UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN MEDICINA MOLECOLARE
CICLO XXXIV

Salivary diagnostics.

Biomarkers identification and validation for systemic and oral diseases.

Coordinatore:
Chiar.mo Prof. Prisco Mirandola

Tutore:
Chiar.mo Prof. Marco Meleti

Dottorando: Dott. Giacomo Setti

Anni Accademici 2018/2019 – 2020/2021

The PhD program and part of the scientific activity related to this Thesis were conducted also under the co-supervision of Prof.ssa Cristina Magnoni, MD, PhD, University of Modena and Reggio Emilia.

INDEX

CHAPTER 1

Overview and aim of the study

The late diagnosis of a disease is usually associated to a decrease of treatment success rates, an increase of complications and to a worsening of prognosis. Ideal tools for early diagnosis should detect “footprints” of a disease when it is still clinically undetectable.

A biomarker is defined by the WHO International Program on Chemical Safety Biomarkers in Risk Assessment as “any substance, structure, or process that can be measured in the body or its products and that can influence or predict the incidence of outcome or disease” (http://www.inchem.org/documents/ehc/ehc/ehc222.htmas). Such entities might also be used to tailor treatments for individual patients. In 2017 also the US White House has advocated translational and clinical research in this field. Aggregating new data sources concerning the manifestation and treatment of disease, by identifying specific genes, biomarkers or other information about disease predisposition, researchers will be able to design early and precise interventions.

The last 20 years have been characterized by substantial progression in the science and technologies applied to salivary diagnostics. The incorporation of such discipline into the clinical practice of medicine and dentistry mainly depends on the development of point-of-care (POC) and lab-on-a-chip (LOC) diagnostics; these two paradigms would likely allow patients and healthcare providers to gain rapid and cost-effective medical analytical information by use of bedside or biochip integrated devices, for diseases diagnosis and monitoring.

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 2. Latest literature confirms the fluid high potential as a matrix for multipurpose analysis with several different technological platforms; the large number of recently published studies confirms the increasing interest in saliva. However, in terms of use as a diagnostic tool into clinical setting, little evidence is still available.

Chapter 3 reports data about the exploration of the metabolic profile of human saliva. The salivary fluid has been widely studied and genomic, transcriptomic, and proteomic profiles have been reported. Conversely, its metabolic composition is a topic of research: metabolites in submandibular/sublingual saliva have never been analyzed systematically. By the application of ^{1}H nuclear magnetic resonance

(^{1}H-NMR), samples of whole, parotid, and submandibular/sublingual saliva from 20 healthy donors were studied. The aim of this chapter is to describe salivary gland-specific metabolic composition, providing basis to a further identification of salivary biomarkers in oral and systemic diseases.

The progression from normal mucosa to different grades of dysplasia is explored in relation to salivary metabolome in an experimental case-control study (Chapter 4). Qualitative and quantitative metabolomics is performed on liquid matrix using ^{1}H-NMR. A cohort of voluntary patients with histologically confirmed oral leukoplakia was enrolled and salivary specimens were collected before surgical procedures (incisional/excisional biopsies). Expressed metabolome was compared between patients with dysplastic and non-dysplastic leukoplakia and healthy controls.

Pilot research and its preliminary results are presented in chapter 5; the aim of the study was to compare the salivary metabolome of primary Sjögren's syndrome (pSS) cases and healthy controls (HC). Only a few metabolomic studies have explored the salivary fluids in such syndromic patients and furtherer well conducted research is needed to provide evidence of potential biomarkers. Through ^{1}H-NMR analysis, salivary samples of 7 pSS and 6 HC female patients were analyzed. A high metabolite variability was observed. Normalized spectral matrix multivariate statistical analysis (PCA and PLS-DA) returned promising results, describing significant differences of metabolites expressions between groups.

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

Acute myocardial infarction (AMI) accounts for about 7 million deaths per year worldwide; the early identification of its signs and symptoms and the detection of specific serological markers are mandatory to reach a prompt diagnosis and to start a possibly life-saving treatment. POC technologies applied to salivary diagnostics can provide rapid, simple, low-cost and accurate measurements of specific markers, also in emergency settings. The first systematic review was developed to answer the question "Are salivary biomarkers useful to identify patients with acute myocardial infarction?" (Chapter 6).

Aberrant expression of microRNAs (miRNAs) has been associated with several diseases, including cancer, inflammatory, and autoimmune conditions. Interest for salivary miRNAs as non-invasive tools for diagnosis of malignancies and systemic diseases is rapidly increasing. The second systematic

review was developed for answering the question "Are salivary miRNAs reliable biomarkers for diagnosis of cancer and systemic diseases?" (Chapter 7).

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

Conclusions are provided in chapter 8.

CHAPTER 2

Salivary diagnostics – An Update

Human saliva is an aqueous mixture of secretions from major and minor salivary glands, such as parotid, submandibular, sublingual and diffused glandular tissue beneath the oral mucosa, as well as gingival crevicular fluid (GCF). Components of the non-aqueous fraction (about 1% overall) are both inorganic and organic. Sodium, chloride, calcium, potassium, magnesium, bicarbonate, sulphate, thiocyanate, phosphate and fluoride ions are the most representative inorganic parts, while organic components consist of proteins, enzymes, mucins and immunoglobulins (e.g., IgA) (Kaczor-Urbanowicz, Martin Carreras-Presas et al. 2017). This liquid combination maintains oral cavity homeostasis and contributes to organic functions such as food digestion, lubrication of tissues, swallowing and tasting, buffering of pH variations, antimicrobial activity; reduced secretion of saliva is associated to development of periodontal diseases, dental caries, mucositis and opportunistic infections (Pappa, Kousvelari et al. 2019).

Active and passive transportation of molecules from serum to salivary glands parenchyma and eventually to saliva, continuously take place; consequently, molecules and compounds circulating in bloodstream can be secreted through saliva (Williamson, Munro et al. 2012). Moreover, plasmatic leakage from bloodstream occurs through cervical gingiva of the periodontal sulcus, where GCF is released. The whole saliva composition is also affected by the presence of microbiota and cellular debris from the oral cavity mucosa (Meleti, Quartieri et al. 2020).

Mean daily salivary flow ranges from approximately 500ml to 1500ml, with an average 1ml volume always persistent in the oral cavity. Salivary flow and total volume are influenced by systemic health conditions, age, water intake levels, medications and dietary alterations. Moreover, circadian rhythm and neural/hormonal pathways influence on salivary secretion (Amerongen and Veerman 2002, Helmerhorst, Dawes et al. 2018).

Saliva diagnostic potential, particularly regarding its qualitative composition was explored from the late ‘90. For its strict relationship to teeth and periodontal tissues, caries and periodontal disease the first pathological processes investigated in relation to saliva quality and quantity (Spielmann and Wong 2011). Consistently with the development of diagnostic technologies, numerous analytes have emerged as potential salivary biomarkers. In addition, the interest in the relationship between such complex fluid and non-oral diseases raised (Roda, Mirasoli et al. 2018, Zangheri, Mirasoli et al. 2019).

The detection of salivary molecules associated with pathological and physiological alterations has encouraged the search of novel and non-invasive diagnostic biomarkers and diagnostic tools for oral and systemic health evaluation (Dawes and Wong 2019). The term “salivomics” encompasses all the –omics approaches (genomics, transcriptomics, proteomics, microbiomics and metabolomics) to salivary analysis (Denny, Hagen et al. 2008, Spielmann and Wong 2011, Mikkonen, Singh et al. 2016, Ferrari, Pezzi et al. 2021, Hyvarinen, Savolainen et al. 2021).

The genomic study of saliva refers to the whole high-quality DNA molecules that can be extracted from the oral fluid. Following the proper collection, handling and purification procedures, harvested salivary DNA is qualitatively comparable to blood-derived one (Zhang, Cheng et al. 2016). Besides the identification of genes and their mutations, the salivary genome was also studied to understand its epigenetic behavior. Such investigation highlights how the genome itself is environmentally regulated and capable of adaptation over time, through chemical and structural modification, without double-strain alteration. Epigenetic inheritance includes DNA methylation, RNA-mediated silencing and telomere length measure (Mascolo, Siano et al. 2012). Methylation process consist of addition of a methyl group to DNA nucleotides, directly influencing structural organization and binding of transcription factors. The study of this process is an important part of the salivary epigenome because it reflects the mechanisms through which gene expression changes over time. Salivary DNA has been successfully studied for methylation changes regarding to systemic conditions such as diabetes or muscular dystrophy, as well as cancer. Telomer length is considered the biological clock of the organism; is influenced by genetic factors, with studies reporting heritability estimates ranging from 34 to 82% but also, with not conclusive evidence, by chemical and environmental factors, social and behavioral activities, psychiatric and psychological conditions (Cerveira de Baumont, Hoffmann et al. 2021). The recent application of multiplexed monochrome qPCR to salivary fluid has made possible to measure the telomere length of human DNA isolated from the genome obtained from whole unstimulated saliva (Han, Kubota et al. 2021).

The study of the salivary transcriptome is committed to the identification of salivary mRNA and non-coding RNA (microRNA or miRNAs) and their clinical applications in disease diagnostics as well as for follow-up (Nonaka and Wong 2018). Detection of altered transcriptome could be helpful in disease

classification and progression monitoring. More than 3000 mRNA and 300 miRNAs have been discovered in salivary fluids, both from healthy controls and patients, suggesting their potential role as biomarkers (Park, Li et al. 2006, Park, Zhou et al. 2009). Such RNA fragments directly originate from cells by discharge trough micro-vesicles or from degradation process. Epithelial cells turnover, salivary secretion and GCF disseminate transcriptomic molecules into oral cavity. Identification is commonly performed by application of real-time polymerase chain reaction (RT-PCR), microarrays and RNA sequencing (Li, Zhou et al. 2004, Spielmann and Wong 2011). Periodontal disease, Sjögren's syndrome, diabetes, oral- and non-oral cancer were mainly investigated (El-Sakka, Kujan et al. 2018, Kaczor-Urbanowicz, Trivedi et al. 2018).

Siqueira and Dawes illustrated potential of saliva for potential proteinic markers identification. It is reported than about the 20 most abundant salivary proteins represents only the 40% of salivary protein content, simplifying biomarkers detection from the remaining 60% (Siqueira and Dawes 2011) Different analytical platforms are widely accepted for protein identification (e.g. gel base application, western blot, liquid chromatography, mass spectrometry), providing both qualitative and quantitative results (Hegde, White et al. 2003). Human salivary proteins concentration is extremely variable (Denny, Hagen et al. 2008). Immunoaffinity depletion may be effective to unmask low-abundance proteins in saliva by removing highly abundant proteins, such as amylase and proline-rich proteins. Some salivary proteins are heavily glycosylated, configuring homotypic or heterotypic complexes. For example, heavily glycosylated mucins often selectively form heterotypic complexes with amylase and histatins to concentrate those antimicrobial proteins at tissue interfaces for oral protection (Hu, Loo et al. 2007).

Microbiomics is the study of the microbiota, the whole of the microorganisms belonging to a given community. It is performed through bacterial DNA analysis, with extraction from target samples (e.g., saliva, GCF, soft and hard tissues) and PCR amplification. Sequence of resulting genetic fingerprint can be matched with databases for microorganism identification (Belstrom 2020). The identification and the behavior of a bacterial community can be monitored in time, also considering environmental alteration of the harvesting site (Shaw, Ribeiro et al. 2017). Salivary fluid is sterile until the intraoral secretion; then enters in contact with oral cavity bacterial reservoirs (e.g. tonsillar pits, lingual

specialized epithelium, dental and periodontal tissue), being “contaminated” by microorganism from those different ecological niches (Mark Welch, Rossetti et al. 2016) The salivary microbiome appears to be strictly related to the individual and its health condition. Moreover, it seems to appear quite stable in its representativeness during time. On the other hand, it is mostly influenced by presence and status of dentition, environmental changes and diet (Belstrom 2020). Salivary microbiomics was widely applied in the study of periodontitis, caries and systemic diseases (Simon-Soro, Sherriff et al. 2018, Damgaard, Danielsen et al. 2019). Even considering the limitations of published data, salivary microbiome seems to be a very promising source of information.

The analysis of metabolites, the small, hydrophilic molecular intermediate and end-product of metabolic pathways, is called metabolomic (or sometimes metabonomic). Such as small molecules can rapidly diffuse through cells membranes and being eliminated into biofluids as well as blood, urine and also saliva (Dame, Aziat et al. 2015). From a thorough literature evaluation emerge that different approaches can be employed for metabolites analysis, depending on the aim of the study, type of investigation set up, funding, number of samples and characteristics of the disease to be investigated. Such process can be distinguished into untargeted and targeted metabolomics; the first refers to the exploration and description of the whole metabolome in a certain condition, qualitatively and/or quantitatively. Targeted metabolomics is otherwise the research and quantification of specific molecules in the reference environment. Different analytical platforms, like mass spectrometry (MS), ultraperformance liquid chromatography MS (UPLC-MS), gas chromatography MS (GC-MS) and nuclear magnetic resonance (NMR), are mostly used to investigate the liquid phase matrix. A recently published review indicates oral cancer and periodontitis as the most investigated diseases with salivary metabolomics, in addition to systemic conditions like Sjögren’s syndrome, breast cancer and diabetes (Hyvarinen, Savolainen et al. 2021).

2.1 Caries

There is growing evidence supporting salivary testing for identification of caries risk. However, to the best of our knowledge, no single salivary molecule has sufficient specificity and sensibility to recognize patients with elevated caries susceptibility. On the other hand, a reliable diagnostic-

prognostic approach can be based on a combination of different analysis. chair-side diagnostic salivary tests are under development.(Gao, Jiang et al. 2016). It is worth to mention that besides the qualitative variation of salivary fluids (e.g. microbial contamination, metabolites expression and proteomic pattern), reduction of salivary flow is the main predisposing factor for caries development, followed by pH level and impairment of buffering potential (Leone and Oppenheim 2001).

Proteomic analysis of salivary fluid in type 1 diabetes children has recently been performed. Results highlighted how down-regulation of usually expressed proteins may explain the increased incidence of caries. Defense response to the bacterium, beta-defensin and proline-rich protein activities, oxygen binding, calcium binding and glycosylation were deregulated in these patients, thus leading to increasing caries risk for a failure in the process of tooth remineralization (Pappa, Vougas et al. 2021). Peculiar proteomic profile related to caries risk and progression was also described in children, suggesting a synergistic action of the mentioned group of molecules; such proteomic expression seems to demonstrate an abnormal oral condition of young children susceptible to dental caries (Wang, Wang et al. 2018).

Identification and concentration of specific bacteria in saliva (such as *mutans streptococci* and *lactobacilli spp.*) was also related to high risk of caries development, in a study conducted on Australian indigenous population (Fernando, Tadakamadla et al. 2021).

Early childhood caries (ECC) disease is a non-self-limiting infectious disease. It can affect deciduous teeth in a particularly virulent form, with caries beginning barely after dental eruption and involving all surfaces indistinctly; it can be rapidly progressing and damaging dentition irreversibly. Early childhood caries disease is related to *mutans streptococci*, *prevotella and candida spp.*; in addition, salivary proteins like IgA and IgG, proline-rich proteins (PRP) and histatin peptides are involved in pathogenesis. Such bacterial and molecular factors were analyzed regarding salivary testing and their concentrations compared with caries-free individuals. An higher caries prevalence of caries was reported suggesting that salivary IgA, IgG, PRP and histatin could be useful as ECC biomarkers (Hemadi, Huang et al. 2017).

2.2 Periodontal diseases

Since the earliest interest in salivary testing for periodontal disease, a variety of oral biomarkers in saliva and GCF were investigated for screening, diagnosis and follow-up (Giannobile, Beikler et al. 2009, Sexton, Lin et al. 2011). Proteomic analysis of oral fluids has detected molecules such as IL-1β and TNFα, associated both with early manifestation and progression of periodontitis, and therefore potentially useful as biomarkers (Shin, Kim et al. 2019) . The salivary detection of IL-8 protein although, was not significantly associated with chronic periodontitis forms, while its expression in biopsied gingival tissue was positively correlated with the disease (Finoti, Nepomuceno et al. 2017). Matrix metalloproteinase-8 (MMP-8), a host-derived collagenase, can destroy collagen I; such enzyme is important in normal activity of periodontal tissue and in disease associated tissue degradation. Studies on MMP-8 indicates that the protein is one of the most interesting markers in discrimination between periodontal diseases and healthy tissues. Several studies have tested saliva for MMP-8 detection, with high variability in sensitivity and specificity in results, also based on the analytical approach used (e.g., ELISA, lateral flow immunoassay, immunofluorometric assay, multiplex cytometry assay) (Deng, Pelekos et al. 2021).

Metabolomics has also been used to explore the salivary fluid of periodontally compromised patients. A few human metabolites potentially candidate as biomarkers were detected, the most of small molecules identified being of bacterial origin. Aimetti *et* al. reported good performance in discriminating general chronic periodontitis (GCP) patient from healthy controls (Aimetti, Cacciatore et al. 2011). The same authors reported how metabolites expression of GCP remain unaltered after non-surgical therapy despite of major a clinical improvement (Romano, Meoni et al. 2019, Citterio, Romano et al. 2020).

2.3 Non-oral cancers

The purpose of a liquid biopsy matrix alternative to blood collection has moved research towards the application of different analytical techniques to saliva, in search of potential biomarkers. For its advantages in collection and handling, the oral fluid was widely explored for the detection of both of oral and systemic diseases (Kaur, Jacobs et al. 2018, Rapado-Gonzalez, Martinez-Reglero et al. 2020, de Sa Alves, de Sa Rodrigues et al. 2021, Goldoni, Scolaro et al. 2021).

Various Authors have investigated the potential of human salivary microRNAs, cell-free nucleic acids, mRNA, cytokines and peptides for cancers diagnosis, therapy efficacy evaluation and follow-up. Such molecules should be furtherly tested before validation as biomarkers; in addition, where reviews has systematically evaluated quality assessment, a majority of low/intermediate quality clinical studies were reported and future investigations should avoid related potential risk of biases (El-Sakka, Kujan et al. 2018, Setti, Pezzi et al. 2020, Chiamulera, Zancan et al. 2021, Ferrari, Pezzi et al. 2021).

A recent review reported how cancer salivary molecular footprint was widely investigated in the last 20 years, with the purpose of identify prospective biomarkers. Even with questionable evidence, a wide group of potential molecules (such as C-erbB2, CA 15-3, Cathepsin D, sialic acid and P53, EGF, VEGF and the CEA), were identified in breast cancer patients' saliva through the application of MS, Raman Spectroscopy and proteomic technologies. Similarly, salivary DNA and proteome were explored to discover markers of lung cancer, as well as RNA was the main target for the detection of both lung (10 studies) and pancreatic malignancies (6). The two diseases share a higher mortality due to the late diagnosis. Lung cancer was associate to EGFR, mRNAs, and a variety of proteins while pancreatic cancer was related to the detection of salivary mRNA and miRNAs. The evidence reported is remarkable, suggesting that the incorporation of such markers, alone or in association, could be a reliable diagnostic tool. Statistically significant results characterized the salivary testing for gastric cancer; the neoplasm is frequently asymptomatic disease (80%) late diagnosed and particularly aggressive; 7 studies explored miRNAs, mRNAs, proteins and salivary bacteria. The promising findings addresses saliva as a good substrate matrix for diagnostic delay prevention (Meleti, Cassi et al. 2020).

To point out on the level of reliability of salivary markers for cancer diagnosis, Rapado-Gonzalez *et* al. performed a systematic review and meta-analysis on 155 data sets obtained from 29 papers (Rapado-Gonzalez, Martinez-Reglero et al. 2020), published between 1990 and 2018. This wide data-output comprises 11,153 adult subjects (cancer patients and healthy controls) tested for biomarkers identification. Eleven proteomic, 9 epigenomic, 4 transcriptomic studies, 2 metabolomic, 2 genomic and 2 microbiome studies were selected. Encouraging results emerged about potential salivary markers; authors reported an overall high sensitivity (73–91%) and specificity (71–89%) for detecting distinct tumor location. However, biases and potential fatal flaws has emerged such as studies with limited sample size, absence of validation phase related to specificity and sensitivity results and missing relationship with TNM classification of tumor stage.

Besides the generally supporting results, further and well conducted studies (e.g., larger and randomized controlled studies) are needed.

2.4 Oral cancer

Oral squamous cell carcinoma (OSCC) accounts for 90–95% of oral cavity cancers and its survival rate is still related to the stage at diagnosis. To avoid diagnostic delay and possibly detect subclinical forms, tools platforms and biomarkers for malignancy identification either than histology on a biopsy, which remains the current gold standard, have been proposed (Nikitakis, Pentenero et al. 2018).

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 tumor 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 (Guerrero-Preston, Soudry et al. 2011). Moreover, somatic mutations of TP53 in saliva identified OSCC in a whole of oral cancer patients (Wang, Springer et al. 2015).

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 were

reported in saliva of OSCC, with evidence of decrease of the latter after disease treatment (Park, Zhou et al. 2009, Liu, Lin et al. 2012). In addition, a pilot study reports a comparison between OSCC patients and HC which returned diminished concentrations of miR-139–5p in cancer group (Duz, Karatas et al. 2016).

Several authors investigated the alteration of metabolic pathways and their consequent metabolites expression between OSCC and HC through targeted and untargeted metabolomic procedures (Mikkonen, Singh et al. 2016, de Sa Alves, de Sa Rodrigues et al. 2021, Hyvarinen, Savolainen et al. 2021) Therefore, the comparison between saliva of OSCC patients and affected by others diseases, such as dental caries and periodontitis, highlights how marker specificity is still uncertain (Gardner, Carpenter et al. 2020). The definition of reproducible inclusion/exclusion criteria, case and control selection and clear definition of study population is mandatory to avoid inconsistent results. The majority of the metabolomic study on salivary samples from OSCC cases grossly report about decreased concentrations of valine, leucine, isoleucine, and phenylalanine, as well as elevated levels of porphyrin (Vitório, Duarte-Andrade et al. 2020).

2.5 COVID-19

During the COVID-19 pandemic, interest in salivary testing has raised. The potential of oral fluid as non-invasive and easily accessible matrix for massive population analysis was explored. It has been reported that SARS-CoV-2 RNA virus was present in saliva at measurable concentration both in symptomatic and asymptomatic patients (Michailidou, Poulopoulos et al. 2020, Bastos, Perlman-Arrow et al. 2021). Taking the nasopharyngeal swab (NPS) and polymerase chain reaction (PCR) as a reference tool, the salivary PCR analysis performed with just a slight lower sensitivity (98% vs. 91%). Even in a few reported cases, saliva specimen with viral load were detected, despite a negative NPS (Azzi, Carcano et al. 2021). On the other hand, a recent review has reported how global performances of NPS is still superior when compared to salivary testing; the latter performed at best when viral loads were at their highest peak. Despite of the easy access, the cost reduction and the possibility to perform mass screening, it is important to underline how salivary collection and samples handling should follow specific recommendations to obtain reliable results. For example, collection in

the morning daytime is indicated due to the highest viral load, as well as the indication to immediately store samples at -80°C (Michailidou, Poulopoulos et al. 2020, Canete, Valenzuela et al. 2021).

References

1. Aimetti, M., Cacciatore, S., Graziano, A. and Tenori, L. (2011). "Metabonomic analysis of saliva reveals generalized chronic periodontitis signature." Metabolomics **8**(3): 465-474.
2. Amerongen, A. V. and Veerman, E. C. (2002). "Saliva--the defender of the oral cavity." Oral Dis **8**(1): 12-22.
3. Azzi, L., Carcano, G., Dalla Gasperina, D., Sessa, F., Maurino, V. and Baj, A. (2021). "Two cases of COVID-19 with positive salivary and negative pharyngeal or respiratory swabs at hospital discharge: A rising concern." Oral Dis **27 Suppl 3**: 707-709.
4. Bastos, M. L., Perlman-Arrow, S., Menzies, D. and Campbell, J. R. (2021). "The Sensitivity and Costs of Testing for SARS-CoV-2 Infection With Saliva Versus Nasopharyngeal Swabs : A Systematic Review and Meta-analysis." Ann Intern Med **174**(4): 501-510.
5. Belstrom, D. (2020). "The salivary microbiota in health and disease." J Oral Microbiol **12**(1): 1723975.
6. Canete, M. G., Valenzuela, I. M., Garces, P. C., Masso, I. C., Gonzalez, M. J. and Providell, S. G. (2021). "Saliva sample for the massive screening of SARS-CoV-2 infection: a systematic review." Oral Surg Oral Med Oral Pathol Oral Radiol **131**(5): 540-548.
7. Cerveira de Baumont, A., Hoffmann, M. S., Bortoluzzi, A., Fries, G. R., Lavandoski, P., Grun, L. K., Guimaraes, L. S. P., Guma, F., Salum, G. A., Barbe-Tuana, F. M. and Manfro, G. G. (2021). "Telomere length and epigenetic age acceleration in adolescents with anxiety disorders." Sci Rep **11**(1): 7716.
8. Chiamulera, M. M. A., Zancan, C. B., Remor, A. P., Cordeiro, M. F., Gleber-Netto, F. O. and Baptistella, A. R. (2021). "Salivary cytokines as biomarkers of oral cancer: a systematic review and meta-analysis." BMC Cancer **21**(1): 205.
9. Citterio, F., Romano, F., Meoni, G., Iaderosa, G., Grossi, S., Sobrero, A., Dego, F., Corana, M., Berta, G. N., Tenori, L. and Aimetti, M. (2020). "Changes in the Salivary Metabolic Profile of Generalized Periodontitis Patients after Non-surgical Periodontal Therapy: A Metabolomic Analysis Using Nuclear Magnetic Resonance Spectroscopy." J Clin Med **9**(12).
10. Dame, Z. T., Aziat, F., Mandal, R., Krishnamurthy, R., Bouatra, S., Borzouie, S., Guo, A. C., Sajed, T., Deng, L., Lin, H., Liu, P., Dong, E. and Wishart, D. S. (2015). "The human saliva metabolome." Metabolomics **11**(6): 1864-1883.
11. Damgaard, C., Danielsen, A. K., Enevold, C., Massarenti, L., Nielsen, C. H., Holmstrup, P. and Belstrom, D. (2019). "Porphyromonas gingivalis in saliva associates with chronic and aggressive periodontitis." J Oral Microbiol **11**(1): 1653123.
12. Dawes, C. and Wong, D. T. W. (2019). "Role of Saliva and Salivary Diagnostics in the Advancement of Oral Health." J Dent Res **98**(2): 133-141.
13. de Sa Alves, M., de Sa Rodrigues, N., Bandeira, C. M., Chagas, J. F. S., Pascoal, M. B. N., Nepomuceno, G., da Silva Martinho, H., Alves, M. G. O., Mendes, M. A., Dias, M., Alves, L. A. C. and Almeida, J. D. (2021). "Identification of Possible Salivary Metabolic Biomarkers and Altered Metabolic Pathways in South American Patients Diagnosed with Oral Squamous Cell Carcinoma." Metabolites **11**(10).
14. Deng, K., Pelekos, G., Jin, L. and Tonetti, M. S. (2021). "Diagnostic accuracy of a point-of-care aMMP-8 test in the discrimination of periodontal health and disease." J Clin Periodontol **48**(8): 1051-1065.
15. Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G. S., Boontheung, P., Cociorva, D., Delahunty, C. M., Denny, T., Dunsmore, J., Faull, K. F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S. C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J. A., Ogorzalek Loo, R. R., Malamud, D., Melvin, J. E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S. K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M. A., Takashima, J., Than, S., Wang, J., Whitelegge, J. P., Witkowska, H. E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D. T., Yates, J. R., 3rd and Fisher, S. J. (2008). "The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions." J Proteome Res **7**(5): 1994-2006.

16. Duz, M. B., Karatas, O. F., Guzel, E., Turgut, N. F., Yilmaz, M., Creighton, C. J. and Ozen, M. (2016). "Identification of miR-139-5p as a saliva biomarker for tongue squamous cell carcinoma: a pilot study." Cell Oncol (Dordr) **39**(2): 187-193.
17. El-Sakka, H., Kujan, O. and Farah, C. S. (2018). "Assessing miRNAs profile expression as a risk stratification biomarker in oral potentially malignant disorders: A systematic review." Oral Oncol **77**: 57-82.
18. Fernando, S., Tadakamadla, S., Kroon, J., Lalloo, R. and Johnson, N. W. (2021). "Predicting dental caries increment using salivary biomarkers in a remote Indigenous Australian child population." BMC Oral Health **21**(1): 372.
19. Ferrari, E., Pezzi, M. E., Cassi, D., Pertinhez, T. A., Spisni, A. and Meleti, M. (2021). "Salivary Cytokines as Biomarkers for Oral Squamous Cell Carcinoma: A Systematic Review." Int J Mol Sci **22**(13).
20. Finoti, L. S., Nepomuceno, R., Pigossi, S. C., Corbi, S. C., Secolin, R. and Scarel-Caminaga, R. M. (2017). "Association between interleukin-8 levels and chronic periodontal disease: A PRISMA-compliant systematic review and meta-analysis." Medicine (Baltimore) **96**(22): e6932.
21. Gao, X., Jiang, S., Koh, D. and Hsu, C. Y. (2016). "Salivary biomarkers for dental caries." Periodontol 2000 **70**(1): 128-141.
22. Gardner, A., Carpenter, G. and So, P. W. (2020). "Salivary metabolomics: From diagnostic biomarker discovery to investigating biological function." Metabolites **10**(2).
23. Giannobile, W. V., Beikler, T., Kinney, J. S., Ramseier, C. A., Morelli, T. and Wong, D. T. (2009). "Saliva as a diagnostic tool for periodontal disease: current state and future directions." Periodontol 2000 **50**: 52-64.
24. Goldoni, R., Scolaro, A., Boccalari, E., Dolci, C., Scarano, A., Inchingolo, F., Ravazzani, P., Muti, P. and Tartaglia, G. (2021). "Malignancies and Biosensors: A Focus on Oral Cancer Detection through Salivary Biomarkers." Biosensors (Basel) **11**(10).
25. Guerrero-Preston, R., Soudry, E., Acero, J., Orera, M., Moreno-Lopez, L., Macia-Colon, G., Jaffe, A., Berdasco, M., Ili-Gangas, C., Brebi-Mieville, P., Fu, Y., Engstrom, C., Irizarry, R. A., Esteller, M., Westra, W., Koch, W., Califano, J. and Sidransky, D. (2011). "NID2 and HOXA9 promoter hypermethylation as biomarkers for prevention and early detection in oral cavity squamous cell carcinoma tissues and saliva." Cancer Prev Res (Phila) **4**(7): 1061-1072.
26. Han, X., Kubota, R., Tanaka, K. I., Hayashi, H., Seki, M., Sakai, N., Kawaguchi-Ihara, N., Arakawa, K. and Murohashi, I. (2021). "The correlation of salivary telomere length and single nucleotide polymorphisms of the ADIPOQ, SIRT1 and FOXO3A genes with lifestyle-related diseases in a Japanese population." PLoS One **16**(1): e0243745.
27. Hegde, P. S., White, I. R. and Debouck, C. (2003). "Interplay of transcriptomics and proteomics." Curr Opin Biotechnol **14**(6): 647-651.
28. Helmerhorst, E. J., Dawes, C. and Oppenheim, F. G. (2018). "The complexity of oral physiology and its impact on salivary diagnostics." Oral Dis **24**(3): 363-371.
29. Hemadi, A. S., Huang, R., Zhou, Y. and Zou, J. (2017). "Salivary proteins and microbiota as biomarkers for early childhood caries risk assessment." Int J Oral Sci **9**(11): e1.
30. Hu, S., Loo, J. A. and Wong, D. T. (2007). "Human saliva proteome analysis." Ann N Y Acad Sci **1098**: 323-329.
31. Hyvarinen, E., Savolainen, M., Mikkonen, J. J. W. and Kullaa, A. M. (2021). "Salivary Metabolomics for Diagnosis and Monitoring Diseases: Challenges and Possibilities." Metabolites **11**(9).
32. Kaczor-Urbanowicz, K. E., Martin Carreras-Presas, C., Aro, K., Tu, M., Garcia-Godoy, F. and Wong, D. T. (2017). "Saliva diagnostics - Current views and directions." Exp Biol Med (Maywood) **242**(5): 459-472.
33. Kaczor-Urbanowicz, K. E., Trivedi, H. M., Lima, P. O., Camargo, P. M., Giannobile, W. V., Grogan, T. R., Gleber-Netto, F. O., Whiteman, Y., Li, F., Lee, H. J., Dharia, K., Aro, K., Martin Carreras-Presas, C., Amuthan, S., Vartak, M., Akin, D., Al-Adbullah, H., Bembey, K., Klokkevold, P. R., Elashoff, D., Barnes, V. M., Richter, R., DeVizio, W., Masters, J. G. and Wong, D. T. W. (2018). "Salivary exRNA biomarkers to detect gingivitis and monitor disease regression." J Clin Periodontol **45**(7): 806-817.

