handling: before analysis, 3 μL of water containing 2 mM methionine sulfone and 2 mM 3-aminopyrrolidineas internal standards was added Centrifugation: 2600g for 15 min at 4 °C and spun for another 20 min for incomplete separation	CE-TOF-MS.
[49]	Whole Unstimulated	Saliva was collected 9–10am under aseptic conditions by the drooling method in a collecting jar. The subjects were asked to refrain from eating, drinking, smoking, or using oral hygiene products for at least 1.5 h before saliva was collected	Storing: 80 °C Handling and centrifugation: 10,000 rpm for 10 min (1 mL). Supernatent, taken was lypolized in lyophilizer. Lypholised samples were dissolved in hexane (1 mL). Samples were again centrifuged at 10,000 rpm for 10 min	GC-MS
[50]	Whole Unstimulated Spitting method	Collection 8–9.30am. Mouth rinse with water prior collecting. 5 min of passive accumulation in mouth and collection in a 50 mL tube kept on ice	Storing: 80 °C Handling: supernatant was collected in aliquots, and protease inhibitors were added Centrifugation: 2600 rpm for 15 min at 4 °C	Total protein: Lowry method (750 nm spectrophotometry). Total sialic acid: Thiobarbituric acid method (549 nm spectrophotometry) α-L-fucosidase: p-nitrophenol measure (410 nm spectrophotometry)
[51]	Whole Unstimulated	The participants were requested not to drink (except for water) or eat within 1 h of sample collection. Participants who consumed alcohol or smoked within 24 h of sample collection were excluded from the study	Storing: 80 °C Handling: 3 mL of unstimulated whole saliva was collected in a 50 mL sterile centrifuge tube on ice and then transferred to the laboratory within an hour The supernatant was aliquoted into 0.5 mL sterile cryotubes Centrifugation:7000 rpm in a refrigerated centrifuge at 4 °C for 30 min	Untargeted metabolomics analysis UPLC-MS Targeted metabolomics analysis TPLC-MS
[52]	Whole Unstimulated	Collection 9–11am, No smoking, eating, drinking or oral hygiene procedures for at least 1 h before sample collection. They rinsed their mouths thoroughly with water	Storing: 40 °C Handling: a mixture of acetonitrile/methanol was added to 400 μL aliquots of saliva in a 1.5 mL Centrifugation: 2500 rpm for 15 min at 4 °C (whole samples); 12000 rpm for 20 min at 4 °C (without supernatant)	UPLC-TOF/MS base peak intensity chromatograms
[53]	Whole Unstimulated	Collection 9–10am; subjects refrained from eating, drinking, smoking or oral hygiene procedures for at least 1.5 h before the collection	Storing: 80 °C Handling: ultrapure water (200 μL) and acetonitrile (200 μL) were added to 200 μL of saliva and vortexed for 1 min The mixture was allowed to stand for 10 min and centrifuged. The supernatant was filtered through a syringe filter (0.22 μm) Centrifugation: 3500g for 20 min at 4 °C (whole sample); 15.400g for 15 min (the mixture)	UPLC-Q-TOF-MS
[54]	Whole Unstimulated	Before the collection, no eating, drinking, smoking or using any oral hygiene products; the patients were asked to gargle the oral cavity with clean water. After 15 min the secreted saliva was collected on a sterile container	Storing: NR Handling: NR Centrifugation: 1500 rpm for 5 min	AFS at 405 nm excitation

Legend: MDA: malondialdehyde; TBA: thiobarbituric acid; 8-OHdG: 8-hydroxy-2-deoxyguanosine; HPLC: high-performance liquid chromatography; CE-TOF MS: capillary electrophoresis time-of-flight mass spectrometry; DPA: Diphenylamine; CE-MS: capillary electrophoresis-mass spectrometry; HILIC-UPLC-MS: Hydrophilic interaction chromatography column - ultra performance liquid chromatography – mass spectrometry; UPLC–ESI–MS: ultra-performance liquid chromatography–electrospray ionization–mass spectrometry; UPLC-TOF/MS: ultra-performance liquid chromatography combining with time of flight mass spectrometer; TQLC-MS: Triple Quadrupole Liquid Chromatography Mass Spectrometer; NMR: nuclear magnetic resonance; LC-MS: liquid chromatography mass spectrometry; LC-Q-TOF: liquid chromatography quadrupole time-of-flight; AFS: Autofluorescence spectroscopy; CG: control group; OCG: oral cancer group; VOCs: volatile organic compounds; ZSM-5/PDMS: Zeolite Socony Mobil-5/polydimethylsiloxane; CG-MS: chromatographic analysis with mass spectrometric; CPSI-MS: conductive polymer spray ionization mass spectrometry; ML: machine learning.

OSCC group ($P < 0.0005$).

Hemalatha et al. described comparable results by exploring molecular expression between HC, OPMDs and OSCC groups; samples from neoplastic and pre-malignant patients showed higher concentrations of free sialic acid than HCs, as well as histological well-differentiated OSCC group than moderate/poor tumors [34]. Conversely, staging did not influence any difference in concentration. The salivary total sialic acid/total protein ratio (TSP/TP) and p-nitrophenol (PNP) concentration were evaluated following the methods described by Lowry et al. [58], Skoza et al. [59] and Wiederschain et al. [60], differences between HC, OPMDs and OSCC were described by Vajaria et al. [50]. The combination of salivary TSA/TP and PNP levels in HC and OPMDs was statistically significant ($P < 0.001$ and $P < 0.005$) and can also discriminate between HC and OSCC patients ($P < 0.001$). It is worth mentioning that such results were obtained by setting the receiver operator curve (ROC) to a specificity of 50 %.

Similarly, Shivashankara et al. [45] compared TSA/TP, free salivary sialic acid and malondialdehyde concentrations (MDA) between HCs, submucosal fibrosis patients and OSCC cases (without any further specification regarding grade or stage). All the investigated targets

Table 4
Statistical association of salivary metabolites and oral squamous cell carcinoma.

Ref.	Salivary metabolites	Targeted/ Untargeted	Statistical association with OSCC
[28]	Sialic acid	Targeted	Significant - patients with OC and OPMDs showed levels higher than ~~to~~ healthy controls ($P < 0.001$).
[29]	Cortisol	Targeted	*Significant* - levels in the OSCC group two times higher than that in the healthy ($P < 0.005$), risk ($P < 0.002$) and leukoplakia ($P < 0.002$) groups; - patients with clinical stages III and IV showed levels higher ($P = 0.015$) than those in patients with clinical stages I and II; - male patients with OSCC show higher salivary cortisol levels ($P = 0.04$) than women.
[30]	Pyruvic acid	Targeted	Significant - levels in cancer subjects were statistically higher in contrast to healthy subjects ($P < 0.05$).
[31]	Sialic acid (free sialic acid - FSA and protein-bound sialic acid - PBSA)	Targeted	Significant - mean levels of salivary FSA and PBSA higher in malignant lesions than premalignant, followed by the control group ($P < 0.05$); - mean FSA and PBSA levels progressively increased from Grade I OSCC to Grade III OSCC with a statistically significant difference ($P < 0.05$) between Grade I, Grade II, and Grade III; - mean salivary sialic acid levels were less in stage II as compared to stage I followed by stage III and stage IV.
[32]	Sialic acid	Targeted	Significant levels in the OPMDs and OC groups were higher than the CG group ($P < 0.0005$).
[33]	Pool of 108 metabolites	Untargeted	Significant - up-regulated in OSCC ($P < 0.5$): malic acid, methionine, maltose, protocate- chuic acid, inosine, pantothenic acid, dihydroxyacetone phosphate, hydroxyphenylatic acid, galacturonic acid, indole-3-acetic acid, uracil, isocitric acid, ribose-5-phosphate, o-phosphoserine, lactitol, gluconic acid, hippuric acid, 3-hydroxypropionic acid and spermidine; - down-regulated in OSCC ($P < 0.5$): lactose, catechol, 2-ketoadipic acid, leucine, urea, maleic acid, palmitic acid, ornithine, margaric acid, sucrose, octadecanol, threitol, acetoacetic acid, methionine sulfone, phosphoric acid, elaidic acid, mannose, sorbitol, citric acid, 3-aminopropanoic acid.
[34]	Sialic acid	Targeted	Significant - significantly increased in OSCC and OL, when compared with CG (free and protein bound), - protein bound salivary sialic acid significantly increased in OSCC, when compared with OL ($P < 0.004$) - significantly higher levels of free sialic acid and protein-bound sialic acid in well-differentiated OSCC when compared to the other two grades ($P < 0.000$ and $P < 0.002$).
[35]	Pool of 14 metabolites	Untargeted	Significant - up-regulated in OSCC than in OLP group ($P < 0.05$): trimethylamine *N*-oxide, putrescine, creatinine, 5-aminovalerate, pipecolate, *N*-acetylputrescine, gamma-butyrobetaine, indole-3-acetate, N_1-acetylspermine, 2′-deoxyinosine, ethanolamine phosphate and *N*-acetylglucosamine; - down-regulated in OSCC ($P < 0.05$): *N*-acetylhistidine and *o*-acetylcarnitine.
[36]	Pool of 6 metabolites	Untargeted	Significant - down-regulated in the OSCC/OED than in the PSOML group ($P < 0.05$): ornithine, carnitine, arginine, hydroxybenzoate, N-acetylglucosamine-1-phosphate and ribose 5-phosphate (R5P).
[23]	Pool of 4 metabolites	Untargeted	Significant – 43 saliva metabolites showed significantly different concentrations between OC and controls ($P < 0.05$); – 17 metabolites showed consistent differences in both saliva and tissue-based comparisons.
[37]	8-OHdG MDA Vitamin C Vitamin E	Targeted	Significant - 8-OHdG and MDA higher in contrast to healthy subjects ($P < 0.005$); - vitamins C and E are lower in contrast to healthy subjects ($P < 0.005$).
[38]	Pool of 80 metabolites	Untargeted	Significant - glycine and proline are significantly lower in patients with OSCC than in controls; - glycine, proline, citrulline and ornithine associated with early-stage OSCC; - cystathionine ketamine significantly differentiated early-stage from late-stage tumor.
[39]	Pool of 19 metabolites	Untargeted	Significant - up-regulated in the HNSCC group than in the CG group ($P < 0.05$): fucose, 1,2-propanediol; - down-regulated in the HNSCC group than in the CG group ($P < 0.05$): proline.
[40]	Pool of 25 metabolites	Untargeted	Significant - choline showed the greatest statistically significant difference between patients with OSCC and healthy controls ($P < 0.05$); - urea was the only metabolite with a lower level in patients with OSCC compared with healthy controls.
[41]	Neopterin	Targeted	Significant - pre-operative concentration was significantly higher in patients compared to controls ($P = 0.004$); - concentrations in 16 patients decreased significantly after the surgery ($P = 0.001$).
[42]	Sialic acid	Targeted	Significant - a significant difference was recorded between healthy controls and moderately-poorly differentiated OSCC; - a significant difference was found between well-differentiated OSCC and moderately poorly differentiated OSCC.
[43]	Pool of 15 amino acids	Untargeted	Significant - significantly higher in OSCCs than in CG groups: histidine, threonine, valine, isoleucine, methionine, phenylalanine, leucine, lysine, tyrosine, arginine, alanine, glycine, serine and aspartic acid; - glutamic acid lower in well-differentiated OSCC.
[44]	Pool of 100 metabolites (volatile organic compounds, VOCs):	Untargeted	Significant 22 compounds with statistically significant differences:

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Table 4 (*continued*)

Ref.	Salivary metabolites	Targeted/ Untargeted	Statistical association with OSCC
	80 with a good day reproducibility −7 independently present in the healthy group; −38 independently present in OC group; −35 with overlap between healthy and OC group		- up-regulated in OSCC ($P < 0.05$): 4-methyl-3-penten-2-one, cyclohexanone; - down-regulated in OSCC ($P < 0.05$): ethanol, 2-pentanone, 1-propanol, hexanal, thiocyanic acid, 1-hydroxy-2-propanone, 2-hydroxy-propanoic acid, propylene glycol, 1-hexanol, ethanedioic acid, hydroxy-acetic acid, 1-ccten-3-ol, dimethyl sulfone, phenylethyl alcohol, phenol, 4-methyl-phenol, 2-piperidinone, hexadecanoic acid, docosanoic acid, indole.
[45]	Total protein Total sialic acid Malondialdehyde GSH	Targeted	Significant - levels of total proteins, free sialic acid, protein-bound sialic acid and malondialdehyde higher in OSCC than controls; - glutathione levels are lower in OSCC than controls.
[46]	Pool of 627 metabolites	Untargeted	Significant - significantly changed metabolites between OPMDs and OSCC: 4-hydroxybutyric acid, palmitic acid, propionyl carnitine, guanosine, 3-hydroxyphenylacetate, adenosine, serine, lactate, phosphocreatine, pentadecanoyl carnitine, inosine, indoleacetic acid, MG(14:0/0:0/0:0), 5-aminopentanoic acid, leucine, ketoleucine, 1-methylhistidine, oleic acid, cadaverine, 8-oxoguanine, deoxycholic acid, urocanic acid, linoleic acid, 2-ketobutyric acid, decenoic acid, palmitic amide, phytosphingosine, carnitine, N-acetylneuraminic acid, MG(16:0/0:0/0:0), methionine, spermidine, 8-hydroxy-7-methylguanine, N-acetylglucosamine 2-hydroxyvaleric acid, hydroxyoctanoic acid, desaminotyrosine, phosphoserine, hippuric acid, leucic acid, pipecolic acid, 1,3-dimethyluracil, arginine, acetyl carnitine, creatine, 3-hydroxydodecanedioic acid, creatinine, tryptophan, butyrylcarnitine, N-glycolylneuraminic acid, adenosine monophosphate, glucose, MG(18:1/0:0/0:0), hypoxanthine, piperidine, ribulose, thymidine, uridine, N-acetylserine, glutamate, MG(16:1/0:0/0:0), glycerol, glutaric acid, 3-hydroxydodecanoic acid, MG(18:0/0:0/0:0), dihydrothymine, adipic acid, methyladenine, aspartate, 4-aminobutyrate, citrulline, threonine, putrescine iminoaspartic acid, phenylalanine, acetylcarnosine, betaine, urea, N-acetyl cadaverine, ricinoleic acid, sphingosine, adrenic acid, proline, hydroxyarachidonic acid, glycerol-3-phosphate, glutamine, phosphoethanolamine, caprylic acid, MG(20:4/0:0/0:0), ornithine, taurine, histidine, cytosine, glycerophosphocholine; - significantly changed metabolites between CG and OPMDs: phosphoethanolamine, adenosine, N-acetyl cadaverine, putrescine, N-glycolylneuraminic acid, piperidine, ethanolamine, cadaverine, 1,3-dimethyluracil, glutarate, niacinamide, propionyl carnitine, N-Acetyl putrescine, phenylalanine, lysine, thymidine, N-acetylglucosamine, choline, N-acetylneuraminic acid, histidine, allantoin, arginine, acetylcholine, N1-acetyl spermidine, acetyl carnitine, proline, linolenic acid, sphinganine, hypoxanthine 1-methylhistidine, phosphoserine, phosphorylcholine, aspartate, lactate, palmitic acid, MG(14:0/0:0/0:0), linoleic acid, sphingosine, glucose, 8-hydroxy-7- methylguanine, pentadecanoyl carnitine, sucrose.
[47]	Pool of 196 metabolites	Untargeted	Significant - up-regulated in OSCC and OL ($P < 0.05$): deoxycholic acid disulfate, etoposide glucuronide, 6-beta-hydroxytriamcinolone acetonide, sativic acid, 13-cis-retinol, 16-iodo-hexadecanoic acid, prephytoene diphosphate, PGF1a alcohol, hexadecanedioic acid, tetradecanedioic acid, 17beta-estradiol, hydroquinine, 1-methylhistidine, inositol, pseudouridine, spaglumic acid, d-glycerate 2-phosphate, 2-hydroxymestranol, octopine, fumarylacetoacetic acid, 4-nitroquinoline-1-oxide, estrone 3-sulfate, etidronic acid, dihydroisolysergic acid II, 2-oxoarginine, octadecanoic acid trichloroethyl ester, norcocaine nitroxide, gamma-aminobutyryl- lysine, 9-chloro-10-hydroxy- decanoic acid, dextrorphan sulfate, sphinganine 1-phosphate, 4-hydroxyaminoquinoline N-oxide, n-histidyl-2-aminonaphthalene, undecaprenyl diphosphate, d-urobilinogen, estrone 3-glucuronide, (S)ureidoglycolic acid; - down-regulated in OSCC and OL ($P < 0.05$): 12-amino-octadecanoic acid, ubiquinone, deoxypodophyllotoxin, zolpidem metabolite II, estradiol valerate, neuraminic acid, L-homocysteic acid, isosorbide dinitrate, muramic acid, retinol phosphate, 3-hydroxylidocaine glucuronide.
[48]	Pool of 28 metabolites	Untargeted	Significant - $P < 0.00$: Pyrroline hydroxycarboxylic acid, leucine plus isoleucine, choline, tryptophan, valine, threonine, histidine, pipecolic acid, glutamic acid, carnitine, alanine, piperidine, taurine, and two other metabolites; - $P < 0.0$: piperidine, alpha-aminobutyric acid, phenylalanine; - $P < 0.05$: betaine, serine, tyrosine, glutamine, beta-alanine, cadaverine.
[49]	Pool of 90 metabolites	Untargeted	Significant - up-regulated in OSCC ($P < 0.5$): decanedioic acid, 2-methyloctacosane, Octane, 3,5-dimethyl, pentadecane, hentriacontane, 5,5-diethylpentadecane, nonadecane, oxalic acid, 6-phenylundecanea, L-proline, 2-furancarboxamide, 2-isopropyl-5-methyl-1-heptanol, pentanoic acid, docosane; - up-regulated in OL ($P < 0.5$): eicosane.
[50]	Total sialic acid and total protein ratios, α-L-fucosidase.	Targeted	Significant - comparison of TSA/TP ratio in controls and patients with PMDs: statistically significant ($P < 0.001$ and $P < 0.005$); - comparison of serum and salivary α -L-fucosidase activity in controls, patients with OPC, and oral cancer patients: higher in patients with OPMDs and oral cancer patients as compared to the controls ($P < 0.088$ and $P < 0.001$, respectively); - TSA/TP ratio and α -L-fucosidase activity significantly discriminated between controls and oral cancer patients ($P < 0.001$ and $P < 0.0001$, respectively); - α-L-fucosidase activity significantly discriminated between controls and patients with OPMDs ($P < 0.014$) and patients with OPMD and oral cancer patients ($P < 0.003$).
[51]	Pool of 130 metabolites N-acetyl-D-glucosamine, L-pipecolic acid, L-carnitine, phosphorylcholine, deoxyguanosine	Untargeted Targeted	Significant - overexpressed in the SCCT than in the healthy control group; N-acetyl-L-phenylalanine, phosphorylcholine, D-alanyl-D-alanine, deoxyguanosine, cytidine, N-acetyl-D-glucos- amine, L-pipecolic acid, palmitoyl-L-carnitine, N-glycyl-L-proline and L-carnitine. Significant - up-regulated in SCCT than the CG ($P < 0.5$).

