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DOTTORATO DI RICERCA IN
Medicina Molecolare

CICLO XXXIV

SALIVA: THE POTENTIAL OF A DIAGNOSTIC BIOFLUID

SALIVA: IL POTENZIALE DIAGNOSTICO DI UN BIOFLUIDO

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ABSTRACT

Metabolomics is the systematic and comprehensive analysis of metabolites in a biological system and provides a functional snapshot of an organism's condition. It can be used to discover biomarkers for diagnosis and for staging the disease's progression.

Nuclear Magnetic Resonance (NMR) spectroscopy, one of the main analytical platforms used in metabolomics, has been employed in this Thesis exploiting its reproducibility, the possibility to measure all metabolites at once, and absolute metabolite quantification.

This Thesis aims to: optimize salivary sample preparation for metabolomics analysis; determine physiologic metabolic profiles of a cohort of healthy subjects, identify salivary biomarkers associated with oral pathologies such as inflammation, potentially malignant disorders, and oral carcinoma.

The optimization of saliva samples preparation for ^{1}H-NMR analysis includes an ultrafiltration step followed by freeze-drying which allows a 5-fold gain of metabolite's concentration. The method has been validated, and the LOQ and LOD were determined.

Three different types of saliva were collected (whole, parotid, and submandibular/sublingual) from 20 healthy volunteers without oral cavity diseases. Metabolites derived from endogenous host metabolism and oral bacterial microflora and differently distributed within the three saliva subtypes were identified and quantified.

The periodontal health status of our study cohort was assessed by the "Full Mouth Bleeding Score" (FMBS). Multivariate statistical analysis of the whole saliva highlighted the correlation between some metabolites and FMBS. The identified metabolites represent a dysbiotic oral bacterial colonization that can induce inflammation and gingival bleeding.

These findings are the starting point to set up an early diagnostic tool for oral inflammation preceding periodontitis.

The last part of the study was the determination of the metabolic profiles of the whole saliva from patients with oral cancer (OSCC) and patients with potentially malignant oral disorders (leukoplakia and lichen planus).

Preliminary data showed metabolic alterations associated with the progressive transformation of potentially malignant lesions to neoplastic cells. Noteworthy, the metabolic data allowed also to distinguish, in the leukoplakia cases, different stages of dysplasia degeneration of the oral mucosa. Further investigations will be needed to correlate metabolomic data with subjects' clinical and epidemiological features.

To conclude, ^{1}H-NMR metabolomics analysis of saliva revealed its potential for developing design protocols for the early diagnostic of oral pathologies.

RIASSUNTO

La metabolomica è l'analisi sistematica dei metaboliti presenti in un sistema biologico ed è in grado di fornire un'istantanea delle condizioni di salute di un organismo. Può essere utilizzata per la ricerca di biomarcatori utili nella diagnosi e nella stadiazione della progressione di diverse patologie.

La spettroscopia di Risonanza Magnetica Nucleare (NMR), una delle principali tecniche analitiche utilizzate in metabolomica, è stata impiegata in questa tesi per tutti i suoi vantaggi, tra cui la riproducibilità, la possibilità di misurare tutti i metaboliti contemporaneamente e la capacità di fornire una quantificazione assoluta dei metaboliti.

Questa tesi si propone di: ottimizzare la preparazione del campione salivare per l'analisi metabolomica tramite tecnica ^{1}H-NMR; determinare i profili metabolici di una coorte di soggetti sani in condizioni fisiologiche; identificare i metaboliti salivari che possano espletare la funzione di biomarkers per patologie orali come l'infiammazione, lesioni orali potenzialmente maligne e il carcinoma orale.

L'ottimizzazione della preparazione dei campioni di saliva per l'analisi ^{1}H-NMR ha previsto una fase di ultrafiltrazione seguita da una fase di liofilizzazione, che ha consentito un aumento di 5 volte della concentrazione nativa dei metaboliti, permettendone la completa quantificazione. Il metodo è stato convalidato analiticamente e ne sono stati determinati LOQ (limite di quantificazione) e LOD (limite di rilevamento).

Sono stati raccolti tre diversi tipi di saliva (intera, parotidea e sottomandibolare/sublinguale) da 20 volontari sani, senza malattie del cavo orale. Sono stati identificati e quantificati metaboliti derivanti sia dal metabolismo endogeno che dalla microflora batterica che popola il cavo orale, presenti in concentrazioni diverse nei tre sottotipi di saliva.

Lo stato di salute paradontale della nostra coorte di studio è stato valutato tramite il "Full Mouth Bleeding Score" (FMBS): l'indice di sanguinamento delle mucose orali. L'analisi statistica multivariata del metaboloma della saliva intera ha evidenziato la correlazione tra alcuni metaboliti e FMBS.

Questi risultati sono il punto di partenza per lo sviluppo di uno strumento diagnostico che possa rilevare precocemente le condizioni di infiammazione orale, prima che evolvano a stadi più severi, come la parodontite.

L'ultima parte dello studio è stata la determinazione dei profili metabolici della saliva intera di pazienti con cancro orale (OSCC) e di pazienti con lesioni orali potenzialmente maligne (leucoplachia e lichen planus).

Dati preliminari hanno mostrato alterazioni metaboliche associate alla progressiva trasformazione di lesioni potenzialmente maligne in fenotipi più aggressivi. In particolare, i dati metabolici hanno consentito di distinguere, nei casi di leucoplachia, diversi stadi di displasia epiteliale degenerativa della mucosa orale. Saranno necessarie ulteriori indagini per correlare i dati metabolomici con le caratteristiche cliniche ed epidemiologiche dei pazienti.

Per concludere, l'analisi metabolomica ^{1}H-NMR della saliva ha rivelato il potenziale di questo biofluido per lo sviluppo di protocolli e dispositivi per la diagnosi precoce delle patologie orali.

I. INTRODUCTION

1. WHAT IS METABOLOMICS

1.1. Metabolomics: A field of Omics sciences

The *Omics sciences* encompass disciplines that, unlike traditional biological sciences that focus on selected biological processes, aim to study the ensemble of genes (genomics), transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics) expressed by the cells (Figure I.1). *Omics sciences*, therefore, analyse cells and tissues from a different perspective, probably best suited to describe biological systems characterized by a high degree of complexity. This change in biology was possible thanks to the introduction of new technologies and produces a considerable amount of information.

Genomics, the first member of the *Omics* family introduced in the 80' of the 20th century, focus on the elucidation of the entire genome of an organism, allowing the characterization and quantification of all genes at once. DNA sequencing and genetic variant information gained by genomics approaches have enabled the identification of gene mutations and chromosomal rearrangements related to specific genetic syndromes (Olivier et al, 2019).

Transcriptomics investigates the set of coding and non-coding RNA measuring the direct activity and functional characteristics of the genome. Strongly linked to genomics transcriptomics captures gene transcription changes in a precise moment under different conditions. Understanding "how" and "why" gene expression profiles change is required to identify molecular mechanisms underlying pathological conditions (Jiang et al, 2015).

Proteomics identifies and quantifies the expressed proteins in the cells or tissues: the proteome. Proteins, responsible for cellular processes control, are altered by innumerable factors, external or internal perturbations leading to proteome adaptation. The proteomics approach provides the means to interpret metabolic pathways alterations in response to pathogenic conditions (Aslam et al, 2017).

Metabolomics is the *Omics* discipline that aims to provide a quantitative measure of low molecular weight metabolites present in a cell, tissue, organ, or organism. Metabolites are the substrates, intermediates, and end products of metabolic pathways. Their levels' alteration reflects the dynamic measure of the multiparametric response of a living organism to a pathophysiological perturbation or gene variation (Nicholson & Lindon, 2008; Liu et al, 2017).

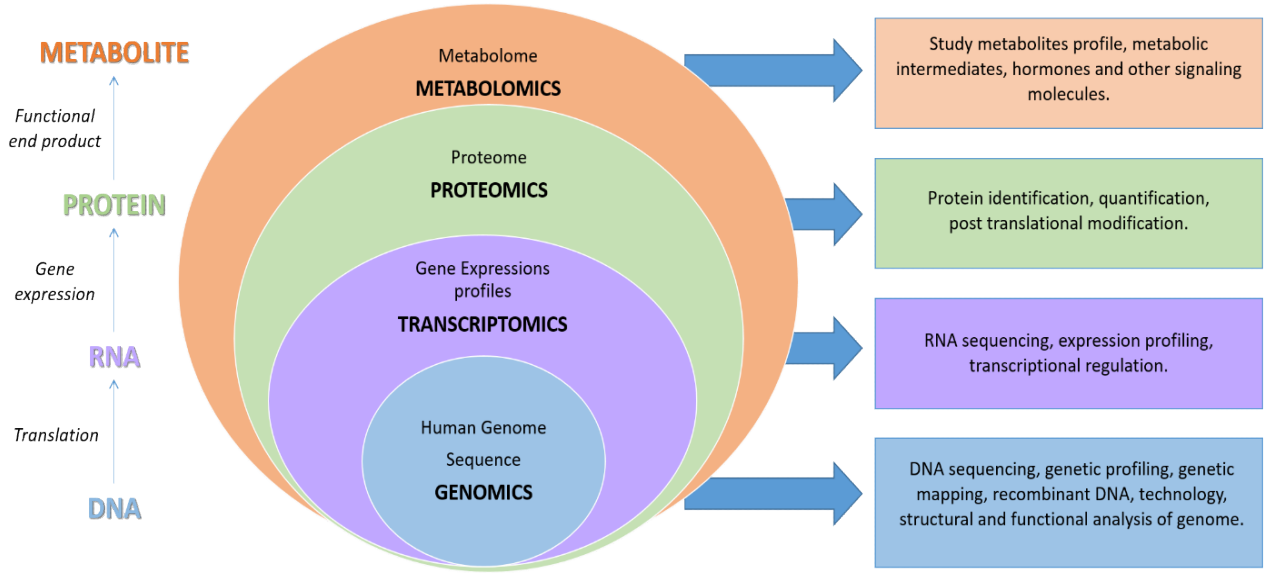


Figure I.1: Diagram of *Omics* sciences (Rai et al, 2018).

Two emerging *Omics* strategies are fluxomics and lipidomics. Fluxomics explores metabolic pathways using precursors enriched with stable isotopes (^{13}C, ^{15}N). It overrides the limitations of metabolite analyses at steady-state and allows to monitor metabolic dynamics and pathways variations by quantifying metabolites consumption/generation rates (Giraudeau, 2020). Lipidomics attempts to comprehensively identify and quantify all kinds of lipid molecular species. Lipids cover a central role in many biological processes and their level imbalance can underline many pathological conditions (Züllig et al, 2020).

The *Omics* family is in continuous expansion and the new disciplines include metagenomics (Riesenfeld et al, 2004), glycomics (Raman et al, 2005), connectomics (Sporns, 2005), cellomics (Primiceri et al, 2013), and even foodomics (Braconi et al, 2018).

The integration of all *Omics* sciences opens new scenarios in the understanding of systems biology and the use of the *multi-omics* approach is envisaged as an important support to precision medicine in clinics (Olivier et al, 2019).

The power of metabolomics, among the *Omics*, derives from the recognition that subtle changes in genes or protein levels can lead to substantial variations in metabolome levels. Metabolome, the combination of more than 1.000.000 metabolites, is the dynamic measure of the phenotype at the molecular level and this places metabolomics at the top of *Omics* sciences for pathophysiological biomarker discovery (Wishart, 2019). Compared to other *Omics*, metabolomics can provide direct information with a low quantity of material and an easy sample preparation (Fuhrer et al, 2015).

Metabolites are defined as small molecules (<1500 Da) (lipids, amino acids, short peptides, sugars, etc) produced directly from endogenous processes or derived from exogenous sources, such as plant or microbial-derived compounds, xenobiotics and drugs (Hocher et al, 2017).

Historically, the basic principle of metabolomics, the relation between biochemical pathways and biological events, can be dated back to Middle Ages, where urine colours, tastes, or smells, from the metabolic origin, were tested to diagnose diabetes (Nicholson & Lindon, 2008). However, systematic metabolomics studies in the 1970s by Horning & Horning (Horning & Horning, 1971) and by Pauling and cols. (Pauling et al, 1971) started a new age in metabolomics research. The modern approach to metabolomics, as we know it today, was then developed at the end of the '90s and early 2000s when Nicholson and colleagues defined metabolomics as "*the study of the quantitative complement of metabolites in a biological system and changes in metabolite concentrations or fluxes related to genetic or environmental perturbations. Studies are typically holistic in nature through targeted studies are also encompassed in the term metabolomics*"(Nicholson et al, 1999). Even if at the beginning two terms were used: metabolomics and metabonomics, today, they are utilized indifferently. For the record, metabolomics is the qualitative and quantitative analysis of metabolites levels under various conditions, instead, metabonomics refers to metabolic changes related to stressful conditions, such as diseases or toxic exposures (Krastanov, 2010).

Metabolomics analysis can be made on different types of matrixes of human or animal origin, including body fluids (urine, serum, plasma, saliva, cerebrospinal, sweat, milk, tears, or seminal fluid), tissue (including biopsy samples), and cell culture (Bollard et al, 2005). A contemporary sampling of tissue and biofluids derived from the same organ offers an evaluation of global organ metabolic activity.

In the literature one can find several handling protocols and metabolome characterization of major human biofluids, in physiological conditions, revealing their distinct and characteristic metabolic composition (Beckonert et al, 2007; Luque de Castro et al, 2018; www.hmdb.ca).

Metabolomics is applied in medical sciences to identify biomarkers to facilitate early diagnosis, accurate prognosis, monitoring of diseases stage, and therapy effects. It is also employed to characterize the interactions of organisms with their environment for new risk biomarkers, from pollution to microbial colonization. In addition, metabolomics has a considerable scope in the pharmaceutical industry for the optimization of drugs development, from the validation of biomarkers efficacy to the determination of new drug targets. Not only, but the application of metabolomics includes also nutritional research and the plant industry (Peng et al, 2015).

1.2. Human samples for metabolomics

Metabolomics can be performed on a wide range of biological matrices, including biofluids, cells, and tissues. The well-established biofluids are serum, plasma, urine, and saliva widely employed in clinical studies aiming to discover new biomarkers linked to several pathologies. The advantage of biofluids is their ability to reflect the metabolic activity of specific organs or anatomical districts: for example, urine offers information about kidney function, cerebrospinal fluid on the brain metabolism and saliva provides evidence of the oral cavity state (Lindon et al, 2000; Wishart, 2019). In the last decade, the group of Prof. David Wishart of the University of Alberta (Canada) has developed systematic databases for different biofluids using data present in the literature. These databases contain countless information on metabolites: structure, chemical characterization, concentration in healthy and pathological conditions, as well as on enzymes and metabolic pathways involved in their production or consumption: all information is freely accessible. That wealth of data is organized by biofluid: the human serum metabolome (4229 metabolites, Psychogios et al, 2011), the human urine metabolome (445 metabolites, Bouatra et al, 2013), the human cerebrospinal fluid metabolome (308 metabolites, Wishart et al, 2008) and the human saliva metabolome (853 metabolites, Dame et al, 2015). The assembling of all information is accessible at "The Human Metabolome Database" (www.hmdb.ca, with 220,945 metabolites, Wishart et al, 2007).