34. Kaur, J., Jacobs, R., Huang, Y., Salvo, N. and Politis, C. (2018). "Salivary biomarkers for oral cancer and pre-cancer screening: a review." Clin Oral Investig **22**(2): 633-640.
35. Leone, C. W. and Oppenheim, F. G. (2001). "Physical and chemical aspects of saliva as indicators of risk for dental caries in humans." J Dent Educ **65**(10): 1054-1062.
36. Li, Y., Zhou, X., St John, M. A. and Wong, D. T. (2004). "RNA profiling of cell-free saliva using microarray technology." J Dent Res **83**(3): 199-203.
37. Liu, C. J., Lin, S. C., Yang, C. C., Cheng, H. W. and Chang, K. W. (2012). "Exploiting salivary miR-31 as a clinical biomarker of oral squamous cell carcinoma." Head Neck **34**(2): 219-224.
38. Mark Welch, J. L., Rossetti, B. J., Rieken, C. W., Dewhirst, F. E. and Borisy, G. G. (2016). "Biogeography of a human oral microbiome at the micron scale." Proc Natl Acad Sci U S A **113**(6): E791-800.
39. Mascolo, M., Siano, M., Ilardi, G., Russo, D., Merolla, F., De Rosa, G. and Staibano, S. (2012). "Epigenetic disregulation in oral cancer." Int J Mol Sci **13**(2): 2331-2353.
40. Meleti, M., Cassi, D., Vescovi, P., Setti, G., Pertinhez, T. A. and Pezzi, M. E. (2020). "Salivary biomarkers for diagnosis of systemic diseases and malignant tumors. A systematic review." Med Oral Patol Oral Cir Bucal **25**(2): e299-e310.
41. Meleti, M., Quartieri, E., Antonelli, R., Pezzi, M. E., Ghezzi, B., Viani, M. V., Setti, G., Casali, E., Ferrari, E., Ciociola, T., Spisni, A. and Pertinhez, T. A. (2020). "Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva." Metabolites **10**(8).
42. Michailidou, E., Poulopoulos, A. and Tzimagiorgis, G. (2020). "Salivary diagnostics of the novel coronavirus SARS-CoV-2 (COVID-19)." Oral Dis.
43. Mikkonen, J. J., Singh, S. P., Herrala, M., Lappalainen, R., Myllymaa, S. and Kullaa, A. M. (2016). "Salivary metabolomics in the diagnosis of oral cancer and periodontal diseases." J Periodontal Res **51**(4): 431-437.
44. Nikitakis, N. G., Pentenero, M., Georgaki, M., Poh, C. F., Peterson, D. E., Edwards, P., Lingen, M. and Sauk, J. J. (2018). "Molecular markers associated with development and progression of potentially premalignant oral epithelial lesions: Current knowledge and future implications." Oral Surg Oral Med Oral Pathol Oral Radiol 125(6): 650-669.
45. Nonaka, T. and Wong, D. T. W. (2018). "Liquid Biopsy in Head and Neck Cancer: Promises and Challenges." J Dent Res **97**(6): 701-708.
46. Pappa, E., Kousvelari, E. and Vastardis, H. (2019). "Saliva in the "Omics" era: A promising tool in paediatrics." Oral Dis **25**(1): 16-25.
47. Pappa, E., Vougas, K., Zoidakis, J., Papaioannou, W., Rahiotis, C. and Vastardis, H. (2021). "Downregulation of Salivary Proteins, Protective against Dental Caries, in Type 1 Diabetes." Proteomes **9**(3).
48. Park, N. J., Li, Y., Yu, T., Brinkman, B. M. and Wong, D. T. (2006). "Characterization of RNA in saliva." Clin Chem **52**(6): 988-994.
49. Park, N. J., Zhou, H., Elashoff, D., Henson, B. S., Kastratovic, D. A., Abemayor, E. and Wong, D. T. (2009). "Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection." Clin Cancer Res **15**(17): 5473-5477.
50. Rapado-Gonzalez, O., Martinez-Reglero, C., Salgado-Barreira, A., Takkouche, B., Lopez-Lopez, R., Suarez-Cunqueiro, M. M. and Muinelo-Romay, L. (2020). "Salivary biomarkers for cancer diagnosis: a meta-analysis." Ann Med **52**(3-4): 131-144.
51. Roda, A., Mirasoli, M., Guardigli, M., Zangheri, M., Caliceti, C., Calabria, D. and Simoni, P. (2018). "Advanced biosensors for monitoring astronauts' health during long-duration space missions." Biosens Bioelectron **111**: 18-26.
52. Romano, F., Meoni, G., Manavella, V., Baima, G., Mariani, G. M., Cacciatore, S., Tenori, L. and Aimetti, M. (2019). "Effect of non-surgical periodontal therapy on salivary metabolic fingerprint of generalized chronic periodontitis using nuclear magnetic resonance spectroscopy." Arch Oral Biol **97**: 208-214.
53. Setti, G., Pezzi, M. E., Viani, M. V., Pertinhez, T. A., Cassi, D., Magnoni, C., Bellini, P., Musolino, A., Vescovi, P. and Meleti, M. (2020). "Salivary MicroRNA for Diagnosis of Cancer and Systemic Diseases: A Systematic Review." Int J Mol Sci **21**(3).

54. Sexton, W. M., Lin, Y., Kryscio, R. J., Dawson, D. R., 3rd, Ebersole, J. L. and Miller, C. S. (2011). "Salivary biomarkers of periodontal disease in response to treatment." J Clin Periodontol **38**(5): 434-441.
55. Shaw, L., Ribeiro, A. L. R., Levine, A. P., Pontikos, N., Balloux, F., Segal, A. W., Roberts, A. P. and Smith, A. M. (2017). "The Human Salivary Microbiome Is Shaped by Shared Environment Rather than Genetics: Evidence from a Large Family of Closely Related Individuals." mBio **8**(5).
56. Shin, M. S., Kim, Y. G., Shin, Y. J., Ko, B. J., Kim, S. and Kim, H. D. (2019). "Deep sequencing salivary proteins for periodontitis using proteomics." Clin Oral Investig **23**(9): 3571-3580.
57. Simon-Soro, A., Sherriff, A., Sadique, S., Ramage, G., Macpherson, L., Mira, A., Culshaw, S. and Malcolm, J. (2018). "Combined analysis of the salivary microbiome and host defence peptides predicts dental disease." Sci Rep **8**(1): 1484.
58. Siqueira, W. L. and Dawes, C. (2011). "The salivary proteome: challenges and perspectives." Proteomics Clin Appl **5**(11-12): 575-579.
59. Spielmann, N. and Wong, D. T. (2011). "Saliva: diagnostics and therapeutic perspectives." Oral Dis **17**(4): 345-354.
60. Vitório, J. G., Duarte-Andrade, F. F., Dos Santos Fontes Pereira, T., Fonseca, F. P., Amorim, L. S. D., Martins-Chaves, R. R., Gomes, C. C., Canuto, G. A. B. and Gomez, R. S. (2020). "Metabolic landscape of oral squamous cell carcinoma." Metabolomics **16**(10): 105.
61. Wang, K., Wang, Y., Wang, X., Ren, Q., Han, S., Ding, L., Li, Z., Zhou, X., Li, W. and Zhang, L. (2018). "Comparative salivary proteomics analysis of children with and without dental caries using the iTRAQ/MRM approach." J Transl Med **16**(1): 11.
62. Wang, Y., Springer, S., Mulvey, C. L., Silliman, N., Schaefer, J., Sausen, M., James, N., Rettig, E. M., Guo, T., Pickering, C. R., Bishop, J. A., Chung, C. H., Califano, J. A., Eisele, D. W., Fakhry, C., Gourin, C. G., Ha, P. K., Kang, H., Kiess, A., Koch, W. M., Myers, J. N., Quon, H., Richmon, J. D., Sidransky, D., Tufano, R. P., Westra, W. H., Bettegowda, C., Diaz, L. A., Jr., Papadopoulos, N., Kinzler, K. W., Vogelstein, B. and Agrawal, N. (2015). "Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas." Sci Transl Med **7**(293): 293ra104.
63. Williamson, S., Munro, C., Pickler, R., Grap, M. J. and Elswick, R. K., Jr. (2012). "Comparison of biomarkers in blood and saliva in healthy adults." Nurs Res Pract **2012**: 246178.
64. Zangheri, M., Mirasoli, M., Guardigli, M., Di Nardo, F., Anfossi, L., Baggiani, C., Simoni, P., Benassai, M. and Roda, A. (2019). "Chemiluminescence-based biosensor for monitoring astronauts' health status during space missions: Results from the International Space Station." Biosens Bioelectron **129**: 260-268.
65. Zhang, C. Z., Cheng, X. Q., Li, J. Y., Zhang, P., Yi, P., Xu, X. and Zhou, X. D. (2016). "Saliva in the diagnosis of diseases." Int J Oral Sci **8**(3): 133-137.

CHAPTER 3

Metabolic profiles of whole, parotid and submandibular / sublingual saliva

Published on Metabolites 2020, 10, 318.

3.1 Introduction

Human whole saliva (WS) is a mixture of fluids produced by parotid (20%), submandibular (65 to 70%), sublingual (7% to 8%), minor (<10%) salivary glands, and by gingival sulcus (crevicular fluid) (De Almeida, Grégio et al. 2008).

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 (Slavish, Graham-Engeland et al. 2015, Wallner-Liebmann, Tenori et al. 2016, Setti, Pezzi et al. 2020). Variations in salivary flow (e.g., by stimulation with citric acid) it is associated in 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 (Figueira, Gouveia-Figueira et al. 2017).

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 (Wallner-Liebmann, Tenori et al. 2016).

Despite the relatively well-described composition of WS, parotid saliva (PS) and submandibular/sublingual saliva (SM/SL), in terms of nucleic acids and proteins (Schipper, Silletti et al. 2007, Fábian, Fejérdy et al. 2008), 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 (Dame, Aziat et al. 2015, Wallner-Liebmann, Tenori et al. 2016, Gardner, Parkes et al. 2019, Pereira, Duarte et al. 2019).

Being unlikely a contamination by exogenous and microbial molecules, the majority of the metabolites in PS (Figueira, Gouveia-Figueira et al. 2017)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 (Gardner, Parkes et al. 2019).

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 (Gardner, Carpenter et al. 2020). In particular, Yamada-Nosaka and co-workers (Yamada-Nosaka, Fukutomi et al. 1991) 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 (Gardner, Carpenter et al. 2020) 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 (Lohavanichbutr, Zhang et al. 2018, Mikkonen, Singh et al. 2018, Kumari, Goyal et al. 2020, Meleti, Cassi et al. 2020) 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) (Cicero, Di Marino et al. 2016, Pitti, Petrella et al. 2019).

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.

3.2 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.

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) (Gomes Pde, Juodzbalys et al. 2012). None of them revealed a full-mouth plaque score (FMPS) and/or full-mouth bleeding score (FMBS) higher than 25%.

3.2.1 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 45min 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 NaN3 (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.

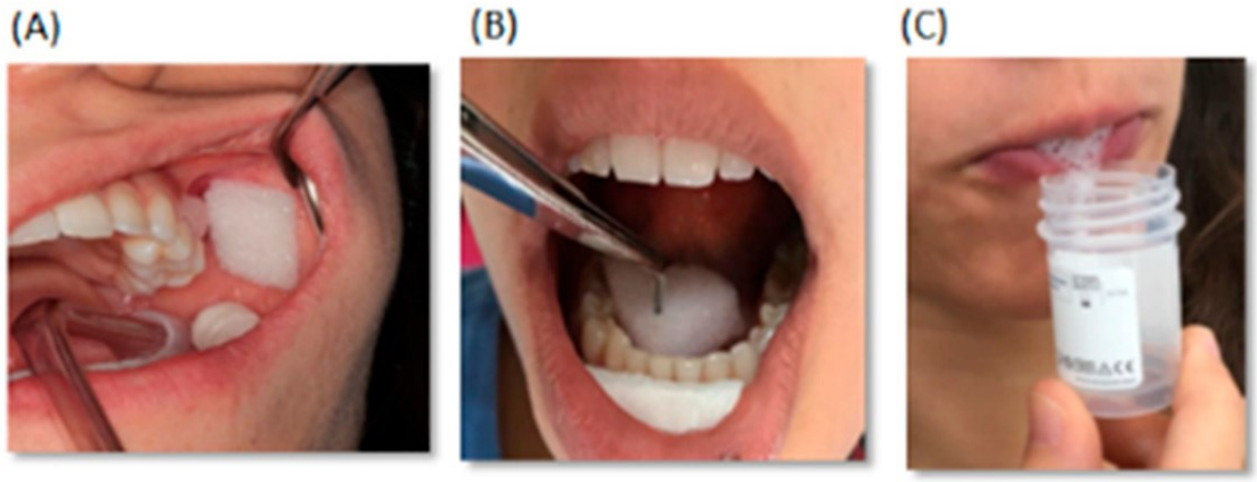

Figure 1. Experimental set-up used for parotid (A), submandibular/sublingual (B), and whole saliva (C) collection.

3.2.2 Sample Preparation and 1H-NMR Spectra Collection and Analysis

Each frozen saliva sample was thawed at room temperature and centrifuged at 15,000x g for 10 min at 4 °C to remove eukaryotic and prokaryotic cells, cellular debris, and mucins, according to Gardner et al. (Gardner, Parkes et al. 2018). The supernatants were protein-depleted by ultra-filtration, using Amicon Ultra-4 Centrifugal filters (3000 MWCO, Merck Millipore) at 4000x g for 120 min at 10 °C, and lyophilized.

For 1H-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) 1H-NMR spectra acquisition and processing were carried out according to Pertinhez *et* al. (Pertinhez, Casali et al. 2014). 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) (Chong, Wishart et al. 2019), with normalization referenced to TSP and autoscaling.

3.2.3 Cell Counting

Eukaryotic cells (oral epithelial cells and leucocytes) and prokaryotic cells were counted according to Gardner et al. (Gardner, Parkes et al. 2018), to estimate their possible contribution to the metabolic profile of WS, SM/SL, and PS.

3.2.4 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.

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), resulting in unsupervised multivariate analysis.

3.3 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).

The salivary flow rates (0.15 ± 0.16mL/min for PS; 0.20 ± 0.09mL/min forSM/SL; 0.49 ± 0.28mL/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 (Percival, Challacombe et al. 1994, Ship, Nolan et al. 1995). 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.

3.3.1 Metabolite Content of Saliva Subtypes: Emerging Differences

The 1D 1H-NMR spectra of WS, SM/PS samples (Figure 2) highlight different and characteristic metabolites patterns. Separation between WS and the other two salivary subtypes is accounted for by lactate, propionate, maltose, 2-aminoadipate, and taurine.

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.

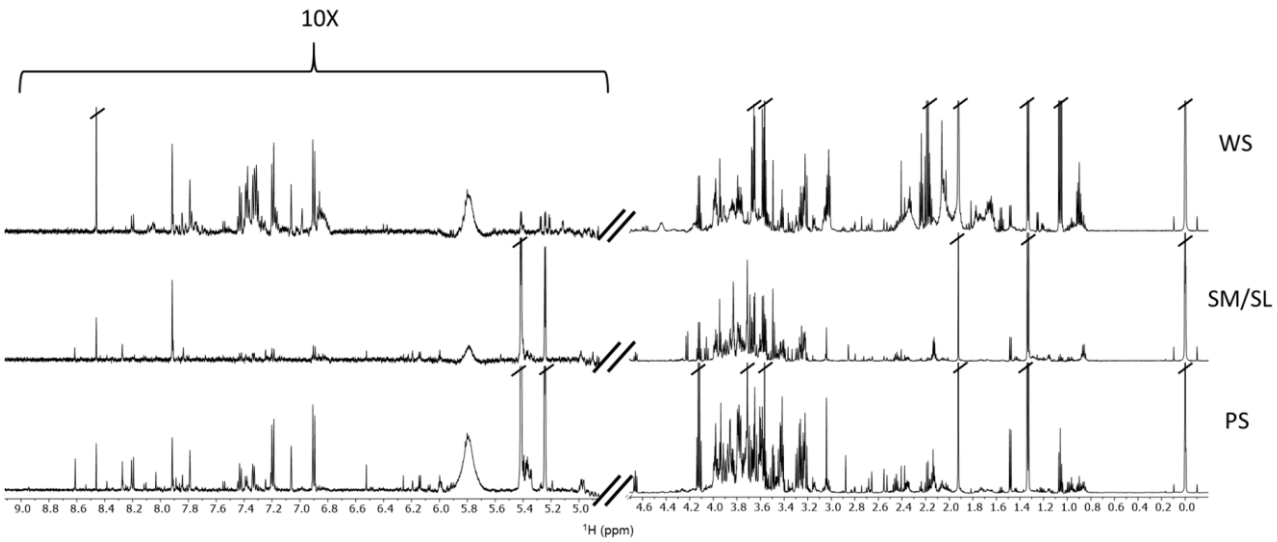

Figure 2. 1H-NMR spectra of unstimulated WS, PS, and SM/SL saliva from the same participant, acquired at 25 °C. The left region of the spectra shows the vertical scale increased by a factor of 10.

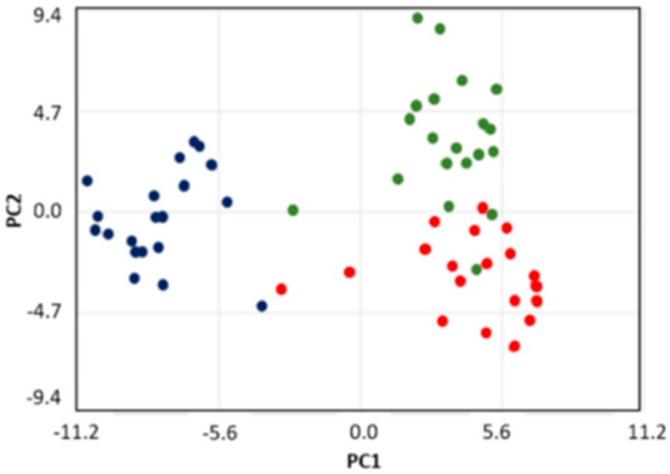

Figure 3 PCA score plot of 1H-NMR spectra of whole saliva (blue), parotid saliva (red), and submandibular/sublingual saliva (green) samples. The contribution of the three principal components of the total variance are PC1 = 62.6%, PC2 = 6.8%, and PC3 = 5.1%.

3.3.2 Salivary Metabolites

We identified 66 metabolites with average concentrations higher than 5 M. 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 few 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.

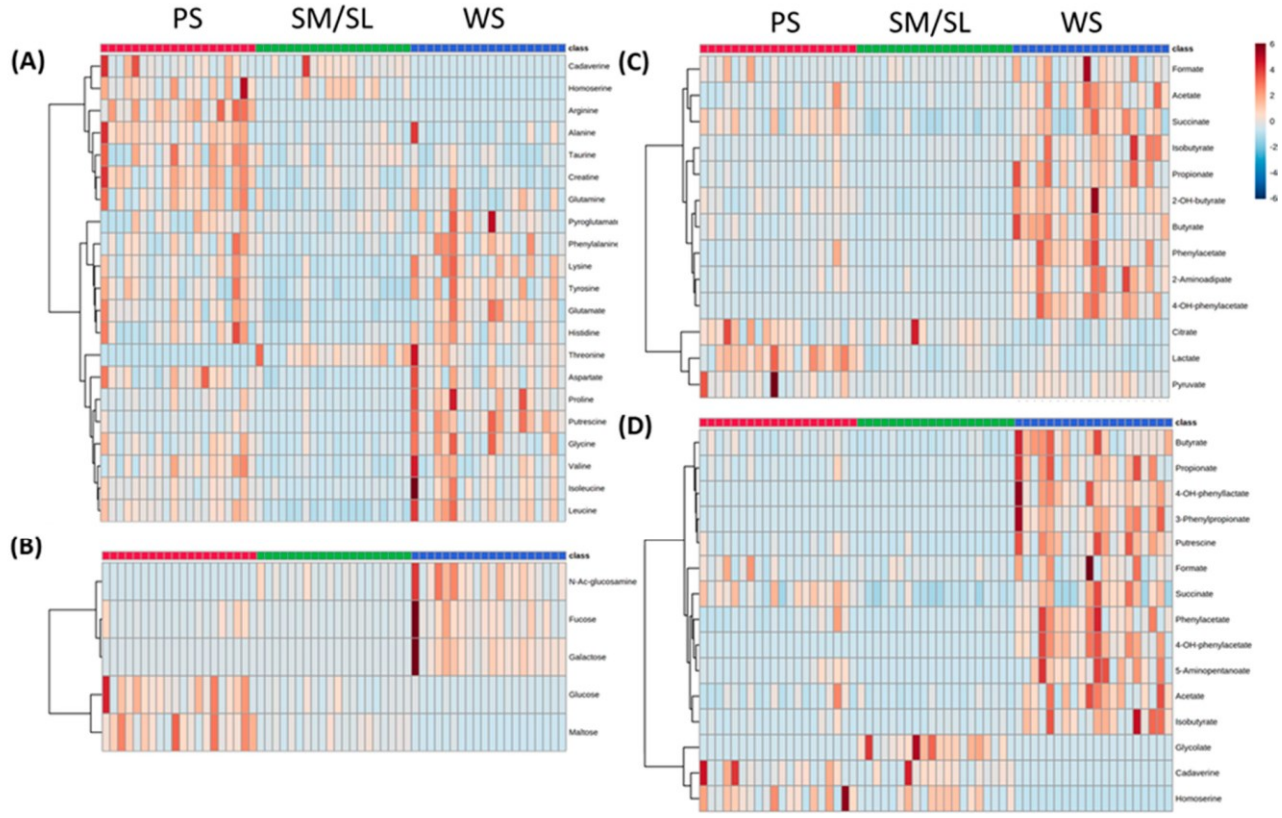

Figure 4. Heatmap analysis of metabolites grouped according to the categories: (A) amino acids, (B) carbohydrates, (C) organic acids, and (D) selected prokaryotic metabolites. PS (red), SMS (green), and WS (blue) samples.

3.3.3 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 most 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.

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)

Note:
[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.

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). 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 (Smith and Macfarlane 1997). 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.

3.3.4 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. (Sender, Fuchs et al. 2016). In all types of saliva, eukaryotic cell counts range between 0.1 and 0.5 × 106 cells/mL, and they are approximately three orders of magnitude lower than the prokaryotic ones.

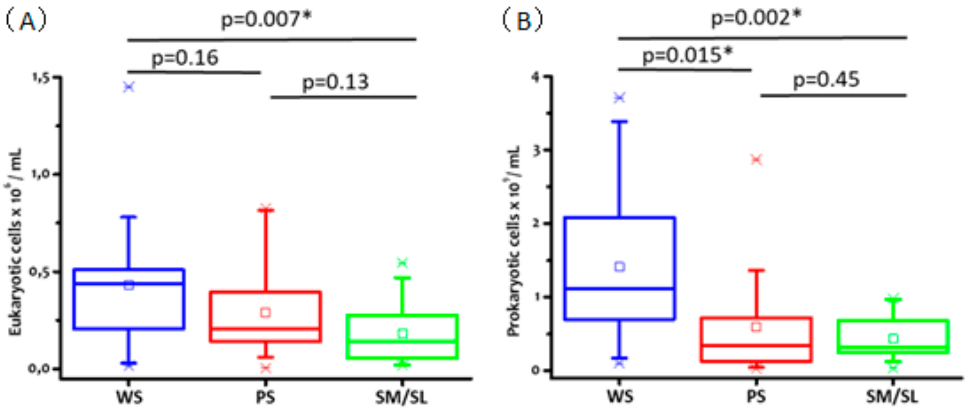

Figure 5 Eukaryotic (A) and prokaryotic (B) cell count distributions obtained with the three salivary types of all the subjects of the study. The significance level of the independent t-tests is set at 0.05 (p < 0.05).*

3.4 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 (Thomadaki, Helmerhorst et al. 2011). Proteolytic amino acid-degrading bacteria dissect proteins and peptides into amino acids and convert them in short chain fatty acids (Takahashi 2015), 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) (Levine 2011), 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 (Zalewska, Zwierz et al. 2000). 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 (Cooke, Leeves et al. 2003). 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 (Vanwuyckhuyse, Bowen et al. 1995) 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 (Evelyn, Javier et al. 2014). 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 (Revenga-Parra, Martínez-Periñán et al. 2017), 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 (Mozaffari and Borke 2002) and, in human saliva, appears to be correlated to physical stress (Cicero, Di Marino et al. 2016).

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 (Gupta, Nayak et al. 2017).

Our study shows that glucose level in PS not only is considerably higher than in WS, as reported by Wang and co-workers (Wang, Du et al. 2017), 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 (Kejriwal, Bhandary et al. 2014). Because glycoproteins degradation is the result of the combined action of various microbial glucosidases (Takahashi 2015), 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

amylase, an enzyme produced by serous cells of parotid glands, with minor contributions from other glands' enzymes (Nikitkova, Haase et al. 2013).

Based on the results reported here, we can hypothesize that the concentrations of maltose in glandular saliva reflect the concentration of gland 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 (Takahashi 2005, Takahashi 2015). 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 (Takahashi 2005, Takahashi 2015).

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 (Gardner, Parkes et al. 2019), 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 (Gardner, Parkes et al. 2019). However, WS lactate concentration may reflect also the contribution of microorganisms and oral mucosa cells (Nagler, Lischinsky et al. 2001). 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 (Gardner, Parkes et al. 2019). 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) (Albandar and Kingman 1999), 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.

References

1. Albandar, J. M. and Kingman, A. (1999). "Gingival Recession, Gingival Bleeding, and Dental Calculus in Adults 30 Years of Age and Older in the United States, 1988-1994." Journal of Periodontology **70**(1): 30-43.
2. Chong, J., Wishart, D. S. and Xia, J. (2019). "Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis." Current Protocols in Bioinformatics **68**(1).
3. Cicero, D. O., Di Marino, S., Dinallo, M., Pieri, M., Summa, V., Desideri, A., Bernardini, F., Perondi, S. and D'Ottavio, S. (2016). "A small sided game session affects salivary metabolite levels in young soccer players." BSI **5**: 55-70.
4. Cooke, M., Leeves, N. and White, C. (2003). "Time profile of putrescine, cadaverine, indole and skatole in human saliva." Archives of Oral Biology **48**(4): 323-327.
5. Dame, Z. T., Aziat, F., Mandal, R., Krishnamurthy, R., Bouatra, S., Borzouie, S., Guo, A. C., Sajed, T., Deng, L., Lin, H., Liu, P., Dong, E. and Wishart, D. S. (2015). "The human saliva metabolome." Metabolomics **11**(6): 1864-1883.
6. De Almeida, P. D. V., Grégio, A. M. T., Machado, M. Â. N., De Lima, A. A. S. and Azevedo, L. R. (2008). "Saliva composition and functions: A comprehensive review." Journal of Contemporary Dental Practice **9**(3): 072-080.
7. Evelyn, R., Javier, M., Gustavo, M., Miguel, N., Patricia, P., Valeria, G., Juan, E. O. and Ismael, Y. (2014). "Caries-free subjects have high levels of urease and arginine deiminase activity." Journal of Applied Oral Science **22**(3): 235-240.
8. Fábian, T. K., Fejérdy, P. and Csermely, P. (2008). "Salivary genomics, transcriptomics and proteomics: The emerging concept of the oral ecosystem and their use in the early diagnosis of cancer and other diseases." Current Genomics **9**(1): 11-21.
9. Figueira, J., Gouveia-Figueira, S., Öhman, C., Lif Holgerson, P., Nording, M. L. and Öhman, A. (2017). "Metabolite quantification by NMR and LC-MS/MS reveals differences between unstimulated, stimulated, and pure parotid saliva." Journal of Pharmaceutical and Biomedical Analysis **140**: 295-300.
10. Gardner, A., Carpenter, G. and So, P. W. (2020). "Salivary metabolomics: From diagnostic biomarker discovery to investigating biological function." Metabolites **10**(2).
11. Gardner, A., Parkes, H. G., Carpenter, G. H. and So, P. W. (2018). "Developing and Standardizing a Protocol for Quantitative Proton Nuclear Magnetic Resonance (1 H NMR) Spectroscopy of Saliva." Journal of Proteome Research **17**(4): 1521-1531.
12. Gardner, A., Parkes, H. G., So, P. W. and Carpenter, G. H. (2019). "Determining bacterial and host contributions to the human salivary metabolome." Journal of Oral Microbiology **11**(1).
13. Gomes Pde, S., Juodzbalys, G., Fernandes, M. H. and Guobis, Z. (2012). "Diagnostic approaches to Sjögren's syndrome: A literature review and own clinical experience." J Oral Maxillofac Res **3**(1).
14. Gupta, S., Nayak, M., Sunitha, J., Dawar, G., Sinha, N. and Rallan, N. (2017). "Correlation of salivary glucose level with blood glucose level in diabetes mellitus." Journal of Oral and Maxillofacial Pathology **21**(3): 334-339.
15. Kejriwal, S., Bhandary, R., Thomas, B. and Kumari, S. (2014). "Estimation of levels of salivary mucin, amylase and total protein in gingivitis and chronic periodontitis patients." J Clin Diagn Res **8**(10): ZC56-ZC60.
16. Kumari, S., Goyal, V., Kumaran, S. S., Dwivedi, S. N., Srivastava, A. and Jagannathan, N. R. (2020). "Quantitative metabolomics of saliva using proton NMR spectroscopy in patients with Parkinson's disease and healthy controls." Neurological Sciences **41**(5): 1201-1210.
17. Levine, M. (2011). "Susceptibility to dental caries and the salivary proline-rich proteins." International Journal of Dentistry.
18. Lohavanichbutr, P., Zhang, Y., Wang, P., Gu, H., Nagana Gowda, G. A., Djukovic, D., Buas, M. F., Raftery, D. and Chen, C. (2018). "Salivary metabolite profiling distinguishes patients with oral cavity squamous cell carcinoma from normal controls." PLoS ONE **13**(9).

19. Meleti, M., Cassi, D., Vescovi, P., Setti, G., Pertinhez, T. A. and Pezzi, M. E. (2020). "Salivary biomarkers for diagnosis of systemic diseases and malignant tumors. A systematic review." Medicina Oral Patologia Oral y Cirugia Bucal **25**(2): e299-e310.
20. Mikkonen, J. J. W., Singh, S. P., Akhi, R., Salo, T., Lappalainen, R., González-Arriagada, W. A., Lopes, M. A., Kullaa, A. M. and Myllymaa, S. (2018). "Potential role of nuclear magnetic resonance spectroscopy to identify salivary metabolite alterations in patients with head and neck cancer." Oncology Letters **16**(5): 6795-6800.
21. Mozaffari, M. S. and Borke, J. L. (2002). "Taurine in submandibular gland of the rat: Effect of muscarinic drugs." Journal of Histochemistry and Cytochemistry **50**(4): 527-532.
22. Nagler, R. M., Lischinsky, S., Diamond, E., Klein, I. and Reznick, A. Z. (2001). "New insights into salivary lactate dehydrogenase of human subjects." Journal of Laboratory and Clinical Medicine **137**(5): 363-369.
23. Nikitkova, A. E., Haase, E. M. and Scannapieco, F. A. (2013). "Taking the starch out of oral biofilm formation: Molecular basis and functional significance of salivary α-amylase binding to oral streptococci." Applied and Environmental Microbiology **79**(2): 416-423.
24. Percival, R. S., Challacombe, S. and Marsh, P. D. (1994). "Flow Rates of Resting Whole and Stimulated Parotid Saliva in Relation to Age and Gender." Journal of Dental Research **73**(8): 1416-1420.
25. Pereira, J. L., Duarte, D., Carneiro, T. J., Ferreira, S., Cunha, B., Soares, D., Costa, A. L. and Gil, A. M. (2019). "Saliva NMR metabolomics: Analytical issues in pediatric oral health research." Oral Diseases **25**(6): 1545-1554.
26. Pertinhez, T. A., Casali, E., Lindner, L., Spisni, A., Baricchi, R. and Berni, P. (2014). "Biochemical assessment of red blood cells during storage by 1H nuclear magnetic resonance spectroscopy. Identification of a biomarker of their level of protection against oxidative stress." Blood Transfusion **12**(4): 548-556.
27. Pitti, E., Petrella, G., Di Marino, S., Summa, V., Perrone, M., D'ottavio, S., Bernardini, A. and Cicero, D. O. (2019). "Salivary metabolome and soccer match: Challenges for understanding exercise induced changes." Metabolites **9**(7).
28. Revenga-Parra, M., Martínez-Periñán, E., Moreno, B., Pariente, F. and Lorenzo, E. (2017). "Rapid taurine and lactate biomarkers determination with disposable electrochemical detectors." Electrochimica Acta **240**: 506-513.
29. Schipper, R. G., Silletti, E. and Vingerhoeds, M. H. (2007). "Saliva as research material: Biochemical, physicochemical and practical aspects." Archives of Oral Biology **52**(12): 1114-1135.
30. Sender, R., Fuchs, S. and Milo, R. (2016). "Revised Estimates for the Number of Human and Bacteria Cells in the Body." PLoS Biology **14**(8).
31. Setti, G., Pezzi, M. E., Viani, M. V., Pertinhez, T. A., Cassi, D., Magnoni, C., Bellini, P., Musolino, A., Vescovi, P. and Meleti, M. (2020). "Salivary microRNA for diagnosis of cancer and systemic diseases: A systematic review." International Journal of Molecular Sciences **21**(3).
32. Ship, J. A., Nolan, N. E. and Puckett, S. A. (1995). "Longitudinal analysis of parotid and submandibular salivary flow rates in healthy, different-aged adults." Journals of Gerontology - Series A Biological Sciences and Medical Sciences **50A**(5): M285-M289.
33. Slavish, D. C., Graham-Engeland, J. E., Smyth, J. M. and Engeland, C. G. (2015). "Salivary markers of inflammation in response to acute stress." Brain, Behavior, and Immunity **44**: 253-269.
34. Smith, E. A. and Macfarlane, G. T. (1997). "Formation of phenolic and indolic compounds by anaerobic bacteria in the human large intestine." Microbial Ecology **33**(3): 180-188.
35. Takahashi, N. (2005). Microbial ecosystem in the oral cavity: Metabolic diversity in an ecological niche and its relationship with oral diseases. International Congress Series. **1284:** 103-112.
36. Takahashi, N. (2015). "Oral microbiome metabolism: From "who are they?" to "what are they doing?"." Journal of Dental Research **94**(12): 1628-1637.

37. Thomadaki, K., Helmerhorst, E. J., Tian, N., Sun, X., Siqueira, W. L., Walt, D. R. and Oppenheim, F. G. (2011). "Whole-saliva proteolysis and its impact on salivary diagnostics." Journal of Dental Research **90**(11): 1325-1330.
38. Vanwuyckhuyse, B. C., Bowen, W. H., Perinpanayagam, H. E. R., Tabak, L. A., Bevacqua, D. and Billings, R. J. (1995). "Association of Free Arginine and Lysine Concentrations in Human Parotid Saliva with Caries Experience." Journal of Dental Research **74**(2): 686-690.
39. Wallner-Liebmann, S., Tenori, L., Mazzoleni, A., Dieber-Rotheneder, M., Konrad, M., Hofmann, P., Luchinat, C., Turano, P. and Zatloukal, K. (2016). "Individual human metabolic phenotype analyzed by 1H NMR of saliva samples." Journal of Proteome Research **15**(6): 1787-1793.
40. Wang, B., Du, J., Zhu, Z., Ma, Z., Wang, S. and Shan, Z. (2017). "Evaluation of parotid salivary glucose level for clinical diagnosis and monitoring type 2 diabetes mellitus patients." BioMed Research International **2017**.
41. Yamada-Nosaka, A., Fukutomi, S., Uemura, S., Hashida, T., Fujishita, M., Kobayashi, Y. and Kyogoku, Y. (1991). "Preliminary nuclear magnetic resonance studies on human saliva." Archives of Oral Biology **36**(9): 697-701.
42. Zalewska, A., Zwierz, K., Zółkowski, K. and Gindzieński, A. (2000). "Structure and biosynthesis of human salivary mucins." Acta Biochimica Polonica **47**(4): 1067-1079.

CHAPTER 4

Metabolic profile of saliva in patients with oral dysplastic and non-dysplastic leukoplakia.