(*continued on next page*)

Table 4 (*continued*)

Ref.	Salivary metabolites	Targeted/ Untargeted	Statistical association with OSCC
[52]	Pool of 15 metabolites	Untargeted	Significant - up-regulated in OSCC ($P < 0.05$): lactic acid, hydroxyphenyllactic acid, N-nonanoylglycine, 5-succinic acid, ornithine, hexanoylcarnitine, propionylcholine; - down-regulated in OSCC ($P < 0.05$): carnitine, 4-hydroxy-L-glutamic acid, acetylphenylalanine, sphinganine, phytosphingosine and S-carboxymethyl-L-cysteine.
[53]	41 salivary metabolites in OSCC relative to the CG 61 metabolites in OSCC relative to OLK 27 metabolites in OLK relative to the CG	Untargeted	Significant - up-regulated in OSCC than in CG ($P < 0.05$): lactic acid, *n*-eicosanoic acid; - down-regulated in OSCC than in CG ($P < 0.05$): valine, γ-aminobutyric acid, phenylalanine; - up-regulated in OSCC than in OL ($P < 0.05$): alanine, lactic acid, 3-indolepropionic acid, *n*-eicosanoic acid; - down-regulated in OSCC than in OL ($P < 0.05$): valine, γ-aminobutyric acid, isoleucine, leucine, n-tetradecanoic acid, proline, phenylalanine, threonine, n-dodecanoic acid; - up-regulated in OL than in CG ($P < 0.05$): isoleucine, n-tetradecanoic acid, phenylalanine, threonine, homocysteine, 4-methoxyphenylacetic acid.
[54]	Endogenous porphyrin	Targeted	SIGNIFICANT The overall fluorescence emission intensity of OSCC saliva was found to be significantly higher than CG.

Legend: 8-OHdG: 8-hydroxy-2-deoxyguanosine; MDA: malondialdehyde; OC: oral cancer; SSC: squamous cell carcinoma; OSCC: oral squamous cell carcinoma; OPMDs: oral potentially malignant disorders; OPC: oropharyngeal cancer; OLP: oral lichen planus; OED: oral epithelial dysplasia; PSOML: persistent suspicious oral mucosal lesions; HNSCC: head and neck squamous cell carcinoma; CG: control group; OL: oral leukoplakia; TSA/TP: total sialic acid/total protein; SCCT: squamous cell carcinoma of the tongue.

resulted in statistical differences between OSCC cases and controls. ($P < 0.001$).

Some targeted metabolites, such as cortisol and neopterin, have been studied for their role in modifying the immune response [30,41].

Cortisol is a steroid hormone secreted in response to physical and psychological stress. In some types of tumors, such as breast, ovary, kidney, lung and colon cancer, high cortisol levels may induce immunosuppression, facilitate tumor progression and be associated with a worse prognosis [61–64]. Salivary cortisol concentrations were studied by Bernabè et al. [29] in relationship with OSCC, in different groups of patients. OSCC group cortisol was approximately two times higher than in the HC ($P < 0.005$), at risk ($P < 0.002$) and leukoplakia ($P < 0.002$) groups. Patients with advanced disease (clinical stage III and IV) showed salivary cortisol levels higher ($P = 0.015$) than concentrations at early stage (clinical stages I and II).

Neopterin is the catabolic product of guanosine triphosphate, a purine nucleotide. It is known to be a marker of immune system activation; increased neopterin concentrations are established in patients with an activated cellular immune response (Th1), including allograft rejection, viral infection, autoimmune disorders, and various malignant tumors [65]. Salivary neopterin in OSCC was investigated by Pink et al. [41]. Pre-operative salivary neopterin concentration was significantly higher than HC ($P = 0.004$), decreasing significantly after the surgery ($P = 0.001$). No significant differences were observed in salivary post-operative neopterin concentration compared to HC ($P = 0.353$).

Pyruvic acid is the product of glucose degradation through glycolysis in aerobic conditions. Indeed, Bhat et al. described how levels were high in cancer subjects to healthy subjects and showed a statistically significant difference between the groups ($P < 0.05$) [30].

Malondialdehyde salivary concentration was also investigated together with 8-hydroxy-2' -deoxyguanosine (8-OHdG), which likewise is a biomarker of oxidative stress and carcinogenesis [66], by authors Kaur et al. [37]. Salivary concentrations were statistically significantly higher in OSCC patients compared to HC ($P < 0.005$), with a combination of markers sensitivity and specificity at 82 % and 81 %, respectively. Moreover, salivary MDA and 8-OHdG were able to distinguish OPMD patients from HC ($P < 0.005$, 81 % and 80 %) and OSCC from OPMDs ($P < 0.005$, 80 % and 79 %).

Salivary endogenous porphyrins concentrations were investigated by Yuvaraj et al. [54]. The elevated porphyrin level in OSCC may depend on an altered amino acid degradative pathway and may be released from the malignant cells through pinocytosis. The overall fluorescence emission intensity of OSCC saliva was found to be significantly higher than that of healthy subjects, with 85.7 % sensitivity and 93.3 % specificity on the classification of OSCC from healthy; distinct fluorescence emission peaks at 625 nm and 687 nm relative to endogenous porphyrin were 3.4 and 2 times higher than that of normal saliva respectively.

3.5. Untargeted metabolomic

The untargeted approach was the technique chosen in 16 studies [23,25,33,35,36,38–40,43,44,46,47,49,51,53,67] (Table 4).

Wei et al. detected lower levels of salivary valine, leucine and isoleucine (branched-chain amino acids, BCAA) in OSCC patients than HC and report how valine, as a marker, can discriminate disease cases with 82.4 % sensitivity and 75.7 % specificity [53]. In addition, isoleucine was also found significantly downregulated in OSCC than OL cases [38], and Ohshima et al. (valine $P = 0.002$, isoleucine $P = 0.011$, leucine $P = 0.004$), Reddy et al. ($P < 0.05$) and Sugimoto et al. ($P < 0.001$) detected significantly decreased levels of the three BCAAs in cancer patient compared to HC [40,43,48].

Alpha-amino acid alanine was significantly upregulated in OSCC saliva of all the 3 records ($P < 0.05$ [40]; $P < 0.05$ [43]; $P < 0.05$ [48]); in addition, significant alterations emerged comparing OL and HC ($P < 0.01$, [53]).

Aspartate, like glutamate, is an intermediate in the urea cycle and plays a key role in the tricarboxylic acid (TCA) cycle. Both were significantly discriminant between OSCC and OPMDs [46]. Aspartate is significantly elevated in OSCC compared to HC, as reported by Reddy et al. [43].

Song X. et al. [46] highlight how lysine degradation pathways (polyamine metabolism) were significantly changed both in OPMDs and OSCC; lysine was found to be lower in cancer than in its OPMDs, suggesting that it serves as a nitrogen source and that such biochemical alterations are involved in cell hyperproliferation. On the other hand, lysine was found to be significantly upregulated by Reddy et al. 22 ($P < 0.05$) [43].

Pipecolinic acid is a cyclic amino acid, an intermediate of the lysine degradation pathway. It was detected by Song et al. [46], Ishikawa et al. [23,35], Sugimoto et al. [48], showing a significant elevation trend compatible with the increase of lysine metabolism in cancer cells. Wang et al. explored a pool of potential markers, including pipecolinic acid, for OSCC/HC discrimination; through UPLC-MS metabolomics, authors reported similar significant upregulation trends in cancer ($P < 0.0001$) as well as high levels of sensitivity (100 %) and specificity (96.7 %) for disease discrimination when such markers were used in combination [27].

Arginine, ornithine, and citrulline are strictly linked in several bidirectional metabolic pathways, such as the urea cycle (also known as the ornithine cycle) and the synthesis of nitrous oxide.

Arginine stimulates protein translation and anabolic and proliferative functions related to polyamines, which become unregulated in cells after malignant transformation [68]. It is reported how levels were increased between HC and OPMDs while were lower in OSCC as the amino acid degradation for energetical and synthetic purposes proceeds simultaneously with cancer development [69,70]. Reddy et al. report it as significantly upregulated ($P < 0.05$) [43], while Song et al. and Ishikawa et al. [36,36,46] highlighted its downregulation in OSCC and OPMDs other than HC (and persistent suspicious epithelial lesions ($P < 0.015$), respectively.

Ornithine is a major source of polyamines in mammalian physiological systems [71]. It was successfully studied as a biomarker in non-melanoma skin cancer as well as in breast and pancreatic cancer [48,72]. Four records considered this molecule with a statistically significant potential OSCC salivary marker; Song et al. [46] and Lohavanichbutr et al. [38] and Ishikawa et al. [35] found low levels in cancer patients than HC ($P < 0.05$, $P < 0.002$ and $P < 0.05$, respectively), while Wang et al. [25] ($P < 0.03$, 53.8 % sensitivity and 86,7 % specificity) report about higher levels than HC.

Citrulline-involving pathways, such as arginine biosynthesis, were investigated for cancer relationships [73]. In salivary analysis for OSCC, Song et al. [46] report significant differences in citrulline levels between HC, OPMDs and cancer (with a trend of upregulation), as well as Lohavanichbutr et al. [38], that described discriminant capacity and downregulation in disease cases other than HC ($P < 0.010$).

Proline, a proteinogenic amino acid, could be synthesized from ornithine or glutamate. Through salivary metabolomics, it was found to be downregulated between OSCC and HC in the study of Lohavanichbutr et al. ($P < 0.003$ cases vs. HC and $P < 0.001$ T1/T2 vs. HC) and Mikkonen et al. ($P = 0.043$) [38,39].

The role of serine, glycine, and threonine was studied by Redd et al. and Lohavanichbutr et al., which found the 3 amino acids to be upregulated in OSCC than HC [38,43]. Conversely, Lohavanichbutr et al. [38] found significant lower levels of glycine in T1/T2 OSCC saliva compared to controls ($P < 0.003$), with a strong pathway impact (>12 log(P)) on cell metabolism. Threonine was found to be significantly capable of distinguishing OSCC from HC ($P < 0.001$), whereas, together with serine, it was elevated in males with OSCC ($P < 0.046$) [48]; it is worth noting that threonine was downregulated in OSCC compared to OL ($P < 0.05$) [53] as with OPMDs in the work of Song *et* al [46]. The latter also reports a slight upregulation of serine in the OSCC/OPMDs and OSCC/HC ratios ($P < 0.0001$).

Phenylalanine was detected by Song et al. [46], Reddy et al. [43], Wei et al. [53] and Sugimoto et al. [48] with different trends. It was reported that the marker can discriminate between OSCC, OPMDs and HC [46] and OSCC cases from HC ($P < 0.01$) [48]. However, Reddy et al. reported higher levels of the molecule in cancer patient [43], while Reddy et al. [43] described the opposite trend comparing disease to HC.

Three records reported data about the significant presence of choline in the saliva with overlapping trends: Ohshima et al. [40] described choline as the metabolite with the highest discriminant significance between cases and HC ($p < 0.001$), like Sugimoto et al. ($P < 0.001$) [48]. The upregulated trend was also reported by Ishikawa et al. ($P < 0.05$) without any difference related to the histological grade or clinical stage [35]. Choline derivatives, such as phosphorylcholine and propionylcholine, were considered among the main metabolites altered during malignant progression from HC to OSCC. Song et al. report about the upregulation of phosphorylcholine in all OPMDs/HC, OSCC/OPMDs and OSCC/HC ratios ($P < 0.0001$) [46]; similarly, Wang et al. described the upregulation of propionylcholine in OSCC stage I-II vs. HC ($P < 0.0001$, sensitivity 76.9 %, specificity 96.7 %) [25].

Putrescine and cadaverine are products of arginine and lysine breakdown, respectively. The pro-carcinogenic role of putrescine was investigated for a few diseases, such as colon and pancreas [74]. Song et al., Ishikawa et al., and Sugimoto et al. highlight differences in salivary concentration in the presence of OPMDs and OSCC. Song et al. described a trend of upregulation of putrescine from HC to OSCC (OPMDs vs. HC, $P < 0.0075$, OSCC vs. OPMDs $P < 0.0001$ and OSCC vs. HC $P < 0.00001$) [46]. Ishikawa et al., taking into consideration oral lichen planus cases as a baseline, reported an elevated trend in salivary diamine concentration in OSCC, significant for discrimination [35]. A similar trend was identified by Sugimoto et al. in discrimination from HC from oral cancer [48].

Salivary cadaverine was detected by Song et al., Ohshima et al., and Sugimoto *et* al. with an upregulated OSCC/HC ratio trend [40,46,48]. Moreover, Song et al. described a significant peculiar increment of fold change ratio in HC, OPMDs and cancer (OPMDs/HC $P < 0.0006$, OSCC/OPMDs $P < 0.0056$, OSCC/HC $P < 0.0010$). The role of bacterial metabolism and the oral hygiene conditions were probably a contributing factor to this trend [75].

Song et al. and Ishikawa et al. studied the role of salivary spermidine, showing a significant upregulation during OSCC development and progression ($P < 0.010$ and $P < 0.0043$, respectively), supporting the idea that a further exploration of polyamine metabolism (also including spermine, putrescine and their acetylated forms), would be useful for detection of reliable salivary markers.

Salivary lactate is known to be a common host-derived metabolite or of bacterial origin [76]. Its quantitative fluctuation is associated with several diseases, such as OSCC, HIV, periodontal disease, sarcoidosis and B hepatitis. This review identified 3 records reporting a significant trend of upregulation in the saliva of oral cancer patients rather than HC [25,38,53].

Eventually, even if not comparable with other results, it is worth mentioning results by Shigeyama et al., in which salivary fluid analysis was performed to identify volatile organic compounds (VOCs) through GC-MS [44]. Shigheyama et al. identified patterns of VOC characteristics of OSCC compared to HC. Among the 80 compounds detected, 12 were significantly tumor-specific and likely to be candidates as disease biomarkers. The most promising compounds able to distinguish HC and OSCC were 2-pentanone and hexadecanoic acid (most decreased in cancer), undecane (disappeared in cancer) and 1,3-butanediol (newly generated in cancer).

4. Discussion

The metabolome of malignant tumors is intricately linked to the genotype and phenotype of cancer cells, reflecting altered cellular pathways. Oncogenes, protein changes, and abnormal enzymatic activities contribute to metabolic reprogramming in neoplastic cells [46]. Specific metabolites can thus serve as biomarkers, providing insights into tumors at various development stages.

Saliva, or "whole saliva" (WS), is a composite fluid produced by the salivary glands and gingival sulcus. Given its proximity to the development sites of OSCC and potentially malignant disorders, saliva is an ideal medium for detecting neoplastic changes through metabolomic analysis [39,75]. This systematic review identified 16 targeted and over 1000 untargeted salivary metabolites related to OSCC, emphasizing the potential of salivary biomarkers (Table 4).

Among targeted metabolites, sialic acid was the most investigated. It shows promise as a biomarker for different histological grades and distinguishing between OPMDs and healthy controls.

The immune system's role in tumorigenesis and growth control is crucial, as it can both suppress and promote cancer development [77]. Tumors often exhibit high glycolytic activity, known as the Warburg effect, where cancer cells consume large amounts of glucose and produce lactic acid even in the presence of oxygen. This metabolic shift supports the energy and biosynthetic needs of rapidly proliferating tumor cells [78]. Three studies in this review highlighted significant increases in salivary lactic acid in OSCC patients compared to HCs [25,

38,53].

Pyruvate, another key metabolite, was found at significantly higher concentrations in cancer subjects, reflecting its role in supporting glycolysis [30]. Similarly, amino acids are crucial for energy production, protein synthesis, and cell proliferation. Seven studies reported significant changes in amino acids and their pathways, suggesting their potential as biomarkers [36,38,40,43,46,48,68].

Branched-chain amino acids like valine, leucine, and isoleucine are critical for fueling the tricarboxylic acid cycle and oxidative phosphorylation. Increased BCAA levels have been associated with tumor growth inhibition, while deficiencies can promote metastasis [79]. This review found BCAAs to be significantly altered in OSCC saliva [38,40,43,48,53].

Other metabolites, including lysine, pipecolinic acid, arginine, ornithine, citrulline, putrescine, and cadaverine, are involved in the urea cycle and polyamine metabolism. These metabolites suggest that nitrogen metabolism plays a role in cell proliferation and cancer progression [24,25,35,40,43,46,48].