Blood, a systemic fluid, is employed to capture information on the organism's metabolic status and is the choice in clinical analyses. The different collecting procedures and the coagulation cascade influence the concentrations of metabolites, in plasma and serum (Hernandes et al, 2017). Yu and colleagues (Yu et al, 2011) demonstrated good data reproducibility for both blood components and confirmed that serum obtained, after whole blood clotting, is suitable for the metabolomics studies and biomarker detection. In a recent study, the use of whole blood was evaluated as a reliable matrix for cases in which the haemolysis could contribute to the variance of the metabolome (Stringer et al, 2015).

Urine is less complex than other body fluids and is highly employed in biomarker discovery. It can be collected as serial sampling to monitor disease and therapy response (Luque de Castro et al, 2018). Urine metabolomics explores the diseases from cancer (Dinges et al, 2019) to inflammatory bowel diseases (Storr et al, 2013), the interaction host-gut microbiome (Chen et al, 2019), nutritional aspects (Cheung et al, 2017) and provides a signature of the individual metabolic phenotypes

(Assfalg et al, 2008). A considerable effort has been devoted to urinary metabolomics as a diagnostic tool and now is moving from discovery to the validation phase (Bancos et al, 2020).

Saliva, different from blood and urine, only in the last decades has been used to search for biomarkers. It is emerging as a tool to diagnose oral diseases and systemic pathologies, thanks to its non-invasive and inexpensive collection.

Saliva is produced by different types of salivary glands, which contribute differently to its chemical composition, and contains also metabolites derived from the prokaryotic cells that colonize the oral cavity (Ishikawa et al, 2016). The final saliva has a great inter-individual variability, producing specific signature. The salivary samples are also employed to monitor athletic performance (Pitti et al, 2019) and taste perception (Gardner et al, 2020b).

There are also other biofluids used for metabolomic analyses, less common as they require more articulated and/or more impacting (physically or mentally) sampling on the donor: semen (Wang YX et al, 2019), exhaled breath (Ghosh et al, 2021), human milk (Ninonuevo et al, 2006), aqueous humour (Barbosa Breda et al, 2020), sweat (Serag et al, 2021) and amniotic fluid (Bardanzellu et al, 2019).

The biological samples are metabolically active even after their collection, hence, it is necessary to proceed with a quickly quench, by the removal of cellular components or by rapid freezing to preserve the *in vivo* conditions and maintain the metabolites composition at the time of the sampling. Moreover, biofluids are very complex samples that require adequate preparation before analysis: many works have already been published on protocols for best handling practices, especially on the importance of eliminating macromolecules that interfere with the identification and quantification of metabolites (Wishart, 2019; Beckonert et al, 2007; Gardner et al, 2018).

The biofluids report the biochemical processes throughout the body. To get more precise information on the metabolic activity occurring at the site of the disease, metabolomics can be performed in tissues derived from biopsies, both intact, and subjected to a homogenization process. The metabolic profile from intact tissue specimens can be obtained, with a minimum sample manipulation, using the high-resolution magic-angle-spinning (HR-MAS) Nuclear Magnetic Resonance (NMR) spectroscopy (Grinde et al, 2019, Tilgner et al, 2019). On the other hand, the tissue homogenate is subjected to multiple preparation steps to obtain the target sample for metabolomics studies (Römisch-Margl et al, 2012).

1.3. Analytical techniques and experimental strategies

For the characterization of metabolites from the cells or organisms, metabolomics makes use of high throughput technologies.

The high resolution of mass spectrometry (MS) and the reproducibility of nuclear magnetic resonance (NMR) spectroscopy combined with their ability in the elucidation of chemical structures resulted to be the most used analytical technologies for metabolomics studies. NMR and MS methods are complementary procedures (Table I.1).

MS is a highly sensitive method, with high accuracy and resolution: a potent tool for the detection and quantification of thousands of small molecules. MS methods need prior separation techniques such as liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE). The basic principle for metabolite detection and identification is the ionization of molecules: thanks to molecular fragmentation and the molecule's mass-to-charge (m/z) ratio it is possible to recognize a given compound. To perform a MS experiment only a few microliters of sample are required, but the unavoidable ionization process vaporizes the sample, making MS an intrinsically destructive technique (Emwas, 2015).

NMR, instead, is a non-destructive technique, that provides high reproducibility and quantitative results, thanks to the use of internal/external standards. NMR spectroscopy is non-biased, easily quantifiable, and requires minimal sample preparation. NMR is easily automatable, favouring high-throughput experiments. In addition, NMR is particularly suited to detect and characterize compounds that are less discernible with LC-MS analysis such as organic acids, polyols and other highly polar compounds. NMR spectroscopy is perfect for real-time metabolite profiling of living cells and real-time metabolic flux analysis (Emwas, 2015).

However, NMR has also several disadvantages, the most significant of which is low sensitivity, detecting molecules at the micromolar range: compared to LC-MS and GC-MS, NMR spectroscopy is 10 to 100 times less sensitive. Despite sensitivity has increased enormously thanks to technical improvements such as the introduction of cryo-cooled probes (electronics are cooled to near liquid helium temperatures (~20 K) to reduce electronic thermal noise) that lead to a three-to four-fold enhancement of signal, still, it remains a weak point. In general, NMR spectrometers are quite expensive compared to mass spectrometers, requiring highly skilled operators and suitable laboratory spaces: these factors have hindered NMR metabolomics development due to the difficulty of being transferred to clinical uses (Markley et al, 2017; Crook & Powers, 2020).

Table I.1. Summary of advantages and limitations of NMR and MS in metabolomics applications (from Emwas et al, 2019)

	NMR	MASS SPECTROMETRY
Reproducibility	High reproducibility is one of the fundamental advantages of NMR spectroscopy.	Compared to NMR spectroscopy, MS data are less reproducible.
Sensitivity	Intrinsically low but can be improved with multiple scans (time), higher magnet field strength, cryo-cooled probes and micro-probes, and hyperpolarization methods.	High sensitivity is a major advantage of MS; metabolites with nanomolar concentrations can be readily detected.
Selectivity	NMR is generally used for nonselective analysis. Peaks overlap from multiple detected metabolites pose major challenges.	MS is selective. However, in combination with chromatography (such as liquid and gas phase separation), it is a superior tool for targeted analysis.
Sample measurement	Enables relatively fast measurement using 1D ^{1}H-NMR spectroscopy, where all metabolites at a detectable concentration level can be observed in one measurement.	Different ionization methods are required to maximize the number of detected metabolites.
Sample preparation	Involves minimal sample preparation, usually transfer the sample to an NMR tube and addition of a deuterated solvent. The measure can be automated.	It is demanding; it requires chromatography; in some cases sample derivatization for gas chromatography (GC)-MS.
Quantitative analysis	NMR is inherently quantitative as the signal intensity is directly proportional to the concentration of the metabolite.	The intensity of the MS line is often not correlated with metabolite concentrations as the ionization efficiency is also a limiting factor.
Fluxomics Analysis	NMR permits both *in vitro* and *in vivo* metabolic flux analyses. Its inherently quantitative nature also enables precise quantification of precursors and products. Mapping of stable isotope locations in molecules is very easy via NMR.	MS can be used for fluxomics analysis; however, the destructive nature of MS-based methods means it is somewhat more limited than NMR-based fluxomics. *In vivo* fluxomics is not possible with MS, and isotope mapping is more difficult.
Tissue samples	Using high-resolution magic-angle sample spinning (HR-MAS) NMR, it is possible to detect metabolites in tissue samples.	Although some MALDI-TOF approaches can be used to detect metabolites in tissue samples, these approaches are still far from being routine.
Number of detectable metabolites	Depending on spectral resolution, usually less than 200 metabolites can be unambiguously detected and identified in one measurement.	Using different MS techniques, it is possible to detect thousands of different metabolites and identify several hundred.

Target and Untargeted metabolomics

The strategies employed in metabolomics studies, with both MS or NMR techniques, depending on the final objective and the state of knowledge of the system, are two: targeted or untargeted.

A targeted (or hypothesis-driven) approach is carried out when at least some of the metabolites that potentially have a key role in the development of some biological functions or processes are known, or if their possible importance is suspected *a priori*. The experimental determination is focused exclusively on the research and quantification of these compounds. This method provides higher sensitivity and selectivity, ensuring better classification, accuracy, and highly reproducible results (Luque de Castro et al, 2018).

The untargeted analysis uses global metabolic profiling, it analyses all the detectable metabolites present in a sample without *a priori* knowledge of the metabolome, generating the metabolic fingerprint. It is an unbiased analysis. It is used for a screening analysis to provide sample classification and to get the first discrimination between samples from different groups (*i.e.*, disease/healthy) (Ellis et al, 2007). Untargeted approaches are also defined as "hypothesis-generating" since the data are used for the preliminary quantification of the samples to advance hypotheses which are then subsequently analysed with targeted approaches (Schrimpe-Rutledge et al, 2016).

Compared to the targeted approach, the untargeted one takes a more holistic point of view and provides results related to the complexity of cellular metabolism.

1.4. Statistical analysis of metabolic data

The metabolomics analysis produces large datasets like the others "*Omics*". Robust statistical analyses, uni- and multivariate, are required for the accurate elaboration of these complex datasets to extract significant biological information.

Univariate methods analyse data independently, with the disadvantage that do not consider the presence of interactions between metabolites. Commonly, parametric tests such as Student's *t*-test and ANOVA are applied where data normality is assumed; otherwise, non-parametric tests such as Mann-Whitney *U* test or Kruskal-Wallis one-way are preferable (Alonso et al, 2015).

The use of multivariate data analyses allows an exam of the overall differences, their trends in the variations, and the relationships between samples and variables: the metabolites from the same metabolic pathway tend to be highly correlated (Vu et al, 2019). Its application enables also to

determine, whether the examined samples have a tendency to divide into clusters and it highlights the metabolites most responsible for those differences, at last, generating predictive models.

The visualization methods for highlighting the differences between samples and/or between variables can be divided into two groups: unsupervised and supervised. Unsupervised methods are used for a preliminary exploration of data and their purpose is to provide an overall visualization of the data, reducing the variables and trying to maximize the variance between them, without however providing information based on *a priori* knowledge of the data to guide the analysis.

There are several unsupervised methods available, the most used is the Principal Component Analysis (PCA) which allows to evaluate the existence of correlations between variables and their relevance, to identify the possible presence of outliers and clusters, to summarize the description of the data, to eliminate noise and to reduce dimensionality. PCA converts the original dataset into two matrices: loadings and scores plots. The loadings graph allows the analysis of the role of variables in the different components, their direct and inverse correlations, and their importance. The scores plot consent to visualize the behaviour of the object (matrix data) in the different components, their similarities, or to identify groups of similar objects (clusters), the presence of outliers, therefore, is the most important tool for the preliminary data investigation.

The supervised methods are also used to detect the existence of groupings, patterns and to construct predictive models, but in this case, the system is instructed with additional information, such as the number and type of classes to be identified. The Partial least squares-discriminant analysis (PLS-DA), a supervised linear regression, is the most used of these methods, which maximizes the covariance between the matrix of the independent variables (the data matrix) and the matrix containing the dependent variables (Smolinska et al, 2012). This approach is necessary when datasets are made of highly correlated variables.

In the PLS-DA model, the measurement of the importance of a variable is given by the Variable Importance in Projection (VIP) score. The VIP score of a variable is calculated as a weighted sum of the squared correlations between the PLS-DA components and the original variable and is considered important when is close to or greater than one (Figure I.2).

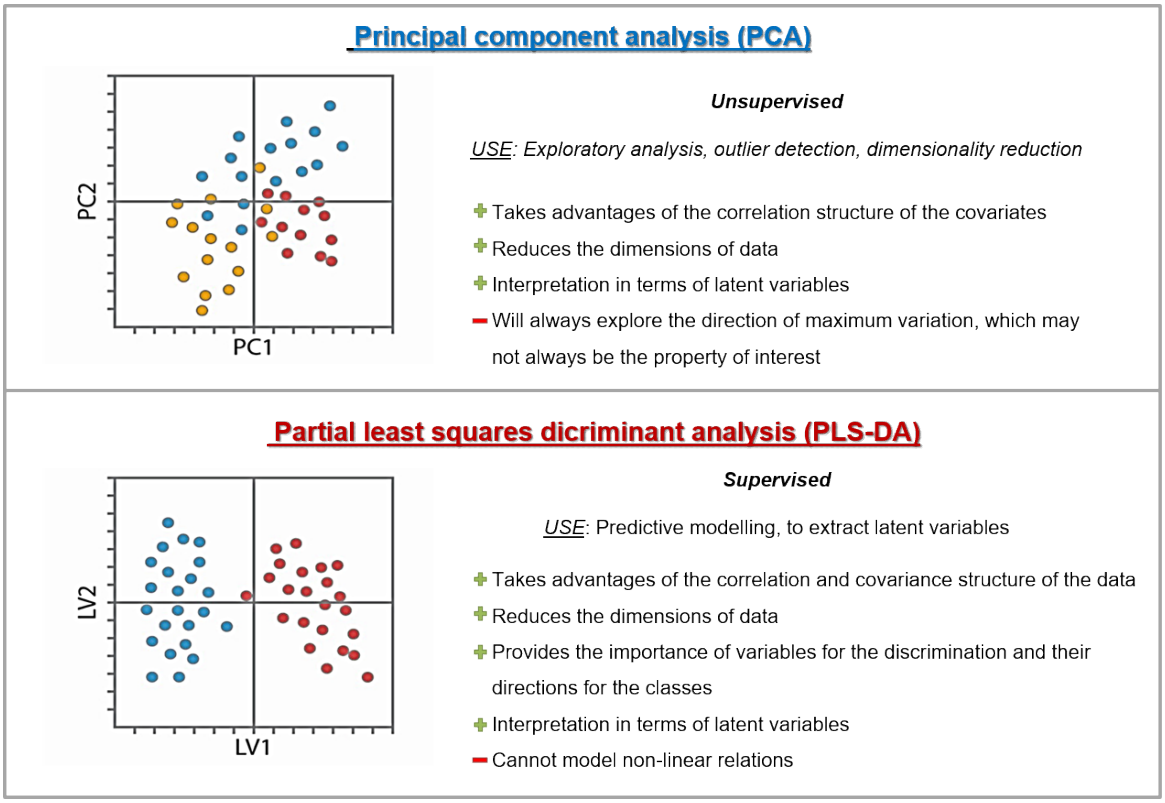


Figure I.2: Overview of the strengths and limitations of PCA and PLS-DA (Debik et al, 2021).