4.1. Introduction

Oral squamous cell carcinoma (OSCC) is a relatively frequent malignant tumor, with an estimated new 377,713 cases per year (2% of all sites; incidence, 4.1%) and 177,757 related deaths (1,9% of all sites; mortality rate 1.9%) occurring in 2020 (Sung, Ferlay et al. 2021). Despite a marked improvement of treatment modalities, the 5-year survival rate is approximately 50%. Stage at diagnosis remains an important indicator for predicting patient survival. OSCC may develop *de novo*, or in areas of pre-existing mucosal pathology (potentially malignant disorders, PMDs, lesions with increased risk of cancer development that can exhibit different grade of dysplasia) and usually spreads locally invading surrounding structures (van der Waal 2009, Warnakulasuriya 2018)

PMDs indicates a group of heterogeneous disorders involving the oral mucosa, including, among others, leukoplakia (OLK), erythroplakia, oral submucous fibrosis, lichen planus (OLP) and oral lichenoid lesions (Warnakulasuriya 2018). The majority of such lesions are asymptomatic in the early stages and the lack of symptoms is possibly associated to diagnostic delay. The risk of malignant transformation depends on numerous variables such as the type of PMDs, the characteristics of disorder such as color, location, size, duration, the presence and grade of dysplasia (Speight 2007, Warnakulasuriya 2020)

4.1.1. Oral dysplasia

Oral dysplasia is defined as an abnormal epithelial growth characterized by a spectrum of cytologic, maturational and architectural changes. Such alterations can affect cell layers from the lowest one-third (basal/parabasal), to middle one-third until the upper one-third. In one of the indexes used, extension of involvement is related to the grade definition: mild (<1/3), moderate (1/3 to 2/3) and severe (>2/3), respectively.

Full thickness changes are also indicated as *carcinoma in situ* (CIS). In these cases, neoplastic keratinocytes stromal invasion is still not appreciable.

Cytological alteration includes anisonucleosis, anisocytosis, nuclear enlargement and hyperchromatism, increased and abnormal mitotic figures. Architectural alterations encompasses

irregular epithelial layering, loss of polarity and development of “drop shape” rete ridges. In addition, dyskeratosis (premature keratinization), keratin pearls, bulbous rete pegs and basal cells hyperplasia are described (Speight 2007, Woo 2019). A recent review has summarized the genetic bases underlying dysplasia and PMDs development. Loss of heterozygosity (LOH) in 3p, 4q, 9p and 17p loci are associated with dysplasia development and progression, while 8p, 11q and 13q are related to evolution from dysplasia to OSCC. Genomic imbalances of CDKN2A (9p21.3), RASSF1 (3p21.31), FHIT (3p14.2) and RB1 (13q14.2), have been reported in dysplastic OLK that progressed to OSCC and in non-progressive dysplasia cases. While in dysplasia with malignant progression copy number alterations (CNA) involved multiple loci, in non-progressive dysplasia alterations are related to a single locus. Increased expression of p53 is detectable in moderate and severe dysplasia as well as CIS. Moreover, DNA ploidy in dysplastic lesions is a good predictor of malignant transformation. Epithelial grow factor receptor (EGFR) copy number increase has been demonstrated in oral epithelial dysplasia (Guimaraes, Diniz et al. 2021).

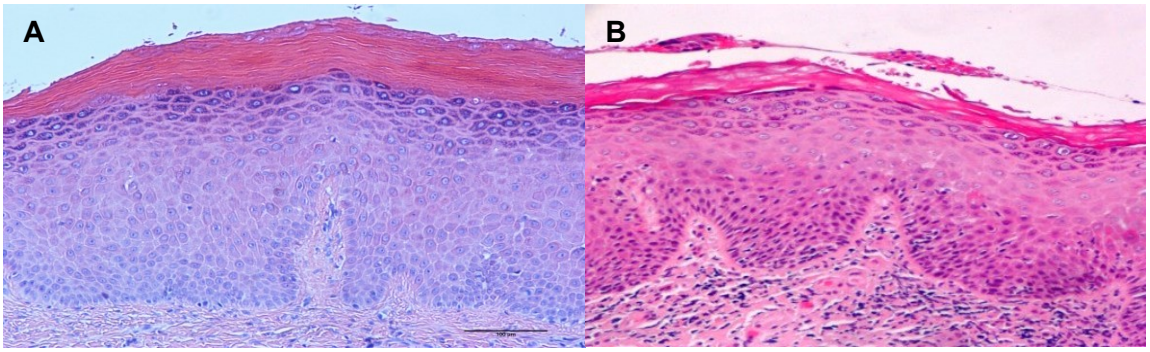

Figure 1. Histological slides from leukoplakia biopsy. A) absence of dysplasia at Hematoxylin & Eosin (H&E) – 50X. B) presence of moderate grade dysplasia at H&E – 50X.

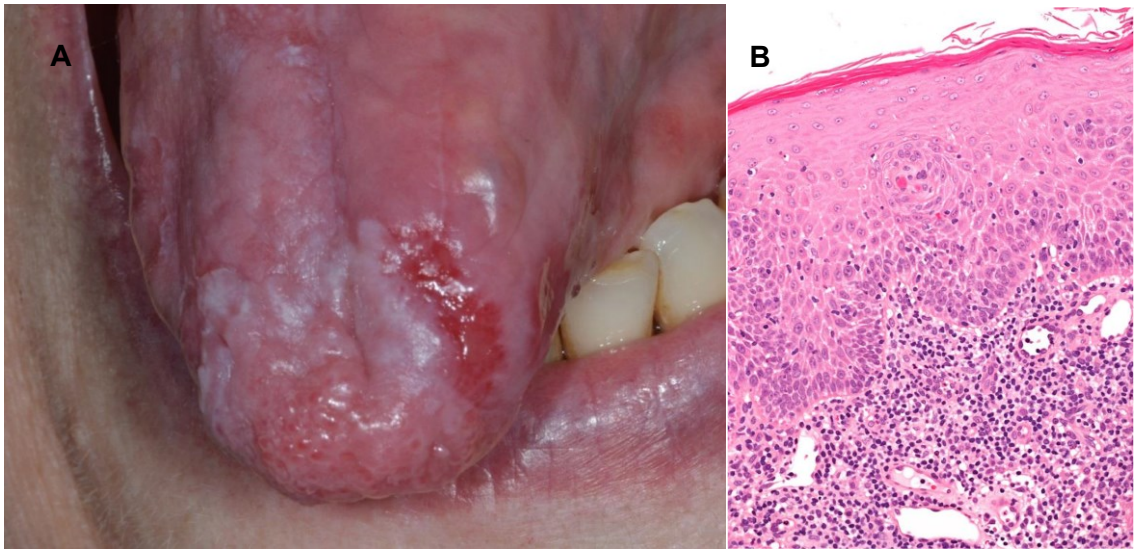

Figure 2. Chronic ulcerative lesion of the tongue in 74 y/o female (A). A biopsy highlighted the presence of severe dysplasia at H&E – 50X (B).

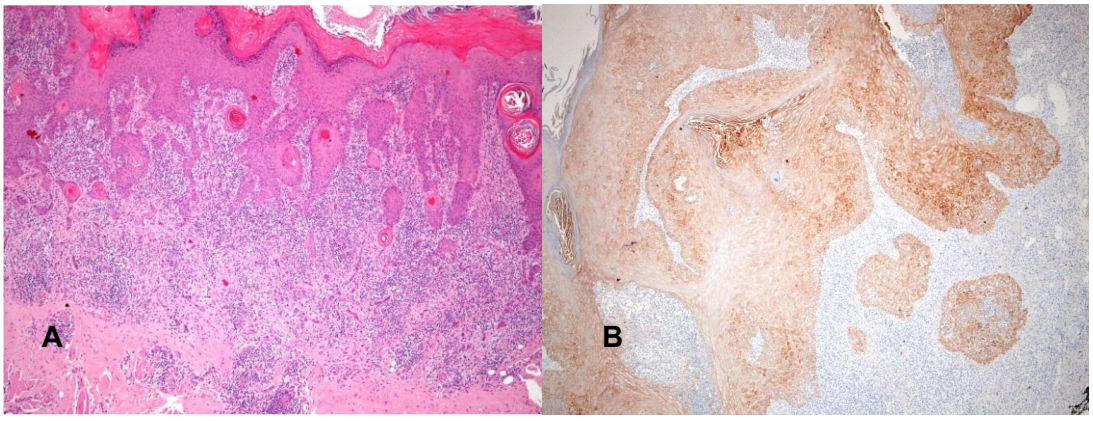

Figure 3. A) Infiltrative low-differentiated OSCC, H&E – 25X. Keratin pearls and inflammatory infiltrate are present in the stroma. B) Stromal infiltration of neoplastic cells marked with MNF116, a broad-spectrum cytokeratin immunostaining which react with high and low molecular weight cytokeratins (e.g., CK 5, 6, 8, 17 and 19) – 25X.

4.1.2. Leukoplakia, pathogenesis and progression to cancer

Oral leukoplakia (OLK) has been defined a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer (Warnakulasuriya, Johnson et al. 2007). It is classified under the ICD code as ICD-10: K13.21. The malignant transformation rate of OLK is influenced by lesion features including location, extension, borders, superficial pattern, macroscopic differentiation from the surrounding tissue and grade of epithelial dysplasia (Villa and Woo 2017).

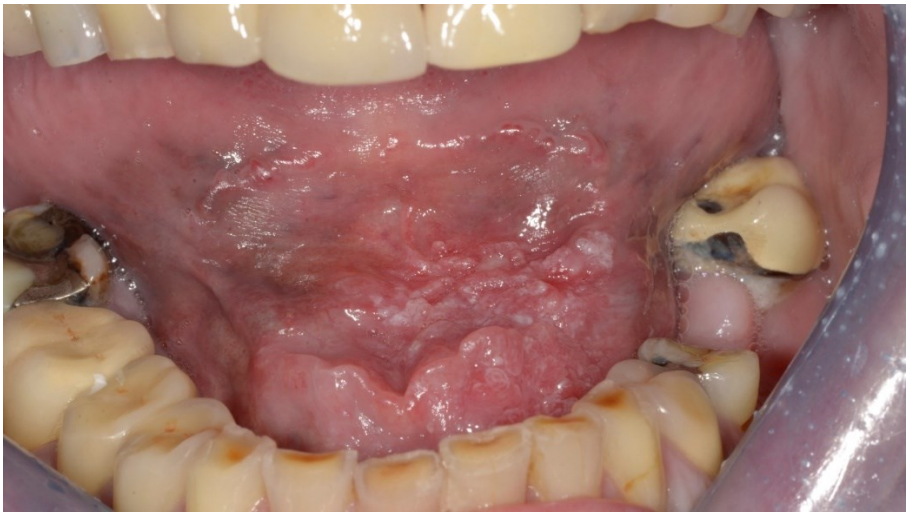

Figure 4. Oral squamous cell carcinoma of the floor of the mouth in a 62 y/o female. Multiple biopsies highlighted the presence of dysplastic alterations in the clinically normal surrounding tissue.

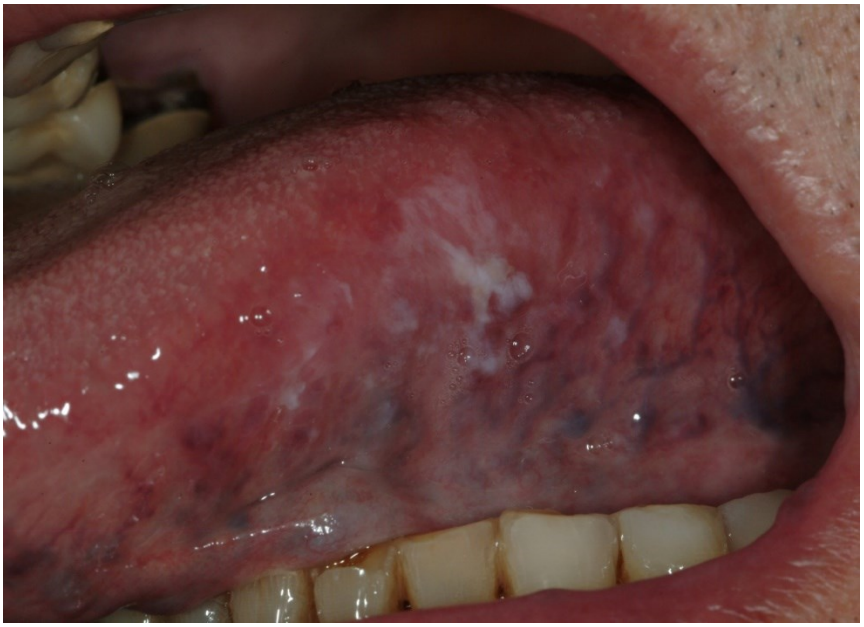

Figure 5. Non-homogeneous leukoplakia (with dysplasia at histological level) of the tongue in a 75 y/o male.

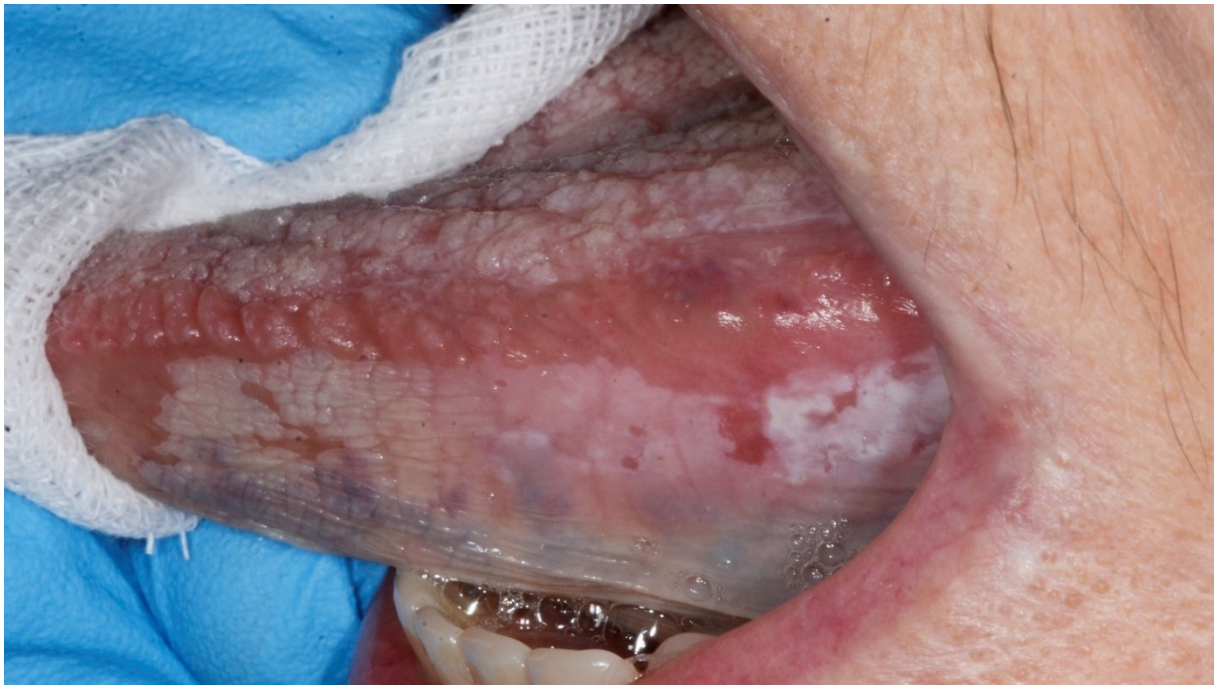

Figure 6. Non-homogeneous oral leukoplakia in a 75 y/o female, extensively involving the tongue.

Prevalence of leukoplakia in general population was reported between 1,5% and 2,6% with M/F ratio 3:1, especially in developing countries. Recently, a meta-analysis on 22 papers updated pooled prevalence at 4,1% for general population. Higher incidence was reported in males of developing countries, related to working and social habits and tobacco/alcohol. Slightly similar incidence in both sex is reported in developed countries, with less difference in tobacco/alcohol exposures (Petti 2003, Mello, Miguel et al. 2018).

An appropriate consistent clinical description of OLK should include the evaluation of texture, margins and color(s). Homogeneous lesions are white, flat, with well-defined boundaries. Non-homogeneous OLK presents with white areas often accompanied by erythema, also possibly associated to areas of nodularity and verrucosity (van der Waal 2010). Those presenting a mix of red and white plaques are defined as erythroleukoplakia; such lesions frequently harbor moderate/severe degree of dysplasia at diagnosis. When single large sites or multifocal concomitant lesions are presents, proliferative verrucous leukoplakia (PVL) should be considered. PVL include a specific subgroup of non-homogenous OLK frequently involving the gingiva, buccal mucosa, and tongue. Presentation of this variant is peculiar: it is infrequent in males (pooled female/male ratio 2,5); it is diagnosed between the

5th and the 7th decades, and it is not strictly associated with smoking habit (Neville and Day 2002, Pentenero, Meleti et al. 2014).

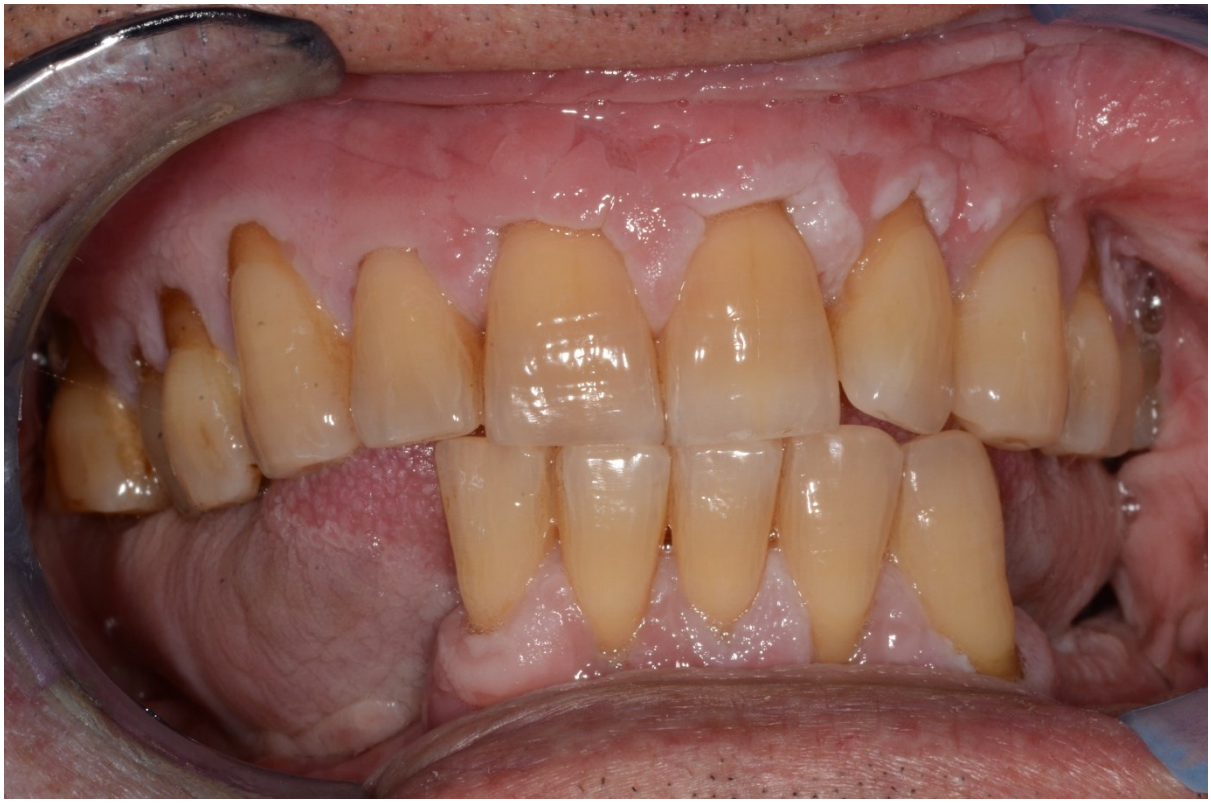

Figure 7. Proliferative verrucous leukoplakia involving maxillary gingiva and lining mucosa in an 82 y/o male.

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. The same review, based only on observational studies, reported evidence of specific determinants to assess OLK 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, which have the highest transformation rate (about 61%, mean follow-up 7 years). Moreover, oral sites associated with a higher risk of dysplasia and/or cancer are the ventral tongue, floor of the mouth and soft palate (Warnakulasuriya and Ariyawardana 2016).

4.1.3. Histological and molecular features

Leukoplakia is a clinical term; hence an appropriate diagnosis should be confirmed only after a histopathologic evaluation. While small homogeneous lesions can be easily biopsied or radically excised, PVLs or large OLK require multiple biopsies for “field mapping” the involved area, to detect different grades of dysplasia or cancer localization (Lee, Hung et al. 2007, van der Waal 2009).

OLK with dysplasia exhibits characteristic architectural and cytological features of keratinizing dysplasia. Such features include hyperkeratosis and parakeratosis, epithelial atrophy or hyperplasia with bulbous rete ridges, basal cell hyperplasia with hyperchromatic nuclei or increased nuclear cytoplasmic ratio, atypical mitoses, dyskeratosis and dyscohesion. Approximately 33% of dysplasia are characterized by an inflammatory infiltrate and should not be misdiagnosed as lichen planus (Fitzpatrick, Honda et al. 2014).

OLK without dysplasia exhibits hyperkeratosis, without histologic features of a frictional/reactive process, such as hyperkeratosis with hyper-granulosis, verrucoid architecture and epithelial atrophy or hyperplasia. Lesions peripheral samples shows, sharply demarcated hyperkeratosis features. Occasionally, OLK without dysplasia exhibits verrucous architecture being reported as verrucous hyperplasia (Woo 2019, Rubert, Bagan et al. 2020).

Although genetic changes are usually present in OLK, they can also happen in the adjacent normal mucosa, taking part in the field cancerization (Ha and Califano 2003). DNA aneuploidy, CNA, LOH and TP53 mutations represent the core of genetic alterations of OLK and PMDs. The presence of DNA aneuploid cells, with an abnormal number of chromosomes, is associated with an increased risk of malignant progression. The detection of CNA and the presence of LOH on chromosomes 3p, 4q, 9p and 17p, where tumor suppressor genes are mapped, are associated with a higher risk of progression to OSCC. TP53 is the most frequently mutated gene in oral dysplasia and PMDs, in agreement with the role of this gene in the oral carcinogenesis process (Perez-Sayans, Somoza-Martin et al. 2009, Guimaraes, Diniz et al. 2021).

Early detection of PMDs and subclinical OSCC disease is an attractive strategy to screen the higher risk population and likely intercept malignant progression. For such disorders the current diagnosis

gold standard is tissue histological examination. However, to improve diagnostic accuracy and to reduce invasiveness of biopsy, techniques such as micro-biopsy, exfoliative cytology, vital staining, light-based detection systems and optical diagnostic technologies have been proposed, with different level of evidence (Macey, Walsh et al. 2015, Giovannacci, Vescovi et al. 2016, Pentenero, Val et al. 2018). Particularly, autofluorescence and vital staining can facilitate the management of OLK and may be useful for biopsy site selection, surgical excision planning or during follow-up. Due to the high sensitivity and low specificity, such supplementary tools are not recommended for screening (Stojanov and Woo 2018, Walsh, Warnakulasuriya et al. 2021).

4.1.4. Salivary metabolomics as potential diagnostic procedure

Genes and proteins are functionally influenced by epigenetic regulations and post-translational modifications. Conversely, metabolites serve as direct signatures of biochemical activity, easily correlate to the phenotype. Hence, metabolite profiling has become an effective methodology that has been largely implemented for clinical diagnostics. (Patti, Yanes et al. 2012)

Unlike non-oral diseases, OLK is 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 (Mikkonen, Singh et al. 2016). 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 (Yakob, Fuentes et al. 2014).

Oral carcinogenesis is multifactorial and involves many genetic processes and molecular pathways that can alter the function of oncogenes, tumor suppressor genes and other related molecules. Such anomalies encompasses the whole hallmarks of cancer onset and progression (Perez-Sayans, Somoza-Martin et al. 2009, Hanahan and Weinberg 2011). Production of growth factors, expression of cell surface receptors and upregulation of signaling or intracellular messengers can select a cellular phenotype capable of overcome immune response. Moreover, such population can express altered metabolic pathways resulting in acquired competitive advantages (Pavlova and Thompson 2016). These changes can cause a loss of tumor suppressor activity and give rise to a phenotype capable

of increasing cellular proliferation, weakening cell cohesion, and causing local infiltration and metastasis. Genetic basis of development of OSCC were widely investigated. Accumulation of genetic alterations concur to the progress of a normal cell into a cancer cell in a multistep carcinogenesis model (Braakhuis, Leemans et al. 2004).

Metabolomics is the study of biochemical processes involving metabolites, molecules with low weight, present within cells, biofluids, tissues or organisms, that provide detailed information on biological systems and their status. The whole of interactions of such small molecules in a biological system describes the metabolome (Shankar, Alex et al. 2014).

Reactions to genetic damages occurs at various levels causing permanent or temporary phenotypic alteration to the cell (Shankar, Alex et al. 2014). The metabolome of a malignant tumor is strictly related to the genotype and phenotype of its neoplastic cells and can provide indication on peculiar altered cellular pathways. Particularly, expression of oncogenes, protein changes and abnormal enzymatic activities, may release traces of metabolic reprogramming of cells within the context of pathologic tissues (Song, Yang et al. 2020). Such a whole of “traces” may therefore serve as potential biomarkers, able to provide information about the tumor at different stages of development, as well as when still clinically undetectable.

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the most two common techniques for metabolomic investigation. The latter is frequently performed by the application of different separation techniques such as capillary electrophoresis (CE), gas chromatography (GC) and liquid chromatography (LC) and their evolutions, to improve capabilities in mass determining and resolving. Evidence exists that metabolomics is suitable for exploration of saliva for PMDs and oral cancer metabolic signature identification (Assad, Mascarenhas et al. 2020). Mass spectrometers operate by converting the analyte molecules to a charged (ionized) state, with subsequent analysis of the ions and their fragments, based on their mass to charge ratio. The wide application of MS technology is a consequence of its high sensitivity, which permits qualitative exploration of salivary metabolome, collecting a limited number of samples. On the other hand, the quantitative description of selected variables requires an additional method. UPLC-MS (Wei, Xie et

al. 2011) and CPSI-MS (Song, Yang et al. 2020) are the most up-to-date MS platforms in terms of performance enhancement, higher resolution, sensitivity increase and fast speed of analysis.

The other technique capable of similar data acquisition is NMR, which is less sensitive than MS, requires many samples to produce results but such data are simultaneously both qualitative and quantitative. To make a gross comparison, MS sensitivity permits the detection of many metabolites (from 300+ to 1000+ if GC-MS or LC-MS is performed), platform costs are cheaper than NMR and is ideal for targeted metabolomics. NMR data are more reproducible than MS's, acquisition execution is faster, requires minimal sample preparation and do not need tissue or matrix extraction. Therefore, the average cost-per sample is cheaper for NMR than MS.

4.2 Material and methods

The present multicentric study was approved by the Ethical Committee of the “Area Vasta Emilia Nord” (AVEN) (protocol number: 38/2017/TESS/AUOMO - 509/2019/TESS/UNIPR). Written consent was obtained from all volunteers who participated in this study, according to the Declaration of Helsinki.

The aim of this work was to describe the salivary metabolome of patients with dysplastic and non-dysplastic OLK and to compare results with healthy controls salivary metabolic profile.

Three centers participated as recruiter, two of them based in Modena (Dermatology Unit and Dentistry Unit, Azienda Ospedaliero Universitaria Policlinico di Modena) and one in Parma (Dentistry University Center, Parma). The present analysis is part of a wider investigation which aims to explore and to compare PMDs and OSCC salivary metabolome. Unstimulated whole saliva (UWS) samples were harvested before mucosal biopsy or a surgical resection. For healthy control (HC) group UWS was collected before minor oral surgery procedure (e.g., third molar extraction, clinically benign oral lesions excision). Cases were then subdivided into groups for data acquisition and comparison with HC: non-dysplastic OLK, dysplastic OLK, OLP and OSCC.

4.2.1 Patient selection and salivary collection

For each enrolled subject, medical history and medications were listed. Smoking and alcohol exposure (quantity and duration) were recorded. A general oral evaluation was performed to disclose potential pathological conditions. Then, to assess oral hygiene, full mouth plaque score (FMPS) and decay-missing-filled index (DMFT) were recorded. Informed consent was obtained, and enrolled patients were anonymized with univocal code.

Criteria for patient inclusion or exclusion are resumed in Table 1.

Saliva samples were collected using standard techniques according to Navazesh (Navazesh 1993). all recruited subjects were asked not to eat, drink, smoke and use oral hygiene products one hour before saliva collecting. Immediately before collection, patients rinsed their mouth with water for 1 1 minute.

Samples from the two groups were collected in the morning (9:00 to 12:00) to minimize the influence of the circadian rhythm on salivary composition.

All subjects were asked to collect saliva on the floor of the mouth and to spit or passive drool the fluid into a sterile container kept on ice. Procedure was repeated once a minute for a maximum of 10 minutes or until a 2ml cuvette was filled. Each sample was immediately fresh-frozen in liquid nitrogen for conservation until -80°C freezer storing.

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

Whole saliva samples were thawed at room temperature and 1 mL of each sample was centrifuged at 15,000 x g for 10 min at 4 °C. The supernatant was ultra-filtered using Amicon Ultra-4 Centrifugal filters (3000 MWCO, Merck Millipore) at 4,000 x g for 60 min at 10 °C. Filters were previously washed several times with water at 4,000 x g for 20 min to remove glycerol from the filter membrane.

After ultra-filtration, at least 800 µL was obtained for 62 samples and a volume lower than 600 µL for 4 samples. All the ultra-filtered saliva samples were frozen and lyophilised overnight: 800 µL for 62 samples, and the total volume obtained for the other samples.

The freeze-dried samples were suspended in phosphate buffer 48.8 mM, pH 7.4, 1.45 mM sodium trimethylsilyl-[2,2,3,3-2H4]-propionate (TSP), and 2.5% D2O. TSP was used as quantitative internal standard and reference for chemical shift (0.00 ppm).

High resolution one-dimensional ^{1}H-NMR spectra were acquired on a JEOL 600 MHz ECZ600R spectrometer at the Interdepartmental Measurement Center “Giuseppe Casnati” of the University of Parma. The spectral parameters used were: first increment of the pulse sequence Noesy, 128 scans, sweep width 20 ppm, 128 k points, and relaxation delay 5 seconds, at 25 °C.

Metabolite identification and quantification were carried out using Chenomx NMR suite 7.6 software (Chenomx Inc., Canada). The statistics analyses were performed using the MetaboAnalystR 5.0 platform (https://www.metaboanalyst.ca).

4.2.3 Statistical analysis

A metabolites database was generated using the metabolites which concentration was equal to or above the limit of quantification of the method (LOQ = 1.1 μM). The outlier samples were excluded from the 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).

Supervised multivariate statistical analysis was performed using Partial Least Squares Discriminant Analysis (PLS-DA) on MetaboAnalystR 5.0. The analysis was performed employing the metabolites data normalized by the median in the log scale (base 10), and auto scaled.

^{1}H-NMR spectra were processed on MestReNova 11.0 software (Mestrelab Research, Santiago de Compostela, Spain) and binning was applied to spectra database, after outliers exclusion. All the spectra were divided into small regions called “bin” and the intensity of each bin is calculated. Binning is an untargeted approach that allow a preliminary global metabolic profiling and sample groups visualization.

The Variable Importance in Projection (VIP) scores estimates the importance of each metabolite in the groups’ aggregation. Higher is the VIP score, higher impact on the cluster. The boxes on the right side are the heatmap: the brightness of each color corresponds to the magnitude of the difference when compared with average value (high concentration in red and low concentration in blue).

Table 1. Inclusion and exclusion criteria for patient enrollment. Standard conditions for saliva collection.

Groups	Inclusion	Exclusion
HC	Tobacco smoking: yes/no Alcohol consumption: yes/no	History of PMDs or OSCC Anemic conditions Uncontrolled diabetes Onco-hematological diseases in the last 12 months
PMDs	Tobacco smoking: yes/no Alcohol consumption: yes/no Clinical and histological diagnosis for PMD.	Clinical and histological diagnosis for OSCC Anemic conditions Uncontrolled diabetes Onco-hematological diseases in the last 12 months
OSCC	Tobacco smoking: yes/no Alcohol consumption: yes/no Clinical and histological diagnosis for OSCC	Anemic conditions Uncontrolled diabetes Onco-hematological diseases in the last 12 months

4.3 Results

A whole population of 67 patients was enrolled; subgroups were composed by 23 with OLK with different dysplasia degrees, 12 OLP, 11 OSCC and 21 HC.

Before analytical phase, a sample from an OSCC case was dropped out due to insufficient salivary volume for proper evaluation. Evaluated samples were 66.

Cases eventually considered for comparison between subgroups were 23 OLK, (13 non-dysplastic and 10 dysplastic) and 21 HC. Characteristics of enrolled subjects are summarized in Table 2.

OLK and HC groups were matched for sex but mean age at enrollment was unbalanced (OLK 61,04 years; HC 44,62 years). Smoking habit was equally distributed between groups; only 10 out of 66 subjects disclose and quantified the alcohol consumption habit (e.g., heavy consumption, social drinker, no consumption). Alveolar mucosa as well as tongue and cheek lining mucosa were the most involved location. Lesions were classified as in prolonged or limited contact to salivary fluid depending on a topographical criterion. Sites of prolonged contact were the lingual portion of mandibular alveolar mucosa; the floor of the mouth; the ventral and the lateral tongue; cheeks lining mucosa. Sites of limited contact were alveolar mucosa, any other site; dorsal tongue; palate; labial mucosa, upper and lower.

Scores plot of PLS-DA (Figure 8, A and B) shows how samples aggregate into three distinct cluster (CON: healthy controls; LK_0: non-dysplastic leukoplakia; LK_1: dysplastic leukoplakia). The algorithm imposed a model of separation to variables (metabolites) resulting in clustering of samples on the basis of variables importance in each group. The clusters of HC and dysplastic leukoplakia (1 severe and 9 mild) are the most separated, with no intersection between groups, manifestation of a specific metabolic profile. An overall of 55 metabolites were identified. VIP diagram reported the most discriminant variables and their importance into the three groups comparison (CON, LK_0, LK_1).

Most representative metabolites (considering a VIP score > 1.5) were isoleucine, glycerol, taurine, lactose, putrescine and 3-hydroxybutyrate.