Citrulline, which appears in proteins through post-translational modification, is linked to processes like apoptosis and differentiation, potentially facilitating cancer development. Its role in pathways like Wnt and androgen receptor signaling further underscores its importance in cancer biology [80].

This review acknowledges limitations due to the heterogeneity of clinical characteristics among study populations and the varying quality of included studies. Saliva's dynamic composition is influenced by factors like oral health, microbiome activity, and dietary habits. External factors, such as sample collection and processing conditions, also impact the stability of the salivary metabolome, posing challenges for diagnostic accuracy [81]Despite these issues, the reviewed studies showed a high degree of consistency in study design and saliva collection procedures.

5. Conclusion

Even though there is a lack of good quality research, salivary metabolomics seems to offer a promising approach for the identification of biomarkers associated with the presence of OSCC at its earliest stage. Most of the studies included in the present review support a diagnostic role of several specific molecules. If confirmed from further studies, identification of sialic acid, neopterine, pyruvate, aspartate, glutamate, and BCAA (valine, leucine and isoleucine) may serve as salivary tests able to promptly discriminate patients with OSCC. Specifically, cortisol appears to play an important role in discriminating OSCC in its early stages.

Financial support and sponsorship

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 7

Salivary Diagnosis of Dental Caries: A Systematic Review

Antonelli R, *Massei V, Ferrari E, Gallo M, Pertinhez TA, Vescovi P, Pizzi S, Meleti M. Current Issues in Molecular Biology. 2024; 46(5):4234-4250. https://doi.org/10.3390/cimb46050258*

Published on Current Issues in Molecular Biology
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Review

Salivary Diagnosis of Dental Caries: A Systematic Review

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Abstract: The activity of dental caries, combined with its multifactorial etiology, alters salivary molecule composition. The present systematic review was developed to answer the following question: "Are salivary biomarkers reliable for diagnosis of dental caries?". Following the "Preferred Reporting Item for Systematic Reviews and Meta-analysis" (PRISMA) guidelines, the review was conducted using multiple database research (Medline, Web of Science, and Scopus). Studies performed on healthy subjects with and without dental caries and providing detailed information concerning the clinical diagnosis of caries (Decayed, Missing, Filled Teeth-DMFT and International Caries Detection and Assessment System-ICDAS criteria) were included. The quality assessment was performed following a modified version of the Joanna Briggs Institute Prevalence Critical Appraisal Checklist. The protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO, ID: CRD42022304505). Sixteen papers were included in the review. All studies reported statistically significant differences in the concentration of salivary molecules between subjects with and without caries ($p < 0.05$). Proteins were the most investigated molecules, in particular alpha-amylase and mucins. Some studies present a risk of bias, such as identifying confounding factors and clearly defining the source population. Nevertheless, the 16 papers were judged to be of moderate to high quality. There is evidence that some salivary compounds studied in this review could play an important diagnostic role for dental caries, such as salivary mucins, glycoproteins (sCD14), interleukins (IL-2RA, 4,-13), urease, carbonic anhydrase VI, and urea.

Keywords: biomarkers; caries detection; cariology; dental caries; salivary diagnostics

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Citation: Antonelli, R.; Massei, V.; Ferrari, E.; Gallo, M.; Pertinhez, T.A.; Vescovi, P.; Pizzi, S.; Meleti, M. Salivary Diagnosis of Dental Caries: A Systematic Review. *Curr. Issues Mol. Biol.* **2024**, *46*, 4234–4250. https://doi.org/10.3390/cimb46050258

Academic Editor: Ru Chih C. Huang

Received: 16 March 2024
Revised: 22 April 2024
Accepted: 30 April 2024
Published: 2 May 2024

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1. Introduction

Dental caries (also known as "tooth decay") is a pathological process consisting of the demineralization of dental hard tissues and the formation of a dental cavity, often causing pain and, if not treated, tooth loss [1].

Even if its incidence in the population between 5 and 12 years and 25 and 44 years in Western countries has decreased in the last four decades [2], dental caries remains one of the most widespread infectious diseases. It has been estimated that there are approximately 2.4 billion people with untreated cavities in permanent teeth, as well as 621 million children with caries in deciduous teeth [3].

Dental caries has a multifactorial etiology. Two primary causal factors are the frequent consumption of free sugars and the metabolism of some commensal tooth-adherent bacteria. Several oral microorganisms, by metabolizing fermentable carbohydrates, produce organic acids. Such acids cause a decrease in salivary pH, inducing tooth tissue demineralization, starting from the external surface of the enamel. A remarkable variety of microorganism species associated with dental caries has been identified, including *Streptococcus mutans*,

which is the most prevalent bacteria found in subjects with dental caries (58.3%) [4]. However, many other host and behavioral factors, such as enamel defects, alterations of salivary pH, flow rate and composition, poor oral hygiene, and low socioeconomic level also play an important role in dental caries pathogenesis [1].

An accurate diagnosis, screening programs, and individual risk assessments are fundamental to preventing this pathology and reducing the number of untreated caries. Currently, the diagnosis of dental caries is mainly based on clinical inspection and, when necessary, on radiographic examination.

The World Health Organization (WHO) recommended using the decayed, missing, and filled teeth (DMFT) index to monitor the distribution and prevalent trends of dental caries. Such an index is based on clinical examination and reflects a patient risk profile for developing dental caries [5].

The International Caries Detection and Assessment System (ICDAS) was developed in 2005 to integrate different systems into a single index that provides information on dental caries stage, activity, and risk profile. The system comprises seven codes, ranging from code 0 (healthy teeth) to codes 5 and 6, indicating caries with exposed dentine [6]. The ICDAS also requires a specific tool (laser-induced fluorescence—Diagnodent™ Pen) to assess tooth decay's stage and gravity.

In the last 20 years, the interest in new non-invasive techniques for the detection of caries, like quantitative light fluorescence (QLF), digital fiber-optic transillumination (DIFOTI—KaVo Diagnocam™), and electric conductance (EC), has significantly increased [7]. At the same time, the interest in the diagnostic role of saliva has significantly increased.

Saliva is a complex biological fluid produced by parotid (20%), submandibular (65–70%), sublingual (7% to 8%), and minor salivary glands located in the lips, tongue, palate, cheeks, and pharynx (<10%), as well as by gingival sulcus (crevicular fluid) [8]. Saliva secretion, its flow rate, and composition depend on several factors, including the type and size of glands, nutritional status, gender, age, and emotional state. Whole saliva (WS) consists of a pool of different organic and inorganic components which represent the physiology of the human body [9]. Besides the massive presence of water, saliva contains metabolites, enzymes, antibodies, hormones, antimicrobial molecules, and cytokines, all potential biomarkers for oral and systemic diseases [10].

Biomarkers include molecules belonging to the genome, epigenome, transcriptome, proteome, and metabolome that may be useful for monitoring health status, disease diagnosis and prognosis, or evaluating response to treatment [11]. Emerging evidence suggests that saliva is an excellent and innovative matrix for the search of molecular markers due to its intrinsic properties (e.g., the complexity of composition, abundance of molecules, fluid availability), combined with its non-invasive sampling and ease of collection, transport, and storage [8].

The present systematic review aims to answer the question: "Are salivary biomarkers reliable for the diagnosis of dental caries?" formulated according to the "Population or problem", "Intervention or exposure", "Comparison", "Outcome" (PICO) worksheet.

2. Materials and Methods

The Preferred Reporting Item for Systematic Reviews and Meta-Analysis (PRISMA) guidelines was used to conduct the present review [12]. The protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO, Centre for Reviews and Dissemination, York, UK, ID: CRD42022304505).

2.1. Search Strategy

We searched Medline, Scopus, and Web of Science databases for original scientific papers published in English after 2000. The search terms were "saliva" or "salivary biomarkers", combined through the Boolean indicator "AND" with "tooth decay" and "dental caries". Periodic screenings of the databases were performed between August 2021 and Oc-

tober 2023. Duplicates were eliminated using End Note X9© software (Clarivate Analytics, Philadelphia, USA, London, UK).

Two independent researchers performed a first-level screening by evaluating titles and abstracts. Conference proceedings, meeting abstracts, short communication, editorials, letters to the editor, and reviews were excluded. Only studies performed on humans, providing detailed information on the clinical diagnosis of dental caries, and specifically applying DMFT and/or ICDAS criteria were included. References in literature reviews were also screened to identify other possible papers of interest. Final eligibility was assessed through full-text evaluation according to the exclusion and inclusion criteria summarized in Table 1.

Table 1. Inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria
English language	Systematic reviews, letters to the editor, editorials, short communications, meeting abstracts
Papers published after June 2000	Studies on pH and buffering capacity evaluation
Studies on humans	Studies on the inorganic composition of saliva (ions)
	Studies on "risk of caries"
Studies applying DMFT and/or ICDAS criteria	Studies on the evaluation of deciduous teeth and/or early childhood caries (ECC)
	Studies on the assessment of the caries level of the child in relation to the mother
	Studies on microbiome and bacteria

Studies focused on the pH, buffering capacity, and inorganic composition of saliva were excluded. Papers evaluating the risk of caries, deciduous teeth, early childhood caries (ECC), and the caries level of the child in relation to the caries experience of the mother were excluded. Additionally, we excluded studies focused on dental plaque and oral microbiome and studies performed on subjects with systemic disorders and/or oral diseases other than caries and on alcohol and/or drug users. Papers on medical devices (for experimental purposes) or involving the administration of products for therapeutic uses were also excluded.

Selected studies underwent data extraction and critical appraisal.

2.2. Data Extraction

Data extracted from each study were summarized into Excel®tables.

Table 2 shows the general characteristics of the selected studies, including the title, authors, year of publication, type of study, and main clinical features of the evaluated subjects.

Table 2. General characteristics of selected studies.

Authors	Title	Design of Study	N° Subjects
Ahmadi-Motamayel et al., 2018 [13]	Salivary and Serum Antioxidant and Oxidative Stress Markers in Dental Caries	Case–control study	56 CG (M:F = 28:28; 17 y) - DMFT = 0 62 AC (M:F = 27:35; 17 y) - DMFT $\geq$ 5
Ashwini et al., 2020 [14]	Dentin degradonomics—The potential role of salivary MMP-8 in dentin caries	Case–control study	25 CG - DMFT = 0 50 AC - 25 (caries not involving more than two teeth) - 25 (caries involving more than three teeth)
Ayad et al., 2000 [15]	The Association of Basic Proline-Rich Peptides from Human Parotid Gland Secretions with Caries Experience	Case–control study	9 CG (M:F = 4:5; 59.2 y) - DMFT = 0 9 AC (M:F = 4:5; 51.2 y) - DMFT = 38.4

Table 2. *Cont.*

Authors	Title	Design of Study	N° Subjects
Banderas-Tarabay et al., 2002 [16]	Electrophoretic Analysis of Whole Saliva and Prevalence of Dental Caries. A Study in Mexican Dental Students	Case–control study	24 CG (19 y) - DMFT < 4 40 AC (19 y) - DMFT > 10
Bilbilova et al., 2012 [17]	Correlation between Salivary Urea Level and Dental Caries	Case–control study	40 CG (16 y) - DMFT = 0–3 40 AC (16 y) - DMFT > 10
Gabryel-Porowska et al., 2014 [18]	Mucin Levels in Saliva of Adolescents with dental caries	Case–control study	8 CG (18 y) - DMFT = 3 27 AC (18 y) - DMFT > 11
Gornowicz et al., 2012 [19]	Pro-Inflammatory Cytokines in Saliva of Adolescents with Dental Caries Disease	Case–control study	10 CG (18 y) - Dmft = 0 27 AC (18 y) - DMFT = 11.33
Gornowicz et al., 2014 [20]	The Assessment of sIgA, Histatin-5, and Lactoperoxidase Levels in Saliva of Adolescents with Dental Caries	Case–control study	8 CG (18 y) - DMFT = 3 27 AC (18 y) - DMFT > 11
Kulhavá et al., 2020 [21]	Proteomic Analysis of Whole Saliva in Relation to Dental Caries Resistance	Case–control study	12 CG (31.8 ± 7.6 y) - DMFT = 0–1 15 AC (38.4 ± 5.6 y) - DMFT = 7–12
Mira et al., 2017 [22]	Salivary Immune and Metabolic Marker Analysis (SIMMA): A Diagnostic Test to Predict Caries Risk	Case–control study	10 CG (19–39 y) 10 AC (19–39 y)
Nireeksha et al., 2017 [23]	Salivary Proteins as Biomarkers in Dental Caries: In Vivo study	Case–control study	20 CG (25–40 y) 60 AC (25–40 y) - 20 DMFT = 1–3 - 20 DMFT = 4–10 - 20 DMFT > 10
Paqué et al., 2021 [24,25]	Salivary Biomarkers for Dental Caries Detection and Personalized Monitoring	Case–control study	18 CG 38 AC
Piekoszewska-Ziertek et al., 2020 [25]	Polymorphism in the CAVI Gene, Salivary Properties and Dental Caries	Case–control study	9 CG (13.25 ± 1.72 y) - DMFT = 0 121 AC (13.25 ± 1.72 y) - DMFT > 0
Prester et al., 2017 [26]	Salivary sCD14 as a Potential Biomarker of Dental Caries Activity in Adults	Case–control study	25 CG (35 y) - DMFT = 16.5 - Cavities = 6.8 30 AC (31 y) - DMFT = 13.8 - Cavities = 0
Reyes et al., 2014 [27]	Caries-Free Subjects Have High Levels of Urease and Arginine Deiminase Activity	Cross-sectional study	10 CG - DMFT = 0 12 AC - DMFT ≥ 4
Yazid et al., 2020 [28]	Caries Detection Analysis in Human Saliva Alpha Amylase	Case–control study	12 CG (18–55 y) - ICDAS = 0 15 AC (18–55 y) - ICDAS ≥ 4

Legend: CG = control group; AC = active caries; F = females; M = males; y = years old; DMFT = decayed, missing, and filled tooth index, ICDAS = International Caries Detection and Assessment System score.

Table 3 displays the type of saliva sample, the conditions for saliva collection, and the analytical procedures used for identifying and quantifying the salivary compounds (e.g., the type of specimen, method of collection, and analysis).

Table 3. Analysis of saliva sample, conditions for saliva collection, and the analytical procedures used.

Authors	Typology of Saliva Sample	Saliva Collection	Biomarker of Analysis
Ahmadi-Motamayel et al., 2018 [13]	Unstimulated whole saliva	Samples were obtained by spitting for 5 min.	Sialo-chemical analysis
Ashwini et al., 2020 [14]	Stimulated saliva	Samples were collected after chewing on a paraffin wax for 5 min.	ELISA
Ayad et al., 2000 [15]	Stimulated parotid (ductal) saliva	Samples were collected in the morning. Subjects were requested not to eat for 2 h before collection. Gustatory stimulated secretions were obtained by means of sugar-free lemon drops.	HPLC
Banderas-Tarabay et al., 2002 [16]	Unstimulated whole saliva	Samples were collected in the morning. Subjects refrained from eating, drinking, smoking, and oral hygiene for at least 2 h prior to saliva collection.	Electrophoresis
Bilbilova et al., 2012 [17]	Unstimulated whole saliva Food-stimulated whole saliva	Samples were collected in the morning, in the fasted state, and without oral hygiene. Samples were taken from all the participants at different time intervals: 5, 30, and 60 min after the meal.	Urase-based enzymatic method
Gabryel-Porowska et al., 2014 [18]	Unstimulated whole saliva	Samples were collected in the morning. Subjects abstained from eating and drinking for 2 h. Unstimulated whole saliva was collected for 10 min by a spitting method.	ELISA
Gornowicz et al., 2012 [19]	Unstimulated whole saliva	Samples were collected in the morning. Subjects abstained from eating and drinking for 2 h. Samples were collected for 10 min by a spitting method.	ELISA
Gornowicz et al., 2014 [20]	Unstimulated whole saliva	Samples were collected in the morning. Subjects abstained from eating and drinking for 2 h. Samples were collected by a standard method in sterilized tubes (placed on ice after collection).	ELISA
Kulhavá et al., 2020 [21]	Unstimulated whole saliva	Samples were collected in the morning. Volunteers were requested not to eat or drink and brush their teeth for 1–2.5 h prior to the trial.	LC-MS
Mira et al., 2017 [22]	Unstimulated whole saliva	Five milliliters of non-stimulated saliva samples were taken by drooling at 30 min, 6, 12, and 24 h after toothbrushing in a sterile 50 mL tube (avoiding spitting or plaque removal with the tongue).	ELISA
Nireeksha et al., 2017 [23]	Unstimulated whole saliva	Samples were collected in the morning. Subjects were asked to abstain from toothbrushing, using mouthwash, and eating/drinking for 2 h prior to sample collection.	PAGE
Paqué et al., 2021 [24,25]	Unstimulated whole saliva	Samples were collected in the morning. The participants were asked not to eat, drink sugary drinks, or perform any oral hygiene measures the night before the saliva donation. Water intake was permitted.	ELISA
Piekoszewska-Ziertek et al., 2020 [25]	Unstimulated whole saliva Buccal smear	Samples were collected in the morning. Subjects were instructed to fast for at least 2 h and not to use antibacterial mouth rinse. The smear was collected for about 2 min using a special buccal swab.	ELISA RT-PCR
Prester et al., 2017 [26]	Unstimulated whole saliva (n = 55) Stimulated whole saliva (n = 55)	Samples were collected between 8–11 a.m. and 2–4 p.m. Two hours before collection, the participants were asked to refrain from eating, drinking, smoking, and toothbrushing to obtain a relatively constant baseline. The resting saliva was collected before chewing 5 g of pure paraffin wax for five minutes without swallowing.	ELISA

Table 3. *Cont.*

Authors	Typology of Saliva Sample	Saliva Collection	Biomarker of Analysis
Reyes et al., 2014 [27]	Unstimulated whole saliva	Saliva sample was collected by expectorating 3 mL of saliva in a sterile plastic tube. Subjects were instructed not to eat for 12 h prior to sample collection and to abstain from any type of oral hygiene.	Biochemical analysis and spectrophotometry (Thermo Spectronic Unicam UV-530 UV–visible)
Yazid et al., 2020 [28]	Unstimulated whole saliva	Subjects were instructed to accumulate saliva and drop it into a cryovial (about 2 mL). A protease inhibitor was added to the saliva samples.	UV-Vis spectroscopy

Legend: ELISA = enzyme-linked immunosorbent assay; HPLC = high-performance liquid chromatography; RT-qPCR = real-time polymerase chain reaction; LC-MS = liquid chromatography–mass spectrometry; PAGE = polyacrylamide gel electrophoresis.