2. SALIVA AS A DIAGNOSTIC TOOL

2.1. Saliva, an overview

Saliva is a dynamic and complex mix of fluids from major and minor salivary glands and gingival crevicular fluid. The major salivary glands include the parotid glands located in the retromandibular fossa, the submandibular glands found on the floor of the mouth, and the sublingual glands sited under the tongue (Figure I.3). Minor glands are found in the lower lip, tongue, palate, and pharynx. The salivary gland tissue is constituted by acinar, duct, and myoepithelial cells irrigated by the capillary network.

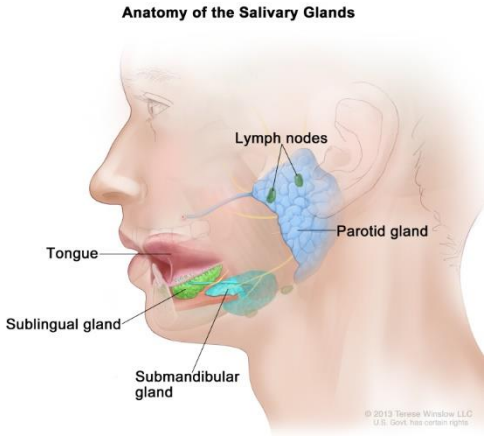


Figure I.3. Anatomy of the salivary glands (PDQ® Adult Treatment Editorial Board).

The daily volume production of saliva fluctuates, in healthy conditions, between 1 and 1.5 L. In the unstimulated condition, the parotid glands contribute to 20% of the flow with parotid saliva (PS), submandibular and sublingual glands to 75% with submandibular/sublingual saliva (SM/SL), and the remaining 5% comes from numerous other minor glands.

The flow rate can also be stimulated with three types of stimuli: mechanical (by chewing paraffin wax), gustatory (with citric acid), and olfactory. Saliva secretion can be highly affected by local or systemic diseases that distress the glands themselves (Humphrey & Williamson, 2001). Saliva secretion could be serous, mucous, or mixed: serous secretions (light and watery) produced mainly by the parotid gland, mucous secretions (slippery solution with mucus) from the minor glands, and mixed one from the sublingual and submandibular glands.

The 99% of saliva content is water, slightly acidic, while the remaining 1% contains many inorganic and organic components (Figure I.4). Inorganic compounds include electrolytes, such as sodium, chloride, potassium, calcium, magnesium, phosphate and bicarbonate, and trace elements. Organic components of saliva are hormones, glucose, lipids (such as cholesterol and fatty acids), amino acids, amines, proteins, RNA, and others. The salivary proteome comprises more than 2500 proteins (Lau et al, 2021), including hormones, antibodies, growth factors, and enzymes. The most represented are proline-rich proteins, amylases, mucins, lysozyme, glycoproteins, and lipoproteins (Cuevas-Córdoba & Santiago-García, 2014). Other components are derived from nasal and bronchial secretions and external sources, like food debris and microbes.

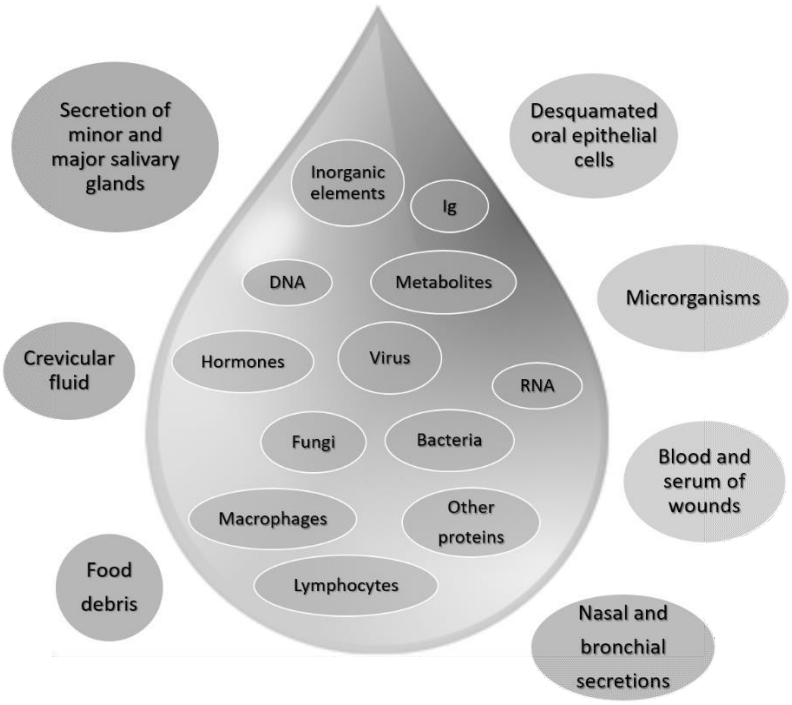


Figure I.4. Schematic representation of the components of whole saliva. (Adapted from Cuevas-Córdoba & Santiago-García, 2014).

Oral microbiota consists of approximately 700 distinct prokaryotic (Deo & Deshmukh, 2019) that affect salivary composition with intrinsic metabolism. The microbial consortia play a role in modulating and maintaining homeostasis and physiological functions in the oral cavity. A balanced microbiota is based on complex interactions between inorganic and organic salivary constituents (Ngamchuea et al, 2017).

In addition, due to a thin layer of epithelial cells separating the salivary ducts from the bloodstream, the saliva also presents some blood components transferred via passive diffusion, active transport, or ultrafiltration (Javaid et al, 2016). The presence of these components allows the achievement of five major saliva functions: lubrication/protection, buffering, maintaining tooth integrity, antibacterial activity, and taste/digestion (Kaplan et al, 1993).

The composition and the concentration of saliva components are influenced by many factors: flow rate, circadian rhythm, size of salivary glands, gender, age, diet, drugs, environment, lifestyle, smoking, physiological/pathological states (Figure I.5). Consequently, saliva can be seen as the reflection of the organism's condition, thus turning into a clinical diagnostics fluid and a potential, less-invasive, surrogate for other biofluids (Schipper et al, 2007).

In clinical application, saliva has important advantages: easy and non-invasive collection, inexpensive storage, and less manipulation than serum, with minimal risks of external contamination. The painless and easy saliva sample collection ensures compliance and alleviates

discomfort even in vulnerable patients with psychiatric disorders and in infant/children populations. The recommended procedure for saliva collection is the passive drool directly into plastic tubes. In the market, different collection devices are available (Khurshid et al, 2016).

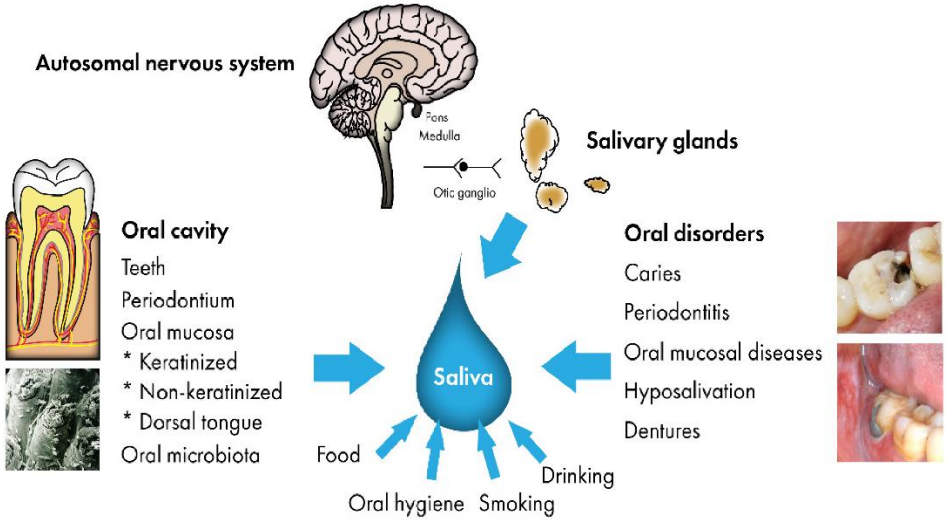


Figure I.5. The complex set of factors involved in the saliva metabolic pathway (Hyvärinen et al, 2021)

2.2. Salivary metabolomics for biomarker discovery

According to the National Institutes of Health, a biomarker is defined as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention*" (Biomarkers Definitions Working Group, 2001).

Overall, a biomarker can be a molecule, or a pattern of molecules, representative of a specific condition, that is not affected by other factors unrelated to the disease or the condition under study. The main concerns against saliva for diagnostic use are that production and composition are influenced by numerous factors: circadian rhythm, gland stimulation, diet, age, gender, exercise, and environment (Cuevas-Córdoba & Santiago-García, 2014). Therefore, saliva has a double-side characteristic since it could be the perfect biofluid for monitoring these variables (Figure I.6).

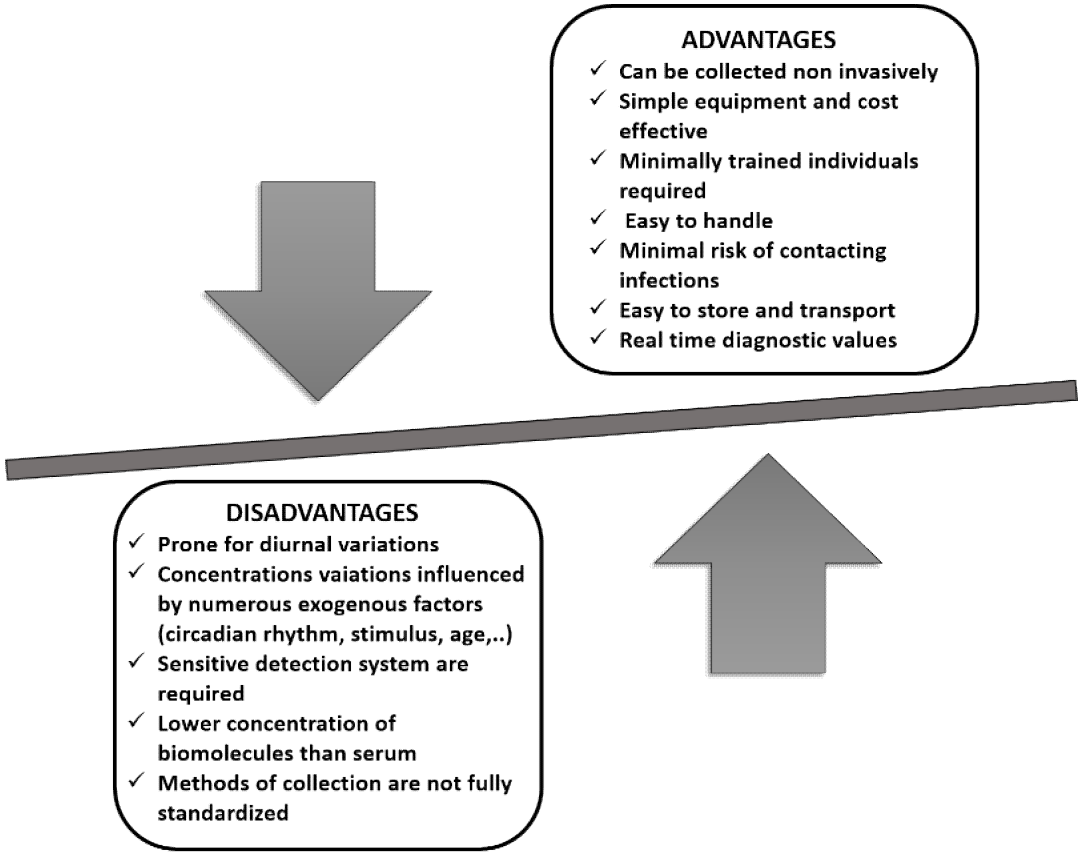


Figure I.6. Counterbalance of saliva as a diagnostic fluid (Javaid et al, 2016).

A candidate biomarker validation process to its applicability in clinical diagnostics requires different steps illustrated in Figure I.7.

Figure I.7. Workflow of biomarker candidate validation process (Nagana et al, 2013).

After discovery, takes place the pre-validation step which is intended to assess the accuracy and the robustness of initial multivariate models to screen false positive biomarker candidates. The most promising metabolites are identified through multivariate statistical analyses, and, individually or in panels, their predictive capability is tested.

The receiver operating characteristic (ROC) curve is the most used tool to assess the statistical performance measure of a classification model. The ROC curve allows the visualization of specificity against the sensitivity at any cut-off value of the model's performance.

The AUC (area under the ROC curve) score gives the degree or measure of separability. Therefore, when AUC is 1.0 the model can distinguish entirely between classes, whereas there is no difference from random chance when AUC is 0.5. An AUC > 0.7 is the minimal score to consider a biomarker clinically useful (Xia et al, 2013). The analytical development stage provides the linearity, sensitivity, limit of detection, recovery, robustness, and reproducibility of the biomarker model. At this point in the process, any interferences are identified and minimized. In the last stage, the single biomarker or biomarkers panel is validated by evaluating its predictability on many new samples, considering confounding factors (biological and technical variances). The final challenge of biomarker discovery is to commercialize the developed technologies for diagnostic purposes in clinics (Nagana Gowda et al, 2013).

Salivary metabolomics is gaining attention because the acquisition of disorder-specific metabolic profiles has facilitated the identification of candidate biomarkers as diagnostic tools (Cuevas-Córdoba & Santiago-García, 2014; Beale et al, 2016).

The salivary biomarkers discovery is growing, as can be appreciated by the increasing number of publications in the last decade (Figure I.8).

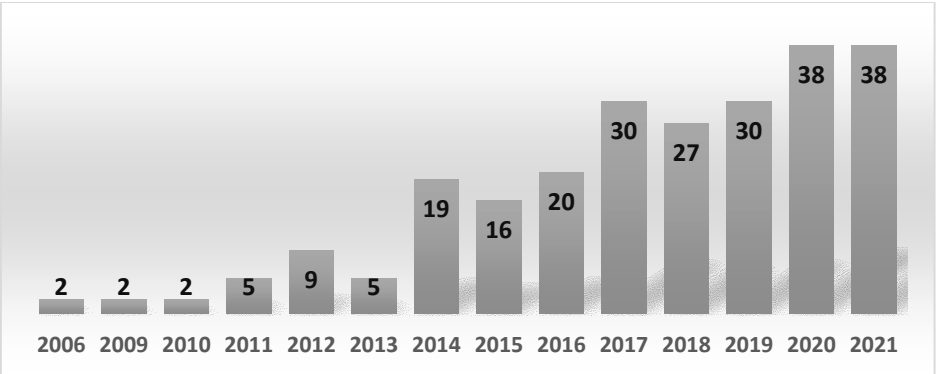


Figure I.8. The number of publications per year is extrapolated from the PubMed portal (www.ncbi.nlm.nih.gov) by entering “saliva AND metabolomics AND biomarkers” as keywords.