Table 2. Descriptive characteristics of enrolled patients

Variables		Subjects	
		OLK	HC
Sex			
	M	10	8
	F	13	13
Age			
	≤50	3	10
	≥50	20	11
Smoker			
	Non-smoker	13	15
	Smoker	10	6
		OLK	
Size (cm^2)			
	≤2	11	-
	≥2	12	-
Location			
	Alveolar mucosa	6	-
	Tongue	4	-
	Cheek	4	-
	Floor of the mouth	2	-
	Palate	2	-
	Lip	1	-
	Lining mucosa	1	-
	Retromolar trigone	3	-
Histopathology			
	Non-dysplastic	13	-
	Low Grade dysplasia	9	-
	High Grade dysplasia	1	-
Contact with saliva			
	Limited	12	-
	Prolonged	11	-

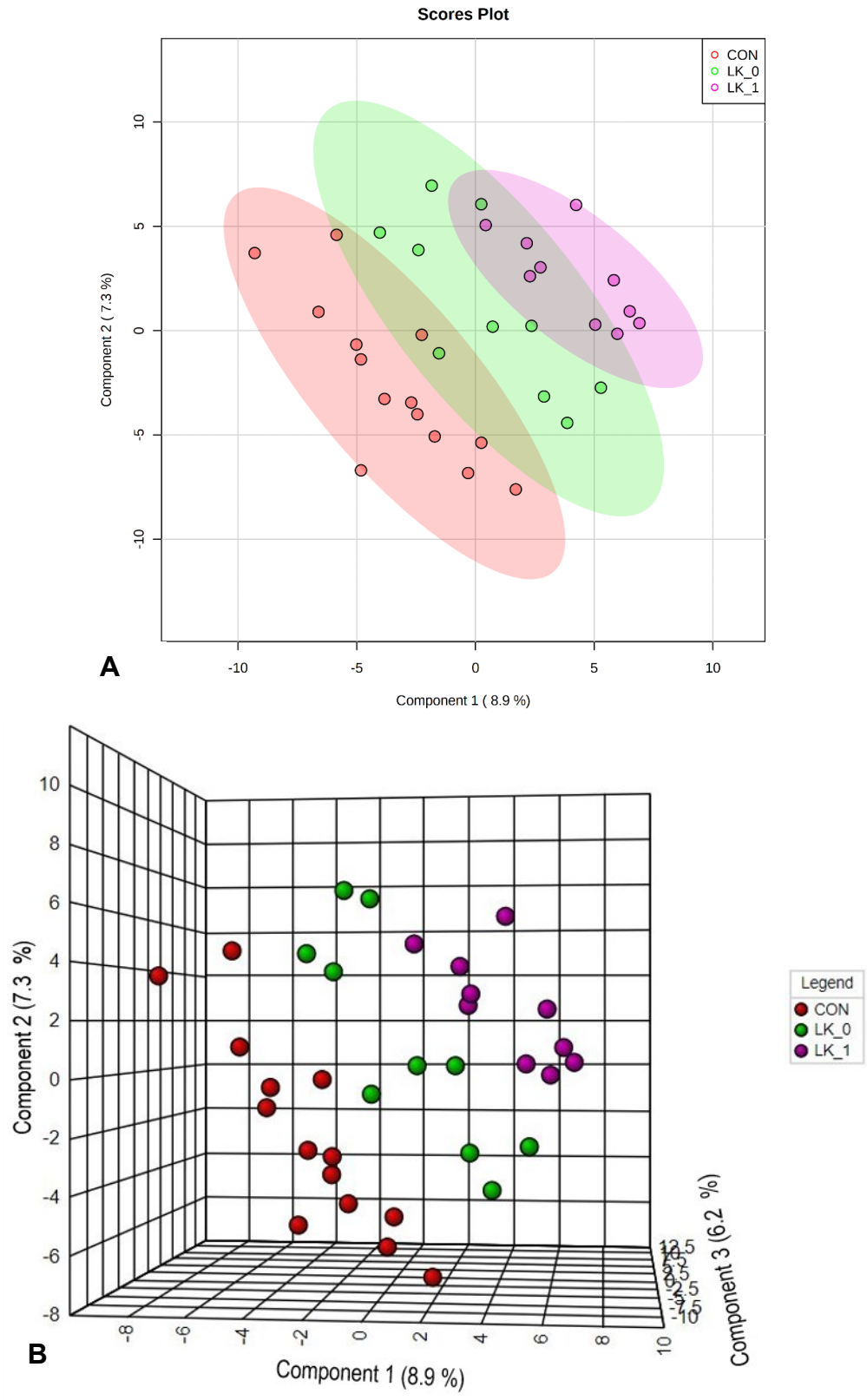

Figure 8, A) PLS-DA scores plot. This statistical analysis allows to distinguish different metabolic profiles and to aggregate similar group of samples into specific set. B) Tridimensional output of PLS-DA.

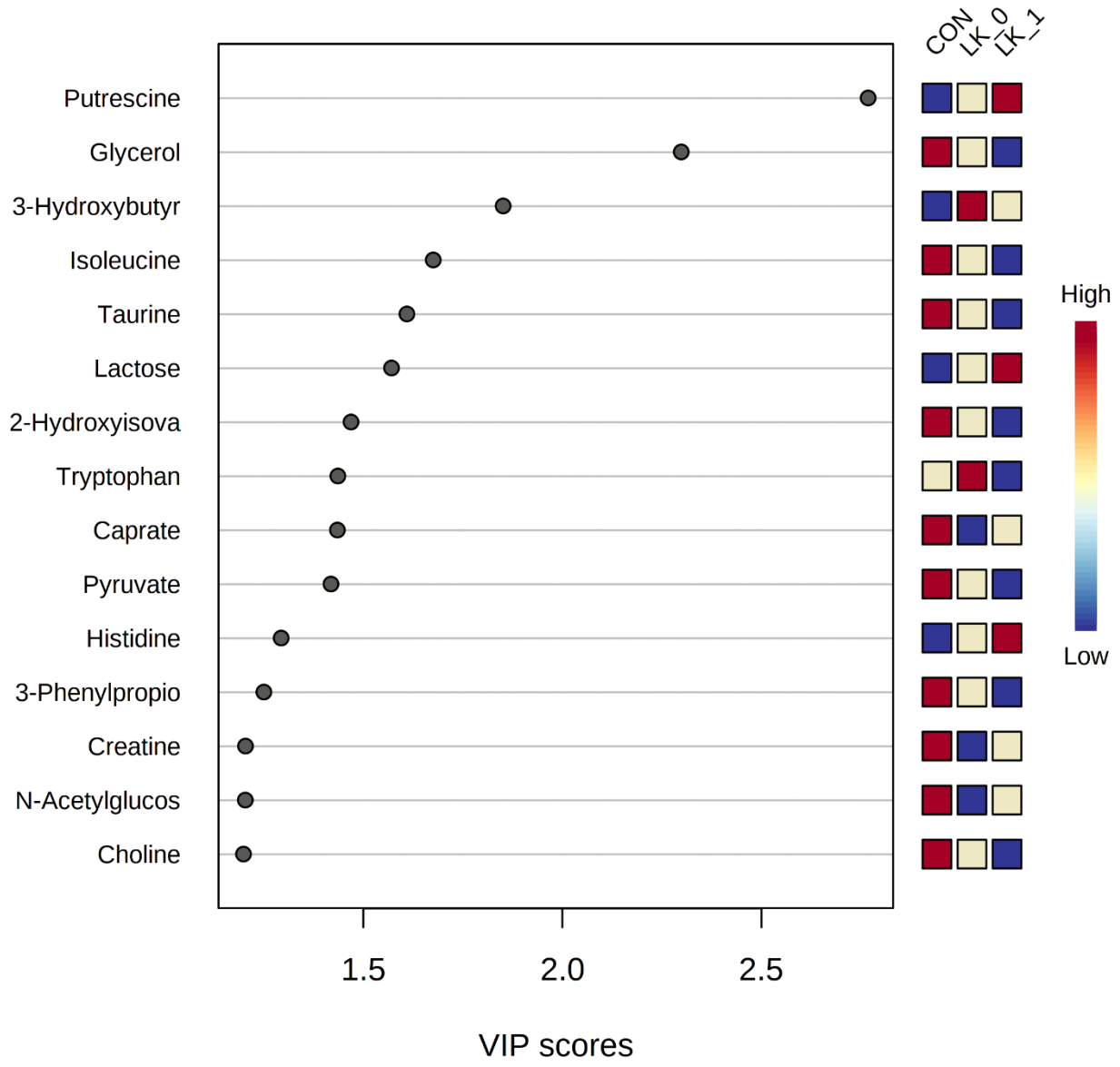

Figure 9. VIP scores indicates which metabolites had more importance in group aggregation. Most important variables had an overall score >1.5. Color shade from deep blue (low) to red (high) is related to metabolite intensity of concentration in each specific group.

4.3 Discussion

The majority of published scientific literature about salivary metabolomics is focused on OSCC, both in targeted and untargeted study settings; the aim was to discover potential biomarkers for early diagnosis and disease monitoring. Hence, salivary metabolomic studies of PMDs are somewhat lacking.

Reports of salivary untargeted metabolomics for exploration of leukoplakia and its grade of dysplasia are particularly limited in literature. A research on multiple databases (Medline, Scopus and World of Science) returned 5 original articles published in English language between 2008 and 2022 (Yan, Wei et al. 2008, Wei, Xie et al. 2011, Ishikawa, Wong et al. 2019, Sridharan, Ramani et al. 2019, Song, Yang et al. 2020); such studies had explored salivary metabolome of leukoplakia or epithelial dysplastic lesions as a group of comparison with OSCC and HC. In addition one more paper was recently published in Chinese language, its content being somewhat problematic to be included here (Xue, Zhu et al. 2021),.

OLK was otherwise investigated through targeted metabolomics procedure such as sialic acid identification; also known as N-acetylneuraminic acid, sialic acid extensively binds to glycoproteins; it is a major component of cells membrane, influencing surface properties and playing a role in cellular invasiveness, adhesiveness, and immunogenicity. With membrane shredding due to neoplastic development, sialic acid become detectable in bloodstream. (Fuster and Esko 2005). Its salivary concentration was studied regarding OSCC diagnosis in comparison to leukoplakia. Several authors report how sialic acid salivary concentration is useful for distinguish between oral leukoplakia and HC with statistical significance, even considering increasing grades of epithelial dysplasia (Hemalatha, Austin et al. 2013, Vajaria, Patel et al. 2013, Dadhich, Prabhu et al. 2014, Chaudhari, Pradeep et al. 2016, Achalli, Madi et al. 2017).

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 9). Following the importance attributed by VIP scores output, the most representative are discussed below. However, it is important to underline that there are significant differences of age

distribution between HC and OLK groups. This may produce false positive results in metabolite marker selection. A further small sample set analysis could be performed to avoid unproper selection. Isoleucine is a branched-chain amino acid (BCAA); in peculiar metabolic condition, like high related energy demand associated to neoplasm development and progression, end-products of branched-chain aminotransferase enzyme, acetyl-CoA and succinyl-CoA, can be used as intermediate of tricarboxylic acid cycle (TCA).

In our samples, lowest level of isoleucine was associated to the group of dysplastic leukoplakia (LK_1) while the highest concentration was related to HC. Literature report similar results regarding the downregulation of isoleucine through the progression from normal epithelia to dysplasia and neoplasm, probably associated with increased glycolysis for energy purposes (Sridharan, Ramani et al. 2019).

Putrescine is a diamine, product of arginine breakdown. The pro-carcinogenic role of putrescine was investigated for a few diseases, such as colon and pancreas (Miller-Fleming, Olin-Sandoval et al. 2015). In addition, one study report about the ability of metabolomics to discriminate OSCC tissue by normal one by the identification of significant levels of putrescine (Hsu, Chen et al. 2019). Song X et al., highlight differences in salivary concentration also in presence of PMDs; the authors described a trend of upregulation of putrescine from HC to OSCC (PMDs vs. HC, P< 0.0075, OSCC vs. PMDs P<0,0001 and OSCC vs. HC P<0,00001), (Song, Yang et al. 2020). The highest level of putrescine in group LK_1 is comparable to results highlighted in literature. Clinically, related lesions were high and moderate leukoplakia of the molar trigone, abundantly over the 2 cm^2 size cut-off, in heavy smokers and drinkers; however patients were classified as having poor oral hygiene (FMPS > 25% and DMFT = 20) so an influence from bacterial metabolism end products should also to be considered (Meleti, Quartieri et al. 2020).

3-Hydroxybutyric acid is a ketone body. It is synthesized in the liver from acetyl-CoA in condition of glucose deficiency. The VIP output showed molecule higher concentration in non-dysplastic leukoplakia rather than HC or dysplastic lesions (Figure 9). It was reported that salivary levels of 3-hydroxybutyric acid were increased in OSCC patients' saliva, likely depending on the metabolic

turnover and membrane synthesis necessity to sustain cell proliferation (Ohshima, Sugahara et al. 2017). However, no other data regarding leukoplakia are available.

The presence of lactose could be explained by an insufficient control of patient dietary intake before salivary collection, resulting in presence of residual food compound. In addition, a mean 65% of FMPS and a DMFT score of 20 were recorded in groups LK_0 and LK_1, which showed the highest presence of the molecule, possibly relating the poor oral hygiene/health overall conditions to such elevated levels.

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. Taurine is a beta-sulfonic amino acid, and probably it is the most abundant free amino acid in mammalian tissues. In this dataset, taurine levels are higher in HC group than others. In human saliva, seems to be correlated to physical stress and a previous study on saliva in healthy patients showed that such metabolite is present with a marked prevalence in parotid saliva, indicating a role in salivary glands function (Meleti, Quartieri et al. 2020). Otherwise, taurine is suggested as a potential salivary biomarker, capable to distinguish HC from OSCC cases, in which appears to be more elevated (Sugimoto, Wong et al. 2010, Ohshima, Sugahara et al. 2017).

Regarding the PLS-DA output, a sharp group clustering is observable (Figure 8, A and B). 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. 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 metabolic

analysis (Figure 10); hence, emerging 27 discriminant metabolites were capable of distinguish OLK group from HC (Wei, Xie et al. 2011).

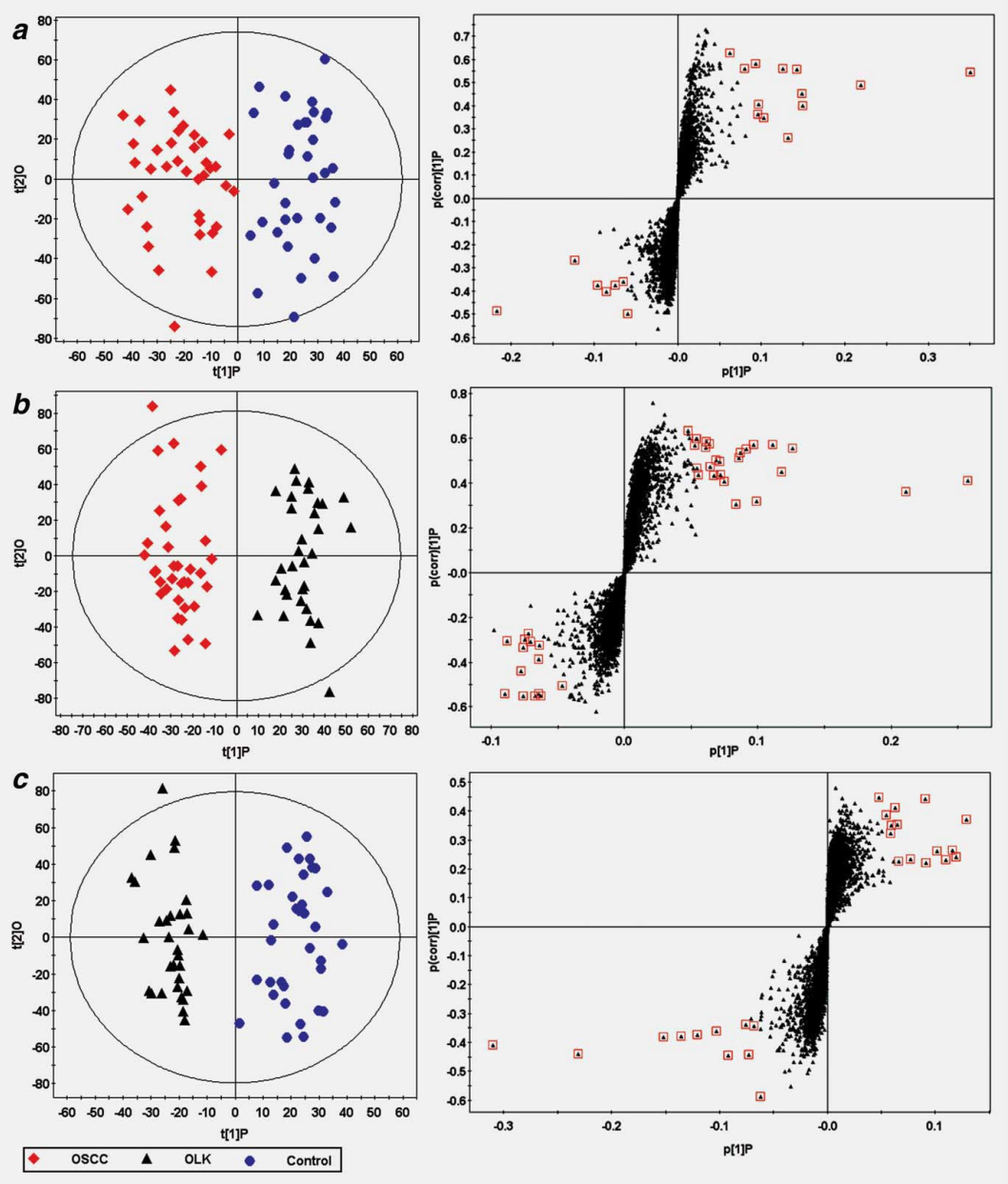

Figure 10. OPLS-DA scores plots and S-plots of metabonomic comparison between (a) OSCC group and the healthy control group, two component model (R2X = 0.149, R2Y = 0.818, Q2(cum) = 0.534); (b) OSCC group and the OLK group, three-component model (R2X = 0.253, R2Y = 0.908, Q2(cum) = 0.466); and (c) OLK group and the healthy control group, three-component model (R2X = 0.23, R2Y = 0.914, Q2(cum) = 0.721). From: Wei, J., Xie, G., Zhou, Z., Shi, P., Qiu, Y., Zheng, X., Chen, T., Su, M., Zhao, A. and Jia, W. (2011). "Salivary metabolite signatures of oral cancer and leukoplakia." Int J Cancer 129(9): 2207-2217.

Within the limitations of this study, salivary metabolic footprint seems to be capable to distinguish patients with 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.

References

1. Achalli, S., Madi, M., Babu, S. G., Shetty, S. R., Kumari, S. and Bhat, S. (2017). "Sialic acid as a biomarker of oral potentially malignant disorders and oral cancer." Indian J Dent Res **28**(4): 395-399.
2. Assad, D. X., Mascarenhas, E. C. P., de Lima, C. L., de Toledo, I. P., Chardin, H., Combes, A., Acevedo, A. C. and Guerra, E. N. S. (2020). "Salivary metabolites to detect patients with cancer: a systematic review." Int J Clin Oncol **25**(6): 1016-1036.
3. Braakhuis, B. J., Leemans, C. R. and Brakenhoff, R. H. (2004). "A genetic progression model of oral cancer: current evidence and clinical implications." J Oral Pathol Med **33**(6): 317-322.
4. Chaudhari, V., Pradeep, G. L., Prakash, N. and Mahajan, A. M. (2016). "Estimation of salivary sialic acid in oral premalignancy and oral squamous cell carcinoma." Contemp Clin Dent **7**(4): 451-456.
5. Dadhich, M., Prabhu, V., Pai, V. R., D'Souza, J., Harish, S. and Jose, M. (2014). "Serum and salivary sialic acid as a biomarker in oral potentially malignant disorders and oral cancer."
6. Fitzpatrick, S. G., Honda, K. S., Sattar, A. and Hirsch, S. A. (2014). "Histologic lichenoid features in oral dysplasia and squamous cell carcinoma." Oral Surg Oral Med Oral Pathol Oral Radiol **117**(4): 511-520.
7. Fuster, M. M. and Esko, J. D. (2005). "The sweet and sour of cancer: glycans as novel therapeutic targets." Nat Rev Cancer **5**(7): 526-542.
8. Giovannacci, I., Vescovi, P., Manfredi, M. and Meleti, M. (2016). "Non-invasive visual tools for diagnosis of oral cancer and dysplasia: A systematic review." Med Oral Patol Oral Cir Bucal **21**(3): e305-315.
9. Guimaraes, L. M., Diniz, M. G., Rogatto, S. R., Gomez, R. S. and Gomes, C. C. (2021). "The genetic basis of oral leukoplakia and its key role in understanding oral carcinogenesis." J Oral Pathol Med **50**(7): 632-638.
10. Ha, P. K. and Califano, J. A. (2003). "The molecular biology of mucosal field cancerization of the head and neck." Crit Rev Oral Biol Med **14**(5): 363-369.
11. Hanahan, D. and Weinberg, R. A. (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.
12. Hemalatha, V. T., Austin, R. D., Manisundar, N., Sarumathi, T. and Aarthi Nisha, V. (2013). "Evaluation of salivary sialic acid in patients with different clinico-pathological stages of oral leukoplakia and oral squamous cell carcinoma - A cross sectional study." Biosciences Biotechnology Research Asia **10**(1): 419-425.
13. Hsu, C. W., Chen, Y. T., Hsieh, Y. J., Chang, K. P., Hsueh, P. C., Chen, T. W., Yu, J. S., Chang, Y. S., Li, L. and Wu, C. C. (2019). "Integrated analyses utilizing metabolomics and transcriptomics reveal perturbation of the polyamine pathway in oral cavity squamous cell carcinoma." Anal Chim Acta **1050**: 113-122.
14. Ishikawa, S., Wong, D. T. W., Sugimoto, M., Gleber-Netto, F. O., Li, F., Tu, M., Zhang, Y., Akin, D. and Iino, M. (2019). "Identification of salivary metabolites for oral squamous cell carcinoma and oral epithelial dysplasia screening from persistent suspicious oral mucosal lesions." Clin Oral Investig **23**(9): 3557-3563.
15. Lee, J. J., Hung, H. C., Cheng, S. J., Chiang, C. P., Liu, B. Y., Yu, C. H., Jeng, J. H., Chang, H. H. and Kok, S. H. (2007). "Factors associated with underdiagnosis from incisional biopsy of oral leukoplakic lesions." Oral Surg Oral Med Oral Pathol Oral Radiol Endod **104**(2): 217-225.
16. Macey, R., Walsh, T., Brocklehurst, P., Kerr, A. R., Liu, J. L., Lingen, M. W., Ogden, G. R., Warnakulasuriya, S. and Scully, C. (2015). "Diagnostic tests for oral cancer and potentially malignant disorders in patients presenting with clinically evident lesions." Cochrane Database Syst Rev(5): CD010276.
17. Meleti, M., Quartieri, E., Antonelli, R., Pezzi, M. E., Ghezzi, B., Viani, M. V., Setti, G., Casali, E., Ferrari, E., Ciociola, T., Spisni, A. and Pertinhez, T. A. (2020). "Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva." Metabolites **10**(8).

18. Mello, F. W., Miguel, A. F. P., Dutra, K. L., Porporatti, A. L., Warnakulasuriya, S., Guerra, E. N. S. and Rivero, E. R. C. (2018). "Prevalence of oral potentially malignant disorders: A systematic review and meta-analysis." J Oral Pathol Med **47**(7): 633-640.
19. Mikkonen, J. J., Singh, S. P., Herrala, M., Lappalainen, R., Myllymaa, S. and Kullaa, A. M. (2016). "Salivary metabolomics in the diagnosis of oral cancer and periodontal diseases." J Periodontal Res **51**(4): 431-437.
20. Miller-Fleming, L., Olin-Sandoval, V., Campbell, K. and Ralser, M. (2015). "Remaining Mysteries of Molecular Biology: The Role of Polyamines in the Cell." J Mol Biol **427**(21): 3389-3406.
21. Neville, B. W. and Day, T. A. (2002). "Oral cancer and precancerous lesions." CA Cancer J Clin **52**(4): 195-215.
22. Ohshima, M., Sugahara, K., Kasahara, K. and Katakura, A. (2017). "Metabolomic analysis of the saliva of Japanese patients with oral squamous cell carcinoma." Oncol Rep **37**(5): 2727-2734.
23. Patti, G. J., Yanes, O. and Siuzdak, G. (2012). "Innovation: Metabolomics: the apogee of the omics trilogy." Nat Rev Mol Cell Biol **13**(4): 263-269.
24. Pavlova, N. N. and Thompson, C. B. (2016). "The Emerging Hallmarks of Cancer Metabolism." Cell Metab **23**(1): 27-47.
25. Pentenero, M., Meleti, M., Vescovi, P. and Gandolfo, S. (2014). "Oral proliferative verrucous leucoplakia: are there particular features for such an ambiguous entity? A systematic review." Br J Dermatol **170**(5): 1039-1047.
26. Pentenero, M., Val, M., Rosso, S. and Gandolfo, S. (2018). "Microbiopsy a first-level diagnostic test to rule out oral dysplasia or carcinoma in general dental practice." Oral Dis **24**(1-2): 109-111.
27. Perez-Sayans, M., Somoza-Martin, J. M., Barros-Angueira, F., Reboiras-Lopez, M. D., Gandara Rey, J. M. and Garcia-Garcia, A. (2009). "Genetic and molecular alterations associated with oral squamous cell cancer (Review)." Oncol Rep **22**(6): 1277-1282.
28. Petti, S. (2003). "Pooled estimate of world leukoplakia prevalence: a systematic review." Oral Oncology **39**(8): 770-780.
29. Rubert, A., Bagan, L. and Bagan, J. V. (2020). "Oral leukoplakia, a clinical-histopathological study in 412 patients." J Clin Exp Dent **12**(6): e540-e546.
30. Shankar, A. A., Alex, S. and Routray, S. (2014). "Incorporation of salivary metabolomics in oral cancer diagnostics." Oral Oncol **50**(10): e53-54.
31. Song, X., Yang, X., Narayanan, R., Shankar, V., Ethiraj, S., Wang, X., Duan, N., Ni, Y. H., Hu, Q. and Zare, R. N. (2020). "Oral squamous cell carcinoma diagnosed from saliva metabolic profiling." Proc Natl Acad Sci U S A.
32. Speight, P. M. (2007). "Update on oral epithelial dysplasia and progression to cancer." Head Neck Pathol **1**(1): 61-66.
33. Sridharan, G., Ramani, P., Patankar, S. and Vijayaraghavan, R. (2019). "Evaluation of salivary metabolomics in oral leukoplakia and oral squamous cell carcinoma." J Oral Pathol Med **48**(4): 299-306.
34. Stojanov, I. J. and Woo, S.-B. (2018). "AAOM clinical practice statement subject: leukoplakia." Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology **126**(4): 331-334.
35. Sugimoto, M., Wong, D. T., Hirayama, A., Soga, T. and Tomita, M. (2010). "Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles." Metabolomics **6**(1): 78-95.
36. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. and Bray, F. (2021). "Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries." CA Cancer J Clin **71**(3): 209-249.
37. Vajaria, B. N., Patel, K. R., Begum, R., Shah, F. D., Patel, J. B., Shukla, S. N. and Patel, P. S. (2013). "Evaluation of serum and salivary total sialic acid and α-l-fucosidase in patients with oral precancerous conditions and oral cancer." Oral Surg Oral Med Oral Pathol Oral Radiol **115**(6): 764-771.

38. van der Waal, I. (2009). "Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management." Oral Oncol **45**(4-5): 317-323.
39. van der Waal, I. (2010). "Potentially malignant disorders of the oral and oropharyngeal mucosa; present concepts of management." Oral Oncol **46**(6): 423-425.
40. Villa, A. and Woo, S. B. (2017). "Leukoplakia-A Diagnostic and Management Algorithm." J Oral Maxillofac Surg **75**(4): 723-734.
41. Walsh, T., Warnakulasuriya, S., Lingen, M. W., Kerr, A. R., Ogden, G. R., Glenny, A. M. and Macey, R. (2021). "Clinical assessment for the detection of oral cavity cancer and potentially malignant disorders in apparently healthy adults." Cochrane Database Syst Rev **12**: CD010173.
42. Warnakulasuriya, S. (2018). "Clinical features and presentation of oral potentially malignant disorders." Oral Surg Oral Med Oral Pathol Oral Radiol **125**(6): 582-590.
43. Warnakulasuriya, S. (2020). "Oral potentially malignant disorders: A comprehensive review on clinical aspects and management." Oral Oncol **102**: 104550.
44. Warnakulasuriya, S. and Ariyawardana, A. (2016). "Malignant transformation of oral leukoplakia: a systematic review of observational studies." J Oral Pathol Med **45**(3): 155-166.
45. Warnakulasuriya, S., Johnson, N. W. and van der Waal, I. (2007). "Nomenclature and classification of potentially malignant disorders of the oral mucosa." J Oral Pathol Med **36**(10): 575-580.
46. Wei, J., Xie, G., Zhou, Z., Shi, P., Qiu, Y., Zheng, X., Chen, T., Su, M., Zhao, A. and Jia, W. (2011). "Salivary metabolite signatures of oral cancer and leukoplakia." Int J Cancer **129**(9): 2207-2217.
47. Woo, S. B. (2019). "Oral Epithelial Dysplasia and Premalignancy." Head Neck Pathol **13**(3): 423-439.
48. Xue, J. Y., Zhu, X. Y., Wei, C. L., Sun, P., Zhang, H. and Deng, J. (2021). "[Study of salivary metabonomics in patients with oral squamous cell carcinoma and leukoplakia]." Shanghai Kou Qiang Yi Xue **30**(2): 201-205.
49. Yakob, M., Fuentes, L., Wang, M. B., Abemayor, E. and Wong, D. T. (2014). "Salivary biomarkers for detection of oral squamous cell carcinoma - current state and recent advances." Curr Oral Health Rep **1**(2): 133-141.
50. Yan, S. K., Wei, B. J., Lin, Z. Y., Yang, Y., Zhou, Z. T. and Zhang, W. D. (2008). "A metabonomic approach to the diagnosis of oral squamous cell carcinoma, oral lichen planus and oral leukoplakia." Oral Oncol **44**(5): 477-483.

CHAPTER 5

Metabolic profile of whole saliva in patients with Sjögren's syndrome

Published on Proceedings 2019, 35, 39.

5.1 Introduction

Primary Sjögren's syndrome (pSS) is a chronic systemic autoimmune rheumatic disease characterized by sicca syndrome and/or systemic manifestations as clinical hallmarks. pSS affects 0.1% to 4.8% of the population with an incidence rate of 6.92 per 100,000 person–years. Age at diagnosis is with a female-male ratio of 9:1 and a 0.5–1% prevalence in the general population (Parisis, Chivasso et al. 2020).

Distinctive recurrent phase are associated to pSS pathogenesis: (1) environmental stimuli (such as viral infections) acts as a trigger in certain condition of genetic predisposition, presence of epigenetic factors and hormonal regulation; (2) overturn of normal exocrine gland function (e.g. salivary, lacrimal and diffuse glandular tissue of esophagus, stomach, bowel, pancreas and bladder), caused by alteration of epithelial cells activity (Ebert 2012); (3) B and T lymphocyte becomes hyperactive and by infiltrates glands tissue with autoantibodies production. This chronic cells behavior worsen the anatomical modification and hypofunction (Konttinen and Kasna-Ronkainen 2002).

Clinical manifestations are consequences of systemic involvement: dryness secondary to exocrinopathy, autoimmune epithelitis with peri-epithelial lymphocytic infiltration of target organs, associated organ-specific autoimmunity with specific autoantibodies, the presence of immune complexes or cryoglobulinemia and clonal lymphocytic expansion (Parisis, Chivasso et al. 2020).

During the natural course of the disease, the 75% of patients will develop at least one extra glandular manifestation, which can occur at diagnosis or during follow-up. In general, the manifestations caused by lymphocytic infiltration of a target organ are indolent and durable (e.g., sicca syndrome, renal tubular acidosis, pulmonary involvement) while the autoimmune disorders linked to immune complexes or autoantibodies have a more unpredictable course, with flares and remissions. Overall, quality of life is strongly reduced by the diverse manifestations of the disease. pSS can be disabling and associated with significant functional status impairment related to oral and/or ocular dryness, systemic activity, pain, fatigue and daytime somnolence, anxiety, psychological distress and depression symptoms (Haldorsen, Moen et al. 2008).

The progression of pSS is favorable with a patient's life expectancy comparable with general population. However, a subgroup of patients will have unfavorable prognosis; specific risk factors are identifiable, such as male sex, old-age, atypical parotid gland imaging (e.g., scintigraphy), extra-glandular involvement, vasculitis, anti-SSB/Ro positivity, reduction of C3, C4 and CH50 inflammatory protein and cryoglobulinemia. The excess mortality is generally attributed to the development of non-Hodgkin lymphoma, solid tumors or to uncommon but severe visceral involvement, such as cardiovascular disease and infections (Singh, Singh et al. 2016).

Diagnostic criteria are resumed in the 2016 ACR/EULAR Consensus of Classification Criteria for pSS, and are based on the sum of weighted scores applied to 5 items: anti-SSA/Ro antibody positivity, focus score of ≥1 foci/4 mm^2, ocular staining score ≥5 (or van Bijsterveld score ≥ 4) in at least one eye, a Schirmer's test result of ≤5 mm/5 min and an unstimulated salivary flow rate (SFR) of ≤0.1 mL/minute (Shiboski, Shiboski et al. 2017).

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, related to the presence and concentration of specific categories of molecules, have been described (de Almeida Pdel, Gregio et al. 2008, Meleti, Quartieri et al. 2020).

Because the salivary glands are a major site of autoimmune destruction in pSS, changes in salivary components could reflect the pathogenesis of the disease. Such changed molecules were investigated trough different technologies (e.g. mass spectrometry) and recently, by taking advantages of the emerging 'omics' technologies, salivary fluid has been explored for the discovery of biomarkers for pSS diagnosis and prognosis (Jonsson, Brokstad et al. 2018).

More than a few proteomic studies have shown differential protein expression in the saliva of pSS patients and healthy control subjects, which could lead to identification of potential biomarkers (Baldini, Giusti et al. 2011) In addition, transcriptome analysis of the saliva from pSS patients and controls revealed differences in mRNA expression levels (Hu, Wang et al. 2007).

Metabolomics has been successfully proposed to characterize pSS saliva compared to healthy controls (HC). However, the limited number of published studies provides a lack of evidence about

the reliability of metabolomics as a diagnostic tool. Different analytical approaches were performed, for instance nuclear magnetic resonance (NMR) and mass spectrometry (MS). Through untargeted ^{1}H-NMR, Mikkonen *et* al. identified 24 metabolites in stimulated whole saliva, with significant variations of specific molecules between group of analysis; differences in concentration were also related to salivary flow rate of cases and HC (Mikkonen 2012, Herrala, Mikkonen et al. 2020). Kageyama *et* al. analyzed the metabolite profiles of the whole saliva from 14 pSS patients with MS. Of the 83 metabolites identified, 41 were significantly decreased in pSS patients compared to HC, most probably reflecting salivary gland damage (Kageyama, Saegusa et al. 2015).

5.2 Material and methods

The aim of this pilot study is to compare the salivary metabolome of pSS and HC, through application of untargeted metabolomics performed with ^{1}H-NMR. The present study was approved by the Ethical Committee of the "Area Vasta Emilia Nord" (AVEN) (protocol number: 1183/2018/SPER/AOUMO). Written consent was obtained from all volunteers who participated in this study, according to the Declaration of Helsinki.