Table 4 shows the statistical association between specific salivary molecules and dental caries.

Table 4. Statistical association between specific salivary molecules and dental caries.

Authors	Biomarker Category	Biomarker	Statistical Association with Dental Caries (P)
Ahmadi-Motamayel et al., 2018 [13]	Metabolite	MDA	0.001a (higher in AC)
Ashwini et al., 2020 [14]	Protein	MMP8	< 0.05a (higher in AC)
Ayad et al., 2000 [15]	Protein	Ps1 (PRB1) Con1 (PRB2) Pmo1 (unassigned gene)	Ps1 = < 009a (higher in CF) Con1 < 009a (higher in CF) Pmo1 < 015a (higher in CS)
Banderas-Tarabay et al., 2002 [16]	Protein	MG1 MG2 PRP 1	< 0.001 (lower in CS)
Bilbilova et al., 2012 [17]	Metabolite	Urea	< 0.01a (higher in CF)
Gabryel-Porowska et al., 2014 [18]	Protein	MUC1 MUC5B MUC7	MUC1 = 0.011 a (higher in AC) MUC5B = 0.06 (higher in AC) MUC7 =0.918 (higher in CG)
Gornowicz et al., 2012 [19]	Protein	IL-6 IL-8 TNF-α	IL-6 < 0.005 [a] (higher in AC) IL-8 < 0.008 [a] (higher in AC) TNF-α < 0.002 [a] (higher in AC)
Gornowicz et al., 2014 [20]	Protein	SIgA Histatin-5 LPO	SIgA = 0.003 [a] (higher in CA) Histatin-5 = 0.015 [a] (higher in CA) LPO = 0.02 [a] (higher in CA)
Kulhavá et al., 2020 [21]	Protein	Proteins in salivary supernatants: α-Amylase 1 Serum albumin Protein S100-A9 Immunoglobulin heavy variable 4–31 Immunoglobulin heavy constant α 1 Immunoglobulin κ constant Apolipoprotein A-I Immunoglobulin heavy variable 1–44 Cystatin B Lysozyme C Annexin A1 Polymeric immunoglobulin receptor Prolactin-inducible protein Proteins in salivary pellets: Annexin A1 Protein ς Cornulin	P not reported [a] (higher in CF) P not reported [a] (higher in AC)

Table 4. *Cont.*

Authors	Biomarker Category	Biomarker	Statistical Association with Dental Caries (P)
Mira et al., 2017 [22]	Protein Metabolite	LL37 IgA Statherin β-defensin 2 Collagen I Fibrinectin Formate	< 0.5 [a]
Nireeksha et al., 2017 [23]	Protein	Total protein IgA Mucin CRP Albumin globulin	Total protein < 0.001 [a] (higher in CF) IgA < 0.001) (higher in CF) Mucine < 0.01 [a*] (higher in AC) CRP < 0.01 [a**] (higher in AC) Albumin globulin < 0.001 [a***] (higher in AC)
Paqué et al., 2021 [24,25]	Protein	IL-4 IL-13 IL-2-RA Eotaxin (CCL11)	IL-4 = 4.1×10^{-13a} IL-13 = 3.1×10^{-12a} IL2-RA = 1.0×10^{-4a} Eotaxin (CCL11) = 4.4×10^{-4a}
Piekoszewska-Ziertek et al., 2020 [25]	Protein / Gene	CA VI rs2274333 A/G	CA VI = 0.014 [a] (lower in AC)rs2274333 A/G < 0.5 [a] (higher in AC)
Prester et al., 2017 [26]	Protein	sCD14	0.004 [a] in resting saliva (higher in AC) 0.001 [a] in stimulated saliva (higher in AC)
Reyes et al., 2014 [27]	Protein	Urease activity ADS activity	Urease activity 0.01 [a] (higher in CF) ADS activity = 0.02 [a] - Higher in CF
Yazid et al., 2020 [28]	Protein	Alpha-amylase	P not reported [a] (higher in AC)

Legend: AC = active caries; ADS = urease and arginine deiminase system; CA VI = carbonic anhydrase isozyme VI; CF = caries-free; CG = control group; CRP = C-reactive protein; CS = caries-susceptible; LPO = human lactoperoxidase; MDA = malondialdehyde; MG1 = high-molecular-weight mucin; MG2 = low-molecular-weight mucin; MUC1 = mucin-1; PRP-1 = acidic proline-rich protein-1; sCD14 = soluble form of CD14 (coreceptor); SI = statistically insignificant; SIgA = salivary IgA; SS = statistically significant. [a] Statistically significant. * Except between CF group and AC group I (DMFT = 1–3). ** Except for CF group and CA group I–group II (DMFT 1–10). *** Except for AC group II and AC group III (DMFT > 4).

2.3. Critical Appraisal

For critical appraisal, we used two modified versions of the Joanna Briggs Institute Prevalence Critical Appraisal Checklist (JBI critical appraisal) adapted to the scopes of the present review, one for case–control studies and another for analytical cross-sectional studies [29]. The detailed description of all questions from the JBI critical appraisal tool is reported in Supplementary Tables S1 and S2.

For case–control studies, because of the multifactorial etiology of dental caries, we excluded question 4 ("Was the exposure measured in a standard, valid, and reliable way?"); question 5 ("Was the exposure measured in the same way for cases and controls?"); and question 9 ("Was the exposure period of interest long enough to be meaningful?"). For the same reason, we did not consider question 3 ("Was the exposure measured in a proper and reliable way?") for the critical appraisal of cross-sectional studies.

An assessment of the risk of bias was performed by two authors. Disagreements were solved by a consultation with a third author.

3. Results

A total of 6940 papers were considered eligible for title and/or abstract screening, after removing duplicates. After further application of inclusion and exclusion criteria, one hundred and fifty articles were qualified for full-text evaluation. Finally, 16 papers were included in the review: 15 were case–control studies, and 1 was a cross-sectional study (Table 2). Figure 1 depicts the PRISMA flowchart of the selection procedure and the reasons for exclusion. The complete list of the excluded papers after full-text reading is reported in Supplementary Table S3.

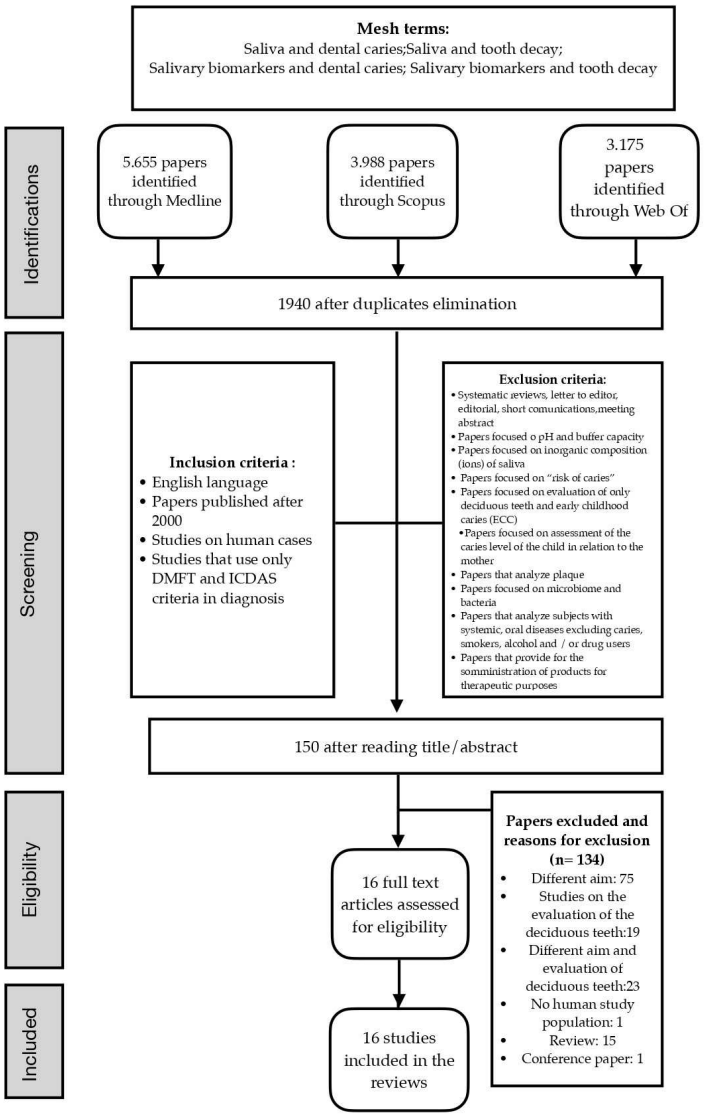

Figure 1. Flowchart diagram for the selection of 16 papers included in the review.

3.1. Study Population (Oral Status; General Health; Diagnosis of Caries)

Eleven papers [13,15,18–20,22–27] reported data on the oral health of the studied population, detailing that all the subjects exhibited good oral health status (e.g., healthy periodontium and oral mucosa, good oral hygiene, the absence of dental plaque accumulation). Prester et al. verified the absence of burning mouth syndrome and dry mouth condition [26], and only three studies reported on the correlation between saliva flow rates and the presence of caries [14,16,26].

Almost all the papers, except for three [14,18,20], reported data on general health status (e.g., the presence and/or absence of systemic diseases such as diabetes, hypertension, autoimmune disease, medications, diet history).

All the papers, except one [14], reported that the diagnosis of tooth decay was performed by an expert operator with a dental explorer. Only in one paper was an additional radiographic evaluation including digital bitewings and panoramic radiography used [14].

In 15 studies, a standardized criterion for diagnosis was adopted (DMFT in 14 studies and ICDAS in 1 study). The remaining study, considering "open caries lesions", did not mention any standard diagnostic criterion [24]. Eleven studies considered a DMFT score ≥ 3 to be indicative of a high risk of caries: two studies considered a DMFT score ≥ 11 [18,20], two a DMFT score ≥ 10 [16,17], one a DMFT score in the range of 7–12 [21], one a DMFT score ≥ 5 [13], two a DMFT score ≥ 4 [27,28], and one a DMFT

score ≥ 3 [22]. Two studies reported average values of DMFT of 38.4 and 11.33 [15,19], and the authors of two papers analyzed salivary biomarkers based on different DMFT score ranges (≤2 and ≥3; 1–3, 4–10, and ≥10) [14,23]. One study included patients with a high DMFT score and analyzed biomarkers based on the presence of dental cavities (a DMFT score of 13.8 with 0 cavities VS a DMFT score of 16.5 with 6.8 cavities—average values) [26]. Only one study considered a DMFT score ≥ 0, with an average value of 2.54 [25]. On the other hand, nine studies considered DMFT = 0 to be indicative of a low risk of caries [14,15,19,22,23,25,27,28,30], one study considered a DMFT score between 0 and 1 as a low risk of caries [21], three a DMFT score ≤ 3 [17,18,20], and only one a DMFT score < 4 [16].

3.2. Saliva Collection and Processing Method

Details on the methods for saliva collection and analysis are summarized in Table 3.

Thirteen studies (81%) reported the specific instructions given to patients before saliva collection. The instructions consisted of refraining from eating, drinking (beverages other than water), smoking, and/or practicing oral hygiene for 1 or 2 h before saliva collection. Paqué et al. [24] extended this period to the night before the procedure and Reyes et al. [27] to the previous 12 h.

Eleven articles (69%) reported information about the time of collection. Saliva was collected in the morning (8–12 a.m.), except for one study (2–4 p.m., in addition to 8–12 a.m.) [26].

Piekoszewska-Ziętek et al. reported the collection of salivary fluid using Salivette® collection tubes (Sarstedt AG&Co., Numbrecht, Germany) [25].

The identification of potential salivary biomarkers for the diagnosis of dental caries was conducted on WS samples in all the studies but one [15]. Ayad et al. [15] performed a gustatory-stimulated saliva collection from the parotid glands, using a modified Lashley cup [31].

Specifically, 12 studies (75%) reported the use of passive drooling or unstimulated spitting [13,16,18–25,27,28]; 2 studies (12.5%) evaluated both stimulated and unstimulated WS [17,26]; and 1 study analyzed stimulated saliva [14]. To stimulate salivation, participants were asked to chew pure paraffin wax for 5 min [14,26]. In one study, salivary secretion was stimulated by food ingestion, and three saliva samples were taken at 5, 30, and 60 min after the meal [17].

All the selected studies reported information about the handling and/or storage of saliva. The centrifugation conditions were heterogenous, and the most common parameters were 10,000× *g*, 15 min, and 4 °C. After centrifugation, refrigerated storage was adopted, with temperatures ranging from −20 °C to −80 °C.

Biochemical–analytical methods for the identification and quantification of salivary biomarkers included the following: (1) enzyme-linked immunosorbent assay (ELISA) [14,18–20,22,24,26]; (2) protein electrophoresis [16,23]; (3) real-time polymerase chain reaction (rt-PCR) [25]; (4) high-performance liquid chromatography (HPLC) [15]; (5) liquid chromatography–mass spectrometry (LC-MS) [21]; (6) spectrophotometry [27,28]; (7) enzymatic methods [17,28]; and (8) sialo-chemical analysis [13].

3.3. Critical Appraisal

Critical appraisals are summarized in Figure 2A,B for case–control and cross-sectional studies, respectively.

In total, 4 out of the 15 case–control studies received seven "yes" answers to seven questions [15,16,25,26]. Two received six "yes" answers [18,24], seven received five "yes" answers [13,17,20–23,28], and two received four "yes" answers [14,19].

Since all 15 case–control studies reported the same specific diagnostic method for all the subjects of the study, as well as an appropriate statistical analysis, they were positively evaluated for questions 3 and 10.

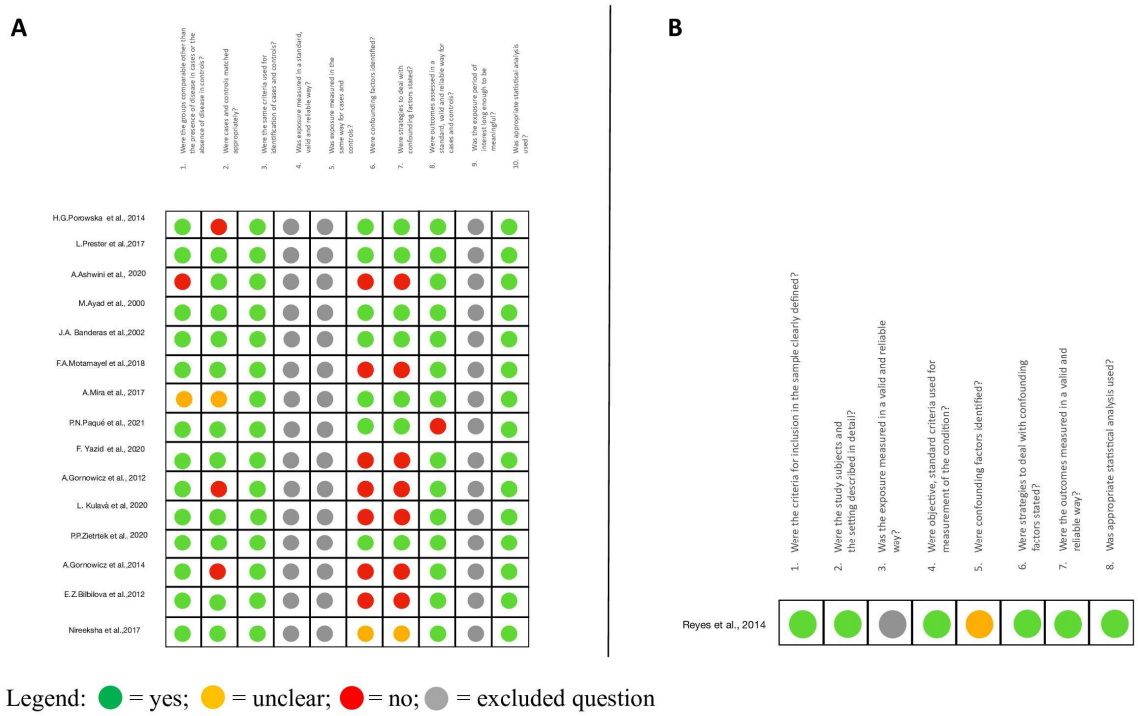

Figure 2. (**A**) Critical Appraisal Checklist of the Joanna Briggs Institute (JBI) based on the specific questionnaire for case–control studies [13–19,21–26,28]. (**B**) Critical Appraisal Checklist of the Joanna Briggs Institute (JBI) based on the specific questionnaire for analytical cross-sectional studies [27].

All the papers but two [14,22], received a "yes" answer to the first question. For question 2, the studies by Porowska et al. and Gornowicz et al. [18,20] were negatively evaluated, based on a discrepancy in sample size. The study of Mira et al. [22] received an "unclear" answer for question 2 due to the limited information relating to the two study groups (cases and controls).