Most of these publications are concentrated on diagnosis, management, and follow-up of several pathologies. The investigation of salivary metabolome has been carried out on patients with various systemic disorders to identify biomarkers for early diagnosis.

Table I.2 reports the recent studies focused on salivary metabolomics and reflects the potential of this strategy on biomarkers identification, pathogenesis, and disease classification.

Table I.2. Recent studies on systemic diseases based on salivary metabolomics approach.

DIFFERENT DISEASES	
Diabetes	Barnes et al. 2014 De Oliveira et al. 2016 Sakanaka et al. 2021
HIV	Shulte et al. 2020
Respiratory diseases	Li et al. 2021
COGNITIVE DISORDERS	
Dementia	Figueira et al. 2016
Cerebral palsy	Symons et al. 2015
Alzheimer's disease	Yilmaz et al. 2017
Parkinson's disease	Kumari et al. 2020
Schizophrenia	Kim et al. 2021b Cui et al. 2021
Temporomandibular disorders	Sanches et al. 2020
CANCER	
Breast cancer	Sugimoto et al. 2010 Murata et al. 2019 Xavier Assad et al. 2020
Hepatocellular carcinoma	Hershberger et al. 2021
Thyroid cancer	Zhang J et al. 2021
Glioblastoma	García-Villaescusa et al. 2018
Head and neck cancer	Mikkonen et al. 2018 Taware et al. 2018

Apart from the systemic disorders, due to saliva *in situ* production and its interaction with the local environment, salivary metabolomics studies are primarily focused on the oral cavity pathologies. Salivary metabolomics analyses are directed to the elucidation of the alterations associated with the presence of periodontal diseases and oral cancer, and particularly for the discovery of early diagnosis biomarkers.

Currently, on commerce are present a few diagnostic kits based on salivary *Omics*: some of them are routinely used in clinical laboratories for pathologies such as human immunodeficiency (HIV), oral human papillomavirus (HPV), and familial hypercholesterolemia (Nunes et al, 2015).

Recently, saliva has been proposed in COVID-19 diagnosis as a promising tool to develop a rapid diagnostic test, easily performed also by non-trained medical staff, for sizeable epidemiological cohort studies (Azzi et al 2020; Costa Dos Santos et al, 2020; Atieh et al, 2021).

2.3. ^{1}H-NMR salivary metabolomics for oral diagnostic

The first ^{1}H-NMR studies on saliva were performed using the whole saliva (WS), the spectra acquired were of low resolution, which allowed the assignments of only a few peaks (Dan et al, 1989; Harada et al, 1987).

Some years later, Yamada-Nosaka and colleagues employed stimulated saliva derived from the parotid glands (PS) and the submandibular and sublingual glands (SM/SL), obtaining preliminary results, on a 360 MHz spectrometer, of the comparison between healthy subjects and patients with sialadenitis (Yamada-Nosaka et al, 1991). They also reported a coarse assignment of some peaks in NMR spectra of PS acquired with a 500 MHz spectrometer.

In 2002 Silwood and colleagues (Silwood & Grootveld, 2002) published NMR spectra of unstimulated whole saliva from a healthy subject, acquired with a 600 MHz spectrometer, with a rough assignment of the main peaks of few carbohydrates, amino acids, and organic acids.

However, the first complete assignment was carried out in the same year, with the same spectrometer, when Silwood and co-workers (Silwood & Lynch, 2002) performed a comprehensive study of WS from healthy subjects. Following a rigorous saliva sampling, comparable to the protocols used today, they were able to identify 63 metabolites in the WS. In that work, they presented also the first applications of two-dimensional (2D) NMR techniques to analyse WS saliva.

In the following years, numerous reports showed a better characterization of the salivary metabolome, together with standard operating procedure (SOP) improvements.

Dame and colleagues produced the most extensive quantitative metabolomics study on the human salivary metabolome in 2015 (Dame et al, 2015): by collecting sixteen WS samples of healthy individuals, they identified and quantified 64 ± 4 metabolites per sample.

Subsequently, several studies on salivary metabolome reported on the variables that can impact saliva metabolic profiles, such as smoking, exercise, diet, and gender (Figueira et al, 2017; Takeda et al, 2009; Pitti et al, 2019; Wallner-Liebmann et al, 2016).

Because saliva is produced and collected directly from the oral cavity, thus allowing the study of metabolome alterations with minimal interference, it has become the focus of much research by clinicians active in the various dentistry field.

Periodontal diseases are usually bacterial-driven inflammatory disorders, affecting tissues supporting the teeth. Disease severity ranges from reversible inflammation to chronic destruction, mainly induced by pathogenic bacteria and individual host immune reactions. The early phase of the disease is called gingivitis, and it is characterized by gingival reddening, bleeding, and swelling

(Liebsch et al, 2019; Aimetti et al, 2012). At present, standard diagnostic is based on visual examination, but there are limitations in predicting probable periodontal tissue destruction, so periodontitis is recognized only in advanced states.

Many studies (Na et al, 2021; Gawron et al, 2019; Kim et al, 2021a) have indicated that the saliva's metabolome might be a valuable tool to estimate periodontal inflammation levels. The metabolites are released in the oral cavity by bacterial metabolism or host-induced inflammatory processes: future perspective is to validate potential biomarkers that reflect the severity of disease and translate these findings into salivary diagnostic devices development. Thanks to saliva easy and safe sampling, salivary metabolomics is also very useful in managing widespread paediatric oral diseases such as caries and early dental problems. Identifying and quantifying salivary metabolites that could be utilized as biomarkers for caries susceptibility and risk assessment can overcome the limitations of other more invasive treatments (Pereira et al, 2019; Pappa et al, 2019).

Metabolomics for diagnostic purposes has been primarily used also for oral cancer detection (Khurshid et al, 2018). Oral cancer is the sixth most common cancer globally, and over 90% is represented by oral squamous cell carcinoma (OSCC). Several oral lesions, such as lichen planus and leukoplakia, are considered potentially malignant oral disorders (Wetzel & Wollenber, 2020). Metabolomics approaches could easily help understand the complex processes of progression from pre-cancer to cancer disorders. The interpretation of metabolomics alterations in oral cancer will be helpful to identify novel biomarkers for a timely diagnosis and to improve patients' survival rates.

Saliva is produced and delivered within the same anatomical site of OSCC, and it seems reasonable to hypothesize that several molecules, directly originating from neoplastic cells, are released within the salivary fluid. Based on such an assumption, it is possible to argue that the metabolic profile of saliva might differ according to the presence of neoplastic cells at a different stage.

Salivary metabolomics has been explored as a practical diagnostic and categorization tool for oral cancer. Numerous studies have investigated how salivary metabolites profiles could distinguish patients with OSCC from normal controls. Most of those studies identified potential salivary biomarkers through MS technique (Shigeyama et al, 2019; Ohshima et al, 2017; Wang Q et al, 2014a; Wang Q et al, 2014b; Ishikawa et al, 2019; Yang, et al, 2020; Ishikawa et al, 2016) while only a few have used NMR to detect pathological salivary metabolic changes (Mikkonen et al, 2018; Lohavanichbutr et al, 2018; Supawat et al, 2021). Both techniques have also been used for the discrimination of OSCC lesions from precancerous lesions, such as oral leukoplakia, oral epithelial

dysplasia, and lichen planus (Ishikawa et al, 2019; Ishikawa et al, 2020; Muthu et al,2021; Sridharan et al, 2019; Yan et al, 2008; Wei et al, 2011; Zhu et al, 2021).

The development of a non-invasive screening to discriminate pre-malignant lesions at an early stage could help dentists, and oral surgery specialists to early detect malignant transformation. Researchers also suggest that integrating both saliva and tumour tissue metabolomics could confirm the metabolites involved in oral cancers. These combined approaches could be the beginning of a clinically viable non-invasive oral cancer screening (Ishikawa et al, 2016).

Though from these studies, a variety of tumour-specific metabolites emerge, there are discrepant results, and, to date, it seems difficult to get a unique consensus. Therefore, it is necessary to perform comprehensive and more extensive studies to evaluate the numerous discriminant salivary to identify precise biomarkers for clinical application (Chen & Yu, 2019).

The final goal of the use of metabolomics for salivary biomarker discovery is to transform preliminary laboratory findings into a simple, rapid, and accurate chairside periodontal toolkit available to clinicians worldwide for periodontal and oral cancer early screening.

II. AIMS OF THE THESIS

This study aims to discover potential salivary biomarkers for early diagnosis and progression monitoring of oral diseases.

To analyse the salivary metabolites, we applied a metabolomic approach using Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy.

The current literature reports a variety of protocols for collecting and handling saliva for metabolomics studies that, makes very difficult a comparison of the results. It appears therefore the desirable definition of a standardized and more sensible protocol.

The first part of this project is dedicated to defining an efficient and reproducible protocol for preparing saliva samples for ^{1}H-NMR analysis. The results are presented in section Results IV.1.

The devised protocol is then used to characterize the metabolic profiles of three different types of saliva from healthy subjects: PS (parotid saliva), WS (whole saliva), and SM/SL (submandibular/sublingual saliva). This step is necessary to define the saliva physiologic metabolic profile. The results are presented in section Results IV.2.

The clinical assessment of the participants forming the healthy cohort, based on plaque and bleeding indexes, allows correlating the salivary metabolic profile to the periodontal health status. The results are presented in section Results IV.3.

The last part of the work is dedicated to a cohort of patients with potentially malignant disorders (PMDs), oral lichen planus (OLP), oral leukoplakia (OLK), and Oral Squamous Cell Carcinoma (OSCC). With this study we plan to characterize the saliva metabolic profile associated to those pathological conditions, as well as to highlight the metabolic changes that take place with the progression of premalignant lesions towards neoplastic conditions. The results are presented in section Results IV.4.

It is our expectation to identify promising diagnostic metabolic biomarkers of oral pathological conditions or potentially malignant disorders. The final goal is to develop an analytical protocol usable in the clinical practice for early diagnosis of risk patients: If successful, it will improve their quality of life and will reduce the mortality rate.

III. MATERIALS AND METHODS

1. SALIVA SAMPLES COLLECTION AND PREPARATION

1.1. *Subjects enrolment*

1.1.1. Healthy cohort

The study *"Valutazione comparativa tra il profilo metabolico di "saliva totale", "saliva parotidea" e "saliva sottomandibolare/sottolinguale" in soggetti sani – Studio pilota (METASAL3)"* 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 participants to the study according to the Declaration of Helsinki.

The inclusion and exclusion criteria of the study are listed in Table III.1.

Table III.1. Study inclusion and exclusion criteria

INCLUSION CRITERIA	EXCLUSION CRITERIA
Adults between 19 and 25 years of age	Hyposalivation (salivary flow less than 0.5 mL/5min)
Signed informed consent	DFMT, PSR, FMPS, FMBS scores out of normal range[a]
	Presence of systemic or oral disease impacting on dental/ periodontal tissue
	Under pharmacological therapy that could affect the salivary function
	Pregnant or lactating

a. DMFT: "Decayed, Missing and Filled Teeth" index; PSR: "Periodontal Screening and Recording" score; FMPS: "Full Mouth Plaque Score"; FMBS: "Full Mouth Bleeding Score".

The enrolment of the participants and the clinical evaluation were carried out by the group of Prof. Marco Meleti of the Centro Universitario di Odontoiatria of the University of Parma. A final cohort of twenty healthy volunteers (10 males, 10 females) was enrolled from March to June 2019. The demographic information and participants' social habits are presented in Table III.2.

Table III.2. Demographic data and participants' social habits.

	MALE (n=10)	**FEMALE** (n=10)
Age (years)	23.7 ± 1.3	23.7 ± 2.0
BMI (kg/m^2)	23.2 ± 1.4	21.3 ± 1.8
Under medication	2^a	4^b
Smokers	2	3
Social drinkers[c]	5	4

a. antihistamine therapy; b. contraceptive therapy; c. less than 7 drinks/week.

The participants underwent an interview to collect data on the general medical history and underwent a dental visit to assess the presence of inflammatory and/or infectious conditions of the oral cavity, Figure III.1.

The oral inspection was performed by carefully examining the teeth, dental support tissues (periodontal), and oral mucosa (cheeks, tongue, floor of the mouth, hard and soft palate).

The periodontal health was assessed through the "periodontal screening and recording (PSR)" index, the "full mouth plaque score (FMPS)", the "full mouth bleeding score (FMBS)" and the "decayed, missing, and filled teeth" (DMFT) index (Dhingra & Vandana, 2011; Landry, 2002), Table III.3. This index comprises the decayed, missing, and filled permanent teeth number. For example, a subject with two decayed, one missing, and one filled tooth has a DMFT of 4 (Shulman & Cappelli, 2008). Two participants with FMPS/FMBS > 25% were treated through a non-surgical periodontal session to remove the plaque and tartar before the saliva collection.

The salivary function (saliva production capacity in the unit of time, mL/min) was evaluated through the quantitative analysis of salivary flow, using the modified Saxon test (Kohler & Winter, 1985), Table III.3. One volunteer was excluded due to hyposalivation (salivary flow less than 0.5 mL/5 min).

Valutazione comparativa tra il profilo metabolico di "saliva totale", "saliva parotidea" e "saliva sottomandibolare/sottolinguale" in soggetti sani – Studio pilota.

SCHEDA RACCOLTA DATI

CODICE IDENTIFICATIVO PAZIENTE ___

Dati anagrafici

Data di nascita_____ Luogo di nascita _____

Età _____ Sesso _____

Altezza_____ Peso_____

Anamnesi prossima e remota

Patologie sistemiche _____

_____

Allergie _____

Farmaci _____

Fumo _____ se si, n/die _____ Ex fumatore _____

Consumo di bevande alcoliche _____

Intensa attività fisica nelle precedenti 12 ore _____

Esame obiettivo orale

1. Denti (indice DMFT)

Elementi cariati _____ Elementi otturati _____ Elementi mancanti (esclusi ottavi) _____

2. Tessuti parodontali (indice PSR)

Sestante 1 _____ Sestante 2 _____

Sestante 3 _____ Sestante 4 _____

Codice finale ☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4

FMBS _____ FMPS _____

Tessuti molli (presenza di lesioni)

_____

_____

Necessità di esecuzione di terapia parodontale professionale prima del prelievo salivare ed istruzione alle manovre di igiene orale domiciliari

☐ SI ☐ NO

Data di esecuzione della visita odontostomatologica

_____

Data di esecuzione di eventuale terapia parodontale professionale

_____

DATI RACCOLTA SALIVA

Data e orario del prelievo_____

Tempo necessario per raccogliere 4 mL

Prelievo parotideo _____ minuti

Prelievo sottomandibolare _____ minuti

Prelievo saliva totale _____ minuti

Figure III.1: Data collection sheet used in the study: personal data, anamnesis, and information on salivary sample collection.