To constitute the cohort of pSS for analysis, histological slides of salivary glands biopsy were re-assessed by a pathologist to standardize the diagnostic criteria (Chisholm and Mason 1968, Shiboski, Shiboski et al. 2017). All patients were, at the time of review, in follow-up with the Rheumatology service of University of Modena and Reggio Emilia, Policlinico di Modena. Their serological status was also evaluated, to assess the level of anti-SSA/Ro and antinuclear antibody (ANA). Moreover, clinical information such as referred symptoms and Schirmer test results were considered. Age and sex matched HC were recruited from a cohort of healthy volunteers.

Strict inclusion and exclusion criteria were applied, to select the most homogeneous study population. Considering the two groups, patients and volunteers with history of head and neck radiotherapy, HIV or HCV infection, history of lymphoma, sarcoidosis, graft versus host disease and use of anticholinergic drugs were excluded. Furthermore, the presence of oral lesions, active oral infections, diabetes, smoking habit and oncological diseases constitutes a criterion of exclusions.

5.2.1 Saliva collection

A general oral evaluation was performed to disclose potential pathological conditions. Saliva samples were collected using standard techniques according to Navazesh (Navazesh 1993). To all recruited patients was asked to not eat, drink, smoke and use oral hygiene products one hour before saliva collecting. Immediately before collection, patients rinsed their mouth with water for 1 minute. Samples from the two groups were collected in the morning (9:00 to 12:00) to minimize the influence of the circadian rhythm on salivary composition. Unstimulated whole saliva was passively collected or spitted into a sterile Eppendorf cuvette, kept on ice during the entire process. Salivary flow rate was

contextually evaluated (Navazesh, Kumar et al. 2008). All samples were immediately frozen into liquid nitrogen for transportation and stored into −80 °C refrigerator until analysis. Specimen anonymization took place at this stage.

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

Each frozen saliva sample was thawed at room temperature and centrifuged at 15,000 rpm for 10 min at 4° C to remove cells, cellular debris, and mucins, according to Gardner et al. (Gardner, Parkes et al. 2018). To equalize the pH measurements, all samples were buffered with a solution containing trisodium phosphate (TSP), to maintain the chemical shift; 500 μL of supernatant were mixed with 100 μL of TSP buffer (pH 7.45), then 500μL of this solution was used for NMR.

The ^{1}H-NMR metabolomics was blindly performed, as the operator was not aware of samples group provenience. An NMR Bruker Avance III HD 600 MHz spectrometer sited at “Centro interdipartimentale Grandi Strumenti (CIGS)”, University of Modena and Reggio Emilia, Modena, was used for data acquisition. Monodimensional (^{1}H) experiment were performed. Software aided data analysis were performed (TopSpin ® 4.0.7, Bruker). Software aided chemometric and statistical analysis (unsupervised or supervised) were executed (Microsoft Excel, Microsoft corp; MNova, Mestrelab; Metaboanalyst 4.0, XiaLab McGill).

5.3 Results

Between 2011 and 2017,124 patients received a biopsy of minor salivary glands for SS suspect. From the retrospective review of histological slides, clinical evaluation and serological status, 60 cases were confirmed as pSS cases. After application of exclusion criteria, 7 female cases were selected for salivary investigation (mean age 65,4). In addition, 6 sex and age HC were concurrently identified between a cohort of volunteers.

Despite reported symptoms of xerostomia in 6 out 7 SS patients, salivary flow rate test was positive in only 2 cases. In such patients, one with gland hypofunction did not report xerostomia as symptoms.

Metabolic analysis focused on 360ms eco interval 1D ^{1}H-NMR CPMG spectra. Exponential function was applied as filter (LB = 0.5) and spectra were corrected at baseline. Chemical shift scale was calibrated at 0 ppm, keeping the trimethylsilyl propanoic acid (TSP) Me_3Si group as reference.

Metabolites were assigned on CPMG spectra, starting from those comprised between 0 and 4,7 ppm. An overall 33 metabolites were detected.

Graphical spectra underwent a preliminary visual inspection to compare data from SS group and HC group. At this level no differences were appreciable. Then, chemometric and statistical software aided analysis were performed. Univariate t-test returned difference between groups for proline and pyruvate ($p<0,01$), both elevated in HCs. Unsupervised PCA analysis also was not able to cluster variables of the two groups.

Of the whole PCA output, PC3 showed the most promising separation behavior between groups (Figure 1). The graphical PC3 loading plots expansion highlighted metabolites potentially able to discriminate samples between groups, based on their statistical importance in each group. A supervised partial least squared-data analysis (PLS-DA) was performed, to maximize the differences between the two groups. The algorithm imposed a model of separation to variables (metabolites) resulting in clustering of samples based on variables importance in each group. PLS-DA graphical output showed a distinct separation between SS cases and HCs. Loading plots returned a pool of metabolites distinctly separating each group (Figure 2).

Table 1. Resume of the most significant metabolites which contribute to group discrimination at PLS-DA

Group 1: Sjögren syndrome	Group 2: Healthy controls
Methylamine	Putrescine
Dimethylamine	Proline
Trimethylamine	Glycine
Propionate	Sarcosine
Butyrate	Fucose
Acetate	Valine

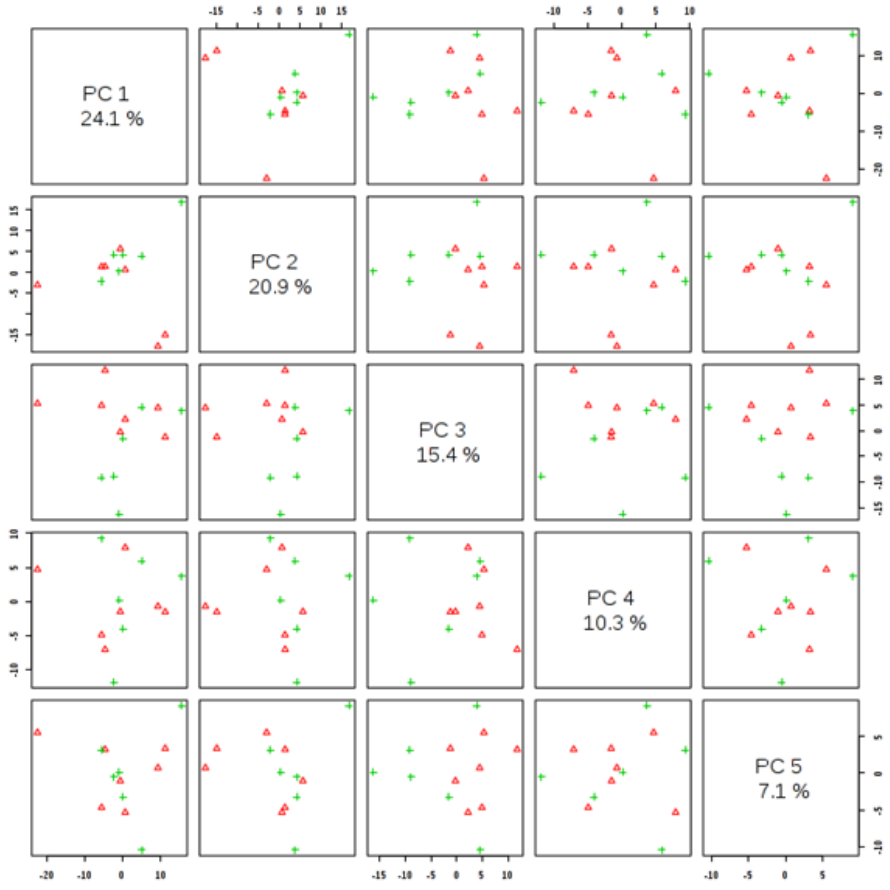

Figure 1. PCA graphical output.

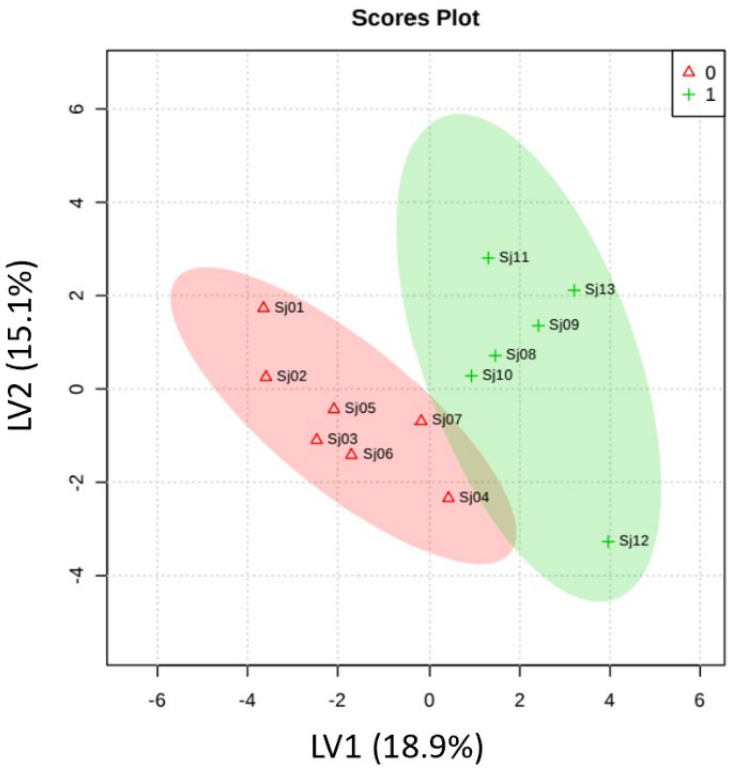

Figure 2, PLS-DA scores plot. Clustering and sample separation is appreciable.

5.4 Discussion

^{1}H-NMR stands out for the high reproducibility of results and for both qualitative and quantitative data output. Conversely, NMR is a less sensitive technique compared to mass spectrometry (MS) and hence requires much larger samples for analysis. Data acquisition procedure is expensive as well as chemometric and statistical analysis were highly time consuming.

Main limitations to this preliminary study were the small sample size and a rough statistical interpretation. A wider population is mandatory to observe groups separation also at the PCA analysis. Otherwise, only PLS-DA can provide good insight. Moreover, in such preliminary phase, no deconvolution or spectra integration was performed, resulting in a less precise analysis of the graphical output area were overlapping of peaks is appreciable.

Sjogren syndrome group salivary metabolome was broad characterized by the presence of simple amines and organic acids while amino acids and complex amines were identified in the control group. This result is coherent with Kageyama G. *et* al. that reported a reduction of fucose and amino acids, such as glycine and tyrosine in saliva of SS cases, studied by gas chromatography mass spectrometry (GC-MS) (Kageyama, Saegusa et al. 2015). Otherwise, the salivary NMR analysis of Mikkonen J*. et* al. reported partial opposite findings: while butyrate was coherently elevated in SS, amino acids such as proline and glycine were elevated in HC population (Mikkonen 2012).

Another pitfall of this study was the assessment of oral hygienical status prior salivary collection. Considering the oral cavity an “open” environment, whole saliva composition is affected by the presence of bacteria (Lindon, Nicholson et al. 2000). It is worth mentioning that we found a significant concentration of three metabolites of bacterial origin (acetate, propionate and trimethylamine) in SS WS compared to HC. We interpreted the presence of those metabolites as an indication of microbial contamination due to plaque, as reported by Aimetti *et* al. (Aimetti, Cacciatore et al. 2011) and Chen *et* al. (Chen, Feng et al. 2015) Moreover, emerging results indicates how acetate, acetate, propionate, trimethylamine and also butyrate were increased in NMR salivary metabolome of chronic periodontitis patients (Aimetti, Cacciatore et al. 2011), as well as in severe gingivitis conditions (Klukowska, Goyal et al. 2015).

Despite such limitations, presented preliminary data are promising. PLS-DA showed a well-defined group separation, suggesting that application of ^{1}H-NMR metabolomics in the study of pSS disease is promising (Figure 2). Stronger evidence and further experiment are needed to consolidate metabolomics diagnostic and prognostic capability.

References

1. Aimetti, M., Cacciatore, S., Graziano, A. and Tenori, L. (2011). "Metabonomic analysis of saliva reveals generalized chronic periodontitis signature." Metabolomics **8**(3): 465-474.
2. Baldini, C., Giusti, L., Ciregia, F., Da Valle, Y., Giacomelli, C., Donadio, E., Sernissi, F., Bazzichi, L., Giannaccini, G., Bombardieri, S. and Lucacchini, A. (2011). "Proteomic analysis of saliva: a unique tool to distinguish primary Sjogren's syndrome from secondary Sjogren's syndrome and other sicca syndromes." Arthritis Res Ther **13**(6): R194.
3. Chen, Z., Feng, S., Pow, E. H., Lam, O. L., Mai, S. and Wang, H. (2015). "Organic anion composition of human whole saliva as determined by ion chromatography." Clin Chim Acta **438**: 231-235.
4. Chisholm, D. M. and Mason, D. K. (1968). "Labial salivary gland biopsy in Sjogren's disease." J Clin Pathol **21**(5): 656-660.
5. de Almeida Pdel, V., Gregio, A. M., Machado, M. A., de Lima, A. A. and Azevedo, L. R. (2008). "Saliva composition and functions: a comprehensive review." J Contemp Dent Pract **9**(3): 72-80.
6. Ebert, E. C. (2012). "Gastrointestinal and hepatic manifestations of Sjogren syndrome." J Clin Gastroenterol **46**(1): 25-30.
7. Gardner, A., Parkes, H. G., Carpenter, G. H. and So, P. W. (2018). "Developing and Standardizing a Protocol for Quantitative Proton Nuclear Magnetic Resonance ((1)H NMR) Spectroscopy of Saliva." J Proteome Res **17**(4): 1521-1531.
8. Haldorsen, K., Moen, K., Jacobsen, H., Jonsson, R. and Brun, J. G. (2008). "Exocrine function in primary Sjogren syndrome: natural course and prognostic factors." Ann Rheum Dis **67**(7): 949-954.
9. Herrala, M., Mikkonen, J. J. W., Pesonen, P., Lappalainen, R., Tjaderhane, L., Niemela, R. K., Seitsalo, H., Salo, T., Myllymaa, S. and Kullaa, A. M. (2020). "Variability of salivary metabolite levels in patients with Sjogren's syndrome." J Oral Sci **63**(1): 22-26.
10. Hu, S., Wang, J., Meijer, J., Ieong, S., Xie, Y., Yu, T., Zhou, H., Henry, S., Vissink, A., Pijpe, J., Kallenberg, C., Elashoff, D., Loo, J. A. and Wong, D. T. (2007). "Salivary proteomic and genomic biomarkers for primary Sjogren's syndrome." Arthritis Rheum **56**(11): 3588-3600.
11. Jonsson, R., Brokstad, K. A., Jonsson, M. V., Delaleu, N. and Skarstein, K. (2018). "Current concepts on Sjogren's syndrome - classification criteria and biomarkers." Eur J Oral Sci **126 Suppl 1**: 37-48.
12. Kageyama, G., Saegusa, J., Irino, Y., Tanaka, S., Tsuda, K., Takahashi, S., Sendo, S. and Morinobu, A. (2015). "Metabolomics analysis of saliva from patients with primary Sjogren's syndrome." Clin Exp Immunol **182**(2): 149-153.
13. Klukowska, M., Goyal, C. R., Khambe, D., Cannon, M., Miner, M., Gurich, N., Circello, B., Huggins, T., Barker, M. L., Furnish, C., Conde, E., Hoke, P., Haught, C., Xie, S. and White, D. J. (2015). "Response of chronic gingivitis to hygiene therapy and experimental gingivitis. Clinical, microbiological and metabonomic changes." Am J Dent **28**(5): 273-284.
14. Konttinen, Y. T. and Kasna-Ronkainen, L. (2002). "Sjögren's syndrome: viewpoint on pathogenesis." Scandinavian Journal of Rheumatology **31**(2): 15-27.
15. Lindon, J. C., Nicholson, J. K., Holmes, E. and Everett, J. R. (2000). "Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids." Concepts in Magnetic Resonance **12**(5): 289-320.
16. Meleti, M., Quartieri, E., Antonelli, R., Pezzi, M. E., Ghezzi, B., Viani, M. V., Setti, G., Casali, E., Ferrari, E., Ciociola, T., Spisni, A. and Pertinhez, T. A. (2020). "Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva." Metabolites **10**(8).
17. Mikkonen, J. J. W. (2012). "Metabolic Profiling of Saliva in Patients with Primary Sj?gren?s syndrome." Journal of Postgenomics Drug & Biomarker Development **03**(03).
18. Navazesh, M. (1993). "Methods for collecting saliva." Ann N Y Acad Sci **694**: 72-77.
19. Navazesh, M., Kumar, S. K. and University of Southern California School of, D. (2008). "Measuring salivary flow: challenges and opportunities." J Am Dent Assoc **139 Suppl**: 35S-40S.

20. Parisis, D., Chivasso, C., Perret, J., Soyfoo, M. S. and Delporte, C. (2020). "Current State of Knowledge on Primary Sjogren's Syndrome, an Autoimmune Exocrinopathy." J Clin Med **9**(7).
21. Shiboski, C. H., Shiboski, S. C., Seror, R., Criswell, L. A., Labetoulle, M., Lietman, T. M., Rasmussen, A., Scofield, H., Vitali, C., Bowman, S. J., Mariette, X. and International Sjogren's Syndrome Criteria Working, G. (2017). "2016 American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjogren's syndrome: A consensus and data-driven methodology involving three international patient cohorts." Ann Rheum Dis **76**(1): 9-16.
22. Singh, A. G., Singh, S. and Matteson, E. L. (2016). "Rate, risk factors and causes of mortality in patients with Sjogren's syndrome: a systematic review and meta-analysis of cohort studies." Rheumatology (Oxford) **55**(3): 450-460.

CHAPTER 6

Salivary biomarkers and acute myocardial infarction

6.1 Introduction

Cardiovascular diseases account for about 30% of deaths globally. Among these, acute myocardial infarction (AMI), a life-threatening complication of coronary heart disease, is the cause of 7.4 million deaths per year. AMI is the leading single cause of mortality in Europe, being associated to approximately 862,000 deaths among men and to 877,000 deaths among women (19% and 20% of all deaths, respectively), per year (Timmis, Townsend et al. 2018).

From the pathologic point of view, AMI is defined as the death of myocardial cells due to prolonged ischemia. The etiopathogenesis lays on multiple causes, including atherothrombotic coronary artery disease, usually precipitated by atherosclerotic plaque rupture or erosion, and the imbalance between blood supply and heart muscle demand (Thygesen, Alpert et al. 2018).

AMI is diagnosed on detection of signs and/or symptoms of myocardial ischemia and biomarkers of myocardial injury detection. The pivotal role of markers of cardiac damage, (e.g., serum cardiac troponin isoforms) is based on their high sensitivity and specificity in the diagnosis of cardiac cell necrosis. Such biomarkers are usually detectable within 1−3 h, having a peak within 24−48 h (Thygesen, Alpert et al. 2018).

Depending on clinical presentation, electrocardiogram findings and serum biomarkers expression, 2 subgroups of AMI can be identified: ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation myocardial infarction (NSTEMI), the latter accounting for about 60% of all AMI (Hamm, Bassand et al. 2011, Ibanez, James et al. 2018).

To reduce morbidity and mortality as well as to improve patients care, it is of major importance to achieve a prompt diagnosis and provide suitable treatments.

The study of saliva for the diagnosis of oral and systemic diseases is a topic of current high interest. Improved analysis tools and selection of potential biomarkers are boosting salivary diagnostics, providing some perspectives also for cardiovascular diseases diagnosis (Meleti, Quartieri et al. 2020, Setti, Pezzi et al. 2020).

As a mixture of salivary glands secretion (parotid, sub-mandibular, sublingual and minor salivary glands), gingival crevicular fluid, together with epithelial cell products and exfoliation, bacterial cells

and their metabolites, making saliva a very complex biofluid. Its composition includes enzymes, antibodies, hormones, antimicrobial elements and proteins. Plasma components present in saliva are mainly found in the gingival crevicular fluid, which enters the oral cavity through the gingival sulcus and through para-cellular outflow from salivary gland tissue. For liquid biopsy, saliva presents many advantages over blood, its collection being easy, safe, non-invasive and cost-effective (Meleti, Cassi et al. 2020).

Several biomarkers associated to AMI can be detected in saliva and their expression can potentially distinguish between patients and healthy controls.

The aim of the present systematic review is to provide some insights on the possible associations between salivary biomarkers and AMI and to highlight potentials and limits of the most reliable markers. Therefore, the question to which the review attempts to answer is: “Are salivary biomarkers useful to identify patients with acute myocardial infarction?” Such question has been formulated according to the “Patient-Intervention-Comparison-Outcome” (PICO) worksheet.

6.2 Materials and methods

The present systematic review has been designed taking into accounts the guidelines of the “Preferred Reporting Item for Systematic Reviews and Meta-analysis”.

A comprehensive search of the scientific English literature published in the last 30 years (1990 – 2020) (Medline, Scopus and Web of Science) has been performed up to April 2020. The entry terms “saliva” and “salivary” were combined through the Boolean indicator “and” with the following words: “angina”, “myocardial”, “atherosclerosis”, “cardiovascular”, “heart”, “vasculopathy”, “STEMI”, “NSTEMI”. After a software-aided elimination of duplicates (EndNote X9, Clarivate Analytics), records were evaluated through a first-level screening (title evaluation). At this level, case reports, conference proceedings, personal communications, letters to editor and reviews were excluded. Papers selected for second-level screening underwent a careful analysis of the abstract and in case of potential interest were considered eligible for full-text evaluation.

All references of review articles emerged from the research were screened, to identify potentially missed studies. Such approach was also applied to the reference section of papers eventually eligible for selection. Search flow is summarized in Figure 1.

Final eligibility was assessed according to the following exclusion and inclusion criteria; inclusion: studies performed on humans, involving patients with AMI (diagnosed with detectable specific serum myocardial-necrosis markers); studies specifically reporting on collection of saliva occurred within the first 7 days from the acute coronary syndrome onset; exclusion: studies without at least one group of patients affected by AMI only (e.g. studies investigating patients with both acute myocardial infarction and periodontal diseases were excluded); studies considering patients affected by unstable angina (due absence of cardiac damage and consequently normal values of serum markers of cardiac damage); silent ischemia (on-set timing hardly identifiable); iatrogenic myocardial damage (such as septal ablation in patients with hypertrophic cardiomyopathy). A comprehensive recap of selection criteria is provided in Table 1.

6.2.1 Data extraction, quality assessment and critical appraisal

Data extracted from each study were summarized into an Excel table (Office Suite, Microsoft Corp.). Such data included: title, authors, year of publication, study aim, type of serum biomarkers and salivary biomarkers investigated, type of analytic device, type of saliva, timeframe for first salivary sample collection and pre-analytical procedures (Table 2).

Two independent reviewers separately evaluated all papers by using the National Institute of Health - Nation Health, Lung and Blood Institute “Study Quality Assessment Tool”, utilizing the specific questionnaire for case-control studies (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools). Such tools were specifically developed for each type of study (controlled intervention studies, systematic reviews with meta-analysis, observational cohort and cross-sectional studies, case-control studies, before–after studies without control group, case series studies). The questionnaire is designed to help the investigator in the identification of potential fatal flaws caused by study design and conduction.

Critical appraisal of studies has been performed through assignation of a score ranging from 0 to 100%, based on the percentage of “yes” choices on the overall number of answers given. Studies having 80–100% score were labeled as having “good” quality; those ranging from 50 to 70% were “fair”; and studies scoring less than 50% were defined as “poor” quality studies. Discrepancies in score assignment were discussed by the two reviewers, with an additional text evaluation and divergencies resolution. Emerging risk of bias were summarized into Figure 2.

The level of evidence was assessed using the classification of the Oxford Center for Evidence Based Medicine levels for diagnosis (2011).

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
English literature Studies published between 1990 and April 2020 Studies considering human saliva samples Studies involving patients with acute myocardial infarction with detectable specific serum myocardial-necrosis markers. Studies specifically reporting a time of saliva collection performed within the first 7 days from the acute coronary syndrome onset.	Publication type: case reports, conference proceedings, personal communications, letters to editor, reviews. Studies mainly describing a technical method for salivary biomarker isolation. Studies different from the case-control setting.

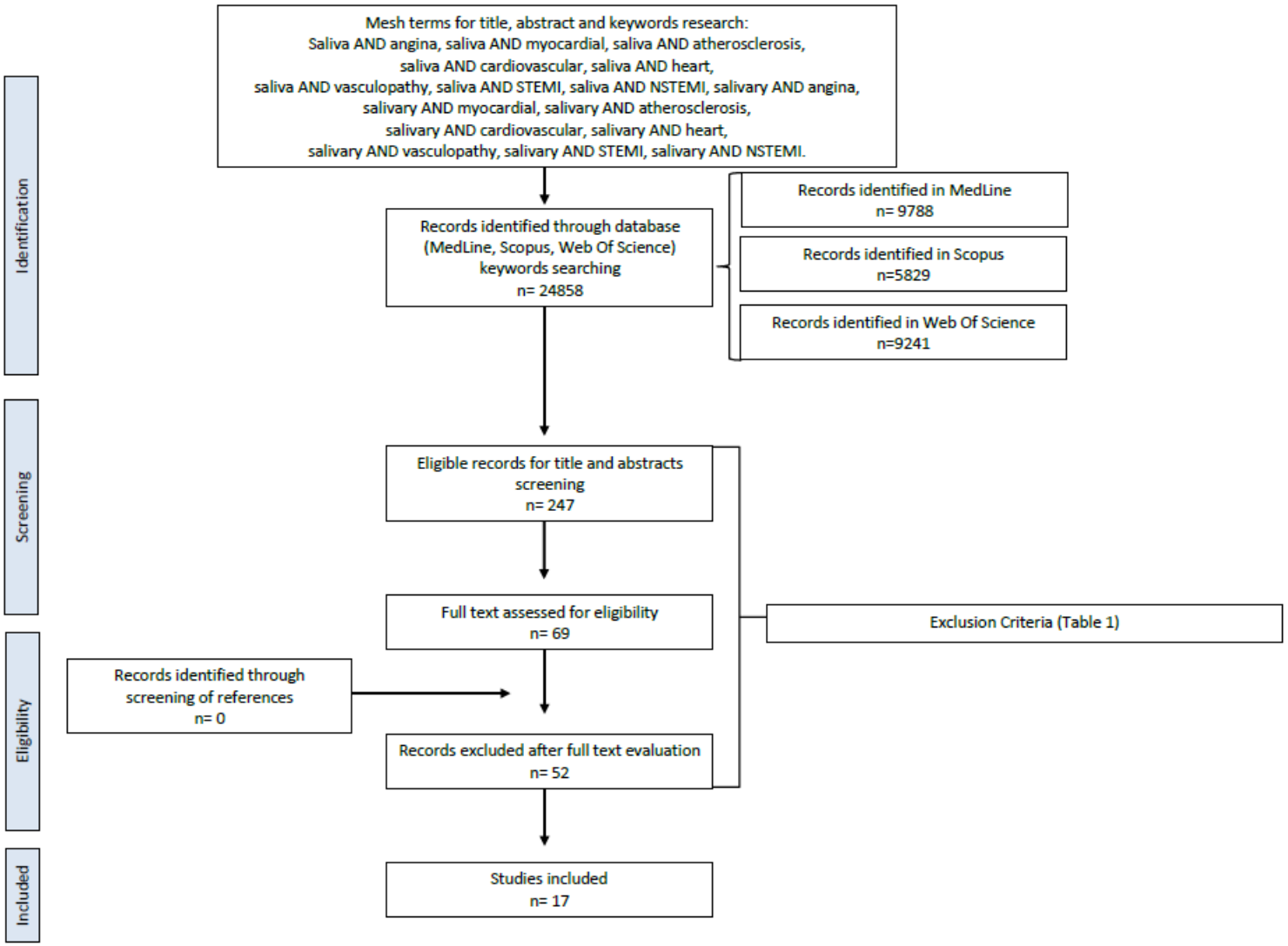

Figure 1. Flow-chart diagram for the selection of 17 papers included in the review.

6.3 Results

The research algorithm is summarized in Figure 1. Multiple databases research for title, abstracts and keywords returned 24858 records; exclusion of repetitions and application of inclusion and exclusion criteria, returned 241 records considered eligible for title and abstract screening. Sixty-nine full-text evaluations were performed, and 52 records were discarded at this level. No records were included from articles references cross-checking. Eventually, 17 papers were included in the review (Jones, Reynolds et al. 1994, Floriano, Christodoulides et al. 2009, Mirzaii-Dizgah and Jafari-Sabet 2011, Mirzaii-Dizgah and Riahi 2011, Mirzaii-Dizgah, Hejazi et al. 2012, Shen, Chen et al. 2012, Y, VS et al. 2012, Mirzaii-Dizgah and Riahi 2013, Mirzaii-Dizgah and Riahi 2013, Rubio, Gonzalez et al. 2013, Toker, Aribas et al. 2013, Aydin, Aydin et al. 2014, Miller, Foley et al. 2014, Aydin, Eren et al. 2017, Ebersole, Kryscio et al. 2017, Mishra, Patil et al. 2018, Omidpanah, Jalilian et al. 2018).

6.3.1 Quality assessment

Disagreement between the two reviewers occurred in 3 cases: a) in paper 1, divergences involved question number 8 (“use of concurrent controls”). Reviewer 1 scored the paper as “intermediate”, reviewer 2 as “good”. After discussion, the study was unanimously scored as “intermediate”; b) paper 13 was scored “poor” by reviewer 1 and “intermediate” by reviewer 2. After discussion about questions 2 (“definition of population”), 4 (“comparable population for recruitment and correspondence of timeframe of recruitment”) and 5 (“definition of inclusion and exclusion criteria”), paper was unanimously scored as “poor”; c) paper 14 was scored as “intermediate” by reviewer 1 and “poor” by reviewer 2. Divergencies about questions 4 (“comparable population for recruitment and correspondence of timeframe of recruitment”) and 12 (“statistical analysis adjusting”). After discussion, paper was scored as “intermediate”. Critical appraisal of the selected papers is summarized in Figure 2.

No “good” quality papers were identified in the present systematic review. Sixteen studies were classified as having “intermediate”, and one was classified as having “poor” quality.

Potential fatal flaws have emerged from the assessment process: studies 3, 6, 9 and 12 (4; 23%) did not describe a comparable population for recruitment and correspondence of timeframe of recruitment. Moreover, study 13, which was the lowest in score assignment, did not clearly describe the study population, the timeframe and the inclusion/exclusion criteria for patient selection. In general, the most frequently encountered potential risk of bias were the absence of data regarding the status of participants (e.g., disease/healthy) to saliva investigators (confirmation of blind evaluation) (17 studies; 100%), the absence of concurrent controls (17 studies; 100%), the lack of sample size justification (16 studies; 94%), and the lack of randomization (10 studies; 59%).

Figure 2. Quality assessment and critical appraisal of the included 17 studies

Table 2. General characteristics of selected papers, information about salivary biomarkers and details on methods for evaluation.

Article	Title	Authors	Year of publication	Aim	Biomarkers	Type of analytic device	Type of saliva	Timeframe for first salivary sample collection	Pre-analytical procedures
1	Evaluation of Salivary Cardiac Troponin-I as Potential Marker for Detection of Acute Myocardial Infarction	Mishra *et al.*	2018	Study the role of salivary troponin-I in AMI, compare salivary and serum concentrations.	cTnI	RayBio cardiac troponin-I ELISA kit	UWS	Within 12-24hrs from clinical symptoms	Water mouth rinses. Centrifuged at 3800 g for 10 minutes.
2	Evaluation of butyryl cholinesterase and acetylcholinesterase activity in serum and saliva of myocardial infarction patients	Omidpanah *et al.*	2018	Compare serum and salivary butyrylcholinesterase and acetylcholinesterase enzyme activity between myocardial infarction and HC.	AChE; BChE.	240nm light absorbance	UWS	1st hour after AMI	Water mouth rinses. Collection into Falcon tubes. Centrifuged at 20.000 rpm for 2-3 minutes, supernatants frozen at -45 ºC
3	Adropin as a potential marker of enzyme-positive acute coronary syndrome	Aydin *et al.*	2017	To determine if saliva contains detectable adropine at diagnostic concentration. Comparing serum and salivary concentrations.	Adropin	EIA, ELISA, 450nm light absorbance	UWS	Within 30-40min after AMI	Water mouth rinses. Centrifuged 15 min at 4.000 rpm. Frozen at -80°C.

4	Salivary and serum adiponectin and C-reactive protein levels in acute myocardial infarction related to body mass index and oral health	Ebersole *et al.*	2017	Describe the relationship between salivary adiponectin and C-reactive protein with AMI.	Adip; CRP	Multiplexed flow cytometry	UWS	Within 48hrs from AMI	Samples were transported on ice, centrifuged and divided into aliquots. Specimens kept on ice before -80°C storage.
5	Decreased saliva/serum irisin concentrations in the acute myocardial infarction promising for being a new candidate biomarker for diagnosis of this pathology	Aydin *et al.*	2014	To measure serum and saliva irisin concentrations in AMI patients	Irisin	EIA, ELISA, 450nm light absorbance	UWS	Within 20-60 min from AMI	Water mouth rinses. Centrifuged 15 min at 4.000 rpm. Frozen at -80°C.

6	Utility of salivary biomarkers for demonstrating acute myocardial infarction	Miller *et al.*	2014	To test the hypothesis that specific salivary biomarkers show clinical utility for the assessment of AMI, especially NSTEMI.	CRP; IL-6; IL-1β; MPO; sCD40L; TNF-α; Adip; sICAM-1; MMP-9.	Luminex IS-100, Multiplexed flow cytometry	UWS	Within 48hrs from AMI	Samples were transported on ice, centrifuged and divided into aliquots. Specimens kept on ice before -80°C storage.
7	Salivary high-sensitivity cardiac troponin T levels in patients with acute myocardial infarction	Mirzaii-Dizgah *et al.*	2013	To identify whole saliva high-sensitivity cardiac troponin T (hs-cTnT) in patients with AMI.	hs-cTnT	ELISA	USW and SWS	Within 10-23hrs after AMI (average 14hrs)	Water mouth rinses. Resting saliva and stimulated whole saliva collection in different tubes. Centrifuged at 3800 g for 10 min, aliquots stored in -70°C.
8	Salivary troponin I as an indicator of myocardial infarction	Mirzaii-Dizgah *et al.*	2013	To assess if the cardiac necrosis biomarker, cTnI, is detectable in saliva of patients with established AMI.	cTnI	ELISA, 450nm light absorbance	USW and SWS	Within 12-24hrs from AMI	Water mouth rinses. Resting saliva and stimulated whole saliva collection in different tubes. Centrifuged at 3800 g for 10 min, aliquots stored in -70°C.