Eight papers [13,14,17,19–21,23,28] received "No" or "Unclear" answers to questions 6 and 7 as they did not mention the potential confounding factors and/or their normalization by means of the statistical analysis.

One paper did not satisfy question 8 [24] because of the absence of a standard and reliable method to identify caries cases (e.g., no mention of the DMFT or ICDAS criteria).

The only cross-sectional study [27] received six "yes" answers to seven questions. The authors did not report on confounding factors ("Unclear" answer to question 6). However, they used the Shapiro–Wilk and Levene tests to determine the normality of data distribution and variance homogeneity (a "yes" answer to question number 6).

With ratings ranging from 4 to 7 on the JBI Critical Appraisal Checklists, the 16 included papers were considered to be of moderate to high quality.

Only one disagreement was raised during the evaluation step for the paper of Ashwini et al. 2020, which was solved by the third reviewer's appraisal.

3.4. Salivary Biomarkers

Overall, 12 papers (75%) analyzed only protein biomarkers [14–16,18–21,23,24,26–28], 2 papers (12.5%) only metabolite biomarkers [13,17], 1 papers (6.25%) both protein and gene biomarkers [25], and 1 paper (6.25%) both protein and metabolite biomarkers [22].

Details on the biomarkers evaluated in each study are summarized in Table 4.

Salivary molecules that are significantly associated with the presence of dental caries were classified as proteins, genes, or metabolites. These molecules are potential biomarkers for dental caries diagnosis.

3.4.1. Protein Biomarkers

Mucins

Nireeksha et al. found that salivary mucin levels were increased in caries-active subjects ($p < 0.05$), but the authors did not report details on specific mucins [18,23]. Instead, Gabryel-Porowska et al. analyzed the concentration of three salivary mucins: mucin-1 (MUC1), mucin-5B (MUC5B), and mucin-7 (MUC7) [18]. Significantly higher MUC1 levels were found in subjects with DMF > 11 when compared to subjects with DMF = 3 ($p = 0.011$), confirming the correlation between MUC1 and the presence of caries. MUC5B and MUC7 salivary concentrations were not significantly associated with this dental pathology ($p = 0.06$ and $p = 0.918$, respectively). In another work, Banderas-Tarabay and colleagues analyzed a series of proteins [16]. Interestingly, subjects with a higher DMFT index (=11.87) showed a significant reduction or the absence of high-molecular-weight mucin glycoprotein-1 (MG1, encoded by MUC5B) and low-molecular-weight mucin glycoprotein-2 (MG2, the translational product of MUC7), ($p \leq 0.001$). Salivary alpha-amylase levels were not statistically associated with caries, whereas subjects with a higher DMFT index (=10.0) presented lower levels of acidic proline-rich protein-1 (PRAP-1) ($p \leq 0.001$).

Glycoproteins, Immunoglobulins, and Enzymes

Prester et al. investigated the role of soluble CD14 (sCD14) in the unstimulated and stimulated saliva of patients with dental caries. Median levels of sCD14 were higher in the active caries than in the caries-free group in both stimulated and unstimulated saliva ($p < 0.01$) [26].

Gornowicz et al. proved that patients with high dental caries activity (DMF > 11) had significantly increased levels of secretory IgA, histatin-5, and lactoperoxidase (LPO) compared to subjects with lower caries activity ($p < 0.05$) [20] Conversely, Nireeksha et al. reported that salivary IgA (sIgA) levels in subjects with active caries were decreased with respect to caries-free subjects ($p < 0.05$) [23].

It is worth detailing the results of the study of Kulhavá et al. [21]. They analyzed the supernatant and pellet fractions of salivary samples obtained from subjects with and without caries. Fourteen proteins showed higher expression levels in the supernatant samples of caries-free subjects compared with subjects with dental caries.

Three proteins (annexin A1, cornulin, and 14-3-3 protein ç) had higher expression in pellet samples than in the supernatants of subjects with caries ($p < 0.5$).

Alpha-amylase was also investigated by Banderas-Tarabay et al. and Yazid et al. [16,28]. Even if Banderas-Tarabay and colleagues did not attribute diagnostic relevance to alpha-amylase, Yazid et al. observed a significant increase in the alpha-amylase absorbance signal (UV-Vis spectroscopy) in patients with caries

In the study performed by Piekoszewska-Ziętek et al., salivary carbonic anhydrase isozyme VI (CA VI) levels were significantly lower in patients with dental caries ($p = 0.014$) [25].

Reyes et al. investigated the role of urease and arginine deiminase system (ADS) activity in saliva and supragingival plaque. Urease activity was significantly higher both in the saliva (3.024 vs 0.437 μmol min^{-1} mg $prot.^{-1}$, $p = 0.010$) and plaque (18.120 vs 0.370, $p = 0.033$) of caries-free subjects. Also, ADS activity in saliva (6.050 vs 1.350, $p = 0.0154$) and plaque (8.830 vs 1.210 μmol min^{-1}mg $prot.^{-1}$, $p = 0.025$) was higher in individuals with DMFT = 0 compared to caries-active patients, having at least four teeth with active caries [27].

Interleukins and Chemokines

Paqué et al. evaluated 19 cytokines, seven chemokines, four growth factors, two metalloproteinases, one metallopeptidase inhibitor, one protease, and the presence of 10 oral bacteria (*P. gingivalis, T. forsythia, T. denticola, F. nucleatum, C. rectus, P. intermedia, A. actinomycetemcomitans, S. mutans, S. sobrinus, and oral lactobacilli*) in healthy individuals and patients with gingivitis or caries. Significantly higher levels were observed for interleukin 13 (IL-13) ($p = 1.5 \times 10^{-15}$ caries/gingivitis, $p = 4.0 \times 10^{-13}$ caries/healthy), interleukin

2-RA (IL-2-RA) ($p = 3.3 \times 10^{-6}$ caries/gingivitis, $p = 1 \times 10^{-4}$ caries/healthy), interleukin 4 (IL-4) ($p = 1.5 \times 10^{-15}$ caries/gingivitis, $p = 4.1 \times 10^{-13}$ caries/healthy), and Eotaxin/CCL11 ($p = 8.1 \times 10^{-5}$ caries/gingivitis, $p = 4.4 \times 10^{-4}$ caries/healthy) in patients with caries when compared to the other groups (healthy and gingivitis) [24].

Gornowicz et al. found a statistically significant increase in IL-6, IL-8, and TNF-alpha levels in the unstimulated WS of subjects with dental caries compared to the controls ($p < 0.05$) [19].

Peptides

Ayad et al. analyzed the phenotypes of 18 genes (proline-rich protein phenotypes—*Pe, Pmf, Ps1, Con2, PmS, Con1, G11, G12, G13, G14, Po, Db, Pa, Pif, PR1, PR2, Pmo1, and Pc2*), which were tested for differences between caries-free and caries-susceptible subjects. *Ps1* and *Con1* peptides were more common in the caries-free group than in the caries-susceptible group ($p = 0.046$). The difference in prevalence for *Pmo 1* was close to statistical significance, thus suggesting that its prevalence might be lower in the caries-free group ($p = 0.06$) [15].

Other Proteins

Ashwini et al. investigated matrix metallopeptidase 8 (MMP-8) and discovered a significantly higher concentration of this molecule in patients with caries compared to the controls ($p < 0.05$) [14].

To test the hypothesis that some molecules were mainly produced after dietary carbohydrate fermentation, Mira et al. compared 25 salivary compounds in caries-free and caries-active individuals at different time points of dental biofilm formation and times of the day. Based on the *p*-values ($p < 0.5$), the following salivary proteins were proven to discriminate between healthy and caries-active individuals: LL-37, IgA, statherin, and fibronectin (statherin only in saliva collected after a sugary solution rinse) at 30 min after toothbrushing (morning sample), and β-defensin 2, LL-37, collagen I, and fibronectin at 6 h after toothbrushing (afternoon sample) [22].

3.4.2. Genes

Piekoszewska-Ziętek et al. examined three single nucleotide polymorphisms (SNPs) of the carbonic anhydrase (CA) VI gene (*rs2274327; rs2274328; rs2274333*) in buccal smear. No association between the increased or decreased risk of caries and the analyzed polymorphisms was found. However, some significant positive correlations were found between the *rs2274333* A/G genotype and the presence of active white spot lesions ($p < 0.05$). Moreover, there were some significant relations concerning SNPs and the salivary buffer capacity and flow rate in *rs2274327* and *rs2274328* [13,25].

3.4.3. Metabolites

Ahmadi-Motamayela and coworkers investigated the salivary and serum malondialdehyde (MDA) levels. Their results showed significantly higher levels of MDA in the case group compared to the healthy control group ($p = 0.001$).

Zabokova Bilbilova et al. examined the values of salivary urea in subjects with different caries activities. The salivary concentration of urea was significantly lower in patients with a high DMFT index (DMFT > 10, from 3.4 to 5.5 mmol/L) compared to subjects with low caries index (DMFT= 0–3, from 5.5 to 9.1 mmol/L). The same result was obtained with the concentrations of salivary urea measured at 5, 30, and 60 min after the meal [17].

Mira et al. found that formate and phosphate at 6 h after toothbrushing (afternoon sample) and phosphate and lactate at 30 min after toothbrushing (morning sample) were able to distinguish healthy subjects from caries-active individuals [22].

4. Discussion

The present systematic review aimed to emphasized the relevance of groups of salivary molecules that are significantly associated with the presence of dental caries by comparing individuals with and without caries experience.

Dental caries represents a complex disease that, if diagnosed and treated early, can be stabilized and, in some cases, reversed with the remineralization of the tooth surface [32].

Caries diagnosis is usually performed during the dental visit by a general dentist [33]. The traditional caries detection method includes a careful visual inspection, dental probing, and radiographic examination if needed. In most cases, this methodology is reliable for detecting caries that have progressed into dentine and need conservative treatments [34]. However, early-stage caries (e.g., those producing small changes in dental enamel) are challenging to diagnose visually and radiographically [6]. Innovative methods for the early diagnosis of carious lesions are currently available [7]. Some of such new approaches could lead to a decrease in invasive treatments and costs for patients and health care systems.

Dental caries is in direct contact with saliva, and some of its components react to the acidic environment induced by bacterial metabolism, contrasting their biofilm's development and adhesion. Indeed, the detection and measurement of salivary caries biomarkers may represent an attractive alternative for the early diagnosis of caries. However, using a single biomarker predictive of disease occurrence appears unsuitable given the multifactorial etiology of caries.

According to the results presented, the most investigated molecules are alpha-amylase [16,21,28] and mucins [16,18,23]).

Salivary alpha-amylase is involved in maintaining oral homeostasis. The alteration of this enzyme is associated with dental caries development, leading to a dysregulation of enamel calcium-binding mechanisms and modifying the capacity of this enzyme to bind to oral streptococci [35]. Even if Banderas-Tarabay et al. [16,21,28] did not attribute diagnostic relevance to alpha-amylase, Yazid et al. and Kulhava et al. highlighted a significant association of the alpha-amylase levels with the presence of dental caries [21,28]. However, these studies showed contrasting results.

The increase in the alpha-amylase absorbance signal in patients with caries is justified by the binding of the enzyme to oral microorganisms, which facilitates starch hydrolysis inside the biofilm and the acid production mechanism of dental plaque, causing dental caries [21,28]. Kulhava and collogues found that salivary alpha-amylase had significantly higher expression levels in the supernatant samples of caries-free subjects [21]. This result could confirm the hypothesis that the binding of alpha-amylase to bacteria in solution may be considered protective if it leads to bacterial clearance from the oral cavity [35]. To date, it is unclear which of these alternate hypotheses is correct.

The reason for such a discrepancy may partly lie in the different biochemical approaches (UV-Vis spectroscopy and LC-MS, respectively) used to evaluate salivary samples.

Mucins are proteins correlated with the formation and progression of dental caries. These molecules constitute an important class of salivary glycoproteins. Notably, they account for approximately 20–30% of the total proteins in unstimulated saliva [36] and play a variety of functions critical to maintaining a stable oral defense. As part of the enamel pellicle, mucins favor the colonization of certain microorganisms while promoting the clearance of others, thus contributing to the formation of a selective/protective barrier; their hydrophilic properties protect oral tissue surfaces against mechanical wear; and they prevent acids access, thus limiting mineral erosion from tooth surfaces [37]. The results of the studies of Gabryel-Porowska et al. and Nireeksha et al. showed a correlation between mucin 1 levels (MUC1) and the DMFT index [18,23]. The increase in mucin levels during caries development could represent a protective mechanism that counteracts acid and bacterial impacts. However, Gabryel-Porowska et al. showed that mucins were not significantly enhanced in cases of an extremely high DMFT index [18]. On the other hand, the results of Banderas-Tarabay et al. showed that a decrease in the salivary level of mucins was associated with a higher DMFT index, a symptom of a serious oral health decline [16].

Based on this incongruity, it can be speculated that different mucin levels might correspond to different stages of caries development.

The results obtained with sIgA salivary levels are also controversial. Gornowicz et al. suggested that a massive presence of caries can be associated with high levels of sIgA, probably increasingly secreted to potentiate their antibacterial effect [20]. Such results are supported by similar studies not included in the present review [38–40]. On the other hand, Nireeksha et al. found that the sIgA level was decreased in active-caries patients [23]. The authors attribute this finding to sIgA's highly specific binding ability to microbial species, resulting in bacterial inactivation and the prevention of adhesion. Some studies have reported a correlation between sIgA levels and the subjects' age. Jafarzadeh et al. demonstrated that mean salivary sIgA levels increased with ages up to 60 years and then slightly decreased in subjects aged 61–70 years [41]. However, the age range of the subjects involved in these two reviewed studies was different (18 yrs in Gornowicz et al. and 25–40 yrs in Nireeksha et al.). It might be relevant to carry out further research correlating the level of salivary sIgA in patients with caries in the same age range.

Among the molecules that have been described singularly, there are proteins with buffering capacity [25] and antimicrobial proteins with enzymatic activity, such as lactoperoxidase (LPO) and lysozyme C. Kulhava et al. reported a significant up-regulation of lysozyme C and other immune proteins dissolved in the salivary supernatants of caries-free subjects and suggested that they could play an important role in caries prevention [21,42]. The authors also analyzed the corresponding salivary pellets and revealed a higher concentration of three calcium-binding proteins (annexin A1, cornulin, and protein ς) in caries-positive subjects. This finding might relate to the decalcification of enamel during the carious process. The concentration of annexin A1 was also significantly higher in the caries-free supernatant. These results appear to be contrasting but might reflect the various and complex roles of annexin A1 (e.g., in innate immune response as an effector of glucocorticoid-mediated responses, a regulator of the inflammatory process, its high affinity for Ca^{2+}).

Urea is part of the saliva buffering system that can neutralize the oral cavity's acids [42]. As reported by Zabokova Bilbilova et al., the salivary level of urea was significantly lower in patients with higher DMFT indexes [17,27]. This result agrees with the study of Reyes et al. [27], which demonstrated higher urease levels in individuals with low DMFT scores. Accordingly, caries-free subjects produced higher ammonia levels because of the salivary urease and arginine deiminase systems. Moreover, increased production of alkaline substances was associated with a low incidence of dental caries, suggesting that they might be investigated as predictive salivary biomarkers for dental caries [27].

One limitation of the present systematic review is that the authors employ different DMFT values to establish a high risk of caries. Despite the fact that the majority of studies (11 out of 16) utilize a DMFT score ≥ 3, there is currently insufficient scientific evidence to consider this as a threshold value.

Due to the complex interactions between salivary components and functions, it may thus be unrealistic to expect any single salivary factor to effectively identify caries-susceptible individuals. In fact, most of the molecules analyzed are found in saliva following the distinct mechanisms of production and release in the oral fluid. It is likely that a pool of salivary biomarkers should be assessed in conjunction with other caries risk factors and indicators, such as diet, exposure to fluoride, individual salivary flow rate, and sociodemographic and behavioral variables, in order to determine caries risk [43].

It is noteworthy that this review highlighted great homogeneity regarding the selection of the study population and the saliva sample collection procedure. All studies included subjects with a good oral and general health status and, except one [15], used whole saliva, centrifuged and fast-frozen at temperatures between -20 °C and -80 °C.

All the included studies, except for two [15,16], were published within the last 10 years, highlighting the recent interest in salivary biomarkers related to dental caries. The critical appraisal of the present systematic review attributed a "moderate" (7 out of 16 studies,

44%) or "good" (9 out of 16 studies, 56%) quality level to the included studies. Furthermore, all case–control studies appropriately matched study groups, using the same identification criteria, and used appropriate statistical analyses to correlate salivary biomarkers with dental caries. These features markedly decrease the risk of bias and support the high quality of all the studies included in this systematic review.

Differently from other systematic reviews, we focused only on permanent dentition and excluded studies on dental caries in children, as this may be due to a different pathogenetic mechanism. Furthermore, most of the published systematic reviews deal with a single category of molecules or a limited number of microorganisms. Indeed, according to the present paper, only a small section of the current literature focuses on molecules other than proteins. A review published in 2022 confirms the important role of some proteins also studied in the present research (e.g., alpha-amylase, histatin-5, lactoperoxidase, and carbonic anhydrase VI) [30]. This review instead considers the entire array of salivary components, including proteins, metabolites, and genes, that have been identified to date [30]. Based on our study selection, some protein levels are more likely to be involved in the occurrence of dental caries with respect to other groups of molecules, albeit the selected studies occasionally gave contradictory results. This occurrence highlights the necessity for further research as well as the development of appropriate and comparable experimental settings and conditions.

Other categories of potential biomarkers have yet to be identified and thoroughly investigated. Future developments in salivary metabolomics, genomics, and transcriptomics may give additional impetus to this research.