Table III.3. Scores periodontal health and salivary flow of the participants to the study.

	MALE (n = 10)	**FEMALE** (n = 10)
% FMPS	12.8 ± 7.1	14.1 ± 7.5
% FMBS	2.6 ± 1.9	4.6 ± 3.4
DMFT	1.8 ± 1.3	1.3 ± 1.9
PSR	0.7 ± 0.5	0.9 ± 0.3
Salivary flow[a]	2.3 ± 1.2	2.2 ± 1.4
Teeth cleaning[b]	1	1

a. Saxon Test; b. treated with a non-surgical periodontal session.

1.1.2. Patients with oral disease

The study "*La progressione della mucosa orale normale verso la displasia epiteliale ed il carcinoma squamocellulare: analisi del profilo metabolico mediante metodica a risonanza magnetica nucleare ad alta risoluzione – magic angle spinnig - HR-MAS NMR*" was approved by the Ethical Committee of the "Area Vasta Emilia Nord" (AVEN) (protocol number: 38/2017/TESS/AUOMO - 509/2019/TESS/UNIPR). The patients were enrolled in 3 centres, two based in Modena (Dermatology Unit and Dentistry Unit, Azienda Ospedaliero Universitaria Policlinico di Modena) and one in Parma (Centro Universitario di Odontoiatria of the University of Parma). Written consent was obtained from all volunteers who participated in this study, according to the Declaration of Helsinki. The inclusion and exclusion criteria of the study are listed in Table III.4.

Table III.4. Inclusion and exclusion criteria to participate in the study.

GROUP	**INCLUSION CRITERIA**	**EXCLUSION CRITERIA**
PMDs	Clinical and histological diagnosis of PMDs	Clinical and histological diagnosis of OSCC Anemia Uncontrolled diabetes Onco-haematological disease in the last 12 months
OSCC	Clinical and histological diagnosis of OSCC	Anemia Uncontrolled diabetes Onco-haematological disease in the last 12 months
CONTROL		History of PMDs or OSCC Anemia Uncontrolled diabetes Onco-haematological disease in the last 12 months
	Signed informed consent	

PMDs: Potentially Malignant Disorders; OSCC: Oral Squamous Cell Carcinoma

The patients enrolled in this study were 46: 35 patients with oral potentially malignant disorders (of which 23 with oral leukoplakia (OLK) with different dysplasia degrees 12 with oral lichen planus (OLP)), and 11 patients with oral carcinoma. A saliva sample from one patient with oral carcinoma was discarded due to insufficient volume. Therefore, the final cohort of patients included 45 subjects.

The control group was composed of 21 healthy volunteers and was matched with the patients by sex and age. The final population of the study was made up of 66 individuals.

All participants were interviewed for the data collection on lifestyle habits (smoke and alcohol consumption) and medical history (pathology, comorbidity, and drug therapy). The study population general data are reported in Table III.5.

Table III.5. Demographic data and social habits of the participants to the study.

	OLP (n=12)	**OLK** (n=23)	**ORAL CARCINOMA** (n=10)	**CONTROL** (n=21)
Age (years)	59.3 ± 11.3	61.0 ± 15.6	65.8 ± 12.3	44.6 ± 16.4
Female (n)	7	13	6	11
Male (n)	5	10	4	10
Smokers (n, %)	3 (25%)	10 (43.5%)	9 (90%)	6 (28.6%)
Social drinkers (n, %) [a]	1 (8.3%)	1 (4.3%)	4 (40%)	6 (28.5%)

a. less than 7 drinks/week

The oral condition was evaluated by “DMFT” and “FMPS” scores. Specific clinical information regarding the lesion was collected: classification, aspect, size, and location. Clinical diagnosis was confirmed by histological analysis. The patients’ clinical data are reported in Table III.6.

Table III.6. Clinical data of patients

	Sex	Age (years)	DMFT	FMPS	Lesion site	Lesion size (cm^2)	Histological diagnosis
OSCC	M	49	13	0.89	Tongue mucosa and oral floor	4.5	OSCC
	M	57	-	-	Left retromolar trigone	15	Verrucous Carcinoma
	M	57	16	0.3	Tongue mucosa	3	OSCC
	M	78	14	0.56	Upper left maxilla	4	Infiltrating squamous cell carcinoma
	M	84	total edentulous	total edentulous	Geniena mucosa	31.5	OSCC
	F	55	total edentulous	total edentulous	Right cheek, right mandibular alveolar ridge, right retromolar trine	4	Dysplastic verrucous carcinoma on proliferative verrucous leukoplakia
	F	58	-	-	Right mand retromolar trine, right mand alveolar ridge	4	Infiltrating squamous cell carcinoma
	F	66	9	0.67	Left mandibular trine, oral floor, left cheek	3	OSCC
	F	74	-	-	Tongue mucosa	3	OSCC
	F	80	26	0.72	Oral floor	2	OSCC
OLK	M	50	16	0.81	Tongue mucosa and floor of the mouth	2	Proliferative leukoplakia with mild dysplasia
	M	53	-	-	Retromolar trigone, tongue dorsum	-	Epithelial hyperplasia with hyperkeratosis. Absent dysplasia
	M	56	-	-	Left retromolar trigone	8	Severe epithelial dysplasia
	M	64	23	0.99	Buccal mucosa	-	Leukoplakia with mild dysplasia
	M	65	-	-	Buccal mucosa	9	Epithelial hyperplasia with hyperkeratosis and notes of inflammation of the chorion. Absent dysplasia
	M	65	-	-	Tongue mucosa	-	Leukoplakia with mild dysplasia
	M	66	-	-	Hard palate	-	Hyperkeratosis and chronic inflammation of the chorion in a lichenoid-like arrangement
	M	66	8 (0-5-3)	0	Left alveolar mucosa (edentulous saddle)	2	Epithelial hyperplasia with hyperchratosis
	M	68	21 (0-9-11)	0.35	Right lingual belly	4	Low-grade lichenoid dysplasia
	M	73	-	-	Alveolar mucosa	3.5	Epithelial hyperplasia with hyperkeratosis, absent dysplasia

	F *	22	-	-	Lingual margin	-	Epithelial hyperplasia with hyperkeratosis and low-grade dysplasia
	F *	24	-	-	Lingual margin	1.5	Hyperkeratosis and microfocula of mild dysplasia of the lining epithelium. Fibrosis and notes of chronic inflammation of the lamina propria
	F	48	16	0.58	Buccal mucosa	0.8	Leucoplakia
	F	54	-	-	Free lingual gingiva at 46	18	Epithelial hyperplasia with hyperkeratosis. Absent dysplasia
	F *	61	20 (0-14-6)	0.55	Alveolar mucosa	1.5	Epithelial hyperplasia with hyperkeratosis and low-grade dysplasia
	F *	61	20 (0-14-6)	0.55	Buccal alveolar mucosa (between 14 and 15)	2.5	Epithelial hyperplasia with hyperkeratosis and low-grade dysplasia
	F *	62	20 (0-14-6)	0.55	Buccal alveolar mucosa (between 14 and 15)	1.5	Fragment of mucosa with hyperkeratosis and fibrosis of the lamina propria
	F	65	-	-	Floor of the mouth	-	Leucoplakia
	F	66	-	-	Lower labial mucosa	-	Epithelial hyperplasia with hyperkeratosis. Absent dysplasia
	F	69	-	-	Buccal alveolar mucosa (between 14 and 15)	-	Hyperkeratosis and fibrosis of the lamina propria
	F	73	-		Left lip commisura	-	Leukoplakia with mild dysplasia
	F	77	-	-	Buccal mucosa	30	Modest epithelial hyperplasia with hyperkeratosis, notes of inflammation of the chorion, absence of dysplasia
	F	96	-	-	Right mandibular fornix, paramedian	3	Proliferative verrucous leukoplakia
OLP	M	45		-	Buccal mucosa	49	Lichen planus, absent dysplasia
	M	50	-	-	Buccal mucosa	-	Lichen planus, absent dysplasia
	M	63	20 (0-20-0)	0	Buccal mucosa	8	Lichen planus, absent dysplasia
	M	63	-	-	Tongue mucosa	4	Lichen planus Graft-Versus-Host Disease related
	M	66	-	-	Lingual margin	4	Lichen planus, absent dysplasia
	F	43	-	-	Buccal mucosa	-	Lichen planus, absent dysplasia
	F	45	-	-	Buccal mucosa	-	Lichen planus, absent dysplasia

	F	59	-	-	Buccal mucosa	20	Lichen planus, absent dysplasia
	F	62	17 (0-4-13)	0.25	Left retromolar trigone	16	Lichen planus, absent dysplasia
	F	67	-	-	Buccal mucosa	-	Lichen planus, absent dysplasia
	F	70	10 (0-6-4)	0.23	Buccal mucosa	2	Lichen planus, absent dysplasia
	F	79	-	-	Buccal mucosa	-	Lichen Planus and adenosalivary ectasia

In the cohort of patients, two subjects have two / three entries in the study due to relapse. The recurrence of the lesion occurred a few months later after the removal and medical treatments. These participants are signalled (*) in Table III.6.

1.2. *Saliva collection*

1.2.1. Healthy cohort

Samples of parotid saliva (PS), submandibular/sublingual saliva (SM/SL), and whole saliva (WS) in the absence of stimulation were collected separately and sequentially for each participant to the study.

Saliva collection took place between 8 and 10 am to minimize the influence of circadian rhythm on saliva composition.

The participants were asked to refrain from eating, drinking other than water, and performing an intense physical activity for the 12 hours prior to salivary collection. Furthermore, it was requested not to carry out oral hygiene procedures (tooth brushing and flossing) during the 45 minutes preceding the collection.

The intake of alcohol, caffeine, nicotine, and drugs 12 hours prior to the salivary collection was recorded. Prior to sampling, patients rinsed their mouths with water for 1 minute.

During collection, the salivary sample was gradually transferred to cryovials containing NaN_3 (0.05% final concentration) and kept on ice (4 °C) in a portable refrigerated box until obtaining 5.4 mL of saliva. The timing required for collecting each type of saliva was recorded (Table III.7). Samples were then immediately frozen at -80 °C.

Table III.7. Mean ± standard deviation (SD) of sampling time of each salivary type

	SAMPLING TIME - mean ± SD (min)	
Saliva subtype	MALE (n=10)	FEMALE (n=10)
PS	56.2 ± 35.1	71.2 ± 23.2
SM/SL	32.1 ± 19.4	32.9 ± 10.6
WS	15.7 ± 9.8	13.3 ± 6.0

Collection of parotid saliva (PS)

The subject was placed in a supine position, with the head slightly rotated, to access the Stensen duct's area easily. Cotton rollers were placed to isolate the field. After gently cleaning the part with sterile gauze, a sterile sponge, capable of absorbing saliva flow, was placed on the Stensen duct papilla (Figure III.2a). The sponge was squeezed every 3 minutes into the cryogenic vials through a sterile syringe (Figure III.2d), until obtaining the final volume of 5.4 mL.

Collection of submandibular/sublingual saliva (SM/SL)

The subject was seated with the head and back perpendicular to the floor. The site was isolated using cotton rollers. After cleaning with sterile gauze, a sterile sponge, capable of absorbing saliva flow, was placed on the floor of the mouth (Figure III.2b). The sponge was squeezed every 3 minutes in the cryovials tube through a sterile syringe until obtaining the final volume of 5.4 mL.

Collection of whole saliva (WS)

The subject was positioned on the dental chair with the back straight, perpendicular to the floor. The participant was instructed not to swallow saliva. Subsequently, the entire amount of fluid present in the mouth was spit (spitting method) into a sterile cooled container (Figure III.2c) and transferred in the cryovials tube through a sterile syringe until 5.4 mL.

(a) (b) (c) (d)

Figure III.2: Salivary sample collection: (a) PS, (b) SM/SL, (c) WS, (d) the sponge absorbed of saliva was squeezed into cryovails containing NaN_3.

1.2.2. Patients with oral disease

The non-stimulated whole saliva (WS) sample was collected in the morning. Prior to sampling, patients rinsed their mouths with water for 1 minute. The experimental protocol did not require a fasting condition. The salivary collection lasted to reach 1 mL volume or non-longer than 15 minutes. The sample volume obtained for the patients was reduced since hyposalivation is a frequent problem related to these pathologies (Mercadante et al, 2015). The salivary sample was gradually transferred from the sterile cooled container to cryovials and kept on ice (4 °C) in a portable refrigerated box. Samples were then immediately frozen at -80 °C.

The volume of the whole saliva collected in the cohort of the healthy volunteers was at least 3 mL.

1.3. *Salivary sample preparation for ^{1}H-NMR analysis*

1.3.1. Protocol optimization

The NMR sample preparation was optimized to improve the identification and quantification of the metabolites in the different salivary types.

The sample preparation workflow is depicted in Figure III.3.

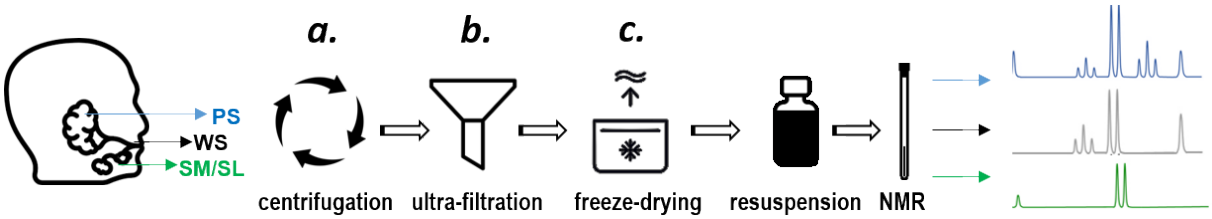


Figure III.3: Saliva sample preparation workflow: ***a*)** centrifugation to obtained the supernatant, ***b*)** ultra-filtration to remove macromolecules and debris, ***c*)** lyophilisation step. For the ^{1}H-NMR samples, the dried pellet was suspended in buffer solution containing TSP (sodium trimethylsilyl-[2,2,3,3-$^{2}H_4$]-propionate).