9	Oxidative stress assessed in saliva from patients with acute myocardial infarction. A preliminary study	Rubio *et al.*	2013	To assess the presence of oxidative stress indices in saliva 24 and 48h after AMI.	DCFHDA; CAT.	Spectro fluorometry 488nm, 525nm and 240nm	UWS	12 hrs after AMI	Centrifuged and stored in 0.5 ml aliquots at -40ºC.
10	Serum and saliva levels of cathepsin L in patients with acute coronary syndrome	Mirzaii-Dizgah *et al.*	2011	To evaluate the serum and saliva levels of cathepsin L in patients with acute coronary syndrome	cathepsin L	ELISA, 450nm light absorbance	UWS and SWS	Within 12-24hrs from AMI	Water mouth rinses. Resting saliva and stimulated whole saliva collection in different tubes. Centrifuged at 3800 g for 10 min, aliquots stored in -70°C.
11	Unstimulated whole saliva creatine phosphokinase in acute myocardial infarction	Mirzaii-Dizgah *et al.*	2011	To examine the relationship between serum and saliva levels of CPK and to compare saliva CPK between HC and patients with AMI	CPK	IFCC method, kinetic UV method	UWS	1st morning after AMI	Water mouth rinses. Centrifuged at 3800 g for 10 min, aliquots stored in -70°C.

12	Diagnostic performance of initial salivary alpha-amylase activity for acute myocardial infarction in patients with acute chest pain	Shen *et al.*	2012	To evaluate the diagnostic value of measuring salivary alpha-amylase (sAA) activity for detecting AMI.	sAA	Quantitative enzyme kinetic method	Mixed UWS and SWS. sublingual cotton plug	Within 4hrs from clinical symptoms	Centrifuged at 3800 g for 10 min, aliquots stored in -70°C.
13	Salivary PAF in acute myocardial infarction and angina: Changes during hospital treatment and relationship to cardiac enzymes	Jones *et al.*	1994	To determine levels of PAF in the saliva of patients admitted to coronary care with acute myocardial infarction to assess the role if any of PAF in this condition.	PAF	Scintillation proximity immunoassay	sublingual cotton plug	On admission	The plug was centrifuged, saliva was frozen at -70 °C.

14	Saliva-based creatine kinase MB measurement as a potential point-of-care testing for detection of myocardial infarction	Mirzaii-Dizgah *et al.*	2012	To compare salivary and serum levels of CK MB in patients with AMI with those of HC.	CK-MB	Immunoinhibition assay, autoanalyzer at 340nm	UWS	First morning after AMI	Water mouth rinses. Centrifuged at 3800 g for 10 min, aliquots stored in -70°C.
15	Serum and Saliva Levels of Ischemia-Modified Albumin in Patients with Acute Myocardial Infarction	Toker *et al.*	2013	To determine the relationship between serum and salivary IMA levels and compare them between HC and AMI patients.	IMA	Spectrophotometry, 470nm	UWS	24hrs after AMI	Water mouth rinses. Whole saliva was collected and then centrifuged at 10,000 × g for 10 min. Storage at -20°C.
16	Use of Saliva-Based Nano-Biochip Tests for Acute Myocardial Infarction at the Point of Care: A Feasibility Study	Floriano *et al.*	2009	To determine if serum biomarkers commonly associated with AMI diagnosis can be detected reliably using UWS	CRP; MMP-9; IL-1; sICAM-1; MYO; MPO; Adip; MCP-1; Gro-α; TNF-α; Scd40l; IL-6.	Luminex IS-100, Multiplexed flow cytometry	UWS	Within 48hrs from AMI	Samples were transported on ice, centrifuged and divided into aliquots. Specimens kept on ice before -80°C storage.
17	Salivary and serum antioxidant activity in patients with acute coronary syndrome	Gulati *et al.*	2012	To evaluate the redox status of AMI patients prior to and following therapy as compared to healthy individuals by	Antioxidant potential	Antioxidant Activity measure (Koracevic)	UWS	4-6 days fter AMI	Centrifuged at 2,000 g x 10 min and stored at -80°C.

				monitoring the antioxidant activity of UWS.					

Legend:

AChE, acetylcholinesterase; ACS, acute cardiac syndrome; Adip, adiponectin; AMI, acute myocardial infarction; AOA, anti-oxidant activity; BChE, butyryl cholinesterase; B-type natriuretic peptide (BNP); CAT, antioxidant enzyme catalase; CK-MB, creatine kinase-MB; CPK, creatine phosphokinase; CRP, C-reactive protein; cTnI, cardiac troponin-I; CTSL, cathepsin-L; DCFH-DA, dichloro-dihydro-fluorescein diacetate; GRO- α, growth-regulated oncogene α; HC, healthy controls; hs-cTnT, high-sensitivity cardiac troponin T; IL-1, interleukin 1; IL-6, interleukin 6; IMA, ischemia modified-albumin; MCP-1, monocyte chemoattractant protein 1; MMP-9, matrix metallopeptidase 9; MPO, myeloperoxidase; MYO, myoglobin; NR, not reported; NSTEMI, non-ST segment elevated myocardial infarction; PAF, platelet activating factor; SAA, salivary alpha-amylase; sCD40L, soluble cluster of differentiation ligand; sICAM-1, soluble intracellular adhesion molecule-1; STEMI, ST segment elevated myocardial infarction; SWS, stimulated whole saliva.

Table 3. Summarization of results of all papers and their statistical significance.

Article	Disease investigated	Cardiac Biomarkers	Subjects	Matrix Compared	Sensitivity	Specificity	Statistical significance
1	AMI (STEMI)	cTnI	60 subjects: 30 STEMI; 30 HC.	Serum and Saliva	NR	NR	Comparable levels of cTnI with positive relation (r=0.972, p=<0.001) between serum and saliva.
2	AMI	AChE BChE.	60 subjects: 30 AMI; 30 HC.	Serum and Saliva	NR	NR	No significant correlation for AChE and BChE at any time of measure in AMI and HC (24,48 and 72h after AMI), comparing serum and saliva.
3	AMI (EPACS)	Adropin	46 subjects: 22 AMI; 24 HC.	Serum and Saliva	4 h after AMI 91% 6h after AMI 91%	4 h after AMI 57% 6h after AMI 57%	Serum and salivary adropin showed significant positive relation, with comparable concentrations (r = 0.763, p ≤ 0.01).
4	AMI (STEMI and NSTEMI)	Adip; CRP	203 subjects: 58 STEMI, 34 NSTEMI 111 HC	Serum and Saliva	NR	NR	CRP levels were significantly elevated in both serum and saliva of the patients with AMI (p < 0.0001, in each case). STEMI showed significantly higher CRP concentrations (serum and saliva) compared to patients with NSTEMI (p = 0.0035). Salivary adiponectin levels were not significantly different among the three groups (p = 0.62).
5	AMI	Irisin	25 subjects: 11 AMI; 14 HC.	Serum and Saliva	NR	NR	Salivary irisin concentration increases 72h after AMI compared to HC (P < 0.05)
6	AMI (STEMI and NSTEMI)	CRP; IL-6; IL-1β; MPO; sCD40L; TNF-α; Adip; sICAM-1; MMP-9.	158 subjects: 73 AMI; 85 HC.	Serum and Saliva	NR	NR	Discrimination of AMIs from HCs represented by significant differences in concentrations of CRP, sICAM, and adiponectin
7	AMI (STEMI)	hs-cTnT	60 subjects: 30 STEMI; 30 HC.	Serum and Saliva	UWS 83% SWS 79%	UWS 70% SWS 65%	Correlation between the UWS concentration of hs-cTnT and its serum concentration (r = 0.415, P < 0.023) and between the SWS concentration of hs-cTn and its serum concentration (r = 0.466, P < 0.021).
8	AMI (STEMI)	cTnI	58 subjects: 30 AMI; 28 HC	Serum and Saliva	NR	NR	Serum cTnI levels showed good correlation with UWS cTnI concentrations (spearman rho =0.45, P=0.001) in the AMI group.
9	AMI (STEMI)	DCFHDA; CAT.	20 subjects: 10 STEMI; 10 HC.	Serum and Saliva	NR	NR	AMI group demonstrated significantly higher salivary DCFH-DA levels as compared to HC (p=0.03) and fair significant correlation was found at 48h (r=0.39; p=0.053).

10	ACS	CTSL	67 subjects: 39 AMI; 28 HC	Serum and Saliva	NR	NR	No significant differences in serum and UWS. SWS 12h after admission showed significant CTSL decrease compared with HC (p = 0.0003)
11	AMI	CPK	60 subjects: 30 STEMI; 30 HC.	Serum and Saliva	NR	NR	Salivary CPK concentration higher in AMI vs. HC. Correlation between salivary and serum CPK in the first day ((r = 0.442, P < 0.01)) and in the second day (r = 0.268, P < 0.01) in AMI group.
12	AMI (STEMI and NSTEMI)	sAA	473 subjects: 53 STEMI; 32 NSTEMI; 388 HC.	Serum and Saliva	78.8%	74.5%	sAA activity in AMI was significantly higher than HC (p < 0.001). STEMI vs. NSTEMI, higher sAA activity (p < 0.001) When combined with initial troponin I, sensitivity and specificity prediction of AMI increase to 88.3% and 78.4%, respectively.
13	AMI	PAF	57 subjects: 16 AMI; 9 non cardiac chest pain; 5 anginas; 8 HC; 19 other diseases.	Serum and Saliva	NR	NR	Salivary PAF concentration is raised in ischemic diseases vs. non-AMI chest pain.
14	AMI (STEMI)	CK-MB	60 subjects: 30 STEMI; 30 HC.	Serum and Saliva	NR	NR	Salivary CK-MB higher in AMI compared to HC (72.41±14.15 vs. 8.16±0.54 U/l, respectively; P=0.004).
15	AMI	IMA	100 subjects: 60 AMI; 40 HC	Serum and Saliva	NR	NR	Salivary IMA levels were significantly higher only in the first day of AMI patients than HC. No differences between serum and salivary IMA levels in the first day and second day of AMI patients
16	AMI (STEMI and NSTEMI)	CRP; MMP-9; IL-1; sICAM-1; MYO; MPO; Adip; MCP-1; Gro-α; TNF-α; sCD40L; IL-6.	84 subjects: 16 NSTEMI; 25 STEMI; 43 HC.	Serum and Saliva	NR	NR	Sensitivity values about 90%–100% for MYO-CRP and MYO-CRP-MPO combinations.
17	AMI (STEMI and NSTEMI)	Antioxidant potential	49 subjects: 24 STEMI; 9 NSTEMI; 16 HC.	Serum and Saliva	NR	NR	The total AOA in saliva of STEMI + NSTEMI was significantly lower (p<0.01) than HC. The salivary AOA in STEMI, and NSTEMI was significantly lower than HC (p< 0.01 and < 0.05, respectively).

Legend:

AChE, acetylcholinesterase; ACS, acute cardiac syndrome; Adip, adiponectin; AMI, acute myocardial infarction; AOA, anti-oxidant activity; BChE, butyryl cholinesterase; B-type natriuretic peptide (BNP); CAT, antioxidant enzyme catalase; CK-MB, creatine kinase-MB; CPK, creatine phosphokinase; CRP, C-reactive protein; cTnI, cardiac troponin-I; CTSL, cathepsin-L; DCFH-DA, dichloro-dihydro-fluorescein diacetate; GRO- α, growth-regulated oncogene α; HC, healthy controls; hs-cTnT, high-sensitivity cardiac troponin T; IL-1, interleukin 1; IL-6, interleukin 6; IMA, ischemia modified-albumin; MCP-1, monocyte chemoattractant protein 1; MMP-9, matrix metallopeptidase 9; MPO, myeloperoxidase; MYO, myoglobin; NR, not reported; NSTEMI, non-ST segment elevated myocardial infarction; PAF, platelet activating factor; SAA, salivary alpha-amylase; sCD40L, soluble cluster of differentiation ligand; sICAM-1, soluble intracellular adhesion molecule-1; STEMI, ST segment elevated myocardial infarction; SWS, stimulated whole saliva.

6.3.2 Level of evidence

Application of the Oxford Center for Evidence-based Medicine guidelines highlighted that all the selected papers have a low level of evidence (4 on 5, the fifth level being the lowest), because of their case-control design.

6.3.3 Type of saliva, methods of collection and analytic device

Details on methods for saliva evaluation are summarized in Table 2.

Potential salivary myocardial infarction biomarkers identification was conducted onto whole saliva samples in each study except one. Specifically, 12 studies detailed the use of passive or spitted unstimulated whole saliva (studies 1,2,3,4,5,6,9,11,14,15,16,17; 70%), 4 evaluated both stimulated and unstimulated whole saliva (studies 7,8,10,12). Paper 13 report about the collection of fluid using a sublingual cotton plug (Salivette saliva collecting tubes, Sarstedt, Nümbrecht, Germany), suggesting that whole saliva was sampled but not clearly disclosed; more to the point, the same harvesting method was used in study 12, for both stimulated and unstimulated whole saliva collection.

Data regarding precise instructions given to patients before saliva collection were available in 12 studies (70%); no instructions were mentioned in studies 9, 12, 13, 16 and 17 (29%). Study number 4 refers to a modification of the collecting protocol published by Navazesh (Navazesh 1993); however, changes adopted were not disclosed in the text. Instructions given to patients were to refrain from eating, smoking and drink anything except water from 1 to 2 hours before saliva collection. Studies 3 and 5 had extended the period to the night before the procedure. All the 11 studies reported the use of mouth rinses with tap water before fluid harvest procedure. Saliva was taken in the morning (from 8:00 to 10:00) in 3 papers (studies 2, 3 and 5). No other information about time of collection comes from remaining papers.

About patient selection, articles 12 and 15 (Shen, Chen et al. 2012, Toker, Aribas et al. 2013) specified how the presence of oral diseases, recent dental works, caries, active periodontitis or mucosal bleeding lesions implicate exclusion from study population. Studies 4, 6 and 16 (Floriano, Christodoulides et al. 2009, Miller, Foley et al. 2014, Ebersole, Kryscio et al. 2017) visually assessed

oral health to not interfere with saliva collection, using a modified scoring system from Burke et. *al* 2003, (Burke, Busby et al. 2003). Evaluation focused on of mucosal inflammation, decays, obvious tooth or gum infection or reports teeth mobility. The number of teeth was recorded. Scoring was stated as poor, fair or good.

All studies reported at least one information about handling (including centrifugation) or storing of saliva. Particularly, centrifugation methods were rather heterogenous, the most frequently used values ranging between 3800×g for 10 minutes (studies number 1,7,8,10,11,12 and 14; 41%). Centrifugation at 4000 rpm for 15 minutes was also performed in studies number 3 and 5 (12%). Occasionally, faster centrifugation methods were used in studies 2 and 15 (12%), respectively for 20000 rpm for 2 minutes and 9500 rpm, 10 minutes. Most of the papers reported on fluid temperature control during collection (salivary samples kept on ice). After centrifugation, refrigerated storage was adopted by all authors, with temperature ranging from -20°C to –80°C.

Biochemical analytic methods included: 1) enzyme linked immunosorbent assay (ELISA), used in 6 articles (studies 1,3,5,7,8,10); 2) multiplexed flow cytometry (studies 4,6,16); 3) quantitative enzyme immunoassay (studies 11, 12).

6.3.4 Salivary biomarkers

Overall, 26 salivary biomarkers were investigated in the studies included in the present systematic review. Molecules significantly associated with AMI or evaluated in more than one study are described below. Summarization of results of all papers is reported in Table 3.

Troponins

The correlation between concentrations of salivary and serum cardiac troponins were evaluated in studies 1,7 and 8. Mishra et al., found that in patients with AMI, serum levels of I isoform of cardiac troponin were directly associated with salivary concentrations with a high significant positive relation ($r=0.972$; $p<0.001$) (Mishra, Patil et al. 2018). Similar results were reported by Mirzaii-Dizgah et al. (Mirzaii-Dizgah and Riahi 2013); serum levels showed good correlation with unstimulated whole saliva concentrations ($p<0.001$). The same authors (Mirzaii-Dizgah and Riahi 2013) reported that salivary T isoform of cardiac troponin concentrations were higher both in stimulated and unstimulated whole

saliva of patients with infarction. Of major importance, serum and salivary levels of the protein were significantly related (both in unstimulated whole saliva ($p<0.023$) and stimulate whole saliva ($p<0.021$)), giving support the use of salivary matrix for diagnosis and monitoring of AMI.

Butyrylcholinesterase and acetylcholinesterase

Butyrylcholinesterase and acetylcholinesterase concentrations in serum and saliva were evaluated and compared in a group of patients, immediately after the occurrence of AMI and after 24 and 72 hours (Omidpanah, Jalilian et al. 2018). No significant correlation was found between the serum and salivary levels of these molecules, immediately ($r= 0.08$, $p= 0.676$ and $r= 0.233$, $p= 0.242$ respectively), at 24 hours ($r= 0.017$, $p= 0.929$ and $r= 0.06$, $p= 0.771$ respectively) and at 72 hours from AMI occurrence ($r= 0.141$, $p= 0.457$ and $r= 0.105$, $p= 0.642$ respectively). However, salivary acetylcholinesterase concentrations were statistically different in patients with AMI compared to healthy controls at any timepoint ($p<0.001$ immediately, $p<0.001$ at 24 hours, and $p<0.001$ at 72 hours after AMI).

Adropin and irisin

The usefulness of adropin as a salivary biomarker was investigated in enzyme positive acute coronary syndrome (Aydin, Eren et al. 2017). Its presence was evaluated in serum and saliva at 4 and 6 hours after syndrome onset. Salivary and serum adropin concentration shared similar sensitivity and specificity (91.7% and 57% at both timepoint) about the possibility to differentiate patients from healthy controls.

Concentrations of salivary and serum irisin were evaluated in study 5 (Aydin, Aydin et al. 2014). Levels of salivary irisin significantly decreased between 12 and 48 hours after myocardial infarction onset, when compared to those in healthy controls. Moreover, after 72 hours from cardiac ischemic event, both salivary and serum concentrations significantly increased, in comparison to that of healthy controls ($p<0.05$).

C-reactive protein

Salivary C-reactive protein was investigated in studies 4, 6 and 16 and its concentration was compared to that of serum. Such molecule is increased in both biofluids of patients with AMI ($p<0.0001$) (Ebersole, Kryscio et al. 2017), and salivary can therefore be potentially useful for

discriminating patients from controls (Miller, Foley et al. 2014). Moreover, when combined to other potential markers (myoglobin and myeloperoxidase), C-reactive protein showed a 91-100% sensitivity (Floriano, Christodoulides et al. 2009).

Adiponectin

The utility of salivary adiponectin as biomarker for acute myocardial infarction was evaluated in studies 4,6 and 16.

In paper 4, serum adiponectin levels showed a decrease in control subjects respect to patients with STEMI ($p = 0.0035$, control *vs.* STEMI and $p = 0.082$ control vs. NSTEMI). In contrast, saliva adiponectin levels were higher (even though not statistically different ($p = 0.62$)) in patients with STEMI and NSTEMI than in controls (Ebersole, Kryscio et al. 2017). Surprisingly, the same authors reported in another paper (Miller, Foley et al. 2014) that the levels of adiponectin in unstimulated whole saliva were significantly higher in patients with AMI than in healthy controls ($p<0.0046$). Again, in paper 16 adiponectin in unstimulated whole saliva reached the 9th position out of 10 markers ($p= 0.052$) for AMI screening accuracy when not used in combination with other molecules (Floriano, Christodoulides et al. 2009).

Creatine phosphokinase

Concentrations of creatine phosphokinase (CPK) and creatine kinase-myocardial band were measured in saliva (Mirzaii-Dizgah and Jafari-Sabet 2011, Mirzaii-Dizgah, Hejazi et al. 2012). Both markers were significantly higher in patients with AMI than in controls ($p<0.01$ and $p<0.004$, respectively).

Others

Soluble cluster of differentiation ligand (sCD40L), tumor necrosis factor-α (TNF-α,) and soluble intracellular adhesion molecule-1 (sICAM-1) in saliva were studied by different authors (Floriano, Christodoulides et al. 2009, Miller, Foley et al. 2014). sICAM-1 levels in unstimulated whole saliva were significantly higher in patients with AMI than in controls in both studies (paper 6: $p=0.0029$; paper 16: $p=0.0001$). Concentrations of sCD40L and TNF- α in unstimulated whole saliva were downregulated in patients with AMI when compared to healthy controls ($p<0.038$ and $p<0.0005$ respectively) in study 16 (Floriano, Christodoulides et al. 2009).

Salivary dichloro-dihydro-fluorescein diacetate was useful to discriminate between patients with AMI and controls in study 9, its concentration being significantly different between the 2 groups at ischemic event baseline ($p=0.03$). The same authors (Rubio, Gonzalez et al. 2013) reported no difference in salivary antioxidant enzyme catalase activity between patients with AMI and controls at 24 and 48 hours after diagnosis ($p=0.15$).

Salivary α-amylase activity was evaluated in study 12. The enzyme showed significantly higher activity in patients with cardiac event than in healthy controls ($p<0.001$); moreover, considering patients with STEMI versus patients with NSTEMI, a higher enzymatic activity was detected among STEMI ($p < 0.001$) (Shen, Chen et al. 2012).

Differences between cases and controls were reported in study 15, regarding salivary ischemic modified albumin concentrations. Its levels were significantly higher during the first day in patients with AMI compared to healthy controls ($p<0.001$). Additionally, there was a positive correlation between salivary and serum levels both in the first and second day (both $p<0.05$, respectively) (Toker, Aribas et al. 2013).

6.4 Discussion

In the last 15 years, there has been a tremendous reconsideration for the role of saliva as diagnostic tool (Derruau, Robinet et al. 2020, Setti, Pezzi et al. 2020). The traditional approach, mainly based on the quantitative evaluation of saliva for the identification of xerostomia and associated conditions, has been complemented by qualitative analytical approaches, which have been the basis for the development of salivary tests for a range of physiologic and pathologic conditions (e.g. hormonal changes, diabetes, *etc.*) (Fares, Said et al. 2019, Pappa, Vougas et al. 2020). Particularly, the massive advancements of the so-called “omics” sciences has led to a renewed research interest for the oral fluid as biologic specimen for diagnosis of systemic diseases (Meleti, Cassi et al. 2020).

At this regard, the neologism “salivaomics” has been introduced to indicate an approach for the evaluation of saliva based on the use of either genomics, transcriptomics proteomics, metabolomics, or a combination of these (Shah 2018).

While salivary diagnostics has shown to be useful for the screening, identification and follow-up of several chronic conditions (e.g. gastric and pancreatic cancers), the usefulness of saliva for prompt identification of acute, life-threatening, conditions (including myocardial infarction) is still matter of controversy (Wang, Kaczor-Urbanowicz et al. 2017, Sturque, Berquet et al. 2019). Such relative lack of studies may partially be explained by the objective difficulties to collect specimens and data within emergency settings.

All studies except two (Jones, Reynolds et al. 1994, Floriano, Christodoulides et al. 2009) included in the present systematic review have been published in the last 10 years, such data demonstrating the novelty of the concept of salivary diagnostics applied to patients with AMI.

All studies took into consideration whole saliva as biological specimen for salivary analysis.

Despite the relatively high number of researches published, the present systematic review has highlighted a lack of “good” quality studies on the association between the occurrence of disease and the presence of related biomarkers within the saliva. Most of the studies do not report adequate sample size justifications, which is crucial for case-controls researches trying to establish statistical correlations between diseases and markers. Other risk of bias identified in all studies are the absence

of both concurrent controls and blinding of case/controls status to saliva evaluators. It seems worthy to highlight that it is however somewhat difficult to select and include concurrent controls within emergency settings, this risk of bias being therefore difficult to be overcome.

As expected, multiple studies (Mirzaii-Dizgah and Jafari-Sabet 2011, Mirzaii-Dizgah, Hejazi et al. 2012, Mirzaii-Dizgah and Riahi 2013, Mirzaii-Dizgah and Riahi 2013, Mishra, Patil et al. 2018) investigated those salivary molecules (troponins and creatine kinases) which are widely used as serological markers for acute myocardial infarction.

Unlike troponin C, cardiac isoforms I and T are genetically different in cardiomyocytes than in skeletal muscle cells (Thygesen, Mair et al. 2012). When infarction and myocardial necrosis occurs, such proteins are typically detectable in the serum; they normally rise within 4 to 12 hours from the acute event, reaching the peak 12 to 48 hours from symptoms onset. The significant association between troponins in serum and saliva has been confirmed by three different studies (Mirzaii-Dizgah and Riahi 2013, Mirzaii-Dizgah and Riahi 2013, Mishra, Patil et al. 2018) altogether with an apparently high diagnostic accuracy of I isoform. Based on such findings, salivary isoforms of troponins may be regarded as the ideal target for the development of a salivary test for AMI diagnosis.

Butyrylcholinesterase and acetylcholinesterase, commonly referred to as “serum cholinesterase” are two very similar nonspecific cholinesterase able to hydrolyze different choline-based esters (La Du and Lockridge 1986). Evaluation of butyrylcholinesterase in plasma are used as liver function test (Pohanka 2013). Surprisingly, salivary acetylcholinesterase seems able to discriminate between patients with disease and healthy controls, even though the correlation between serum and saliva of both butyrylcholinesterase and acetylcholinesterase in not significant at any time points evaluation, after AMI (Omidpanah, Jalilian et al. 2018).

Adropin is a hormonal peptide that plays a protective role in the pathogenesis and development of cardiovascular diseases. Its role as salivary marker of enzyme-positive heart disease was evaluated by Aydin et al. (Aydin, Eren et al. 2017). Even though the results of the study show a significant association between serum and saliva adropin level shortly after an acute cardiac event, the very low specificity (57%) makes it not acceptable to qualify as reliable biomarker for AMI.

Irisin is an exercised derived hormone, typically produced in skeletal muscles and known to be synthetized in cardiac tissue (Peng, Ding et al. 2021). The only study analyzing salivary irisin does not report data on its diagnostic accuracy. Moreover, irisin level both in blood and saliva increases after 72 hours, such a length of time being long for a prompt diagnosis of a life-threatening disease (Aydin, Aydin et al. 2014).

Similarly, to troponins, salivary C-reactive protein was investigated in 3 studies (Floriano, Christodoulides et al. 2009, Miller, Foley et al. 2014, Ebersole, Kryscio et al. 2017). Being C-reactive protein a blood base non-specific inflammation marker, its serological level can increase in several inflammation-related disorders such as diabetes, hypertension and cardiovascular disease (e.g., coronary heart disease risk) (Mendall, Patel et al. 1996). All the three studies demonstrated the capacity of C-reactive protein to discriminate patients with AMI from healthy controls. It is worthy to highlight that a significant increase in sensitivity of such protein can be obtained further biomarker evaluations as myoglobin and myeloperoxidase.

Adiponectin is a protein hormone involved in the regulation of glucose and fatty acid breakdown. According to recent studies, adiponectin can affect the protection from endothelial disfunction, particularly in atherosclerosis plaques formation (Fang and Judd 2018). The studies included in the present review highlight a somewhat controversial role of salivary adiponectin regarding diagnosis of AMI (Miller, Foley et al. 2014, Ebersole, Kryscio et al. 2017). In fact, the same group of authors reported that adiponectin is higher in patients with AMI than in healthy controls, even though in one study (Ebersole, Kryscio et al. 2017) the values are not statistically significant and in the other (Miller, Foley et al. 2014) there is a strong statistical significance. Moreover, when compared to other possible salivary biomarkers, adiponectin alone is not very accurate for diagnosis.

Similarly to what already well-established for its serum counterpart and for troponins, salivary CPK demonstrated an apparently strong potential in differentiating patients with disease from healthy controls (Mirzaii-Dizgah and Jafari-Sabet 2011).

There are several limitations in the present systematic review that should be considered. First, it was not possible to perform a meta-analysis of the reported results, as they are extremely heterogeneous, particularly about the salivary molecules investigated as well as to the analytical approaches used.

Other factors which precluded the statistical synthesis of the results lay on the differences in study designs, purposes, subjects, outcome measures and follow-up. Secondly, all the studies involved limited series of patients, with complete lack of randomized procedures, thus decreasing the overall quality and evidence of the findings reported.

Even though there is a current lack of good quality research on salivary biomarkers for acute myocardial infarction, most of the studies included in the present review support a diagnostic role of several specific molecules. If confirmed from further studies, identification of troponins, C-reactive protein and creatine phosphokinases may serve as salivary tests able to promptly discriminate patients with acute myocardial infarction.

The expected low-cost and low invasiveness of a point-of-care salivary test, altogether with a presumptive reduced analytical time, could potentially revolutionize the diagnostic process of acute myocardial infarction.

References

1. Aydin, S., Aydin, S., Kobat, M. A., Kalayci, M., Eren, M. N., Yilmaz, M., Kuloglu, T., Gul, E., Secen, O., Alatas, O. D. and Baydas, A. (2014). "Decreased saliva/serum irisin concentrations in the acute myocardial infarction promising for being a new candidate biomarker for diagnosis of this pathology." Peptides **56**: 141-145.
2. Aydin, S., Eren, M. N., Yilmaz, M., Kalayci, M., Yardim, M., Alatas, O. D., Kuloglu, T., Balaban, H., Cakmak, T., Kobalt, M. A., Celik, A. and Aydin, S. (2017). "Adropin as a potential marker of enzyme-positive acute coronary syndrome." Cardiovasc J Afr **28**(1): 40-47.
3. Burke, F. J., Busby, M., McHugh, S., Delargy, S., Mullins, A. and Matthews, R. (2003). "Evaluation of an oral health scoring system by dentists in general dental practice." Br Dent J **194**(4): 215-218; discussion 205.
4. Derruau, S., Robinet, J., Untereiner, V., Piot, O., Sockalingum, G. D. and Lorimier, S. (2020). "Vibrational Spectroscopy Saliva Profiling as Biometric Tool for Disease Diagnostics: A Systematic Literature." Molecules **25**(18).
5. Ebersole, J. L., Kryscio, R. J., Campbell, C., Kinane, D. F., McDevitt, J., Christodoulides, N., Floriano, P. N. and Miller, C. S. (2017). "Salivary and serum adiponectin and C-reactive protein levels in acute myocardial infarction related to body mass index and oral health." J Periodontal Res **52**(3): 419-427.
6. Fang, H. and Judd, R. L. (2018). "Adiponectin Regulation and Function." Compr Physiol **8**(3): 1031-1063.
7. Fares, S., Said, M. S. M., Ibrahim, W., Amin, T. T. and Saad, N. E. S. (2019). "Accuracy of salivary glucose assessment in diagnosis of diabetes and prediabestes." Diabetes Metab Syndr **13**(2): 1543-1547.
8. Floriano, P. N., Christodoulides, N., Miller, C. S., Ebersole, J. L., Spertus, J., Rose, B. G., Kinane, D. F., Novak, M. J., Steinhubl, S., Acosta, S., Mohanty, S., Dharshan, P., Yeh, C. K., Redding, S., Furmaga, W. and McDevitt, J. T. (2009). "Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: a feasibility study." Clin Chem **55**(8): 1530-1538.
9. Hamm, C. W., Bassand, J. P., Agewall, S., Bax, J., Boersma, E., Bueno, H., Caso, P., Dudek, D., Gielen, S., Huber, K., Ohman, M., Petrie, M. C., Sonntag, F., Uva, M. S., Storey, R. F., Wijns, W., Zahger, D. and Guidelines, E. S. C. C. f. P. (2011). "ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC)." Eur Heart J **32**(23): 2999-3054.
10. Ibanez, B., James, S., Agewall, S., Antunes, M. J., Bucciarelli-Ducci, C., Bueno, H., Caforio, A. L. P., Crea, F., Goudevenos, J. A., Halvorsen, S., Hindricks, G., Kastrati, A., Lenzen, M. J., Prescott, E., Roffi, M., Valgimigli, M., Varenhorst, C., Vranckx, P., Widimsky, P. and Group, E. S. C. S. D. (2018). "2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC)." Eur Heart J **39**(2): 119-177.
11. Jones, K. P., Reynolds, S. P., Gray, M., Hughes, K. T., Rolf, S. and Davies, B. H. (1994). "Salivary PAF in acute myocardial infarction and angina: changes during hospital treatment and relationship to cardiac enzymes." Thromb Res **75**(5): 503-511.
12. La Du, B. N. and Lockridge, O. (1986). "Molecular biology of human serum cholinesterase." Fed Proc **45**(13): 2965-2969.
13. Meleti, M., Cassi, D., Vescovi, P., Setti, G., Pertinhez, T. A. and Pezzi, M. E. (2020). "Salivary biomarkers for diagnosis of systemic diseases and malignant tumors. A systematic review." Med Oral Patol Oral Cir Bucal **25**(2): e299-e310.
14. Meleti, M., Quartieri, E., Antonelli, R., Pezzi, M. E., Ghezzi, B., Viani, M. V., Setti, G., Casali, E., Ferrari, E., Ciociola, T., Spisni, A. and Pertinhez, T. A. (2020). "Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva." Metabolites **10**(8).