5. Conclusions

Most of the salivary molecules presented in this review might potentially play an important diagnostic or predictive role. According to the "good" quality studies, salivary mucins, glycoproteins (sCD14), interleukins (IL-2RA, 4,-13), urease, carbonic anhydrase VI, and urea appear to exhibit significant different levels in healthy and active-caries subjects [15,17,18,24–27]. These salivary molecules should be the target of clinical research to validate or exclude their relevance as biomarkers for dental caries.

Acknowledging its non-invasiveness and ubiquitous applications, saliva as a probing biofluid sample remains highly attractive. Indeed, salivary diagnostic/prognostic tools are less invasive and less harmful than current tools and enable physicians to intervene early, possibly altering the course of the disease and significantly reducing suffering and disability in patients. In dental practice, a salivary test may be useful to assess the presence of dental caries when it is challenging to perform an X-ray validation (e.g., pregnant woman, patient with disability) or when X-ray is not predictable (e.g., interproximal caries with dental overlap).

From a future perspective, the early self-diagnosis of dental caries might be accomplished through salivary tests potentially available for those categories of patients considered to be at a high risk of caries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb46050258/s1. Table S1. "JBJ Critical appraisal Checklist" for case control studies. Table S2. "JBJ Critical appraisal Checklist" for cross sectional studies. Table S3. Papers excluded and the reasons for exclusion.

Author Contributions: R.A., V.M. and M.M. contributed to the conceptualization, design, and data interpretation and drafted and critically revised the manuscript. T.A.P., E.F. and M.G. contributed in designing the work and to the interpretation of data and critically revised the manuscript. S.P. and P.V. contributed to critically revising the manuscript. All the authors have confirmed the final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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CHAPTER 8

Conclusion

The challenge of saliva diagnostics is to discover the diagnostic potential and optimize engineering technologies for this biofluid. To fully understand the diagnostic potential of saliva, we need to establish the scientific basis and obtain clinical validations. The research presented in this Tesis aims to contribute to the exploration of potential application of salivary diagnostics for systemic and oral diseases.

There are many reasons to use saliva as a diagnostic fluid for monitoring health and diseases. As a clinical tool, saliva has many advantages over serum. It is easy to collect, store and ship and can be obtained at low cost in sufficient quantities for analysis. For patients, the non-invasive collecting techniques dramatically reduce anxiety and discomfort and simplify procurement of repeated samples for longitudinal monitoring over time. For professionals, saliva collection is safer than venepuncture, which could expose health care providers to HIV or hepatitis virus. Saliva-based diagnostics are therefore less invasive, less expensive and present less risk to both the patient and the provider.

The next decades will be crucial for basic and translational research; salivary diagnostics is demanding its role into daily clinical setting, promising to eliminate missed or delayed diagnosis pitfalls and to provide fast, cheap and non-invasive examination. Major advantages and disadvantages of such highly complex fluid analysis should be kept in mind, to improve quality of experiments in order to deliver trustworthy and evidence-based testing platforms.

Annex n° 1

Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva

*Meleti M, Quartieri E, **Antonelli R,** Pezzi ME, Ghezzi B, Viani MV, Setti G, Casali E, Ferrari E, Ciociola T, Spisni A, Pertinhez TA.*
Metabolites, 2020 Aug 6;10(8):318. doi: 10.3390/metabo10080318. PMID: 32781584; PMCID: PMC7466076.

Published on Metabolites 2020, 10, 318.
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Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva

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Received: 15 July 2020; Accepted: 5 August 2020; Published: 6 August 2020

Abstract: The detection of salivary molecules associated with pathological and physiological alterations has encouraged the search of novel and non-invasive diagnostic biomarkers for oral health evaluation. While genomic, transcriptomic, and proteomic profiles of human saliva have been reported, its metabolic composition is a topic of research: metabolites in submandibular/sublingual saliva have never been analyzed systematically. In this study, samples of whole, parotid, and submandibular/sublingual saliva from 20 healthy donors, without dental or periodontal diseases, were examined by nuclear magnetic resonance. We identified metabolites which are differently distributed within the three saliva subtypes (54 in whole, 49 in parotid, and 36 in submandibular/sublingual saliva). Principal component analysis revealed a distinct cluster for whole saliva and a partial overlap for parotid and submandibular/sublingual metabolites. We found exclusive metabolites for each subtype: 2-hydroxy-3-methylvalerate, 3-methyl-glutarate, 3-phenylpropionate, 4-hydroxyphenylacetate, 4-hydroxyphenyllactate, galactose, and isocaproate in whole saliva; caprylate and glycolate in submandibular/sublingual saliva; arginine in parotid saliva. Salivary metabolites were classified into standard and non-proteinogenic amino acids and amines; simple carbohydrates; organic acids; bacterial-derived metabolites. The identification of a salivary gland-specific metabolic composition in healthy people provides the basis to invigorate the search for salivary biomarkers associated with oral and systemic diseases.

Keywords: saliva; metabolomics; salivary gland; parotid; submandibular/sublingual

1. Introduction

Human whole saliva (WS) is a mixture of fluids produced by parotid (20%), submandibular (65–70%), sublingual (7% to 8%), minor (<10%) salivary glands, and by gingival sulcus (crevicular fluid) [1].

Chemical-physical properties and volume of WS can grossly vary among people, as well as in the same person, according to endogenous and exogenous factors (e.g., age, gender, circadian rhythm, psychological state, nutrition, diseases, drugs, and environmental exposures). Moreover, qualitative variations of saliva, mostly related to the presence and concentration of specific categories of molecules, have been reported [2–4].

Variation in salivary flow (e.g., by stimulation with citric acid) is associated with changes in most metabolites' concentrations. The concentration of acetate in unstimulated saliva is markedly higher than in the stimulated one. By contrast, lactate has more elevated levels in stimulated than in unstimulated saliva. Glucose has a similar concentration in the two types of fluids [5].

It is important to highlight that the inter-individual variability of salivary metabolic profiles seems to be higher than the intra-individual one. Such observation has led to hypothesize that under standardized conditions, an individual metabolic phenotype is relatively stable [2].

Despite the relatively well-described composition of WS, parotid saliva (PS) and submandibular/sublingual saliva (SM/SL), in terms of nucleic acids and proteins [6,7], the metabolites composition of these fluids is still subject of research.

The origin of the metabolites in WS is quite diversified: some molecules are produced by human metabolic processes, others by oral microorganisms, and several are of exogenous origin [2,8–10]. Being unlikely a contamination by exogenous and microbial molecules, the majority of the metabolites in PS [5] and SM/SL are presumably host-derived. As for the metabolites of human origin in WS, they either originate within the salivary glands or are released from alive or desquamated oral mucosal cells [8].

To the best of our knowledge, SM/SL metabolites have never been systematically described: only few examples of metabolic profiles have been published so far [11]. In particular, Yamada-Nosaka and co-workers [12] recorded broad and not well resolved proton Nuclear Magnetic Resonance (NMR) spectra of SM/SL, most likely due to the presence of the viscous mucous component.

Currently, the increasing number of studies on salivary metabolites [11] point to interpret the metabolite content of each type of saliva, in light of physiological and pathological changes that characterize each salivary gland.

Indeed, in-depth analysis of the human salivary metabolome may significantly boost: (1) the research of salivary biomarkers for oral and systemic diseases [13–16] and (2) the interpretation of the metabolic alterations occurring in physiological conditions (e.g., effects of physical exercise, weight changes, activation of specific metabolic pathways) [17,18].

Here, we report and compare the metabolic composition of human unstimulated PS, SM/SL, and WS derived from a cohort of young and healthy volunteers in physiological conditions. Quantitative and qualitative differences between salivary types are discussed, with special emphasis on resident microflora contribution.

2. Results

For each participant (n = 20), a sample of unstimulated PS, SM/SL, and WS was collected separately, in this exact order, as described in the Material and Methods section (Figure 1).

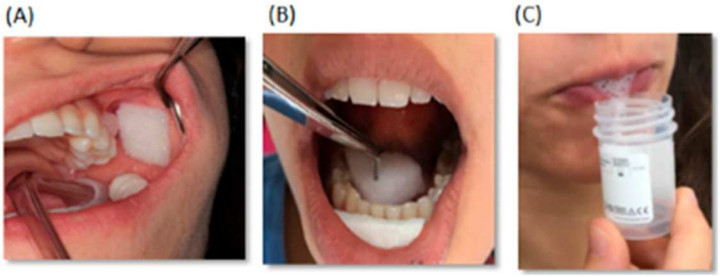

Figure 1. Experimental set-up used for parotid (**A**), submandibular/sublingual (**B**), and whole saliva (**C**) collection.

The salivary flow rates (0.15 ± 0.16 mL/min for PS; 0.20 ± 0.09 mL/min for SM/SL; 0.49 ± 0.28 mL/min for WS, expressed as mean value ± SD) show a wide inter-individual variability, mainly for PS. Our flow rate values of unstimulated saliva are in accordance with flow rates reported in the literature: 0.1, 0.1, and 0.6 mL/min for PS, SM/SL, and WS, respectively [19,20]. Using a sialometry test, we preliminary measured WS flow rate obtaining a good correlation (r = 0.86) with the value measured at the end

of the whole sampling procedure. This fact indicates that the flow rate is not significantly altered, even after the prolonged sample collection required for some participants.

2.1. Metabolite Content of Saliva Subtypes: Emerging Differences

The 1D ^{1}H-NMR spectra of WS, SM/SL, and PS samples (Figure 2) highlight different and characteristic metabolites patterns.

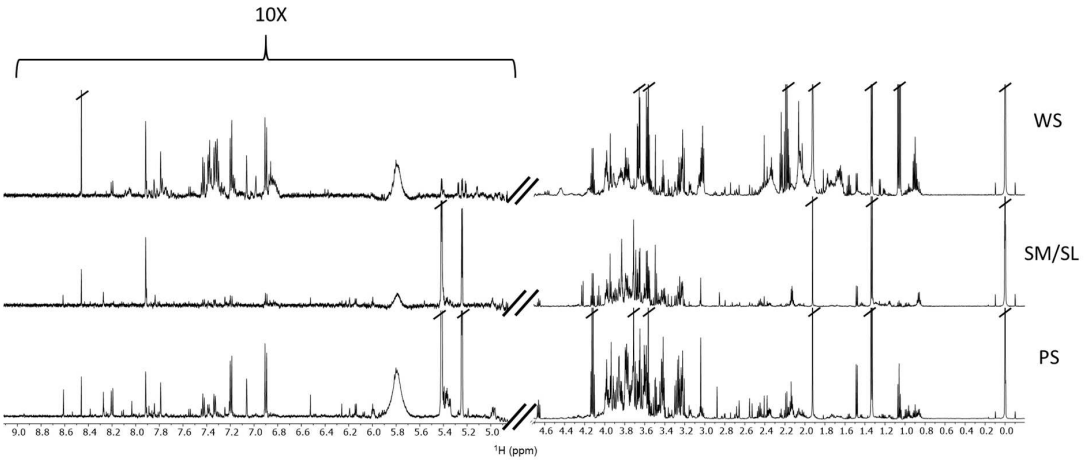

Figure 2. ^{1}H-NMR spectra of unstimulated WS, PS, and SM/SL saliva from the same participant, acquired at 25 °C. The left region of the spectra shows the vertical scale increased by a factor of 10.

Since the total signal area under each NMR spectrum is proportional to the total metabolite content, the comparison of the values derived from the whole cohort reveals a similar total metabolite content for WS and PS and a sensibly smaller value for SM/SL, with a ratio of 1:1:0.3 (WS:PS:SM/SL).

Principal component analysis (PCA) of WS, PS, and SM/SL was applied to binned NMR spectra of all samples (20 samples for each of the 3 salivary types). In Figure 3, WS appears as a well separated cluster of scores, while PS and SM/SL display a partial cluster overlap that might suggest some similarity between their metabolic profiles. As expected, all clusters are characterized by a spread of scores, very likely due to the contribution of inter-individual variability. The principal data variance is given by PC1, and the separation between WS and the other two salivary subtypes is accounted for by lactate, propionate, maltose, 2-aminoadipate, and taurine.

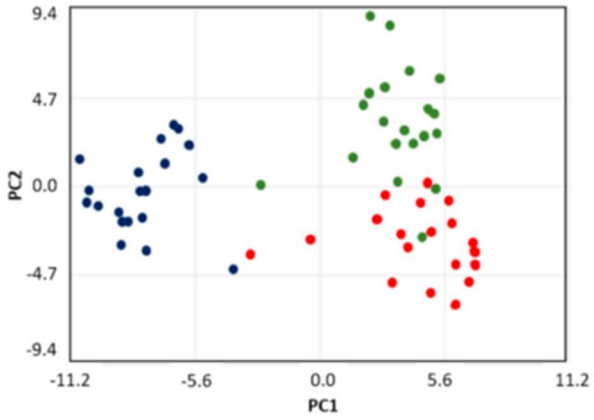

Figure 3. PCA score plot of ^{1}H-NMR spectra of whole saliva (blue), parotid saliva (red), and submandibular/sublingual saliva (green) samples. The contribution of the three principal components of the total variance are PC1 = 62.6%, PC2 = 6.8%, and PC3 = 5.1%.

2.2. Salivary Metabolites

We identified 66 metabolites with average concentrations higher than 5 µM. Heatmap analysis of all salivary metabolites profiles highlights the heterogeneity of WS, PS, and SM/SL composition and shows that 54 are in WS, 49 in PS, and 36 in SM/SL (Figure 4). Overall, 32 metabolites (48%) are common to the three saliva subtypes. Notably, it has been possible to single out a number of metabolites uniquely present in each salivary subtype: 2-hydroxy-3-methylvalerate, 3-methyl-glutarate, 3-phenylpropionate, 4-hydroxyphenylacetate, 4-hydroxyphenyllactate, galactose, and isocaproate in WS; arginine in PS; caprylate and glycolate in SM/SL.

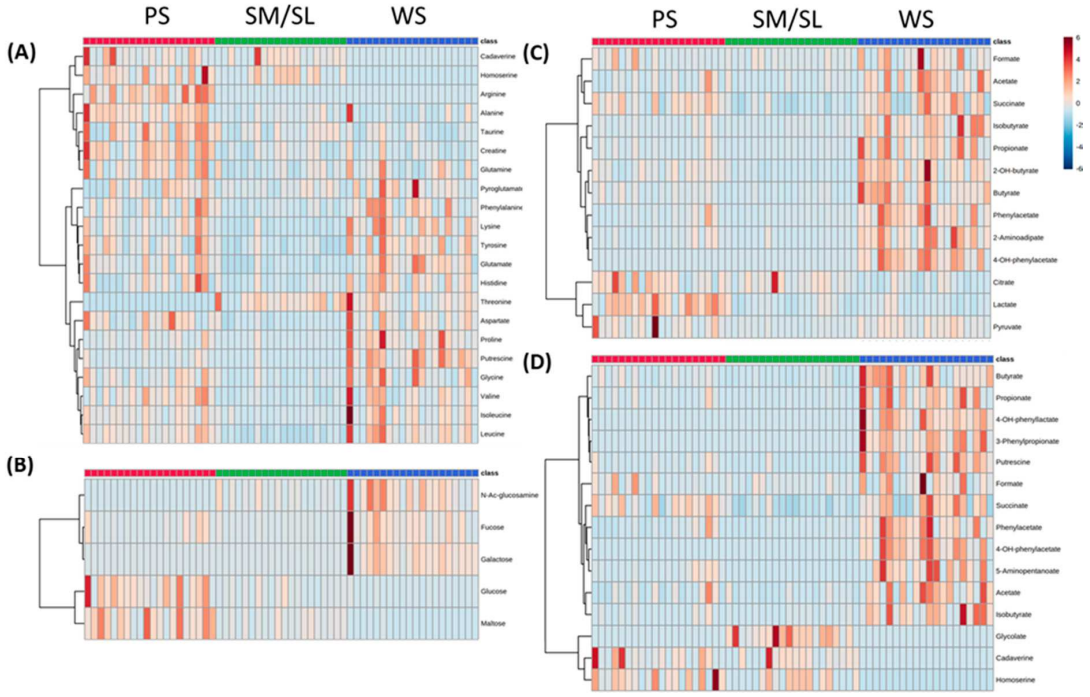

Figure 4. Heatmap analysis of metabolites grouped according to the categories: (**A**) amino acids, (**B**) carbohydrates, (**C**) organic acids, and (**D**) selected prokaryotic metabolites. PS (red), SMS (green), and WS (blue) samples.

2.3. Classes of Metabolites

To describe quantitatively each type of saliva, we selected an ensemble of metabolites, focusing on molecules with relevant concentrations and/or differential expression in the three salivary types. The median value and the range of concentration for each of the selected metabolites are reported in Table 1. Table 1—Section A and Figure 4A indicate the presence of the majority of the standard amino acids, non-proteinogenic amino acids, e.g., pyroglutamate and taurine, and biogenic amines, e.g., cadaverine, creatine, homoserine, and putrescine. When the highest median value (Table 1, in bold) is found in WS, most frequently, we observe that the related metabolite concentrations are significantly higher than in SM/SL. Only for lysine, phenylalanine, proline, and putrescine, their WS concentrations are significantly higher also than the ones found for PS. In the case of alanine, creatine, glutamine, and taurine, instead, the PS concentrations result significantly higher than in WS and SM/SL.

In Table 1—Section B, the relevant simple carbohydrates are listed. PS presents high levels of glucose and maltose that are significantly higher than in WS and SM/SL (Figure 4B).

Finally, Table 1—Section C summarizes the main organic acids. Once more, the highest median value belongs more frequently to WS. Exceptions are lactate and citrate that are significantly higher in PS than in WS and SM/SL (Figure 4C).

Table 1. Concentrations of selected metabolites in whole, parotid, and submandibular/sublingual saliva.