The protocol steps are:

a) Supernatant samples - each sample was thawed at room temperature and centrifuged at 15,000 x g for 10 min at 4 °C. The supernatant was separated from the pellet and kept on ice until use.

b) Ultra-filtered samples - supernatants were ultra-filtered using Amicon Ultra-4 Centrifugal filters (3000 MWCO, Merck Millipore) at 4,000 x g at 10 °C for 120 min. The filters were washed several times with water at 4,000 x g for 20 min to remove glycerol from the filter membrane.

c) Lyophilised samples - immediately after ultra-filtration, 3.0 mL of each sample were frozen and lyophilized overnight (Edwards, Modulyo Freeze Dryed).

To quantify the improvement of each protocol step, the SM/SL samples, the ones which contain the lower metabolic content, were prepared as follow:

1. The ultra-filtered samples (Figure III.3b) were prepared with 575 µL of the ultra-filtered supernatant with the addition of 10 µL of 1 M potassium phosphate buffer (pH 7.4) and 15 µL of 1% sodium trimethylsilyl-[2,2,3,3-$^{2}H_4$]-propionate (TSP) in deuterium oxide (D_2O) - final conditions: final volume 600 µL, 1.45 mM TSP, 2.5% D_2O in 16 mM phosphate buffer.

2. The lyophilized samples (Figure III.3c) were prepared by dissolving the dried pellets in 600 µL of 50 mM phosphate buffer (pH 7.4), containing 1.45 mM TSP and 2.5% D_2O.

TSP was used as chemical shift reference (0.00 ppm) and quantitative internal standard.

The analytical results are described in section IV. Results - 1. *Sample optimization for saliva ^{1}H-NMR metabolic profiling.*

1.3.2. ^{1}H-NMR sample preparation of healthy and oral disease cohorts

The PS, SM/SL, and WS saliva ^{1}H-NMR samples from the study of 20 healthy subjects were prepared using the complete protocol illustrated in Figure III.3.

The ^{1}H-NMR samples of the patients were prepared using some modifications to the protocol. The WS was 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 at 10 °C for 60 min. All the samples reached a final ultra-filtered volume of at least 800 µL, except 4 samples whose final volume was lower than 600 µL. 800 µL of ultra-filtered saliva (n=62) and the total volume available for the 4 outliers were frozen and lyophilized overnight.

After lyophilization, the pellets were dissolved in 200 µL of a 48.8 mM phosphate buffer solution (pH 7.4) containing 1.45 mM TSP and 2.5% D_2O. The samples were loaded in 3 mm outer diameter NMR tubes.

1.4. ***Saliva samples cell count***

Eukaryotic cells (oral epithelial cells and leucocytes) and prokaryotic cells were counted in accordance with Gardner and colleagues (Gardner et al, 2018).

The eukaryotic cells were counted on the same collection day. To 20 µL of each saliva sample was added 20 µL of 0.4% Trypan blue (Sigma-Aldrich, Poole, Dorset, UK) and observed with an inverted microscope (Nikon Eclipse TS100) in a counting chamber (100× magnification) at the laboratory of Centro Universitario di Odontoiatria.

After sample thawing, the prokaryotic cells were counted by heat-fixing 2 µL of sample to a glass slide and stained with Gram staining (Coico, 2005). The samples were viewed at 100× magnification with an optical microscope (Nikon Eclipse 80i) at the Laboratorio di Morfologia of the Department of Medicine and Surgery of the University of Parma. The ratio between the area of one field of view and the whole sample area was calculated, and data were reported as numbers of cells for mL of saliva.

2. ^{1}H-NMR ACQUISITION AND PROCESSING

One dimensional ^{1}H-NMR spectra were acquired at 25 °C with a JEOL 600 MHz ECZ600R spectrometer at the Interdepartmental Measurement Centre "*Giuseppe Casnati*" (CIM) of the University of Parma.

The first increment of the 1DNOESY pulse sequence was used for the acquisition, with 128 scans, a spectral window of 20 ppm, 128 k points, and a relaxation delay of 5 seconds.

The spectra were processed and analysed with Chenomx NMR suite 7.6 software (Chenomx Inc., Edmonton, Alberta, Canada), applying zero-filling to 256 k points, line broadening of 0.5 Hz, and baseline correction using Whittaker spline function.

3. IDENTIFICATION AND QUANTIFICATION OF THE METABOLITES

The metabolites identification and quantification were carried out using Chenomx NMR suite 7.6 software (Chenomx Inc., Edmonton, Alberta, Canada). This software contains a spectral reference library of over 300 metabolites belonging to several metabolic pathways and different biological classes (alcohols, polyols, amides, amines, amino acids, carboxylic acids, hydroxyl acids, drug components, monosaccharides, disaccharides, purine, pyrimidines, food components, etc.). Chenomx is a semi-automated tool for metabolite profiling. It uses the fit of individual library compounds, through the interactive superimposing of the metabolite peaks, upon the experimental spectrum peaks. The concentration of each metabolite was estimated by comparing the area under the metabolite's peaks with the TSP peak, whose concentration is known.

To calculate the total metabolite content of each salivary sample, the spectral areas were manually integrated using MestReNova 11.0 software MestReNova 11.0 software (Mestrelab Research, Santiago de Compostela, Spain). This measurement also considers the areas of those peaks that have not been identified with the Chenomx software.

4. STATISTICAL ANALYSIS

After the profiling of all spectra, it was considered, for the statistical analysis, only the metabolites whose concentration was higher than 5 µM for one saliva subtype.

The statistical analyses were performed using the MetaboAnalystR 5.0 platform (https://www.metaboanalyst.ca).

The metabolites concentrations were normalized using the internal standard (TSP) and scaled prior to analysis. The normalization step is necessary: *i)* when metabolites in the different samples have

significant concentration variations to reduce systematic biased variation, *ii)* to separate biological variation from the ones introduced with the experimental process, and *iii)* to improve the performance of the downstream statistical analysis. The scaling step was performed using autoscaling method, which employs the standard deviation as the scaling factor. The variance across the multiple metabolites and the correlation patterns are illustrated by the heatmaps generated by the MetaboAnalystR 5.0 platform. The colour gradient from warm-to-cool represents the variation in the metabolite concentration, with the warm colours representing high concentration and the cool colours representing low ones.

OriginPro8.5 (OriginLab Corporation, Northampton, MA, USA) was used for graphics creation.

4.1. ***Univariate analysis***

To compare the metabolite composition of each saliva subtype, univariate statistical analysis was conducted, applying the non-parametric upper-tailed Mann–Whitney test (Origin 2019 software) and considering results with a $p \leq 0.05$ statistically significant.

A volcano plot was employed to compare the size of the fold change to the statistical significance level. It reports the *p*-value as a negative log on the y-axis and the fold-change between two conditions on the x-axis. The values set as thresholds were fold change greater than 2.0 and p-value higher 0.05.

4.2. ***Multivariate analysis***

Unsupervised multivariate analysis, Principal Component Analysis (PCA), was performed using the PCA module of MestreNova 11.0 software (Mestrelab Research, Santiago de Compostela, Spain).

PCA provides an overview of the overall variability and reduces data dimension. The related score plot summarizes sample clustering patterns projected into three dimensions to explain the maximal variance of data (Vu et al, 2019).

Supervised multivariate statistical analysis was performed using Partial Least Squares Discriminant Analysis (PLS-DA) on MetaboAnalystR 5.0.

PLS-DA reduces the number of the variables, and it finds a predictive model that describes the direction of maximum covariance between a dataset and the class membership.

The VIP (Variable Importance in Projection) score gives the measurement of the variable's importance in the PLS-DA model. The VIP scores estimate the importance of each metabolite in

groups clustering, summarize the contribution that this makes to the model and show the most efficient metabolites in the classification (Vu et al, 2019).

The predictive ability of the metabolites as a biomarker was tested through the non-parametric analysis of the receiver operating characteristic (ROC). For each identified metabolite was calculated, a ROC curve and the selection of the potential biomarkers was based on the highest AUC (area under the curve) and with an acceptable confidence level.

AUC score gives the probability that a discriminant can distinguish between the different classes: when AUC is 1.0 the model can distinguish completely, whereas there is no difference from random chance when AUC is 0.5. AUC is widely used to compare different biomarker models (Xia et al, 2013).

IV. RESULTS

1. SAMPLE OPTIMIZATION FOR SALIVA ^{1}H-NMR METABOLIC PROFILING

The use of ^{1}H-NMR metabolomics analysis of saliva has been exploited mainly for clinical biomarker discovery in numerous disorders (Gardner et al, 2020a), including systemic pathologies, such as head and neck cancer (Mikkonen et al, 2018), dementia (Figueira et al, 2016), glioblastoma (García-Villaescusa et al, 2018), as well as in some oral states such as paediatric oral health (Pereira et al, 2019) and chronic periodontitis (Romano et al, 2018). Noticeably, all these studies have been performed on whole saliva (WS). Although most of the metabolites in parotid saliva (PS) and submandibular and sublingual saliva (SM/SL) are mainly host-derived with low contamination by exogenous and microbial molecules, studies in selected gland saliva types are limited. This is partially due to the low concentrations of metabolites in PS and especially in SM/SL. Low NMR sensitivity has represented a significant obstacle in investigating the metabolome of saliva from different glands. Therefore, the use of NMR for identifying and quantifying salivary gland metabolites requires the development of a reproducible sample preparation protocol specifically designed to quantify low concentration metabolites.

Though a wide variety of sample preparation methods have been used in previous studies (Gardner et al, 2018), nonetheless it appears evident the need for a standardized method that may allow a reliable comparison of the results obtained in the laboratories worldwide. Because saliva macromolecules can affect the quality of NMR spectra and interfere with metabolite identification and quantification it is necessary to remove cellular debris, bacteria, and high molecular weight proteins. Dame and colleagues (Dame et al, 2015) added an ultra-filtration (3kDa filters) step after the centrifugation. Ultrafiltration is very reproducible and does not generate side reactions with biofluid metabolites (Psychogios et al, 2011). Gardner and colleagues (Gardner et al, 2018) evaluated the effect of a freeze-thaw cycle in the preparation of whole saliva samples.

In the present study, we develop a reproducible saliva preparation protocol designed to identify and quantify low concentration metabolites and suitable for the three saliva subtypes: WS, PS, and SM/SL. This method involves ultra-filtration for protein depletion and introduces a freeze-drying step before the final sample dissolution: this final step overtakes the limited NMR sensibility by increasing the native metabolites' concentration. Note that the freeze-drying step had already been reported for metabolomics studies performed with the LC-MS/MS technique (Fernandez-Peralbo et al, 2015) producing an enhancement of the number of detected metabolites. Nonetheless, though

the freeze-drying step significantly increases the sensitivity of the measure, whether it is carried out by NMR or LC-MS/MS, it is affected by a critical drawback: the loss of volatile metabolites. The scheme of the proposed protocol for saliva samples preparation for NMR metabolic profiling is depicted in Figure III.3 (Section III. Materials and Methods).

1.1. ***Analytical validation of the protocol***

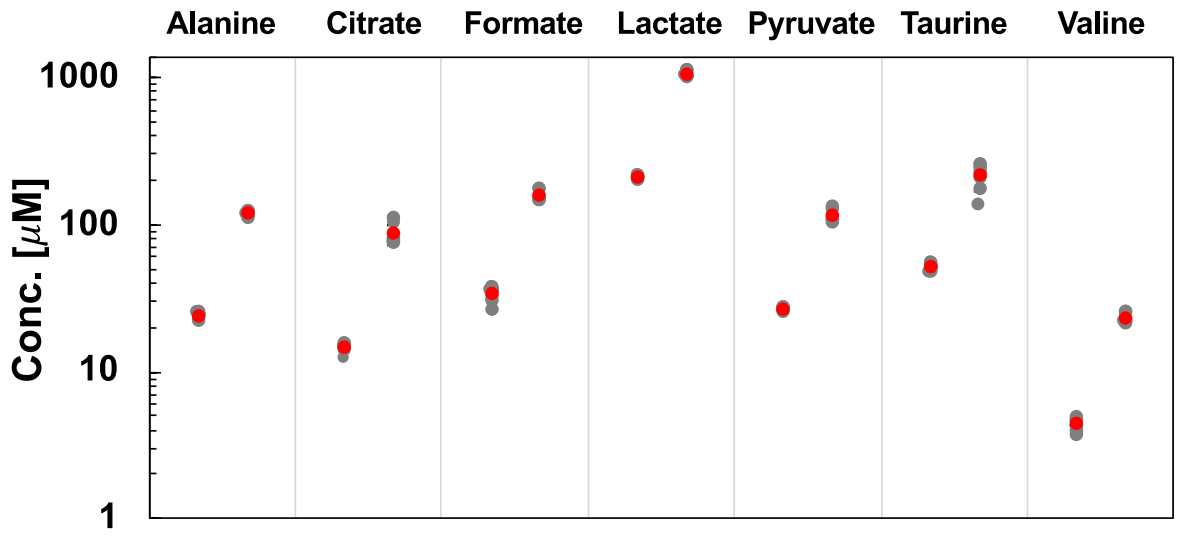
Once the sample preparation protocol is defined, the next step is the analytical validation. This step is necessary to test the technique's performance and the method's capacity to provide solid and reproducible results (Naz et al, 2014; Rao, 2018). The analytical validation of our protocol was carried out by analysing a pool of SM/SL saliva, the most diluted saliva subtype, collected from three different healthy persons. The rationale was that, if the protocol is reliable on the most diluted saliva type, it should also be suitable for WS and PS where metabolites are already more concentrated. For the pooled SM/SL sample we evaluated, as relevant overall performance indicators, the following parameters:

- Precision
- Accuracy
- Limit of detection (LOD)
- Limit of quantification (LOQ)

- *Precision*

Precision is the degree of agreement among results of individual measures performed on multiple samplings. It is expressed as the relative standard deviation (coefficient of variation) of a series of measures.

The SM/SL pool was split into two aliquots. The first aliquot was used to prepare three equivalent ultra-filtered saliva samples (**F**). The second aliquot was used to prepare three equivalent ultra-filtered and lyophilized saliva samples (**L**). Three 1D ^{1}H-NMR spectra were acquired of each sample, obtaining nine replicates of NMR spectra of the **F** and nine replicates of the **L** samples. We selected a set of seven metabolites with different salivary concentrations: alanine, citrate, formate, lactate, pyruvate, taurine, and valine, with concentrations ranging from 4.7 to 187.3 µM in **L** samples, and we measured their NMR concentrations in each spectrum. Results are shown in Figure IV.1.