15. Mendall, M. A., Patel, P., Ballam, L., Strachan, D. and Northfield, T. C. (1996). "C reactive protein and its relation to cardiovascular risk factors: a population based cross sectional study." BMJ **312**(7038): 1061-1065.
16. Miller, C. S., Foley, J. D., 3rd, Floriano, P. N., Christodoulides, N., Ebersole, J. L., Campbell, C. L., Bailey, A. L., Rose, B. G., Kinane, D. F., Novak, M. J., McDevitt, J. T., Ding, X. and Kryscio, R. J. (2014). "Utility of salivary biomarkers for demonstrating acute myocardial infarction." J Dent Res **93**(7 Suppl): 72S-79S.
17. Mirzaii-Dizgah, I., Hejazi, S. F., Riahi, E. and Salehi, M. M. (2012). "Saliva-based creatine kinase MB measurement as a potential point-of-care testing for detection of myocardial infarction." Clin Oral Investig **16**(3): 775-779.
18. Mirzaii-Dizgah, I. and Jafari-Sabet, M. (2011). "Unstimulated whole saliva creatine phosphokinase in acute myocardial infarction." Oral Dis **17**(6): 597-600.
19. Mirzaii-Dizgah, I. and Riahi, E. (2011). "Serum and saliva levels of cathepsin L in patients with acute coronary syndrome." J Contemp Dent Pract **12**(2): 114-119.
20. Mirzaii-Dizgah, I. and Riahi, E. (2013). "Salivary high-sensitivity cardiac troponin T levels in patients with acute myocardial infarction." Oral Dis **19**(2): 180-184.
21. Mirzaii-Dizgah, I. and Riahi, E. (2013). "Salivary troponin I as an indicator of myocardial infarction." Indian J Med Res **138**(6): 861-865.
22. Mishra, V., Patil, R. N., Khanna, V., Tripathi, A., Singh, V., Pandey, S. and Chaurasia, A. (2018). "Evaluation of salivary cardiac troponin-1 as potential marker for detection of acute myocardial infarction." Journal of Clinical and Diagnostic Research **12**(7): 4.
23. Navazesh, M. (1993). "Methods for collecting saliva." Ann N Y Acad Sci **694**: 72-77.
24. Omidpanah, N., Jalilian, N., Vaisi-Raygani, A., Sadeghi, M. and Mozaffari, H. R. (2018). "Evaluation of butyrylcholinesterase and acetylcholinesterase activity in serum and saliva of myocardial infarction patients." Biomedical Research and Therapy **5**(10): 2762-2767.
25. Pappa, E., Vougas, K., Zoidakis, J. and Vastardis, H. (2020). "Proteomic advances in salivary diagnostics." Biochim Biophys Acta Proteins Proteom **1868**(11): 140494.
26. Peng, Q., Ding, R., Wang, X., Yang, P., Jiang, F. and Chen, X. (2021). "Effect of Irisin on Pressure Overload-Induced Cardiac Remodeling." Arch Med Res **52**(2): 182-190.
27. Pohanka, M. (2013). "Butyrylcholinesterase as a biochemical marker." Bratisl Lek Listy **114**(12): 726-734.
28. Rubio, M. C., Gonzalez, P. M., Ramos, C., Lewin, P. G., Friedman, S. M., Puntarulo, S. and Nicolosi, L. N. (2013). "Oxidative stress assessed in saliva from patients with acute myocardial infarction. A preliminary study." Acta Odontol Latinoam **26**(2): 116-120.
29. Setti, G., Pezzi, M. E., Viani, M. V., Pertinhez, T. A., Cassi, D., Magnoni, C., Bellini, P., Musolino, A., Vescovi, P. and Meleti, M. (2020). "Salivary MicroRNA for Diagnosis of Cancer and Systemic Diseases: A Systematic Review." Int J Mol Sci **21**(3).
30. Shah, S. (2018). "Salivaomics: The current scenario." J Oral Maxillofac Pathol **22**(3): 375-381.
31. Shen, Y. S., Chen, W. L., Chang, H. Y., Kuo, H. Y., Chang, Y. C. and Chu, H. (2012). "Diagnostic performance of initial salivary alpha-amylase activity for acute myocardial infarction in patients with acute chest pain." J Emerg Med **43**(4): 553-560.
32. Sturque, J., Berquet, A., Loison-Robert, L. S., Ahossi, V. and Zwetyenga, N. (2019). "Interest of studying the saliva metabolome, transcriptome and microbiome in screening for pancreatic cancer." J Stomatol Oral Maxillofac Surg **120**(6): 554-558.
33. Thygesen, K., Alpert, J. S., Jaffe, A. S., Chaitman, B. R., Bax, J. J., Morrow, D. A., White, H. D. and Executive Group on behalf of the Joint European Society of Cardiology /American College of Cardiology /American Heart Association /World Heart Federation Task Force for the Universal Definition of Myocardial, I. (2018). "Fourth Universal Definition of Myocardial Infarction (2018)." Circulation **138**(20): e618-e651.
34. Thygesen, K., Mair, J., Giannitsis, E., Mueller, C., Lindahl, B., Blankenberg, S., Huber, K., Plebani, M., Biasucci, L. M., Tubaro, M., Collinson, P., Venge, P., Hasin, Y., Galvani, M., Koenig, W., Hamm, C., Alpert, J. S., Katus, H., Jaffe, A. S. and Study Group on Biomarkers in Cardiology of, E. S. C. W. G. o. A. C. C. (2012). "How to use high-sensitivity cardiac troponins in acute cardiac care." Eur Heart J **33**(18): 2252-2257.

35. Timmis, A., Townsend, N., Gale, C., Grobbee, R., Maniadakis, N., Flather, M., Wilkins, E., Wright, L., Vos, R., Bax, J., Blum, M., Pinto, F., Vardas, P. and Group, E. S. C. S. D. (2018). "European Society of Cardiology: Cardiovascular Disease Statistics 2017." Eur Heart J **39**(7): 508-579.
36. Toker, A., Aribas, A., Yerlikaya, F. H., Tasyurek, E. and Akbuga, K. (2013). "Serum and saliva levels of ischemia-modified albumin in patients with acute myocardial infarction." J Clin Lab Anal **27**(2): 99-104.
37. Wang, X., Kaczor-Urbanowicz, K. E. and Wong, D. T. (2017). "Salivary biomarkers in cancer detection." Med Oncol **34**(1): 7.
38. Gulati, Y., Narain, V.S., Saxena, A.K., Nigam, P.K., Ali, B. (2012). "Salivary and Serum Antioxidant Activity in Patients with Acute Coronary Syndrome." IIUM Medical Journal Malaysia **11**(2).

CHAPTER 7

Salivary microRNA for diagnosis of cancer and systemic diseases

Published on International Journal of Molecular Science, 2020, 21, 907.

7.1 Introduction

MicroRNAs (miRNAs or miRs) are short non-coding RNA molecules (about 19-23 nucleotides long) that are involved in regulation of gene expression at transcriptional and post-translational level. miRNAs constitute one of the most abundant classes of gene-regulatory molecules. They are consistently part of the transcriptome and their biological influence is widespread; over 30% of the genome is predicted to be actively regulated by miRNAs and it has been shown that they are involved in the regulation of gene expression and in a variety of cell processes such as apoptosis, proliferation, and differentiation (Lu, Zhang et al. 2008).

Different mechanisms have been established as responsible of the miRNAs deregulation in systemic disease and cancer. Genetic alterations such as chromosomal rearrangements, genomic amplifications, deletions or point mutations presumptively play an important role in disease initiation and progression through the aberrant expression of miRNAs located in the affected regions and subsequently, by deregulation of their downstream mRNAs targets. Gene regulation and gene expression are obtained through miRNA binding to the 3'-UTR (untranslated region) of mRNAs and consequent degradation or translational repression of targeted gene transcripts (Romano, Veneziano et al. 2017).

Aberrant expression of miRNAs has been associated with a growing number of disease states, including cancer, inflammatory and autoimmune diseases (Lu, Zhang et al. 2008).

In recent years, interest has raised on miRNAs, as potential biomarkers for diagnosis, prognosis, and evaluation of treatment efficacy of several diseases. Profiling of miRNAs expression is likely not only to allow identification of a neoplastic tissue and its histological origin, but also to discriminate between different subtypes of malignant lesions. With regard to inflammation, understanding the role of miRNAs in its regulation might be important for helping the comprehension of pathogenesis of a broad group of diseases (Iorio and Croce 2012).

The possibility of evaluating miRNAs in "liquid biopsy" has made rapid progress since 2008, when circulating miRNAs were detected for the first time in blood of patients with diffuse large B-cell lymphoma and prostate cancer (Lawrie, Gal et al. 2008, Mitchell, Parkin et al. 2008).

Circulating miRNAs have subsequently been found in several other body fluids including urine, saliva, and cerebro-spinal fluid. The detection of these small molecules in a surprisingly stable form is due to their association with subcellular vescicle-free particles (usually protein complexes) or to the packaging into micro-vesicles or exosomes (Sanz-Rubio, Martin-Burriel et al. 2018).

Saliva has a very complex composition including enzymes, antibodies, hormones, antimicrobial elements and cytokines. For liquid biopsy, saliva presents many advantages over blood, its collection being easy, safe, non-invasive and cost-effective. Salivary diagnostics have disclosed the existence of several molecular indicators of local and systemic disorders, including cancer (Bahn, Zhang et al. 2015, Zhang, Cheng et al. 2016).

Interest on salivary miRNAs as novel non-invasive tools for cancer and systemic diseases diagnosis has rapidly increased.

The present systematic review has been designed in order to answer the question “Are salivary microRNAs reliable biomarkers for diagnosis of systemic diseases and cancer?”, formulated according to the “Population or problem”, “Intervention or exposure”, “Comparison”, “Outcome” (PICO) worksheet (Schardt, Adams et al. 2007).

7.2 Materials and Methods

The Preferred Reporting Item for Systematic Reviews and Meta-analysis (PRISMA) statement was used to guide the present work (Moher, Shamseer et al. 2015).

A multiple database research (Medline, Web of Science, Scopus) was set, using as entry terms: saliva and miRNA; saliva and microRNA; salivary miRNA; salivary miR; saliva and cancer; saliva and carcinoma; saliva and malignancies. A periodic screening of the databases was performed, between June 2018 and May 2019.

Only literature in English, published after 2000 was taken into consideration. Duplicates resulting from the initial databases querying were discarded through End Note X9©, Clarivate Analytics, software aid. First level screening was performed on titles, abstracts and keywords by two independent investigators. In case of controversial titles/abstract, full texts were evaluated. At the title/abstract screening level, case reports, conference proceedings, personal communications, letters to editor and reviews, were excluded. Studies specifically reporting on biochemical methods, technological aspects, devices used or proposed for saliva evaluation or detection of specific molecules were excluded. Studies focused on the use of salivary miRNAs in patients with pharyngeal, esophageal or oral cavity diseases, oral manifestations of systemic diseases, microbial infections and drug/hormone dosages were excluded.

Studies considering miRNAs expression in relationship to injuries/trauma and physical activity were not included. Moreover, papers investigating the association between salivary miRNAs and aging, psychiatric diseases, dementia and cognitive evaluation were excluded. Studies on *in vitro* or animal model were also excluded. The use of salivary miRNAs for forensic purposes was not considered.

Inclusion and exclusion criteria are summarized in Table 1.

Literature eligible on the selected inclusion/exclusion criteria and reviews were double-checked, including reference lists to identify papers possibly not considered in the previous selection. Considering study period and recruitment centers, a further assessment was performed to identify possible overlapping series of patients. Wherever multiple studies reported the same set of data in fully detectable series, only the most recent or the most complete series was included in the review.

7.2.1 Data extraction, quality assessment and critical appraisal

Information extracted from each study were summarized into an Excel table (Office Suite, Microsoft Corp.) which considered: title, authors, year of publication, disease investigated, number of involved patients, biomarkers (miRNAs), type of analytic device, type of saliva, statistical significance.

Questionnaires issued by the National Heart, Lung, and Blood Institute (NHLBI) within the National Institute of Health (NIH) and called “Study Quality Assessment Tool”, were used (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools). Such tools are specifically developed for each type of study (controlled intervention studies, systematic reviews with meta-analysis, observational cohort and cross-sectional studies, case-control studies, before-after studies without control group, case series studies) and they were answered by two independent investigators. Additionally, the number of patients enrolled in each study was taken into consideration.

Critical appraisal of studies has been summarized through assignation of a score ranging from 0% to 100%, based on the percentage of “yes” choices on the overall number of answers given. Studies having 80-100% score were labeled as “good”; those ranging from 50 to 70% were “intermediate” and studies scoring less than 50% were defined as “poor”.

Level of evidence was assessed using the classification of the Oxford Center for Evidence-Based Medicine (CEMB) levels for diagnosis (https://www.cebm.net/index.aspx?o=5653, 2011).

Disagreements were resolved by discussion between the reviewers.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
English literature Studies published between 2000 and 2019 Studies considering human saliva samples for miRNAs identification Studies using miRNAs as diagnostic biomarkers	Publication type: case reports, conference proceedings, personal communications, letters to editor, reviews. Studies considering salivary miRNAs for diagnostic tools engineering, testing and validation Studies on the diagnostic use of salivary miRNAs in patients with: • pharyngeal, esophageal and oral cavity diseases • oral manifestations of systemic disease • oral microbial infections • drug/hormone dosages • aging • psychiatric and cognitive diseases

7.3 Results

Search flow is summarized in Figure 1. Multiple databases research for titles, abstracts and keywords returned 25868 records; after exclusion of repetitions, 7181 records were screened for titles and abstracts (see inclusion and exclusion criteria, specified in Table 1).

Thirteen records were eligible for full-text reading. Three records were discarded at this level (Figure 1). Cross-references check returned one more record which was included in the full-text evaluation phase. Eleven papers eventually fulfilled the inclusion and exclusion criteria. Table 2 shows the selected papers and their general characteristics such as title, authors, year of publication, disease investigated, selected biomarkers, analytical techniques used, and type of saliva collected (Gao, Chen et al. 2014, Humeau, Vignolle-Vidoni et al. 2015, Schaefer, Attumi et al. 2015, Xie, Yin et al. 2015, Alemar, Izetti et al. 2016, Li, Wang et al. 2016, Machida, Tomofuji et al. 2016, Masotti, Baldassarre et al. 2017, Sazanov, Kiselyova et al. 2017, Chen, Wu et al. 2018, Li, Yoshizawa et al. 2018).

7.3.1 Quality assessment

Critical appraisal of the selected papers is reported in Figure 2. Disagreement between reviewers occurred in 4 cases (47%) and all were resolved by discussion between the two independent assessors as follows:

a) paper 1 (Schaefer, Attumi et al. 2015): divergences involved NIH question number 5 and after discussion the study was scored as “intermediate”; b) paper 5 (Humeau, Vignolle-Vidoni et al. 2015) was scored as “intermediate” by one reviewer and “poor” by the other. Divergences about the use of a statistical analysis were discussed and the paper was scored as “intermediate”; c) paper 6 (Xie, Yin et al. 2015) was scored as “good” by one reviewer and “intermediate” by the other. After discussion the article was scored as “good”; d) paper 7 (Masotti, Baldassarre et al. 2017) was scored as “poor” and “intermediate” by one and the other assessor, respectively. After discussion, both reviewers chose to score the study as “poor”.

The most frequently encountered risk of bias (ROBs) were the absence of data regarding the status of participants (e.g., disease/healthy) to saliva investigators (blind procedure) (10 studies, 91%), the

lack of sample size justification (9 studies, 82%), the absence of concurrent controls (8 studies, 73%), the lack of randomization (8 studies, 73%). Less frequent ROBs were the absence of comparable population for recruitment and correspondence of timeframe of recruitment (6 studies, 54,5%), non-appropriate study question and objectives in addition to non-well-defined study population and to the choice of non-valid and unreliable inclusion and exclusion criteria (1 study). Two “good” quality researches were identified among the group of the 11 selected studies (18,2%). Eight records (73%) were classified as “intermediate” and one (9%) was classified as having “poor” quality.

7.3.2 Level of evidence

Application of the Oxford CEMB guidelines highlighted that all the selected papers have a low level of evidence (4 on 5, the fifth level being the lowest), because of their case-control or case series design.

7.3.3 Disease investigated

Two studies (1 and 2) were focused on colorectal cancer (CRC) and inflammatory bowel diseases (IBD). Crohn’s disease (CD) was compared with ulcerative colitis (UC), to assess the expression of salivary miRNA target. Distal adenocarcinoma, an anatomical variant of CRC, was studied in paper 2 to compare miRNAs in serum and saliva of cancer patients with those of healthy controls (HC) (Schaefer, Attumi et al. 2015, Sazanov, Kiselyova et al. 2017).

Four studies investigated pancreatic oncological diseases. Specifically, paper 3 investigated the presence of two specific miRNAs in salivary exosomes, through a comparison between pancreatic-biliary tract cancer patients and HC (Machida, Tomofuji et al. 2016).

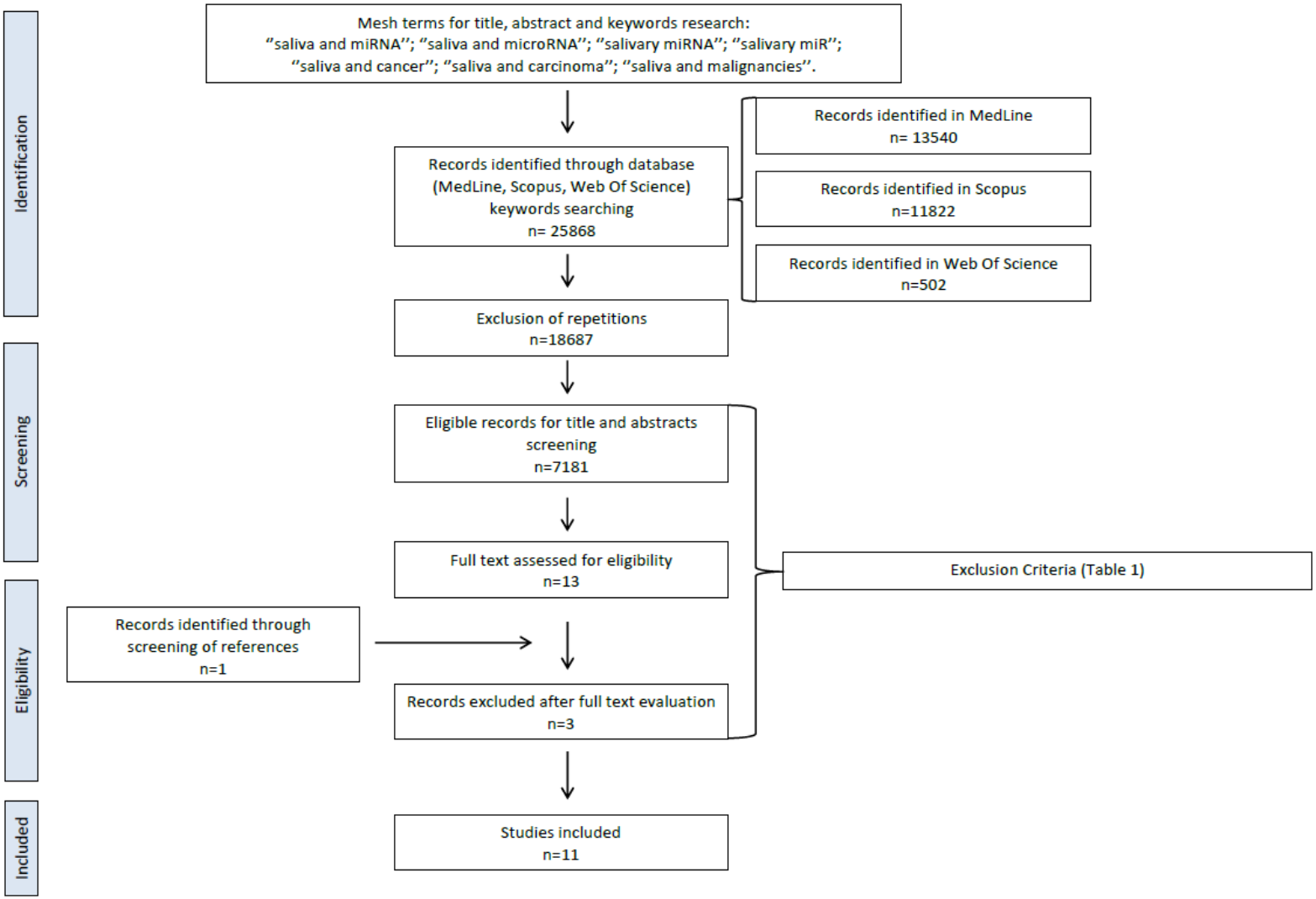

Figure 1. Flow-chart diagram for the selection of 11 papers included in the review.

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

Table 2. General characteristics of the selected papers.

Study Number	Title	Authors	Year of publication	Disease investigated	Number of involved patients	Biomarkers (miRNAs)	Type of analytic device	Type of saliva
1	MicroRNA signatures differentiate Crohn's disease from ulcerative colitis	J.S. Schaefer *et al.*	2015	CD vs. UC	35 HC 42 CD 41 UC 5 saliva samples/group	miR-101,	RT-qPCR + microarray	whole
2	Plasma and saliva miR-21 expression in colorectal cancer patients	A.A Sazanov *et al.*	2016	CRC	31 peripheral blood and 34 saliva samples (CRC stages II–IV) + 34 HC	miR-21	RT-qPCR	whole
3	miR-1246 and miR-4644 in salivary exosome as potential biomarkers for pancreatobiliary tract cancer	T Machida *et al.*	2016	PC	12 PC and 13 HC	miR-1246 and miR-4644	RT-qPCR	whole

4	miRNA-21 and miRNA-34a Are Potential Minimally Invasive Biomarkers for the Diagnosis of Pancreatic Ductal Adenocarcinoma	B Alemar *et al.*	2016	PC	serum and saliva 24PC + HC saliva 10 PC and 10 saliva HC	saliva: miR-21, -34a, -155, -200b, and -376a	RT-qPCR	whole
5	Salivary MicroRNA in Pancreatic Cancer Patients	M Humeau *et al.*	2015	PC	saliva from PC (7) pancreatitis (4) IPMN (2) HC (4)	miR-21, miR-23a, miR-23b and miR-29c	RT-qPCR	whole
6	Salivary microRNAs Show Potential as a Noninvasive Biomarker for Detecting Resectable Pancreatic Cancer	Z Xie *et al.*	2014	PC	40 PC 20 BPT 40 HC	miR-940, miR-3679-5p	qPCR	whole

7	Circulating microRNA Profiles as Liquid Biopsies for the Characterization and Diagnosis of Fibromyalgia Syndrome	A Masotti *et al.*	2016	FM	Blood and saliva, 14 FM, 14 HC.	miR-23a-3p, miR-346, and miR-320b	qPCR	whole
8	Discovery and Validation of Salivary Extracellular RNA Biomarkers for Noninvasive Detection of Gastric Cancer	F Li et al.	2018	GC	Saliva, 63 GC, 31 HC.	miR140-5p, miR374a, miR454 4.61, miR15b, miR28–5p, miR301a	RT-qPCR	whole
9	MicroRNA Expression in Salivary Supernatant of Patients with Pancreatic Cancer and Its Relationship with ZHENG	S Gao *et al*.	2014	PC and ZHENG	30 PC 32 HC	5 miRNA candidates miR-17, miR-21, miR-181a, miR-181b, and miR-196a	RT-qPCR	whole

10	Expression of miR-204 and MMP-9 in Helicobacter pylori-associated gastric ulcer	X Li *et al*.	2016	Helicobacter pylori-associated GU	Ulcer, blood and saliva samples of 46 patients with H. pylori-associated gastritis. Normal stomach mucosa, blood and saliva in 29 HC	miRNA-204	qPCR + microarray + ELISA	whole
11	miRNA Expression Profile of Saliva in Subjects of Yang Deficiency Constitution and Yin Deficiency Constitution	Y Chen *et al*.	2018	Constitution deficit and miRNA	Saliva from 5 balanced individuals, 5 Yin deficiencies, 5 Yang deficiencies	81 miRNA Yang deficiency 98 miRNA Yin deficiency	microarray	whole

Legend:

miRNA: micro-RNA; CD: Crohn's disease; UC: ulcerative colitis; HC: healthy controls; RT qPCR: reverse transcription quantitative polymerase chain reaction; CRC: colon-rectal cancer; PC: pancreatic cancer (including pancreatic-biliary tract cancer, pancreatic ductal adenocarcinoma); BPT: benign pancreatic tumor; IPMN: intraductal papillary mucinous neoplasm; FM: fibromyalgia; GU: gastric ulcer.

Serum and saliva from pancreatic ductal adenocarcinoma (PDAC) patients were compared to HC specimens in study 4: four specific salivary miRNAs targets were investigated (Alemar, Izetti et al. 2016).

Study 5 was focused on pancreatic cancer (PC); salivary miRNAs expression was compared between patients with PC, pancreatitis, inflammatory conditions and HC (Humeau, Vignolle-Vidoni et al. 2015).

The case-control study 6 investigated circulating miRNAs for detecting resectable PC, comparing salivary specimens of PC patients and HC (Xie, Yin et al. 2015).

Relationship between PC and ZHENG (a concept of Traditional Chinese Medicine) was studied in paper 9. Expression of salivary miRNAs was investigated in a case-control setting (Gao, Chen et al. 2014).

In article 7, salivary and blood miRNA detectability was explored patients with fibromyalgia (FM) (Masotti, Baldassarre et al. 2017).

Study 8 was conducted to investigate if salivary miRNAs were reliable biomarkers for diagnosis of gastric cancer (GC) (Li, Yoshizawa et al. 2018).

Study 10 investigated the pathogenesis of gastric ulcer in association to *Helicobacter pylori* by identification of selected miRNAs in saliva, serum and gastric tissue of cases and controls (Li, Wang et al. 2016).

Study 11 explored salivary miRNAs expression in subjects of yang or yin deficiency constitution. Salivary analysis from patients with one or the other constitution category and HC allowed the identification of specific miRNAs (Chen, Wu et al. 2018).

7.3.4 Type of saliva and analytical techniques

It goes beyond the aim of the present systematic review to critically discuss the biochemical methods used in the studies included (see Table 2).

Salivary miRNAs identification was conducted onto whole saliva samples in each study.

Qualitative and quantitative miRNA expression was investigated by Reverse Transcription- real time qualitative Polymerase Chain Reaction (RT-qPCR) in 10 studies. One research was performed

through stand-alone miRNA micro-arrays. Two studies associated micro-arrays to qPCR for miRNAs detection.

Table 3. Statistical significance of salivary miRNAs in association with systemic disease and cancer.

Study	Disease investigated and location	Salivary miRNAs	Subjects	Status	Sensitivity	Specificity	Statistical Significance
1	Intestinal bowel disease	miR-101	CD *vs* HC	overexpressed	NR	NR	p<0,05
		miR-21, miR-31, miR-142-3p	UC *vs* HC	overexpressed	NR	NR	p<0,05
		miR-142-5p	UC *vs* HC	underexpressed	NR	NR	p<0,05
2	Colon-rectal cancer	miR-21	CRC stages II/III/IV *vs* HC	overexpressed	97%	91%	$p = 5e^{-12}$
3	Pancreatic cancer	miR-1246	PC *vs* HC	overexpressed	66,70%	100%	p= 0,008
		miR-4644	PC *vs* HC	overexpressed	75%	76,90%	p= 0.026
		miR-1246 + miR-4644	PC *vs* HC	overexpressed	NR	NR	p=0,005
4	Pancreatic cancer	miR-21	PC *vs* HC	overexpressed	NR	NR	NR
		miR-34a, miR-155, miR-200b, miR-376a	PC *vs* HC	NR	NR	NR	NR
5	Pancreatic cancer	miR-21	PC *vs* HC	overexpressed	71%	100%	p= 0,012
		miR-23a	PC *vs* HC and pre-cancerous	overexpressed	85,70%	100%	p= 0,001

		miR-23b	PC *vs* HC and pre-cancerous	overexpressed	85,70%	100%	p= 0,014
		miR-29c	PC *vs* HC	overexpressed	57%	100%	p= 0,03
		miR-210	Pancreatitis *vs* HC	overexpressed	100%	100%	p= 0,000014
		let-7c	Pancreatitis *vs* HC	overexpressed	75%	80%	p= 0,033
		miR-216	PC *vs* Pancreatitis	overexpressed	50%	100%	p= 0,024
6	Pancreatic cancer	miR-940	PC *vs* HC	overexpressed	90%	40%	p < 0.006
			PC *vs* BPT	overexpressed	62,50%	75%	p < 0.004
			PC *vs* HC + BPT	overexpressed	90%	41,70%	p < 0.001
		miR-3679-5p	PC *vs* HC	underexpressed	83%	45%	p < 0.008
			PC *vs* BPT	underexpressed	90%	45%	p < 0.007
			PC *vs* HC + BPT	underexpressed	85%	45%	p < 0.002
7	Fibromyalgia	miR-23a-3p	FM *vs* HC	NR	NR	NR	NR
		miR-346		NR	NR	NR	NR
		miR-320b		NR	NR	NR	NR
8	Gastric Cancer	miR140-5p	GC *vs* HC	underexpressed	75%	83%	p < 0.05
		miR301a		underexpressed			

		miR374a		underexpressed	NR	NR	
		miR454		underexpressed	NR	NR	
		miR15b		underexpressed	NR	NR	
		miR28-5p		underexpressed	NR	NR	
9	Pancreatic Cancer / ZHENG	miR-17	PC *vs* HC	NR	NR	NR	NR
		miR-21		NR	NR	NR	NR
		miR-181		NR	NR	NR	NR
		miR- 196a		NR	NR	NR	NR
10	Helicobacter pylori-associated Gastric Ulcer	miRNR-204	GU *vs* HC	NR	NR	NR	p < 0,01
11	Constitution deficit / yin yang	miR-4443	Yang deficiency constitution	underexpressed	NR	NR	NR
		miR-2681-3p		overexpressed	NR	NR	NR
		miR-4455	Yin deficiency constitution	underexpressed	NR	NR	NR
		miR-1343-3p		overexpressed	NR	NR	NR

Legend:

NR, not reported; miRNA, micro-RNA; CD, Crohn's disease; UC, ulcerative colitis; HC, healthy controls; RT qPCR, reverse transcription quantitative polymerase chain reaction; CRC, colon-rectal cancer; PC, pancreatic cancer (including pancreatic-biliary tract cancer, pancreatic ductal adenocarcinoma); BPT, benign pancreatic tumor; IPMN, intraductal papillary mucinous neoplasm; FM, fibromyalgia; GU, gastric ulcer.

7.4 Discussion

Salivary analysis is transforming the concept of non-invasive diagnosis, providing novel options in the *panorama* of the so-called “liquid biopsy”.

Evidence exists that several salivary biomarkers (including genetic, transcription molecules, proteins, metabolites and lipids) can accurately describe peculiar pathologic and physiologic states (Farah, Haraty et al. 2018). Examples of diseases that can reliably be diagnosed through salivary analysis are pancreatic (miR-3679-5p and miR-940) (Xie, Yin et al. 2015), lung (cytokines IL1RN, IL1B, CXCL10) (Koizumi, Shetty et al. 2018) and breast cancers (phenylalanine, tryptophan) (Feng, Huang et al. 2015) as well as myocardial infarction (C-reactive protein, myoglobin and myeloperoxidase) (Floriano, Christodoulides et al. 2009).

Among salivary biomarkers, miRNAs seem very promising both for early diagnosis and for understanding the pathogenesis of some diseases (e.g., oral cancer, salivary glands cancer, neurological or psychiatric deficiencies) (Rapado-Gonzalez, Majem et al. 2018). Moreover, it has been demonstrated that salivary transcriptome is very abundant, consisting of thousands of mRNAs and miRNAs (Park, Zhou et al. 2009, Michael, Bajracharya et al. 2010).

Despite the demonstration that the presence of an inflammatory or malignant lesion in a site distant from the oral cavity is related to peculiar miRNAs in the salivary fluid, such occurrence is still poorly understood (Han, Jia et al. 2018). It has been speculated that expression of some miRNAs in saliva could be very similar to those in serum.

There are several hypotheses trying to explain the detection of cell-free nucleic acids (including miRNAs) into saliva. It has been reported that blood-derived molecules, entering salivary glands tissue *via* various cellular mechanisms, such as transcellular (passive intracellular diffusion and active transport) or para-cellular routes (extracellular ultrafiltration) affect the molecular composition of oral fluids. miRNAs could also be produced locally by apoptosis or cell necrosis, and they could also be released by normal epithelial or cancerous cells in exosomes or micro vesicles (Streckfus, Mayorga-Wark et al. 2008, Yoshizawa, Schafer et al. 2013). The present systematic review has highlighted a rapidly increasing interest toward the use of salivary miRNAs for diagnosis of systemic diseases and

cancers. Nevertheless, applying strict inclusion and exclusion criteria for selecting only papers reporting sufficient diagnostic and biochemical information, the number of studies is limited to 11.

As shown in Table 3, most researches (7 out of 11) showed statistically significant correlations between one or more miRNAs and the investigated disease. Even though such results would, in general, indicate that specific salivary miRNAs can contribute to diagnosis of diseases, our critical appraisal highlighted that most of the included studies did not satisfy a relevant percentage of items suggested by the NIH format. Particularly, only 2 studies received a score indicating a "good" quality (Xie, Yin et al. 2015, Li, Yoshizawa et al. 2018). Also, the level of evidence of all the examined studies appears quite low, due to their case-control design.

ROBs most frequently accounting for decreased quality of the study are the absence of information on the status of participants (e.g., disease/healthy) to saliva biochemical analysts (blind procedure) (10 studies: 91%) and the lack of sample size justification (9 studies, 82%).

It is worthy to mention here that one of the studies considered as being of good quality, did not report a sample size justification (study 6). The other (study 8) has been conducted following the principles of the prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE) guidelines, and therefore, even if not clearly reported, it is likely that the Authors calculated a sample size (Pepe, Feng et al. 2008).

Blinding the status of participants (e.g., use of anonymous labels) to specimen evaluators is of paramount importance in research attempting to identify biomarkers. Absence of a blinding procedure increases the probability of subjective interpretation of results, thus leading to misinterpretation of biomarker importance.

The use of untargeted procedures for establishing a relationship between salivary miRNAs and a specific pathology usually leads to the identification of many molecules. Among these, only few, if any, will be validated as biomarkers. The process leading to the identification of one reliable miRNA among the whole group of detected molecules requires a statistically adequate number of cases. Nevertheless, only a few will be able to reliably represent a pathological state, based on their statistical significance.

The sample size justification is connected to the sensitivity and specificity of the potential biomarker. Depending on the number of considered variables, a statistically driven population size selection must be performed during the design stage; the unbalancing of cases *vs* healthy controls, could give rise to confounding results during selection, verification and validation stages.

7.4.1 Pancreatic cancer

The most investigated disease in the present review was pancreatic cancer (5 studies). Such a tumor is the seventh leading cause of cancer-related deaths worldwide. Because patients rarely exhibit symptoms until an advanced stage of the disease, pancreatic carcinoma remains one of the most undiagnosed and lethal malignant neoplasms. Globally, 458,918 new cases have been reported in 2018. Despite advancements in the detection and management of PC, the 5-year survival rate is only 9% (Rawla, Sunkara et al. 2019).

Overall, 18 salivary miRNAs (miR-1246, miR-4644, miR-21, miR-34a, mir-155, miR-200b, miR-376a, miR-23a, miR-23b, miR-29c, miR-210, miR-216, miR-940, miR-3679-5p, miR-17, miR-18b, miR-18a, miR-196a), detected through targeted and untargeted methods, have been studied in relation to the presence of pancreatic cancer, irrespectively to the stage of development of the tumor (Gao, Chen et al. 2014, Humeau, Vignolle-Vidoni et al. 2015, Xie, Yin et al. 2015, Alemar, Izetti et al. 2016, Machida, Tomofuji et al. 2016).

miR-21 is one of the most investigated miRNA in oncology (e.g. colorectal, thyroid, breast, ovarian and cervix cancers) and it is known to inhibit the expression of phosphatases, thus limiting pathways such as AKT and MAPK (Zhu and Xu 2014, Wu, Li et al. 2015). This molecule appears to be constantly upregulated in PC and to be indicative of poor survival, response to treatment and/or metastatic disease (Humeau, Torrisani et al. 2013).