METABOLITE [a]	WS (μM)	PS (μM)	SM/SL (μM)
SECTION A: STANDARD AND NON–PROTEINOGENIC AMINO ACIDS			
Alanine	27.4 (9.4–212.6)	69.1 (41.5–219.1) ° §	20.7 (8.1–74.9)
Arginine	ND	24.2 (5.9–56.3)	ND
Aspartate	19.4 (9.8–76.7)	25.7 (7.9–64.0) §	7.3 (1.8–18.7)
Cadaverine [b]	ND	3.2 (0.7–17.6)	2.5 (0–16.6)
Creatine	10.8 (6.7–49.4)	48.9 (23.7–116.7) ° §	16.6 (6.4–48.1)
Glutamate	108.1 (30.6–250.1) §	57.5 (27.7–224.0)	19.4 (8.6– 71.2)
Glutamine	28.4 (6.6–134.6)	63.3 (9.1–151.4) ° §	21.6 (2.3–70.6)
Glycine	82.5 (12.1–306.6) §	66.6 (3.6–192.3)	14.4 (4.5–76.5)
Histidine	20.1 (4.1–49.9) §	17.6 (7.8–70.7)	5.2 (2.9–21.1)
Homoserine [b]	ND	36.4 (0–158.4)	32.4 (3.2–57.9)
Isoleucine	5.0 (0.9–44.3) §	3.8 (2.0–13.4)	1.2 (0.7–4.1)
Leucine	13.2 (3.5–56.9) §	12.0 (6.3–30.7)	4.8 (2.1–9.5)
Lysine	66.7 (17.6–164.8) * §	19.4 (10.1–97.5)	6.2 (2.2–37.9)
Phenylalanine	16.8 (6.4–48.6) § *	10.9 (5.0–41.1)	4.6 (2.0–12.7)
Proline	64.1 (24.8–446.8) * §	41.1 (10.8–156.0)	7.2 (2.8–74.4)
Putrescine [b]	38.6 (8.5–96.4) * §	5.1 (0.6–27.3)	0.9 (0.5–17.7)
Pyroglutamate	12.9 (2.9–70.5) §	9.4 (0–32.3)	7.6 (3.3–14.7)
Taurine	46.2 (2.8–132.0)	121.4 (0–342.2) ° §	60.2 (3.3–146.9)
Threonine	4.6 (2.4–31.4)	ND	7.3 (2.9–22.3)
Tyrosine	34.9 (10.5–93.5) §	28.2 (14.38–90.9)	10.1 (3.0–30.8)
Valine	9.3 (2.9–59.2)	12.4 (3.0–38.0) §	4.7 (1.1–13.1)
SECTION B: SIMPLE CARBOHYDRATES			
Fucose	34.8 (11.5–275.8) * §	5.6 (0.9–57.9)	4.8 (2.4–16.3)
Galactose	18.9 (6.3–173.9)	ND	ND
Glucose	11.8 (6.8–137.8)	204.6 (81.8–697.8) ° §	46.8 (7.6–211.8)
Maltose	1.3 (0.2–52.8)	296.9 (103.5–1587.9) ° §	76.5 (16.4–420.4)
N-acetylglucosamine	26.3 (2.2–141.5) §	ND	10.5 (1.4–40.8)
SECTION C: ORGANIC ACIDS			
2-Aminoadipate	186.0 (77.2–530.3) * §	25.0 (2.4–117.3)	11.6 (2.0–92.8)
2-Hydroxybutyrate	13.6 (1.4–53.1) * §	7.6 (4.3–12.8)	2.5 (0.9–6.5)
3-Phenylpropionate [b]	10.0 (2.0–38.1)	ND	ND
4-hydroxyphenylacetate [b]	8.6 (1.9–19.0)	ND	ND
4-hydroxyphenyllactate [b]	4.4 (0.7–23.9)	ND	ND
5-Aminopentanoate [b]	100.5 (0–386.3) *	12.1 (0–82.2)	ND
Acetate [b]	2277.9 (734.1–4322.8) * §	470.7 (81.8–3145.0)	237.4 (54.0–1370.4)
Butyrate [b]	20.9 (3.2–77.3) * §	5.1 (0.8–14.8)	4.0 (1.1–18.6)
Citrate	12.7 (0.7–33.0)	35.3 (16.0–125.8) ° §	20.4 (5.9–146.9)
Formate [b]	37.7 (8.7–234.0) * §	15.1 (6.8–106.7)	17.2 (6.5–97.9)
Glycolate [b]	ND	ND	7.8 (1.8–55.4)
Isobutyrate [b]	18.6 (3.76–47.6) * §	1.3 (0.2–9.9)	1.7 (0.3–5.2)
Lactate	123.1 (23.7–517.5)	714.8 (408.8–1683.9) ° §	162.7 (84.0–444.5)
Phenylacetate [b]	15.9 (4.2–46.2) * §	1.3 (0–25.7)	1.6 (0.3–3.3)
Propionate [b]	261.8 (64.7–627.9) * §	31.2 (4.4–200.8)	17.5 (4.3–151.7)
Pyruvate	18.8 (4.0–52.8) §	12.1 (1.4–232.0)	5.1 (2.1–14.2)
Succinate [b]	16.1 (9.9–39.2) * §	12.1 (3.8–23.3)	5.1 (2.2–11.8)

[a] In each section, metabolites are presented in alphanumerical order. The values reported for each metabolite are median concentrations in WS, PS and SM/SL. Numbers in brackets indicate the concentration range. Numbers in bold are the highest median values obtained in the three saliva types; For each metabolite, a Mann–Whitney test has been applied for comparing the concentration pool producing the highest median value with that of the other saliva samples, when detected. The significance level has been set at 0.05; The symbols °, *, and § are associated with the highest median values and indicate that the related metabolite concentration tends to be significantly higher than in WS, PS, or SMS, respectively; [b] Metabolite of bacterial origin, according to the "Human Metabolome Database" (www.hmbd.ca); ND is the abbreviation for not detected.

Figure 4D and Table 1—Section A and C report the distribution of metabolites that, according to the "Human Metabolome Database" (www.hmbd.ca), are mainly referable to bacterial metabolism. Noteworthy, WS presents high abundance of short chain fatty acids (SCFAs, i.e., formate, acetate, propionate, and butyrate), products of amino acid degradation such as putrescine and 5-aminopentanoate, and metabolic products of aromatic amino acids fermentation such as 4-hydroxy-phenyllactate and 3-phenylpropionate [21]. Yet, some of these metabolites are detected

only in gland saliva, possibly reflecting either a microbial contamination or a host gland contribution. In fact, cadaverine and homoserine, detected in PS and SM/SL, are absent in WS; glycolate is detected only in SM/SL.

2.4. Salivary Cell Count

Prokaryotic cell counts are significantly higher in WS than in PS and SM/SL, reflecting the prokaryotic metabolite proportion observed in the three salivary types (Figure 5). The median value of WS cell distribution (Figure 5) is consistent with the value reported by Sender et al. [22].

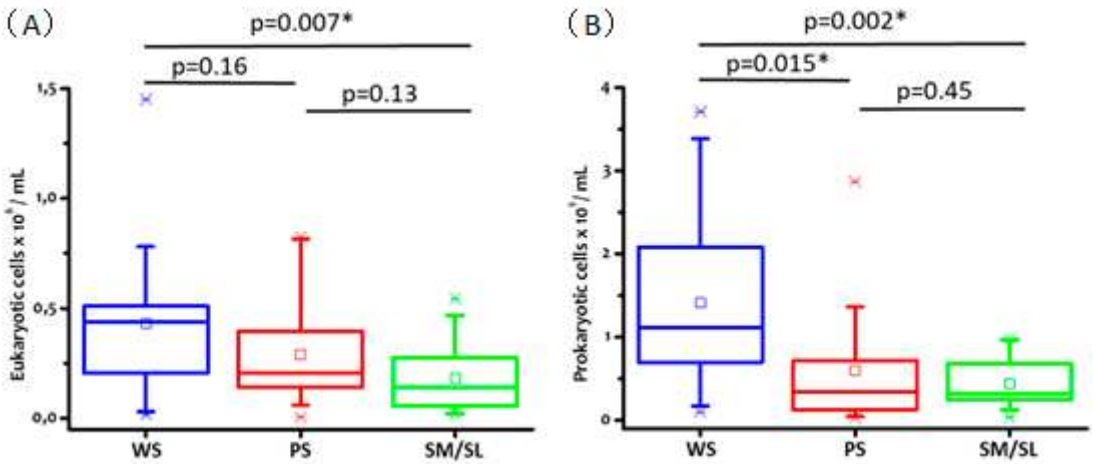

Figure 5. Eukaryotic (**A**) and prokaryotic (**B**) cell count distributions obtained with the three salivary types of all the subjects of the study. The significance level of the independent t-tests is set at 0.05 (* $p < 0.05$).

In all types of saliva, eukaryotic cell counts range between 0.1 and 0.5 × 10^6 cells/mL, and they are approximately three orders of magnitude lower than the prokaryotic ones.

3. Discussion

Mapping the human oral metabolome, with emphasis on the metabolic composition of saliva subtypes, is expected to provide hints to clarify the physiologic and pathologic processes of the salivary glands and oral cavity. In this study, we were able to identify and quantify a considerably high number of metabolites: 54, 49, and 36 in WS, PS, and SM/SL, respectively.

Free salivary amino acids are known to be primarily produced by endogenous and exogenous proteases of salivary glands, exfoliating cells, and oral microflora [23]. Proteolytic amino acid-degrading bacteria dissect proteins and peptides into amino acids and convert them in short chain fatty acids [24], contributing, together with the saccharolytic bacteria, to the organic acid content of saliva.

Thus, we hypothesize that the high concentration of amino acids and organic acids found in WS (Table 1—Sections A and C) may reflect bacterial metabolic pathways.

Indeed, glutamine, glycine, and proline, the most represented residues in salivary Proline-Rich Proteins (PRPs) [25], are among the amino acids found at the highest concentrations (Table 1—Section A) being produced by proteolytic processes. Pyroglutamic acid, the common N-terminal of acidic PRP, is present as well at significant concentrations in saliva samples (Table 1—Section A). Moreover, salivary mucins, a heterogeneous group of glycoproteins synthesized and secreted by the submandibular, sublingual, and minor salivary glands, contribute to proline salivary concentration [26]. Arginine, ornithine, and lysine, which originate from proteins and peptides lysis, are metabolized by oral cavity bacteria, and contribute to the salivary content of putrescine, by decarboxylation of ornithine, an intermediate in the degradation of arginine, and cadaverine, formed by lysine decarboxylation [27]. Our data (Table 1—Section A) point to the presence of cadaverine only in gland salivary types.

We found a significant concentration of arginine only in PS (Table 1—Section A) in agreement with Van Wuyckhuyse and co-workers [28] that found a concentration of free arginine and lysine in PS of caries-free adults significantly higher than in caries-susceptible individuals. Noteworthy, arginine deiminase system is a relevant source of alkali generation by means of ammonia production. Accordingly, this enzymatic system of both saliva and dental plaque results more active in caries-free people when compared to caries-active individuals, likely contributing to the neutralization of plaque acids and to caries resistance [29].

Taurine, a beta-sulfonic amino acid, is probably the most abundant free amino acid in mammalian tissues. As suggested by Revenga-Parra and co-workers [30], the determination of its concentration in various body fluids seems relevant for the early diagnosis of Alzheimer's disease, growth retardation, diabetes mellitus, epilepsy, sepsis, and some types of cancers. In rat SM glands, taurine is suggested to act as a regulator of the saliva ionic strength [31] and, in human saliva, appears to be correlated to physical stress [18]. Our study shows that such metabolite is present in all three types of saliva with a marked prevalence in PS (Table 1—Section A), thus, suggesting a role in salivary glands function.

Glucose, a blood component, passes in saliva through the salivary gland apparatus in proportion to its blood concentration. A statistically significant positive correlation has been found between fasting salivary glucose and fasting blood glucose [32]. Our study shows that glucose level in PS not only is considerably higher than in WS, as reported by Wang and co-workers [33], but is also higher than the concentration found in SM/SL, suggesting that parotid gland is the primary route of entry (Table 1—Section B). Therefore, we can assume that, in fasting conditions, such as in our cohort, the WS glucose level is severely influenced by the oral microorganism's glucose metabolism as well as by fasting blood glucose concentration.

Salivary glycoproteins are a suite of macromolecules that, while contributing with specific functional roles to the oral cavity defense, constitute endogenous nutrients for the resident oral microflora, thus, being also responsible for the microbial plaque growth [34]. Because glycoproteins degradation is the result of the combined action of various microbial glucosidases [24], we conclude that the prevalent presence of monosaccharides such as fucose, N-acetylglucosamine, and galactose in WS samples (Table 1—Section B) should primarily be associated with the microbial saccharolytic activity on the oligosaccharide chains linked to glycoproteins.

Interestingly, the unexpected presence of salivary maltose (Table 1—Section B), a significant source of carbohydrate to oral bacteria, might be attributed to the digestive action of α-amylase, an enzyme produced by serous cells of parotid glands, with minor contributions from other glands' enzymes [35]. On the basis of the results reported here, we can hypothesize that the concentrations of maltose in glandular saliva reflect the concentration of gland α-amylase, while its presence in WS is drastically reduced because of microbial utilization.

Saccharolytic microflora converts sugars to lactic, formic, acetic, succinic, and other organic acids through the glycolytic pathway [24,36]. On the other hand, in subgingival sites, asaccharolytic and/or proteolytic bacteria metabolize nitrogenous compounds derived from gingival crevicular fluid, creating an environment rich in SCFAs and ammonia [24,36].

Organic acids, in our saliva samples, are preferentially present in anionic form and frequently display their highest concentrations in WS (Table 1—Section C).

Consistent with the data reported by Gardner and co-workers [8], we find that acetate is the most abundant metabolite in WS samples, being present at relevant concentrations also in PS and SM/SL saliva. Interestingly, formate, acetate, propionate, and butyrate metabolites are present at a sensibly lower concentration in glandular saliva as compared to WS, a fact that might be due to their reduced bacterial contamination (Figure 5).

Lactate and citrate are more concentrated in PS than in WS and SM/SL, suggesting that the parotid gland is a relevant route of entry of these metabolites into the oral cavity [8]. However, WS lactate concentration may reflect also the contribution of microorganisms and oral mucosa cells [37].

The median lactate concentration that we measured in the WS of individuals with low dental plaque score is in agreement with the value reported by Gardner and colleagues [8].

Eventually, it is worth mentioning that we found a significant concentration of three metabolites of bacterial origin (glycolate, cadaverine, and homoserine) in PS and SM/SL saliva, but not in WS. We interpret the presence of those metabolites as an indication of microbial contamination of saliva. Particularly in the case of the glandular saliva, even if the fluids are collected at the close proximity of the excretory ducts, it is not possible to exclude a bacterial contamination of the outlet of the terminal portion of the salivary ducts, also in the absence of clinical signs of glandular infections. Because the outlets of the Stensen and Wharton ducts are very close to those portions of the dental arches where plaque, calculus, and periodontal diseases are more frequent (vestibular area of maxillary molars and lingual area of mandibular incisors) [38], even following rigorous procedures for saliva collection, it is possible that the area of sponge application is contaminated by dental and periodontal bacterial species. The hypothesis of some bacterial contamination is sustained by the high number of prokaryotic cells measured in all types of saliva (Figure 5). The absence of those metabolites in WS, on the other hand, may be the result of a pronounced dilution effect and/or additional degradation processes.

Overall, we have been able to identify and evaluate the concentration of a relevant number of metabolites in human saliva and to highlight qualitative and quantitative differences between WS, PS, and SM/SL saliva. Particularly, for the first time, we provided a metabolic profile of SM/SL.

We believe that mapping the human salivary metabolome is central for understanding most of the physiologic and pathologic oral metabolic pathways, including those related to the host–microbiome relationships.

4. Materials and Methods

The present study was approved by the Ethical Committee of the "Area Vasta Emilia Nord" (AVEN) (protocol number: 808/2018/SPER/UNIPR METASAL3). Written consent was obtained from all volunteers who participated in this study, according to the Declaration of Helsinki.

4.1. Subjects of the Study

A cohort of twenty healthy volunteers (10 males, 10 females), aged 19–25 years, qualified for saliva collection after oral clinical examination, interview for data acquisition on general medical history, and salivary flow rate assessment by sialometry test (modified Saxon Test) [39]. None of them revealed a full-mouth plaque score (FMPS) and/or full-mouth bleeding score (FMBS) higher than 25%.

4.2. Saliva Collection

For each participant, a sample of PS, SM/SL, and WS was collected separately and in the absence of stimulation. Participants were asked to refrain from eating, smoking, and performing intense physical activity for at least 12 h before salivary sampling and to drink only water. Furthermore, it was requested not to carry out oral hygiene (tooth brushing and flossing) in the 45 min before saliva collection. The procedure took place between 8:00 a.m. and 10:00 a.m. to minimize the influence of the circadian rhythm on salivary composition. Immediately before collection, patients rinsed their mouth with water for 1 min. For PS and SM/SL collection, the outlets of the Stensen and Wharton ducts were isolated and gently cleaned with a sterile gauze. A sterile sponge, capable of absorbing saliva flow, was positioned on the outlet of the ducts. Periodically, the sponge was squeezed and a syringe was used to collect the saliva within a vial. WS was collected by the passive drooling method (Figure 1).

During collection, salivary samples were transferred to a tube containing NaN_3 (0.5% final concentration) and kept on ice until a volume of 5.4 mL, of each salivary type, was obtained and then frozen at −80 °C.

4.3. Sample Preparation and ^{1}H-NMR Spectra Collection and Analysis

Each frozen saliva sample was thawed at room temperature and centrifuged at 15,000× *g* for 10 min at 4 °C to remove eukaryotic and prokaryotic cells, cellular debris, and mucins, according to Gardner et al. [40]. The supernatants were protein-depleted by ultra-filtration, using Amicon Ultra-4 Centrifugal filters (3000 MWCO, Merck Millipore) at 4000× *g* for 120 min at 10 °C, and lyophilized.