	Alanine		Citrate		Formate		Lactate		Pyruvate		Taurine		Valine	
	F	L	F	L	F	L	F	L	F	L	F	L	F	L
Mean [μM]	24.4	119.9	14.8	86	34.6	158	211.2	1073	26.8	116	52.3	216	4.4	23.1
SD [μM]	0.9	5.4	0.9	14	3.7	13	3.2	60	0.7	11	2.8	30	0.4	1.8
RSD %	4	5	6	16	11	8	2	6	3	9	5	14	8	8

Figure IV.1. Precision evaluated for a set of selected metabolites in **F** and **L** samples. Each independent measurement is shown in grey, while the mean value of the nine measurements is in red. In the table below are indicated the mean concentrations (Mean), standard deviations (SD) and relative standard deviation (RSD %) values.

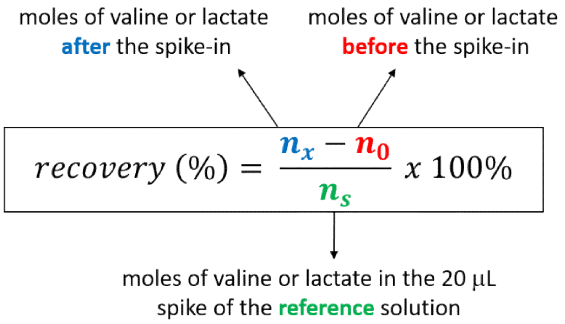
Quantification of the seven selected metabolites leads to a precision of 5 ± 3% for **F** samples and 9 ± 4% for **L** samples, expressed as the relative standard deviation (RSD %).

In addition, for the seven selected metabolites, the ratio between the mean concentrations obtained in the **L** samples with respect to those obtained for the **F** samples turn out to be 4.9 ± 0.6, in agreement with the expected 5-fold increase in concentration upon the lyophilization step.

- *Accuracy*

The accuracy indicates how close the experimental results are to the real value. The accuracy of our protocol was estimated by using the recovery test (defined as = [(method result/ true value) x 100]) for two selected metabolites with different salivary concentrations: valine and lactate (23.1 ± 1.8 µM and 1073 ± 60 µM, respectively). A 20 µL of a reference solution containing known amounts of lactate and valine was added to an SM/SL sample obtained following the complete protocol. The metabolites' concentration before and after the addition of the reference solution was measured using the ^{1}H-NMR methyl signals of both metabolites.

The accuracy was calculated using the following equation:



The experiment was performed in triplicate (Table IV.1).

Table IV.1. Recovery test results

Metabolite	Exp. No.		n_0 [nmol]	n_x [nmol]	n_x-n_0 [nmol]	ns [nmol]	Recovery [%]	Average [%]	SD [%]
Valine	1		13.1	24.1	11.0	10.4	105	**103**	**2**
	2		15.0	25.5	10.5	10.4	101		
	3		15.8	26.6	10.8	10.4	104		
Lactate	1		602.4	983.9	381.5	354.5	108	**106**	**2**
	2		627.2	993.3	366.1	354.5	103		
	3		681.2	1057.8	376.6	354.5	106		

The recovery value obtained is 103 ± 2 % for valine and 106 ± 2 % for lactate. These results show that our method has a good level of accuracy.

- *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The method's sensitivity was evaluated by determining the limits of detection and quantification, parameters related to the smallest concentration of a metabolite that can be reliably measured (Shrivastava & Gupta, 2011). Limit of detection (LOD) is the lowest concentration of a metabolite in a sample that can be detected but not necessarily quantitated under stated experimental conditions. Limit of quantification (LOQ) is the lowest metabolite concentration in a sample which can be quantitatively determined with suitable precision and accuracy.
LOD and LOQ were calculated based upon the IUPAC standard (Shrivastava & Gupta, 2011):

$$LOD = X_N + 3\ SD_N$$
$$LOQ = X_N + 10\ SD_N$$

X_N = intensity of the NMR spectra noise
SD_N = SD of the NMR spectra noise

In our experimental conditions, for the ultra-filtered (**F**) samples, the LOD is 2.5 μM while the LOQ is 5.5 μM. In contrast, for the ultra-filtered and lyophilized (**L**) samples, the corresponding LOD and LOQ values are 0.5 and 1.1 μM, respectively, reflecting the increase of sensitivity obtained by adding the lyophilization step.

The strategy of introducing an additional ultra-filtration step after the centrifugation allows the improvement of the quality of the ^{1}H-NMR spectra by eliminating broad peaks of macromolecules and increasing the signal-to-noise ratio. This step enabled the identification and quantification of 22 metabolites with concentrations ranging from 6 μM to 2.75 μM in a reference SM/SL sample, in comparison with 13 metabolites identified in the same sample, centrifuged but not ultra-filtered.

The following freeze-drying step increases the method's sensitivity by a factor of five, as demonstrated by the LOQ and LOD values obtained in the **F** vs. the **L** samples in the analytical validation of the method. We were able to quantify 47 metabolites, with corresponding concentrations as low as 1.1 μM, in the reference SM/SL sample.

Lyophilization is a conservative process that does not introduce additional peaks or lateral reactions but involves inevitably loss of volatile metabolites (such as methanol, ethanol, and acetone). In addition, the freeze-drying step produces a decrease in precision (~ 9% vs. ~ 5% in the **L** vs. the **F** samples). However, the two-fold increase in quantified metabolites compensates for these drawbacks.

The application of our protocol allowed the characterization of the metabolic profile of the three saliva subtypes (WS, PS, and, for the first time, SM/SL) for a group of 20 healthy subjects (Meleti et al, 2020), quantifying 58 ± 2, 57 ± 2, and 48 ± 2 (mean ± SD) metabolites in WS, PS, and SM/SL, respectively.

In summary, we highlight that the saliva preparation protocol here proposed can generally be applicable in all studies that explore the metabolome of different saliva subtypes: it allows to get a standard operating procedure, which is reproducible, accurate, and sensitive for metabolites quantification. This protocol is suitable for all the saliva subtypes, including PS and SM/SL for which most metabolites are assumed to be host derived thus endowed with high diagnostic power.

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Sample optimization for saliva ^{1}H-NMR metabolic profiling

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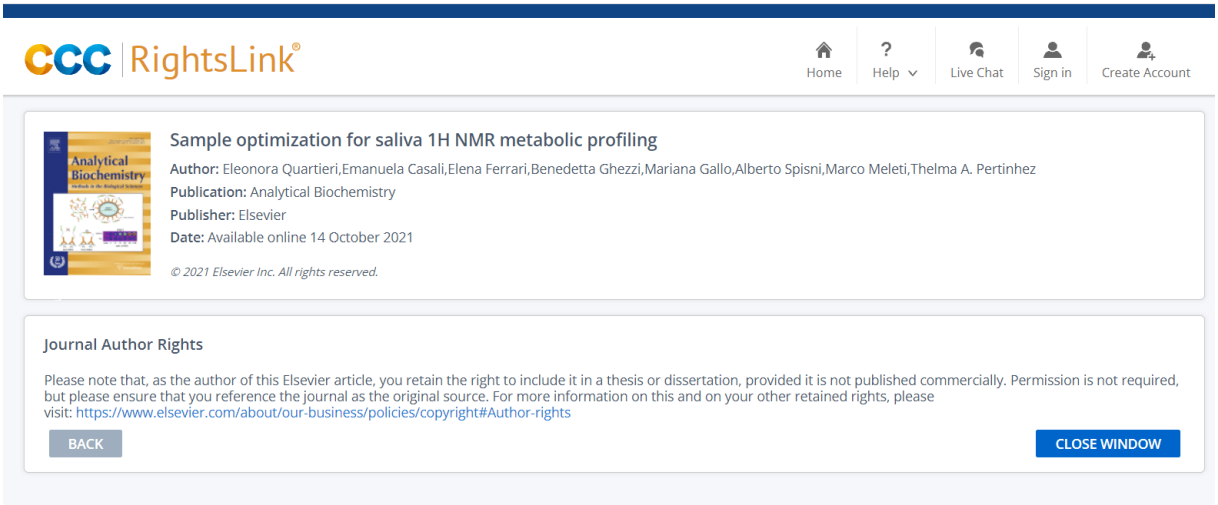

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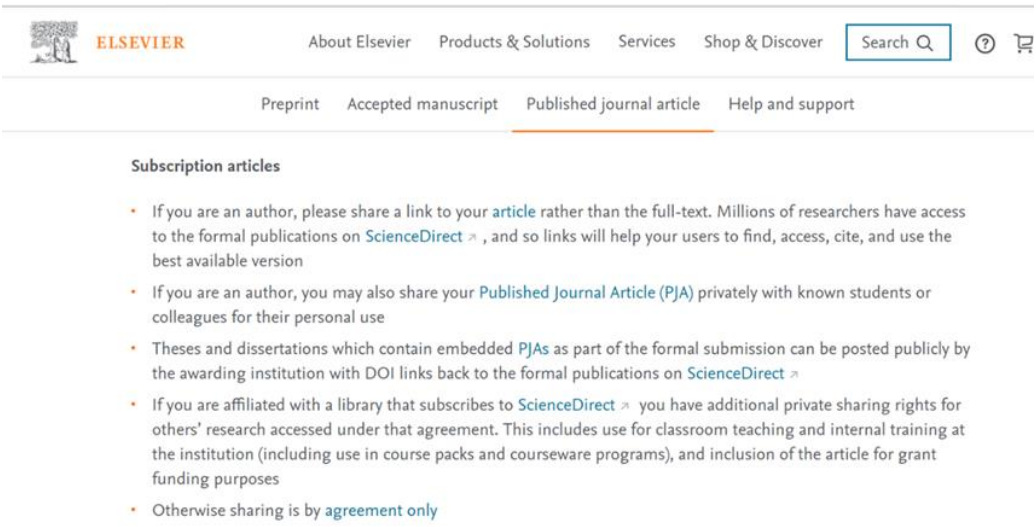

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Sample optimization for saliva ^{1}H-NMR metabolic profiling☆

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ABSTRACT

Nuclear Magnetic Resonance (NMR) based metabolomic analysis of whole saliva has provided potential diagnostic biomarkers for numerous human diseases contributing to a better understanding of their mechanisms. However, a comprehensive interpretation of the significance of metabolites in whole, parotid, and submandibular/sublingual saliva subtypes is still missing. Precision and reproducibility of sample preparation is an essential step. Here, we present a simple and efficient protocol for saliva ^{1}H-NMR metabolic profiling. This procedure has been specifically designed and optimized for the identification and quantification of low concentration metabolites (as low as 1.1 μM) and is suitable for all the saliva subtypes.

Hydrogen Nuclear Magnetic Resonance (^{1}H-NMR) has been extensively used for metabolomic studies on biofluids. Initially, metabolomic research on biofluids focused primarily on blood and urine. In recent years, saliva has been the subject of an increasing number of metabolomic investigations. In fact, even though saliva composition is complex and variable, it is sensitive to metabolic changes, and therefore has a significant potential as a diagnostic fluid [1–3]. Furthermore, saliva exhibits some advantages including simple, inexpensive, and non-invasive collection, ease of multiple sampling and is more respectful of privacy than urine. Those features are driving the search of salivary biomarkers of diseases and the development of saliva based diagnostic tests [4–6].

Saliva contains electrolytes, immunoglobulins, proteins, enzymes, nucleic acids, and metabolites [7–9]. It is known that protein concentration in salivary fluid is approximately 10 times lower than in serum (salivary protein concentration ranges from 0.72 to 2.45 mg/mL[10]). However, an investigation regarding the total metabolites concentration in the different saliva subtypes is still missing.

The term "whole saliva" (WS) refers to a clear mixture of fluids produced by major and minor salivary glands as well as gingival crevicular fluid. Salivary glands contribute to WS in different percentages: 20% parotid, 73% submandibular and sublingual, and less than 10% is due to minor glands secretions [8]. Most of the current metabolomics studies deal with WS because of its easy sampling. Few studies are available on parotid saliva (PS), while submandibular and sublingual saliva (SM/SL) metabolomic investigations turn out to be virtually absent in the literature, likely due to the very low metabolites concentration. Notably, since gland contamination by exogenous and microbial molecules is less probable, most of metabolites in PS and SM/SL are host derived. Recognizing the high scientific and diagnostic impact of the salivary metabolomic research, the knowledge of an accurate qualitative and quantitative metabolic profile of each saliva subtype will be valuable for biochemical studies and effective biomarkers identification.

^{1}H-NMR based metabolomics analysis of WS has provided reliable biomarkers for numerous diseases. Those studies include systemic conditions, such as head and neck cancer [11], dementia [12], glioblastoma [13], as well as oral states in paediatric oral health [14] and in chronic periodontitis [15]. However, the use of ^{1}H-NMR for the identification of salivary gland metabolites as biomarkers requires the development of a reproducible sample preparation protocol specifically designed for the identification and quantification of low concentration metabolites. Bertram et al. without including practically any treatment to saliva, could identify only 16 metabolites in their samples [16]. Duarte and collaborators, using 1D and 2D NMR experiments, identified ca. 50 metabolites, after sample centrifugation. Nevertheless, the authors highlight the presence of broad signals of proteins that makes

☆ 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.

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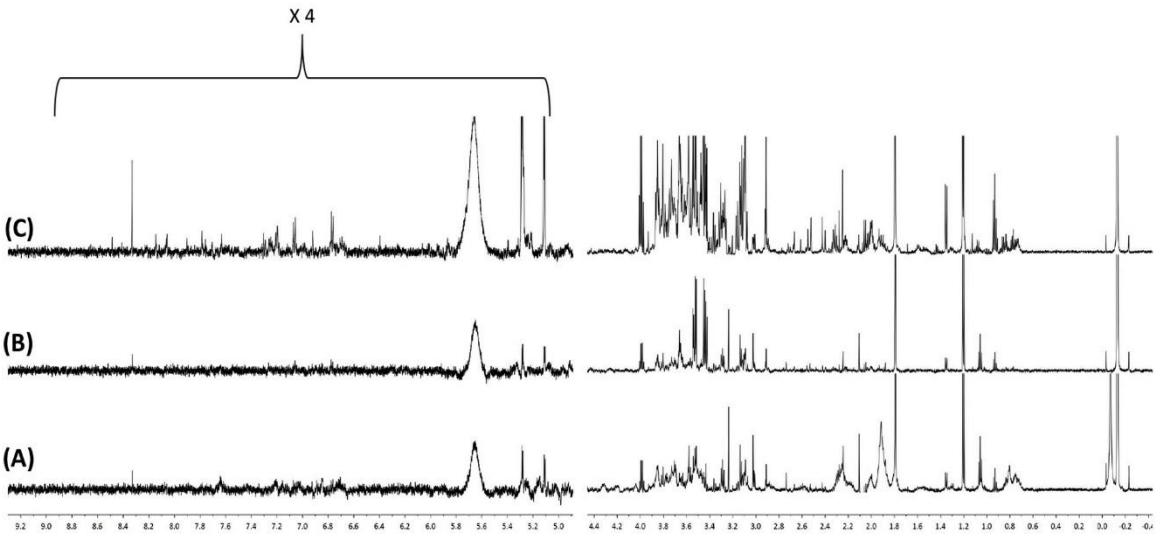

Fig. 1. ^{1}H-NMR spectra obtained with **(A)** supernatant, **(B)** ultra-filtered, and **(C)** ultra-filtered and lyophilised SM/SL saliva samples originated from a single subject. The aromatic region (left panel) is enhanced by a factor of 4.

metabolites quantification difficult [17]. Finally, Gardner and co-workers have evaluated the effect of salivary sample centrifugation in combination with a freeze-thaw cycle, validating a successful protocol for metabolite quantification in WS [18]. In the present study, we improved that protocol with the introduction of ultra-filtration and freeze-drying, before the final sample dissolution, to deplete proteins and increase metabolites concentration. Here we detail this procedure, highlighting the quality improvement of ^{1}H-NMR spectra and the progressive metabolites enrichment of the sample, two features that synergically contribute to enhance the detection of low concentration metabolites. Serum is a widely used biofluid for NMR metabolomics studies and serum sample preparation methods are well validated [19, 20]. Therefore, we evaluated the metabolites content of the three saliva subtypes of 20 healthy donors and compared the results with those obtained for serum samples of the same subjects, used as a reference in terms of the NMR spectra quality and of the number of metabolites.