In the present review, 3 studies have attempted to find a correlation between salivary miR-21 and PC (Gao, Chen et al. 2014, Humeau, Vignolle-Vidoni et al. 2015, Alemar, Izetti et al. 2016), with controversial results. Study 4 demonstrated that miR-21 is overexpressed in patients with PC even though results are not statistically significant and therefore the molecule cannot be used to distinguish patients from healthy controls (Alemar, Izetti et al. 2016). On the other hand, studies 5 and 9 reports

significant results regarding salivary miR-21 sensitivity and specificity for identifying patients with PDAC (Gao, Chen et al. 2014, Humeau, Vignolle-Vidoni et al. 2015). All these 3 studies have been classified has having “intermediate” quality.

miR-1246 and miR-4644 are supposedly involved in tumor initiation, establishment and metastatic process (Zhang, Chin et al. 2016). Particularly, salivary expression of miR-1246 is significantly related to serum levels of the well-known protein CA19-9 (Ballehaninna and Chamberlain 2012, Lin, Huang et al. 2014, Zeng and Tao 2015). It has been speculated that concentration of such miRNA in saliva is directly associated to concentration of the same molecule in PC tissue (Ali, Saleh et al. 2012).

When salivary miR-1246 and miR-4644 are simultaneously taken into consideration, sensitivity and specificity for PC diagnosis are increased with respect to biomarkers analyzed singularly (Alemar, Izetti et al. 2016).

miR-940 regulates the 3’-UTR of the GSK3β and sFRP1 genes expression resulting in Wnt/β-catenin signaling activation. Upregulation of miR-940 suppress GSK3β and sFRP1 and could promote pancreatic carcinoma proliferation and invasion (Yang, Liu et al. 2016). miR-3679-5p seems to activate the cytoskeleton regulator RNA (CYTOR), which plays a pivotal role in development and progression of a variety of cancers (Li, Wang et al. 2019).

According to results of study 6, combined analysis of salivary miR-940 and miR-3679-5p has better discriminatory power than CA19-19 in detecting resectable PC with good sensibility (90% and 83% for miR-940 and miR-3679-5p, respectively) and acceptable sensitivity (40% and 45%, respectively) (Xie, Yin et al. 2015). The positive predictive value of the model combining the two miRNAs was 78.9%, and the negative predictive value was 76.2%. Furthermore, Study 6 was labeled as “good” quality study after critical appraisal. It seems therefore advisable to highlight here the potential important role of salivary miR-940 and miR-3679-5p as reliable markers for PC.

Salivary miR-23a-3p and miR-23b-3p were overexpressed and capable to distinguish PC patients from HC and pancreatitis (study 5, (Humeau, Vignolle-Vidoni et al. 2015)) with appreciable sensitivity and specificity; considering the regulatory role in pancreatic inflammation processes, Grieco *et al*. (Grieco, Sebastiani et al. 2017) described how these miRNAs were found to regulate the expression

of the pro-apoptotic Bcl-2 proteins DP5 and PUMA and consequent human β-cell apoptosis. These results identify a novel cross talk between a key family of miRNAs and pro-apoptotic Bcl-2 proteins in human pancreatic β-cells, broadening our understanding of cytokine-induced β-cell apoptosis in early type 1 diabetes.

7.4.2 Gastric cancer and gastric ulcer

According to the GLOBOCAN 2018 data, gastric cancer is the 5th most common neoplasm and the 3rd most deadly cancer, with an estimated 783,000 deaths in 2018 (Bray, Ferlay et al. 2018).

Among studies of the present review dealing with GC, study 8 was labelled as having “good” quality (Li, Yoshizawa et al. 2018). The research reports the analysis of differences in salivary transcriptomics, including several miRNAs, between a cohort of patients with GC and controls. Particularly, 6 salivary miRNAs (miR-140-5p, miR-301a, miR-374a, miR-454, miR-15b, miR-28-5p), were found to be significantly downregulated ($p<0.05$) in patients without GC, yielding AUC values from 0.63 to 0.70, thus being validated as candidate biomarkers. Among these, only 2 (miR140-5p and miR-301a) yielded an AUC of 0.81, satisfying a prediction model for diagnosis of GC.

Conversely, it has been demonstrated that miR-140-5p expression is decreased in specimens of GC, when compared to healthy mucosal tissue. miR-140-5p is able to suppress the proliferation, migration and invasion of GC by directly targeting the 3’ – UTR of YES1 (Fang, Yin et al. 2017). The possible discrepancy between extracellular salivary and tissue miR-140-5p, seems to deserve more investigation.

miR-301a is an oncogenic miRNA playing an important role in activating tumor cell invasion/migration, promoting cell proliferation, inhibiting apoptosis and enhancing chemosensitivity both *in vivo* and *in vitro* (Zheng, Huang et al. 2018). With specific regard to GC, it has been shown that the abnormal expression of miR-301a is associated with cancer progression and poor prognosis (Xu, He et al. 2013).

GC incidence and mortality are highly variable by region and highly dependent on diet and *Helicobacter pylori* infection (Mera, Bravo et al. 2018). The relationship between infection and expression of salivary miRNA in relationship to MMP-9 in patient with *Helicobacter pylori* associated

gastric ulcer was investigated in study 10, ("intermediate" quality) (Li, Wang et al. 2016). Real-time PCR analysis showed that miR-204 was significantly downregulated in ulcer tissue, blood and saliva samples from *Helicobacter pylori*-associated gastritis patients compared with healthy controls. These results suggested that miR-204 may contribute to the regulation of *Helicobacter pylori*-associated gastritis by targeting MMP-9 mRNA.

Many studies have revealed that miR-204 plays a role as tumor suppressor (Li, Pan et al. 2016). Regarding gastric cancer, validated target genes of miR-204 are BCL-2 (Sacconi, Biagioni et al. 2012), EZRIN (Mao, Zhang et al. 2014), rabb22a (Zhang, Yin et al. 2015), sirt1 (Zhang, Wang et al. 2013), sox4 (Zhou, Li et al. 2014) and usp47 (Zhang, Yin et al. 2015).

7.4.3 Colorectal diseases

Two studies in the present review were focused on 3 colorectal diseases (Chron's disease, ulcerative colitis and colorectal cancer) (Schaefer, Attumi et al. 2015, Sazanov, Kiselyova et al. 2017). Both studies were judged as having "intermediate" quality.

UC induces diffuse inflammation of the colonic mucosa. CD results in transmural ulceration of any portion of the gastrointestinal tract most often the terminal ileum and colon. Patients affected by these inflammatory bowel diseases experience symptoms, including diarrhea, abdominal pain, bloody stools, and vomiting (Fakhoury, Negrulj et al. 2014, Maaser, Sturm et al. 2019).

New cases of CD and UC diagnosed each year are approximately 11 and 12 per 100,000 population, respectively; median age at diagnosis for CD is 34.9 years, while it is 29.5 for UC (Shivashankar, Tremaine et al. 2014).

Authors of study 1 identified 5 salivary miRNAs (miR-21, miR-31, miR142-3p, miR-26a and miR-101) in IBD patients. However, all of such biomarkers failed to show statistical significance after applying the Bonferroni correction for multiple testing (Schaefer, Attumi et al. 2015).

miR-21 was more present in saliva of patients with UC than in HC. Apart from the well-known role in tumor promotion and development, miR-21 is also involved in the deregulation of pathways such as "platelet activation signaling and aggregation". Platelets emerge as key players in the inflammatory cascade. The roles of platelets in platelet-mediated inflammation in CD patients and the critical role

of miRNAs in the control of the mechanism of hemostasis may in part suggest that both inflammation and coagulation impairment could contribute to pathogenesis of UC (Palmieri, Creanza et al. 2017).

As to for miR-21, salivary levels of miR-31 were found to be overexpressed in UC patients compared to HC. miR-31 is implicated in several inflammation-associated disorders; its expression signature could differentiate CD, UC, microscopic colitis and pediatric IBD. MiR-31 is a major regulator of Wnt, BMP, and TGF-β signaling, controlling intestinal stem cells proliferation, intestinal homeostasis, and colorectal cancer progression by binding the 3′-UTR of ERK5, RAS1, TGF-β, β-catenin, and several other mRNAs to control cell migration, cell proliferation *in-vitro* and tumorigenesis and metastasis *in-vivo* (Tian, Ma et al. 2017).

Salivary miR-101 was found to be overexpressed in patients with CD. It has been hypothesized that miR-101 could restrain the migratory potential of cells by repressing the enhancer of zeste homolog 2 (EZH2) (Strillacci, Valerii et al. 2013); In fact, in *vitro* and in *vivo* studies showed how this peculiar miRNA is decreased in colon cancer tissues compared with adjacent non-tumor tissues (Yang, Yu et al. 2019). Moreover, the overexpression of miR-101 suppresses cell proliferation and inhibits cell migration and invasion in HT-29 and RKO colon cancer cell lines (Chen, Xia et al. 2017).

Despite of the scarce importance in IBD differentiation, it is well known the oncogenic role of miR-142-3p, which promotes cellular invasion in colorectal cancer cells by activating RAC1 and the onco-suppressive activity of miR-26a, that inhibits cell aggressiveness by regulating FUT4 in CRC (Li, Sun et al. 2017, Gao, Xu et al. 2018).

Study 2 (Sazanov, Kiselyova et al. 2017) demonstrated the overexpression of salivary miR-21 among patients with CRC. The biomarker was tested both for CRC identification and for discrimination of the stage of disease.

Despite the small sample size, analysis of salivary miR-21 returned a 91% specificity and 97% sensitivity ($p= 5e^{-12}$) distinguishing CRC from HC. However, the study considered, was not able to provide a statistically significant distinction between cancer stages.

7.4.4 Others (Fibromyalgia, Gastritis, Traditional Chinese Medicine)

Fibromyalgia is a somewhat controversial condition characterized by chronic widespread pain, unrefreshing sleep, physical exhaustion and cognitive difficulties. It occurs in all populations throughout the world, with overall prevalence between 2% and 4% (Hauser and Fitzcharles 2018).

In article 7 of the present review (Masotti, Baldassarre et al. 2017), salivary and blood miRNA detectability was explored to study FM by the comparison of serum miRNAs in 14 cases and 14 controls (all females, matched for age). Six serum miRNAs (miR-23a-3p, miR-1, miR-133a, miR-346, miR-139-5p, miR-320b) resulted significantly deregulated. Particularly, among patients with FM, miR-1 showed the greatest downregulation, followed by miR-23a-3p. Six statistically dysregulated target biomarkers were selected for the salivary investigation. Interestingly, only 3 miRNAs were detectable both in serum and saliva (miR-23a-3p, miR-346, and miR-320b). However, though being detectable, salivary miRNA expression values were not statistically different if compared to controls (Masotti, Baldassarre et al. 2017).

The biological effects depending on genes targeted by dysregulated miRNAs in FM patients are, to different levels, involved in muscular atrophy, epilepsy and autism (miR23a-3p), myoblast differentiation and modulation of brain-derived neurotrophic factor (BDNF) expression (miR-1); myoblast differentiation (miR-133a), immune response (miR-346), brain development (miR-139-5p), neuron development, autism and complex regional pain syndrome (miR-320p) (Masotti, Baldassarre et al. 2017).

In the context of Traditional Chinese Medicine, study 11 explored salivary miRNAs expression in subjects of Yang or Yin deficiency constitution (Chen, Wu et al. 2018).

Differences in expression were highlighted for miR-4443 and miR-2681-3p in Yang deficiency constitution (main characteristic: cold intolerance) and for miR-4455 and miR-1343-3p in Yin deficiency constitution (main characteristic: heat intolerance). A role in thermoregulation is played by thyroid hormones T3 and T4 (triiodothyronine and thyroxine). In 2017, Yicheng *et al*. described how T CD4+ cells play a significant role in pathogenesis of Graves' disease, a common immuno-mediate condition; lymphocytic infiltration in thyroid gland leads to the production of autoantibody against thyroid stimulating hormone receptor (TSH-r), which mimics the action of TSH, causing excessive thyroid hormone production and hyperthyroidism. miR-4443 can induce overexpression of cytokines,

chemokines and the proliferation of CD4+ T cells *in vitro*. Authors indicated that miR-4443 was elevated in GD patients and was significantly correlated with GD immune-pathogenesis (Qi, Zhou et al. 2017).

Moreover, Shefler *et al*. recently reported that miR-4443 was present in micro-vesicles derived from activated T cells could and that regulates mast cell activation by targeting PTPRJ gene, suggesting a role of miR-4443 as a regulator of inflammation (Shefler, Salamon et al. 2018).

The heterogeneity of diseases investigated as well as of salivary miRNAs did not allow to perform a metanalysis of the studies included in the present review. Even limiting the analysis to single groups of diseases (e.g., pancreatic cancer) researches report the experience on patients at different stages, therefore limiting the possibility of pooling data.

In conclusion, according to our critical appraisal, only 2 studies on salivary miRNAs have been performed following appropriate methodologies (Xie, Yin et al. 2015, Li, Yoshizawa et al. 2018). According to such studies, salivary miR-940 and miR-3679-5p seem to be reliable markers for pancreatic cancer and miR140-5p and miR301a are promising molecules for salivary diagnosis of gastric cancer.

Further studies, possibly avoiding the ROBs highlighted here, are necessary to consolidate these findings and to identify new reliable salivary biomarkers.

References

1. Alemar, B., Izetti, P., Gregorio, C., Macedo, G. S., Castro, M. A., Osvaldt, A. B., Matte, U. and Ashton-Prolla, P. (2016). "miRNA-21 and miRNA-34a Are Potential Minimally Invasive Biomarkers for the Diagnosis of Pancreatic Ductal Adenocarcinoma." Pancreas **45**(1): 84-92.
2. Ali, S., Saleh, H., Sethi, S., Sarkar, F. H. and Philip, P. A. (2012). "MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer." Br J Cancer **107**(8): 1354-1360.
3. Bahn, J. H., Zhang, Q., Li, F., Chan, T. M., Lin, X., Kim, Y., Wong, D. T. and Xiao, X. (2015). "The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva." Clin Chem **61**(1): 221-230.
4. Ballehaninna, U. K. and Chamberlain, R. S. (2012). "The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: An evidence based appraisal." J Gastrointest Oncol **3**(2): 105-119.
5. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A. and Jemal, A. (2018). "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries." CA Cancer J Clin **68**(6): 394-424.
6. Chen, L. G., Xia, Y. J. and Cui, Y. (2017). "Upregulation of miR-101 enhances the cytotoxic effect of anticancer drugs through inhibition of colon cancer cell proliferation." Oncol Rep **38**(1): 100-108.
7. Chen, Y., Wu, Y., Yao, H., Luo, H., Lin, B., Zhang, X., Liang, X., Sun, R., Zhao, S., Li, Y., Zheng, Y., Li, L. and Wang, Q. (2018). "miRNA Expression Profile of Saliva in Subjects of Yang Deficiency Constitution and Yin Deficiency Constitution." Cell Physiol Biochem **49**(5): 2088-2098.
8. Fakhoury, M., Negrulj, R., Mooranian, A. and Al-Salami, H. (2014). "Inflammatory bowel disease: clinical aspects and treatments." J Inflamm Res **7**: 113-120.
9. Fang, Z., Yin, S., Sun, R., Zhang, S., Fu, M., Wu, Y., Zhang, T., Khaliq, J. and Li, Y. (2017). "miR-140-5p suppresses the proliferation, migration and invasion of gastric cancer by regulating YES1." Mol Cancer **16**(1): 139.
10. Farah, R., Haraty, H., Salame, Z., Fares, Y., Ojcius, D. M. and Said Sadier, N. (2018). "Salivary biomarkers for the diagnosis and monitoring of neurological diseases." Biomed J **41**(2): 63-87.
11. Feng, S., Huang, S., Lin, D., Chen, G., Xu, Y., Li, Y., Huang, Z., Pan, J., Chen, R. and Zeng, H. (2015). "Surface-enhanced Raman spectroscopy of saliva proteins for the noninvasive differentiation of benign and malignant breast tumors." Int J Nanomedicine **10**: 537-547.
12. Floriano, P. N., Christodoulides, N., Miller, C. S., Ebersole, J. L., Spertus, J., Rose, B. G., Kinane, D. F., Novak, M. J., Steinhubl, S., Acosta, S., Mohanty, S., Dharshan, P., Yeh, C. K., Redding, S., Furmaga, W. and McDevitt, J. T. (2009). "Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: a feasibility study." Clin Chem **55**(8): 1530-1538.
13. Gao, S., Chen, L. Y., Wang, P., Liu, L. M. and Chen, Z. (2014). "MicroRNA expression in salivary supernatant of patients with pancreatic cancer and its relationship with ZHENG." Biomed Res Int **2014**: 756347.
14. Gao, X., Xu, W., Lu, T., Zhou, J., Ge, X. and Hua, D. (2018). "MicroRNA-142-3p Promotes Cellular Invasion of Colorectal Cancer Cells by Activation of RAC1." Technol Cancer Res Treat **17**: 1533033818790508.
15. Grieco, F. A., Sebastiani, G., Juan-Mateu, J., Villate, O., Marroqui, L., Ladriere, L., Tugay, K., Regazzi, R., Bugliani, M., Marchetti, P., Dotta, F. and Eizirik, D. L. (2017). "MicroRNAs miR-23a-3p, miR-23b-3p, and miR-149-5p Regulate the Expression of Proapoptotic BH3-Only Proteins DP5 and PUMA in Human Pancreatic beta-Cells." Diabetes **66**(1): 100-112.
16. Han, Y., Jia, L., Zheng, Y. and Li, W. (2018). "Salivary Exosomes: Emerging Roles in Systemic Disease." Int J Biol Sci **14**(6): 633-643.
17. Hauser, W. and Fitzcharles, M. A. (2018). "Facts and myths pertaining to fibromyalgia." Dialogues Clin Neurosci **20**(1): 53-62.

18. Humeau, M., Torrisani, J. and Cordelier, P. (2013). "miRNA in clinical practice: pancreatic cancer." Clin Biochem **46**(10-11): 933-936.
19. Humeau, M., Vignolle-Vidoni, A., Sicard, F., Martins, F., Bournet, B., Buscail, L., Torrisani, J. and Cordelier, P. (2015). "Salivary MicroRNA in Pancreatic Cancer Patients." PLoS One **10**(6): e0130996.
20. Iorio, M. V. and Croce, C. M. (2012). "microRNA involvement in human cancer." Carcinogenesis **33**(6): 1126-1133.
21. Koizumi, T., Shetty, V. and Yamaguchi, M. (2018). "Salivary cytokine panel indicative of non-small cell lung cancer." J Int Med Res **46**(9): 3570-3582.
22. Lawrie, C. H., Gal, S., Dunlop, H. M., Pushkaran, B., Liggins, A. P., Pulford, K., Banham, A. H., Pezzella, F., Boultwood, J., Wainscoat, J. S., Hatton, C. S. and Harris, A. L. (2008). "Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma." Br J Haematol **141**(5): 672-675.
23. Li, F., Yoshizawa, J. M., Kim, K. M., Kanjanapangka, J., Grogan, T. R., Wang, X., Elashoff, D. E., Ishikawa, S., Chia, D., Liao, W., Akin, D., Yan, X., Lee, M. S., Choi, R., Kim, S. M., Kang, S. Y., Bae, J. M., Sohn, T. S., Lee, J. H., Choi, M. G., Min, B. H., Lee, J. H., Kim, J. J., Kim, Y., Kim, S. and Wong, D. T. W. (2018). "Discovery and Validation of Salivary Extracellular RNA Biomarkers for Noninvasive Detection of Gastric Cancer." Clin Chem **64**(10): 1513-1521.
24. Li, M., Wang, Q., Xue, F. and Wu, Y. (2019). "lncRNA-CYTOR Works as an Oncogene Through the CYTOR/miR-3679-5p/MACC1 Axis in Colorectal Cancer." DNA Cell Biol **38**(6): 572-582.
25. Li, T., Pan, H. and Li, R. (2016). "The dual regulatory role of miR-204 in cancer." Tumour Biol **37**(9): 11667-11677.
26. Li, X., Wang, L.-p., Li, G., Zheng, X. and Duan, C. (2016). Expression of miR-204 and MMP-9 in Helicobacter pylori-associated gastric ulcer.
27. Li, Y., Sun, Z., Liu, B., Shan, Y., Zhao, L. and Jia, L. (2017). "Tumor-suppressive miR-26a and miR-26b inhibit cell aggressiveness by regulating FUT4 in colorectal cancer." Cell Death Dis **8**(6): e2892.
28. Lin, M. S., Huang, J. X. and Yu, H. (2014). "Elevated serum level of carbohydrate antigen 19-9 in benign biliary stricture diseases can reduce its value as a tumor marker." Int J Clin Exp Med **7**(3): 744-750.
29. Lu, M., Zhang, Q., Deng, M., Miao, J., Guo, Y., Gao, W. and Cui, Q. (2008). "An analysis of human microRNA and disease associations." PLoS One **3**(10): e3420.
30. Maaser, C., Sturm, A., Vavricka, S. R., Kucharzik, T., Fiorino, G., Annese, V., Calabrese, E., Baumgart, D. C., Bettenworth, D., Borralho Nunes, P., Burisch, J., Castiglione, F., Eliakim, R., Ellul, P., Gonzalez-Lama, Y., Gordon, H., Halligan, S., Katsanos, K., Kopylov, U., Kotze, P. G., Krustins, E., Laghi, A., Limdi, J. K., Rieder, F., Rimola, J., Taylor, S. A., Tolan, D., van Rheenen, P., Verstockt, B., Stoker, J., European, C. s., Colitis, O., the European Society of, G. and Abdominal, R. (2019). "ECCO-ESGAR Guideline for Diagnostic Assessment in IBD Part 1: Initial diagnosis, monitoring of known IBD, detection of complications." J Crohns Colitis **13**(2): 144-164.
31. Machida, T., Tomofuji, T., Maruyama, T., Yoneda, T., Ekuni, D., Azuma, T., Miyai, H., Mizuno, H., Kato, H., Tsutsumi, K., Uchida, D., Takaki, A., Okada, H. and Morita, M. (2016). "miR1246 and miR4644 in salivary exosome as potential biomarkers for pancreatobiliary tract cancer." Oncol Rep **36**(4): 2375-2381.
32. Mao, J., Zhang, M., Zhong, M., Zhang, Y. and Lv, K. (2014). "MicroRNA-204, a direct negative regulator of ezrin gene expression, inhibits glioma cell migration and invasion." Mol Cell Biochem **396**(1-2): 117-128.
33. Masotti, A., Baldassarre, A., Guzzo, M. P., Iannuccelli, C., Barbato, C. and Di Franco, M. (2017). "Circulating microRNA Profiles as Liquid Biopsies for the Characterization and Diagnosis of Fibromyalgia Syndrome." Mol Neurobiol **54**(9): 7129-7136.
34. Mera, R. M., Bravo, L. E., Camargo, M. C., Bravo, J. C., Delgado, A. G., Romero-Gallo, J., Yepez, M. C., Realpe, J. L., Schneider, B. G., Morgan, D. R., Peek, R. M., Jr., Correa, P., Wilson, K. T. and Piazuelo, M. B. (2018). "Dynamics of Helicobacter pylori infection as a

determinant of progression of gastric precancerous lesions: 16-year follow-up of an eradication trial." Gut **67**(7): 1239-1246.
35. Michael, A., Bajracharya, S. D., Yuen, P. S., Zhou, H., Star, R. A., Illei, G. G. and Alevizos, I. (2010). "Exosomes from human saliva as a source of microRNA biomarkers." Oral Dis **16**(1): 34-38.
36. Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. and Tewari, M. (2008). "Circulating microRNAs as stable blood-based markers for cancer detection." Proc Natl Acad Sci U S A **105**(30): 10513-10518.
37. Moher, D., Shamseer, L., Clarke, M., Ghersi, D., Liberati, A., Petticrew, M., Shekelle, P., Stewart, L. A. and Group, P.-P. (2015). "Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement." Syst Rev **4**: 1.
38. Palmieri, O., Creanza, T. M., Bossa, F., Latiano, T., Corritore, G., Palumbo, O., Martino, G., Biscaglia, G., Scimeca, D., Carella, M., Ancona, N., Andriulli, A. and Latiano, A. (2017). "Functional Implications of MicroRNAs in Crohn's Disease Revealed by Integrating MicroRNA and Messenger RNA Expression Profiling." Int J Mol Sci **18**(7).
39. Park, N. J., Zhou, H., Elashoff, D., Henson, B. S., Kastratovic, D. A., Abemayor, E. and Wong, D. T. (2009). "Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection." Clin Cancer Res **15**(17): 5473-5477.
40. Pepe, M. S., Feng, Z., Janes, H., Bossuyt, P. M. and Potter, J. D. (2008). "Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design." J Natl Cancer Inst **100**(20): 1432-1438.
41. Qi, Y., Zhou, Y., Chen, X., Ye, L., Zhang, Q., Huang, F., Cui, B., Lin, D., Ning, G., Wang, W. and Wang, S. (2017). "MicroRNA-4443 Causes CD4+ T Cells Dysfunction by Targeting TNFR-Associated Factor 4 in Graves' Disease." Front Immunol **8**: 1440.
42. Rapado-Gonzalez, O., Majem, B., Muinelo-Romay, L., Alvarez-Castro, A., Santamaria, A., Gil-Moreno, A., Lopez-Lopez, R. and Suarez-Cunqueiro, M. M. (2018). "Human salivary microRNAs in Cancer." J Cancer **9**(4): 638-649.
43. Rawla, P., Sunkara, T. and Gaduputi, V. (2019). "Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors." World J Oncol **10**(1): 10-27.
44. Romano, G., Veneziano, D., Acunzo, M. and Croce, C. M. (2017). "Small non-coding RNA and cancer." Carcinogenesis **38**(5): 485-491.
45. Sacconi, A., Biagioni, F., Canu, V., Mori, F., Di Benedetto, A., Lorenzon, L., Ercolani, C., Di Agostino, S., Cambria, A. M., Germoni, S., Grasso, G., Blandino, R., Panebianco, V., Ziparo, V., Federici, O., Muti, P., Strano, S., Carboni, F., Mottolese, M., Diodoro, M., Pescarmona, E., Garofalo, A. and Blandino, G. (2012). "miR-204 targets Bcl-2 expression and enhances responsiveness of gastric cancer." Cell Death Dis **3**: e423.
46. Sanz-Rubio, D., Martin-Burriel, I., Gil, A., Cubero, P., Forner, M., Khalyfa, A. and Marin, J. M. (2018). "Stability of Circulating Exosomal miRNAs in Healthy Subjects." Sci Rep **8**(1): 10306.
47. Sazanov, A. A., Kiselyova, E. V., Zakharenko, A. A., Romanov, M. N. and Zaraysky, M. I. (2017). "Plasma and saliva miR-21 expression in colorectal cancer patients." J Appl Genet **58**(2): 231-237.
48. Schaefer, J. S., Attumi, T., Opekun, A. R., Abraham, B., Hou, J., Shelby, H., Graham, D. Y., Streckfus, C. and Klein, J. R. (2015). "MicroRNA signatures differentiate Crohn's disease from ulcerative colitis." BMC Immunol **16**: 5.
49. Schardt, C., Adams, M. B., Owens, T., Keitz, S. and Fontelo, P. (2007). "Utilization of the PICO framework to improve searching PubMed for clinical questions." BMC Med Inform Decis Mak **7**: 16.
50. Shefler, I., Salamon, P., Levi-Schaffer, F., Mor, A., Hershko, A. Y. and Mekori, Y. A. (2018). "MicroRNA-4443 regulates mast cell activation by T cell-derived microvesicles." J Allergy Clin Immunol **141**(6): 2132-2141 e2134.
51. Shivashankar, R., Tremaine, W., Harmsen, S., Zinsmeister, A. and Loftus, E. (2014). "Updated Incidence and Prevalence of Crohn's Disease and Ulcerative Colitis in Olmsted County,

Minnesota (1970-2010): ACG IBD Research Award: 1687." Official journal of the American College of Gastroenterology | ACG **109**: S499.
52. Streckfus, C. F., Mayorga-Wark, O., Arreola, D., Edwards, C., Bigler, L. and Dubinsky, W. P. (2008). "Breast cancer related proteins are present in saliva and are modulated secondary to ductal carcinoma in situ of the breast." Cancer Invest **26**(2): 159-167.
53. Strillacci, A., Valerii, M. C., Sansone, P., Caggiano, C., Sgromo, A., Vittori, L., Fiorentino, M., Poggioli, G., Rizzello, F., Campieri, M. and Spisni, E. (2013). "Loss of miR-101 expression promotes Wnt/beta-catenin signalling pathway activation and malignancy in colon cancer cells." J Pathol **229**(3): 379-389.
54. Tian, Y., Ma, X., Lv, C., Sheng, X., Li, X., Zhao, R., Song, Y., Andl, T., Plikus, M. V., Sun, J., Ren, F., Shuai, J., Lengner, C. J., Cui, W. and Yu, Z. (2017). "Stress responsive miR-31 is a major modulator of mouse intestinal stem cells during regeneration and tumorigenesis." Elife **6**.
55. Wu, K., Li, L. and Li, S. (2015). "Circulating microRNA-21 as a biomarker for the detection of various carcinomas: an updated meta-analysis based on 36 studies." Tumour Biol **36**(3): 1973-1981.
56. Xie, Z., Yin, X., Gong, B., Nie, W., Wu, B., Zhang, X., Huang, J., Zhang, P., Zhou, Z. and Li, Z. (2015). "Salivary microRNAs show potential as a noninvasive biomarker for detecting resectable pancreatic cancer." Cancer Prev Res (Phila) **8**(2): 165-173.
57. Xu, X. D., He, X. J., Tao, H. Q., Zhang, W., Wang, Y. Y., Ye, Z. Y. and Zhao, Z. S. (2013). "Abnormal expression of miR-301a in gastric cancer associated with progression and poor prognosis." J Surg Oncol **108**(3): 197-202.
58. Yang, H. W., Liu, G. H., Liu, Y. Q., Zhao, H. C., Yang, Z., Zhao, C. L., Zhang, X. F. and Ye, H. (2016). "Over-expression of microRNA-940 promotes cell proliferation by targeting GSK3beta and sFRP1 in human pancreatic carcinoma." Biomed Pharmacother **83**: 593-601.
59. Yang, Q., Yu, W. and Han, X. (2019). "Overexpression of microRNA101 causes antitumor effects by targeting CREB1 in colon cancer." Mol Med Rep **19**(4): 3159-3167.
60. Yoshizawa, J. M., Schafer, C. A., Schafer, J. J., Farrell, J. J., Paster, B. J. and Wong, D. T. (2013). "Salivary biomarkers: toward future clinical and diagnostic utilities." Clin Microbiol Rev **26**(4): 781-791.
61. Zeng, X. and Tao, H. (2015). "Diagnostic and prognostic serum marker of cholangiocarcinoma (Review)." Oncol Lett **9**(1): 3-8.
62. Zhang, B., Yin, Y., Hu, Y., Zhang, J., Bian, Z., Song, M., Hua, D. and Huang, Z. (2015). "MicroRNA-204-5p inhibits gastric cancer cell proliferation by downregulating USP47 and RAB22A." Med Oncol **32**(1): 331.
63. Zhang, C. Z., Cheng, X. Q., Li, J. Y., Zhang, P., Yi, P., Xu, X. and Zhou, X. D. (2016). "Saliva in the diagnosis of diseases." Int J Oral Sci **8**(3): 133-137.
64. Zhang, L., Wang, X. and Chen, P. (2013). "MiR-204 down regulates SIRT1 and reverts SIRT1-induced epithelial-mesenchymal transition, anoikis resistance and invasion in gastric cancer cells." BMC Cancer **13**: 290.
65. Zhang, W. C., Chin, T. M., Yang, H., Nga, M. E., Lunny, D. P., Lim, E. K., Sun, L. L., Pang, Y. H., Leow, Y. N., Malusay, S. R., Lim, P. X., Lee, J. Z., Tan, B. J., Shyh-Chang, N., Lim, E. H., Lim, W. T., Tan, D. S., Tan, E. H., Tai, B. C., Soo, R. A., Tam, W. L. and Lim, B. (2016). "Tumour-initiating cell-specific miR-1246 and miR-1290 expression converge to promote non-small cell lung cancer progression." Nat Commun **7**: 11702.
66. Zheng, J. Z., Huang, Y. N., Yao, L., Liu, Y. R., Liu, S., Hu, X., Liu, Z. B. and Shao, Z. M. (2018). "Elevated miR-301a expression indicates a poor prognosis for breast cancer patients." Sci Rep **8**(1): 2225.
67. Zhou, X., Li, L., Su, J. and Zhang, G. (2014). "Decreased miR-204 in H. pylori-associated gastric cancer promotes cancer cell proliferation and invasion by targeting SOX4." PLoS One **9**(7): e101457.
68. Zhu, W. and Xu, B. (2014). "MicroRNA-21 identified as predictor of cancer outcome: a meta-analysis." PLoS One **9**(8): e103373.

CHAPTER 8

Conclusions

The work presented in this Thesis aims to contribute to the exploration of potential application of salivary diagnostics in clinical setting, also highlighting caveat and pitfalls emerging from the literature review.

Based on almost three decades of publications, it can be stated that “there is a whole world of wonders in a spit”. 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.

The rushing development of *-omic* sciences contributed to the advancement in point-of-care analysis; in a shortcoming future, it could consistently replace conventional laboratory workflow. The same future will be shared with the improves in automation science, bioinformatics and biostatistics and nanomaterials engineering. An additional boost to salivary diagnostics will be delivered from the “collision” of such entities, resulting into rapid, cost effective and capillary population screening through advanced lab-on-a-chip technologies.

Acknowledgements

Foremost I want to thank my supervisor, Prof. Marco Meleti, for his great support, patience and guidance during this PhD path. He continuously provided encouragement and advices to help me in all the time of my academic research and personal life. He has become more than a true mentor for me.

I would like to express my deepest thanks to Chiar.mo Prof. Ugo Consolo and Prof.ssa Cristina Magnoni, University of Modena and Reggio Emilia for their sight about my career and the opportunity given referencing for this PhD.

I also want to thank Chiar.ma Prof.ssa Thelma Pertinhez for sharing her immense biochemistry knowledge with the group. Her critical opinion has helped me to look at our research from a different point of view. Thanks also to all of her laboratory collaborators who helped for project design and realization. Particularly, I want to thank the PhD candidate Dott.ssa Eleonora Quartieri because I disturbed her with a great number of email and text messages asking for her precious help and competence. I wish her the very best.

My thanks also to Prof.ssa Valeria Righi and Prof.ssa Adele Mucci from University of Modena and Reggio Emilia for having introduced me to NMR and its application to oral diseases 5 year ago.

Thanks to Prof. Gilda Sandri, Dott.ssa Elisabetta Tarentini and Dott.ssa Lucia Panari, University of Modena and Reggio Emilia; they certainly were the core of the clinical activity related to Sjögren syndrome metabolomics investigation.

I'm grateful to Prof. Pierantonio Bellini, University of Modena and Reggio Emilia, which always found time for listen to my problems and ideas besides his many urgent responsibilities.

Lastly, but not of less importance, I want to thank my family. Giulia, Ettore Alberto and Agata Elena, I apologize for the time I took from you during this journey. I love you so much.