For ^{1}H-NMR measurements, each of the lyophilized samples was suspended in potassium phosphate buffer (50 mM, pH 7.4) and 3-trimethylsilyl propanoic acid (TSP) was added as the chemical shift reference (0.00 ppm) and quantitative internal standard.

High-resolution one-dimensional (1D) ^{1}H-NMR spectra acquisition and processing were carried out according to Pertinhez et al. [41]. Metabolites identification and quantification were carried out using Chenomx NMR Suite 8.3 software (Chenomx Inc., Edmonton, AL, Canada).

Heatmap analysis was carried out on targeted metabolites, with concentrations higher than 5 μM at least for one saliva subtype. Heatmaps were generated using MetaboAnalystR (https://www.metaboanalyst.ca) [42], with normalization referenced to TSP and autoscaling.

4.4. Cell Counting

Eukaryotic cells (oral epithelial cells and leucocytes) and prokaryotic cells were counted according to Gardner et al. [40], to estimate their possible contribution to the metabolic profile of WS, SM/SL, and PS.

4.5. Statistical Analysis

To compare the metabolite composition of each saliva subtype, the upper-tailed Mann–Whitney test (Origin 2019 software) was applied. $P < 0.05$ was considered statistically significant. The saliva subtype with the highest median concentration of each metabolite is shown in bold in Table 1.

Unsupervised multivariate analysis. To produce an overview of the overall variability, NMR spectra datasets were analyzed by principal component analysis (PCA), using the PCA module of MestreNova 11.0 software (Mestrelab Research, Santiago de Compostela, Spain).

Author Contributions: M.M.—Contributed to conception, design, data interpretation, drafted and critically revised the manuscript. E.Q.—Contributed to data acquisition and interpretation, drafted the manuscript. R.A.—Contributed to sample collection. M.E.P.—Contributed to sample collection. B.G.—Contributed to data acquisition and interpretation. M.V.V.—Contributed to sample collection. G.S.—Contributed to sample collection. E.C.—Contributed to data acquisition and interpretation and statistical analysis. E.F.—Contributed to data interpretation, statistical analyses, drafted and critically revised the manuscript. T.C.—Contributed to data acquisition and interpretation. A.S.—Contributed to drafted critically revised the manuscript. T.A.P.—Contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: We gratefully acknowledge Paolo Govoni, University of Parma, for the use of microscope.

Conflicts of Interest: The authors declare no conflict of interest.

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PERSONAL INFORMATION

Rita Antonelli
DENTIST, M.Sc, PhD student

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QUALIFICATION

Second Level Degree Course in Dentistry and Dental Prosthesis, cum laude (110/110L) and honorable mention

EDUCATION AND PROFESSIONAL EXPRERIENCE

April-June 2024	Observship program at "Brigham and Women's Hospital" and "Dana Farber Cancer Institute", University of Harvard, Boston (MA)
November 2021 - in progress	PhD course in Molecular Medicine Cod. DR979, Department of Medicine and Surgery, University of Parma.
February 2020 - July 2021	Master's degree in Restorative Dentistry and Aesthetic Prosthesis - Alma Mater Studiorum; University of Bologna.
January - October 2020	Research fellowship (renewed in January 2021) BR - 1/2019 "Evaluation of salivary metabolites in healthy subjects and patients with oral and/or systemic neoplasms; University of Parma.
July 2019	Second Level Degree Course in Dentistry and Dental Prosthesis, with vote 110/100 cum laude and honorable mention, University of Parma.
June - September 2018	Mobility scholarship OVERWORLD - Action 1 of the University of Parma, project "Soggiorno per Tirocinio professionalizzante per il CDCU in Odontoiatria e Protesi Dentaria" for the performance of activities "Attendance of courses and preparation of the thesis of degree", University of Dentistry in Ribeirão Preto, San Paolo USP - Brasile.
2012	Diploma of secondary education at "Liceo Classico Statale Francesca Capece", Maglie (LE) with vote 100/100.

PERSONAL SKILLS

Native Language: Italian

Others Language	LISTENING	SPEAKING	WRITING
English	B2	B2	B2

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Brazilian Portuguese	B1	B1	A2

Levels: A1/A2: Basic - B1/B2: Intermediate - C1/C2: Advanced
Quadro Comune Europeo di Riferimento delle Lingue

ADDITIONAL INFORMATION

Scientific publications

Antonelli R, Pezzi ME, Viani MV, Pertinhez TA, Quartieri E, Ghezzi B, Setti G, Paolo Vescovi P, Meleti M. Salivary Metabolic Analysis in Healthy Subjects and Perspectives for Patients with Oral Cancer: Pilot Study and Systematic Review. Proceedings 2019, 35(1), 44.

Antonelli R, Pertinhez T, Pezzi M, Ghezzi B, Vescovi P and Meleti M (2019). Differences in metabolic profile between submandibular, parotid and whole saliva: a new concept in salivary diagnostics. Front. Physiol. Conference Abstract: 5th National and 1st International Symposium of Italian Society of Oral Pathology and Medicine. doi: 10.3389/conf.fphys.2019.27.00034. IF 3.367

Meleti M, Quartieri E, **Antonelli R,** Pezzi ME, Ghezzi , Viani MV , Setti G , Casali E, Ferrari E, Ciociola T , Spisni A, Pertinhez. Metabolic profiles of whole, parotid and submandibular/sublingual saliva. Metabolites, 2020, 10(8), pp. 1–11, 318. Indexed in Scopus; IF 4.487

Viani MV, Pezzi ME, **Antonelli R,** Manfredi M, Vescovi P, Meleti M. Increase of focalized swelling on the vestibular maxilla | Aumento di volume localizzato sul versante vestibolare mascellare. Dental Cadmos, 2020, 88(7), pp. 409–410. Indexed in Scopus; IF 0.124

Antonelli R., Pezzi M., Viani M.V., Manfredi M, Vescovi P., Meleti M. Pigmented lesion on hard palate | Lesione pigmentata a livello del palato duro. Dental Cadmos, 2020, 88(9), pp. 569–570. Indexed in Scopus; IF 0.12

Meleti M, **Antonelli R,** Viani MV, Pezzi ME, Pertinhez T, Vescovi P. Salivary Biomarkers for diagnosis of oral and systemic diseases. Dental Cadmos, 2021, 89(2), pp. 104–117. Indexed in Scopus; IF 0.124

Antonelli R, Vescovi P, Manfredi M, Ferrari L, Meleti M. Management of recurrent oral leukoplakia with diode laser (445 nm). Dental Cadmos, 2022, 90(6), pp. 489–492. Indexed in Scopus; IF 0.124

Antonelli R, Paes de Almeida O, Bologna-Molina R, Meleti M. Intraoral Sialadenoma Papilliferum: A Comprehensive Review of the Literature with Emphasis on Clinical and Histopathological Diagnostic Features. Oral. 2022; 2(3):242-250. https://doi.org/10.3390/oral2030023

Tuttolomondo D, **Antonelli R,** Setti G, Ardissino D, Pertinhez T, Gallo M, Niccoli G, Nicolini F Georgaki M, Formica F, Borrello B, Meleti M, Cassi D. Salivary biomarkers for diagnosis of acute myocardial infarction: A systematic review. Int J Cardiol. 2022 Sep 24:S0167-5273(22)01393-6. doi: 10.1016/j.ijcard.2022.09.043. Epub ahead of print. PMID: 36167219.

Vescovi P, Giovannacci I, Ruggeri K, Ferarri L, **Antonelli R,** Manfredi M, Meleti M. Laser in the management of jaw bone pathologies. Dental Cadmos, 2023, 91(2), pp. 2–22

Ferrari E, Gallo M, Spisni A, **Antonelli R,** Meleti M, Perthinez T. Human Serum and Salivary Metabolomes: Diversity and Closeness. International Journal of Molecular SciencesThis link is disabled., 2023, 24(23), 16603

Antonelli R; Ferrari E; Gallo E; Ciocola T; Calciolari E; Spisni A; Meleti M; Pertinhez TA. The Association between Salivary Metabolites and Gingival Bleeding Score in Healthy Subjects: A Pilot Study. International Journal of Molecular Sciences DOI: 10.3390/ijms25105448

Antonelli R, Massei V, Ferrari E, Gallo M, Pertinhez TA, Vescovi P, Pizzi S, Meleti M. Salivary Diagnosis of Dental Caries: A Systematic Review. Current Issues in Molecular Biology. 2024; 46(5):4234-4250. https://doi.org/10.3390/cimb46050258

Antonelli R; Setti G; Treister NS; Pertinhez TA; Ferrari E; Gallo M; Bologna-Molina R; Vescovi P; Meleti M. 2024. "Salivary metabolomics in oral cancer: a systematic review." Oral Oncology Reports DOI: 10.1016/j.oor.2024.100657

Awards and honors

September 2024 2° prize CED-IADR Robert Frank Awards, Geneva, Switzerland

June 2024 1° prize Italian CED-IADR Award - Best oral presentation "Identification of salivary miRNAs in women at high risk of breast and ovarian cancer development"; Trieste, Italy

Honorable Mention in Pathology and Oral Medicine for the discussion of poster "Progression from normal oral mucosa to epithelial dysplastic lesions: a metabolic profiling study on saliva", at 31th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Trieste, Italy

June 2022 Best Project "Personalized Medicine" Taobuk Da Vinci Award - Contest 2022, Taormina

April 2022 Honorable Mention in Pathology and Oral Medicine for the discussion of the poster "Identification of salivary miRNAs in women at high risk of breast and ovarian cancer development" at the 29th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Bologna

October 2021 Best Oral Presentation, 1° Research Day, Department of Medicine and Surgery, University of Parma.

September 2020 Honorable Mention in Pathology and Oral Medicine for the discussion of the poster "Analysis of the amino acids present in the whole saliva, parotid and sub-mandibular/sub-lingual saliva" at the 27th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Milan.

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October 2019 Finalist thesis for the Best Thesis Award "Valerio Margiotta 2019" at the 15th National and 3rd International Congress of the Società Italiana di Patologia e Medicina Orale (SIPMO), Bari.

April 2019 Honorable Mention in Pathology and Oral Medicine for the discussion of the poster "Salivary metabolomics in oral cancer: a sistematic review" at the 26th National

Conference presentations and posters

October 2024 Invited speaker at the course "La biologia del microambiente orale: saliva, microbioma, patologie sistemiche" of AIDI (Associazione Italiana Igienisti Dentali), Bologna (Italy)

Oral presentattion "Progression from normal oral mucosa to epithelial dysplastic lesions: a metabolic profiling study on saliva", Congresso Nazionale della Società Italiana di Patologia e Medicina Orale (SIPMO), Naples (Italy)

September 2024 Invited speaker at the course "Patologia e chirurgia orale nel terzo millennio: stato dell'arte e futuri trends" of AIO (Associazione Italiana di Odontoiatria) with the report "Laser applications in odontostomatology", Bari, Italy

September 2024 Oral presentation at "CED-IADR Robert Frank Awards Competitiom", Ginevra (Switzerland)

June 2024 Oral presentation "Identification of salivary miRNAs in women at high risk of breast and ovarian cancer development" for "2nd CED-IADR Award for Italian CED-IADR Award; Trieste

Oral presentation "Diagnostica salivare delle patologie orali e sistemiche" for PhD-day at 31th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Trieste, Italy

Poster discussion "Progression from normal oral mucosa to epithelial dysplastic lesions: a metabolic profiling study on saliva", at 31th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Trieste, Italy

September 2023 Oral presentation at European Association of Oral Medicine (EAOM), 16th biennial congress, London (UK)

May 2023 Invited speaker at International Conference of the Romanian Society of Stomatology and the Romanian Society of Oral Medicine, Bucharest (Romania).

April 2023 Oral presentation "HPV – associated oral lesions. New treatment strategies" at World Federation for Laser Dentistry (WFLD), Wroclaw (Poland).

Poster discussion "Identification of salivary miRNAs in women at high risk of breast and ovarian cancer development" at the 30th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Catania (Italy).

Co-author poster "Menagement of recurrent leukoplakia with diode laser. The importance of careful follow-up during wound healing" at the 30th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Catania (Italy).

Co-author poster "Squamous cell carcinoma (SCC) of the tounge in a 24-year-old patient" at the 30th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Catania (Italy).

Co-author poster "Osteotomy with Er:YAG Laser: a stereomicroscopic evaluation on ex-vivo model" at the 30th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Catania (Italy).

Co-author poster " Unicistic ameloblastoma mimicking a solitary bone cist: report of the controversial case" at the 30th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Catania (Italy).

November 2022 Oral presentation "Salivary diagnostic in oral and systemic diseases" at International symposium of "Società Italiana di Patologia e Medicina Orale – SIPMO" in association with the "European Association of Oral Medicin – EAOM", Parma (Italy).

Poster discussion "Salivary biomarkers for diagnosis of acute myocardial infarction: a systematic review" at International symposium of "Società Italiana di Patologia e Medicina Orale – SIPMO" in association with the "European Association of Oral Medicin – EAOM", Parma (Italy).

Poster discussion "Identification of salivary miRNAs in women at high risk of breast and ovarian cancer development" at International symposium of "Società Italiana di Patologia e Medicina Orale – SIPMO" in association with the "European Association of Oral Medicin – EAOM", Parma (Italy).

Co-author poster "Salivary metabolites in patients with oral leukoplakia" at International symposium of "Società Italiana di Patologia e Medicina Orale – SIPMO" in association with the "European Association of Oral Medicin – EAOM", Parma (Italy).

Co-author poster "Salivary biomarkers for diagnosis of dental caries. Systematic review" at International symposium of "Società Italiana di Patologia e Medicina Orale – SIPMO" in association with the "European Association of Oral Medicin – EAOM", Parma (Italy).

October 2022 Poster discussion "Salivary research group"; 2° Research Day, Department of Medicine and Surgery, University of Parma (Italy).

June 2022 Oral presentation "Identification of salivary miRNAs in women at high risk of breast and ovarian cancer development" at Taobuk Da Vinci Award - Contest 2022, Taormina (Italy).

April 2022 Poster discussion "Identification of salivary miRNAs in women at high risk of breast and ovarian cancer development" at the 29th National Congress - Collegio

dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Bologna (Italy).

Co-author poster " Recurrent Leukoplakia successfully treated with Diode laser: a case report" at the 29th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Bologna (Italy).

Co-author poster "Salivary metabolites in patients with oral leukoplakia" at the 29th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Bologna (Italy).

Co-author poster "Salivary biomarkers for diagnosis of caries: a systematic review" at the 29th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Bologna (Italy).

Co-author poster "A rare case of oral myofibrosarcoma in a young patient" at the 29th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Bologna (Italy).

October 2021 Oral Presentation: "Salivary Diagnostics"; 1° Research Day, Department of Medicine and Surgery, University of Parma (Italy).

April 2021 Poster discussion "Identification of salivary metabolites associated with gingival bleeding: pilot study for early detection of gingival disease" at 28th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Milan (Italy).

Co-author poster "Analysis of salivary microRNAs in a group of patients with mutations BRCA 1-2: a pilot study"- 28th National Congress- Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Milan (Italy).

Co-author poster "An unusual case of mandibular radiotransparency in a young patient" at the 28th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Milan (Italy).

September 2020 Poster discussion "Analysis of the amino acids present in the whole saliva, parotid and sub-mandibular/sub-lingual saliva" at the 27th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Milan (Italy).

Co-author poster "Central giant cell granulomas of the mandible associated with dental implant: a case report" at the 27th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Milan (Italy).

Co-author poster "Salivary cytokines for the diagnosis of oral cancer: a review" at the 27th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Milan (Italy).

Co-autore poster "Characterization of bacterial metabolites in parotid, submandibular/sublingual and whole saliva of healthy subjects" al XV

Congresso Nazionale e III Internazionale della Società Italiana di Patologia e Medicina Orale (SIPMO), Bari (Italy).

October 2019 Discussion poster "Surgical Treatment of Oral Lichen Planus lesions for aesthetic reasons with Diode Laser" at the 7th European Congress WFLD (World Federation for Laser in Dentistry), Parma (Italy).

Co-authors poster "Denture-induced fibrous hyperplasia treated with diode laser: a case report" at the 7th European Congress WFLD (World Federation for Laser in Dentistry), Parma (Italy).

April 2019 Discussion poster "Salivary metabolomics in oral cancer: a sistematic review" at the 26th National Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Naples (Italy).

October 2018 Discussion poster "Differences in metabolic profile between submandibular, parotid and whole saliva: a new concept in salivary diagnostics" at the 5th National and 1st Internation Symposium of the Società Italiana di Patologia e Medicina Orale (SIPMO), Ancona (Italy).

April 2018 Discussion poster "Salivary diagnostics for oral cancer and potentially malignant disorders: a systematic review" at the 25th Nation Congress - Collegio dei Docenti Universitari di discipline Odontostomatologiche (CDUO), Rome (Italy).

Associations

2022 - currently Member of "World Federation for Laser Dentistry" (WFLD)

2022 – currently Member of "European Association of Oral Medicine" (EAOM)

2022 - currently Auditor of Associazione Nazionale Dentisti Italiani di Parma (ANDI – PARMA)

2021 - currently Member of "Società Italiana Laser in Odontostomatologia" (SILO)

2019 - currently Member of "Società Italiana di Patologia e Medicina Orale" (SIPMO)

2018 - currently Member of "Accademia Italiana di Conservativa" (AIC)

2016/2017 - 2017/2018 Local Prophylaxis Officer (LPO) of "Associazione Italiana Studenti di Odontoiatria" (AISO) of the University of Parma.

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