Saliva preliminary treatment

To illustrate the steps of the proposed saliva preparation method, we select SM/SL from a single subject, since it is the subtype with the lowest metabolites content.

Non-stimulated saliva were collected to avoid metabolite's dilution [21].

During collection, the salivary sample was gradually transferred to cryovials containing NaN_3 (0.05% final concentration) and kept on ice. The final volume of saliva was 3.6 mL. According to most of the protocols reported in the literature, we froze at - 80 °C the sample immediately after collection. For the analysis, the saliva sample was thawed at room temperature and centrifuged at 15000×*g* for 10 min at 4 °C; supernatant was separated from the pellet and kept on ice until use. Centrifugation allows a substantial removal of cellular debris, bacteria, mucins, and high molecular weight aggregates [18].

Saliva supernatant sample produces low quality NMR spectrum

For NMR analysis, saliva supernatant sample was prepared as follows: 10 μL of 1 M potassium phosphate buffer (pH 7.4) and 15 μL of 1% 3-trimethylsilyl propionic acid (TSP) in D_2O were added to 575 μL of the salivary supernatant, reaching a final volume of 600 μL (1.45 mM TSP, 2.5% D_2O in 16 mM phosphate buffer). TSP was used as a reference for chemical shift (0.00 ppm) and quantitative internal standard.

One dimensional ^{1}H-NMR spectra were acquired at 25 °C with a JEOL 600 MHz ECZ600R spectrometer using the first increment of the 1DNOESY pulse sequence, 128 scans, sweep window of 20 ppm, 128 k points and relaxation delay of 5 s [22]. The spectra were processed and analysed with Chenomx NMR suite 8.3 software (Chenomx Inc., Edmonton, Alberta, Canada), zero-filling to 256 k points, and line broadening 0.5 Hz.

The saliva supernatant spectrum turned out to be noisy and contained broad signals attributable to residual salivary proteins (Fig. 1A). The broadened signals severely interfere with metabolites identification and quantification. In fact, in this sample, we were able to quantify only 13 metabolites.

Improving NMR spectra quality: the ultra-filtration step

To eliminate the interfering proteins, we introduced an ultra-filtration step, previously suggested by Dame et al. [23]. Thus, the saliva supernatant was ultra-filtered using Amicon Ultra-4 Centrifugal filters (3000 MWCO, Merck Millipore) at 4000×*g* at 10 °C. Filters were previously washed several times with water at 4000×*g* to remove glycerol from the filter membrane. In Fig. 1B is reported the ^{1}H-NMR spectrum of the ultra-filtered SM/SL sample prepared as described above. The disappearance the proteins broad signals improves the quality of the NMR spectrum enabling the identification and quantification of 22 metabolites, ranging from 6.0 μM to 2750.0 μM (vs. 13 metabolites identified in the supernatant saliva sample). For example, we could identify and measure the concentration of glutamate and citrate (42.6 μM and 25.2 μM), which were undetectable in the supernatant sample because of the masking effect of broad protein signals.

Increasing sensitivity: the freeze-drying step

Low NMR sensitivity represents a limit when used to analyse diluted solutions such as saliva, especially SM/SL. To reduce this limitation, we added a freeze-drying step immediately after ultra-filtration. Briefly, 3.0 mL of ultra-filtered SM/SL were frozen and lyophilised, and finally dissolved in 600 μL of 50 mM phosphate buffer (pH 7.4), containing 1.45 mM TSP and 2.5% D_2O. The result is a five-fold increase in metabolites concentration. Indeed, the spectrum in Fig. 1C now allows the quantification of 47 metabolites, with corresponding concentrations as

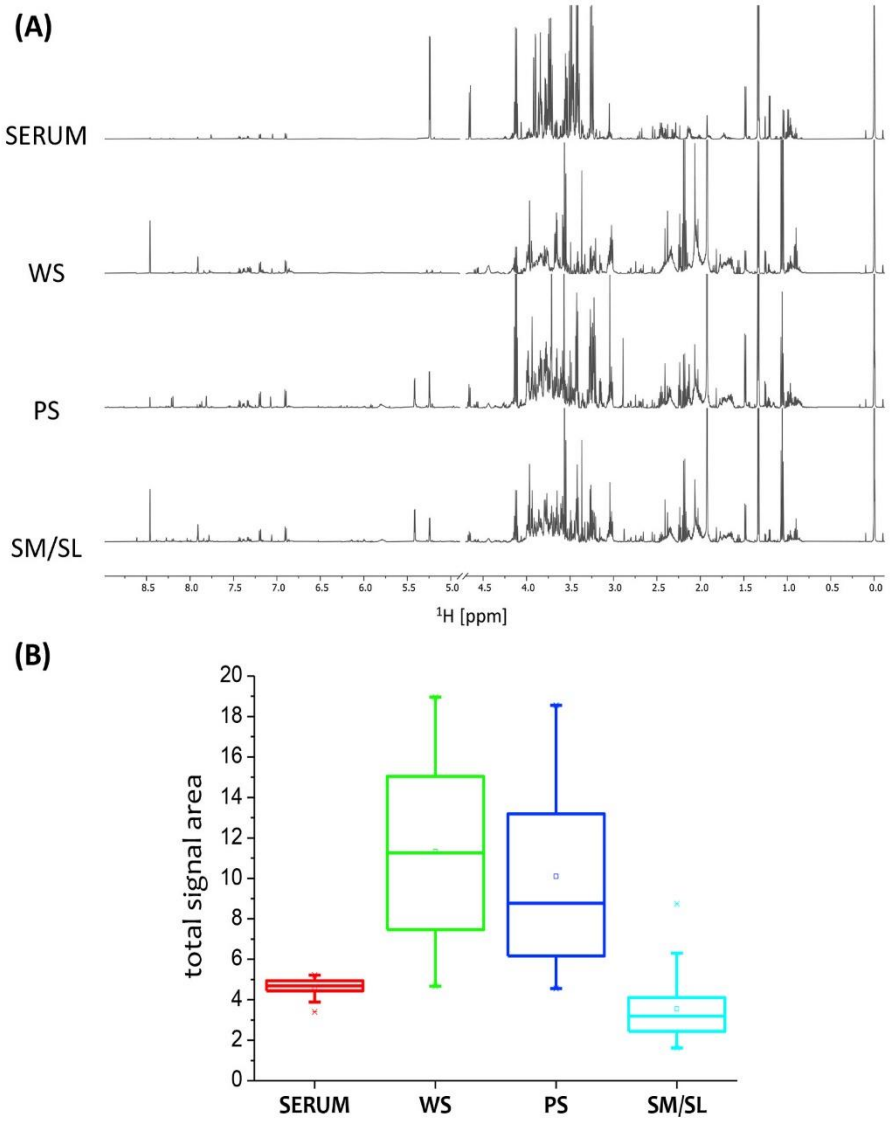

Fig. 2. (A) ^{1}H-NMR spectra of serum, WS, PS, and SM/SL samples obtained from the same subject using the complete proposed protocol. **(B)** Total signal area distribution of the ^{1}H-NMR spectra (n = 20) of WS, PS, and SM/SL ultra-filtered and lyophilised samples. Serum signals area distribution is included as a reference.

low as 1.1 μM in the original SM/SL. Overall, this step increases the metabolites identification by a factor of two with respect to the ultra-filtered saliva and enables the quantification of metabolites undetectable in the ultra-filtered sample, such as the aromatic amino acids phenylalanine and tyrosine. It must be acknowledged that volatile metabolites (such as methanol, ethanol, and acetone) are inevitably lost during the lyophilisation process. Besides this limit, the whole procedure turned out to be conservative and did not introduce additional peaks nor produced side reactions within metabolites.

Furthermore, as reported in Supplementary Material Section S1, the calculated precision and accuracy demonstrate that the protocol is reproducible and accurate, while the LOQ and LOD values evidence its sensitivity.

Evaluation of spectra quality and total metabolite content of the three salivary subtypes

WS, PS, and SM/SL were collected separately from 20 healthy subjects (Supplementary Material Section S2), as previously described [24]. Blood samples from the same donors were also collected and prepared as reported in Ref. [25].

Following the complete protocol here presented, we prepared WS, PS, and SM/SL saliva samples for all the donors. Fig. 2A illustrates the NMR spectra of the three saliva subtypes and serum from one of them. It can be appreciated that the quality of the saliva spectra is comparable to the one of serum. The analysis of the 20 subjects allowed the identification and quantification of 58 ± 2, 57 ± 2, and 48 ± 2 (mean ± SD) metabolites in WS, PS, and SM/SL samples, respectively (Supplementary Material Section S3), while 52 ± 1 metabolites were detected in the corresponding serum samples (data not shown).

To estimate the total metabolite content of saliva and to compare it with the corresponding serum, we used the spectral area of the whole NMR spectrum, which is related to the total observed metabolite concentrations. We used MestReNova 11.0 software (Mestrelab Research, Santiago de Compostela, Spain) to calculate the integral of each spectrum and normalised it by the signal area of the internal standard TSP. The box plots in Fig. 2B show the signal area distributions obtained for each salivary subtype and for serum of the 20 donors. WS and PS

distributions are indicative of a similar total metabolite content and a comparable large variability. The metabolite content of the SM/SL sample is less variable, though markedly lower than WS and PS, with an estimated content ratio of 1:1:0.3 (WS:PS:SM/SL). It is worth noting that the use of our protocol leads to a total metabolite content of WS and PS higher than the one of serum. Even if in SM/SL samples the metabolite total content remains lower than serum, our protocol allows the identification of a significant number of metabolites (48 ± 2).

In this study, we present a method for salivary samples preparation suitable for ^{1}H-NMR metabolic investigations. The introduction of an ultra-filtration and a freeze-drying step overcomes the limited metabolites content of all saliva subtypes. The application of this protocol allowed the characterisation of the metabolic profile of the three saliva subtypes (WS, PS, and, for the first time, SM/SL), for a group of healthy subjects [24]. In this way, we intend to contribute to the definition of a standardised protocol that may enable the comparison of data obtained from saliva samples prepared in different laboratories. We expect that the possibility to reliably identify and quantify low concentration metabolites will favour the discover of biomarkers with diagnostic value.

Author contributions

Eleonora Quartieri - data acquisition.

Emanuela Casali - design, data interpretation, drafted and critically revised the manuscript.

Elena Ferrari – design, data interpretation, drafted and critically revised the manuscript.

Benedetta Ghezzi - sample collection.

Mariana Gallo - data interpretation, statistical analysis, drafted and critically revised the manuscript.

Alberto Spisni – critically revised the manuscript.

Marco Meleti – critically revised the manuscript.

Thelma A. Pertinhez - Conceptualization, data interpretation, and critically revised the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2021.114412.

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2. ^{1}H-NMR METABOLOME OF WHOLE, PAROTID, AND SUBMANDIBULAR/SUBLINGUAL SALIVA FROM HEALTHY VOLUNTEERS

In the last years, the interest in the use of saliva as a biofluid for the discovery of disease-specific biomarkers and the elucidation of metabolic alterations occurring in physiological conditions (e.g., effects of physical exercise, weight changes, smoking) is increasing (Takeda et al, 2009; Pitti et al, 2019). However, the saliva composition is subjected to various factors, including sex, age, circadian cycle, diet, drug effects, stress, oral health, and oral microflora.

The intra-individual variability of whole saliva metabolic composition seems to be smaller when the interindividual variability is considered (Wallner-Liebmann et al, 2016), this observation applies also to the saliva proteome (Jehmlich et al, 2013). Overall these findings lead to the hypothesis that, under standardized conditions, an individual phenotype is relatively stable.

The metabolic characterization of WS in physiological conditions has been extensively investigated by ^{1}H-NMR spectroscopy (Gardner et al, 2018, and cited references). The metabolome of PS has been partially explored (Figueira et al, 2017; Gardner et al, 2019; Rovera et al, 2021), while SM/SL has never been systematically described. Yamada-Nosaka and co-workers (Yamada-Nosaka et al, 1991) recorded broad and low-resolution ^{1}H-NMR spectra of SM/SL. Gardner and colleagues (Gardner et al, 2020a) compared the ^{1}H-NMR spectra obtained from PS and SM/SL, both without the ultrafiltration step, and stated that both salivae showed similar metabolic composition in this condition.

WS metabolome derives from endogenous sources, host oral microorganisms (De Filippis et al, 2014; Takahashi, 2015; Gardner et al, 2019), and exogenous origins (Wallner-Liebmann et al, 2016). On the contrary, PS and SM/SL metabolomes are less affected by microorganism's contaminations, and the majority of their metabolites are likely host-derived (Figueira et al, 2017; Gardner et al, 2019).

The knowledge of the metabolic profiles of the different salivary subtypes, WS, PS, and SM/SL from the healthy adults can provide important information to the interpretation of pathological and physiological changes that characterize each salivary gland.

The metabolic composition of unstimulated WS, PS, and SM/SL derived from a cohort of 20 young and healthy volunteers are presented in Table IV.2. The mean concentration reported is the amounts quantified above the limit of quantification determined by our method (1.1 μM), (see Results IV.1).

Table IV.2. Metabolites, mean concentrations ± SD and concentration ranges measured in the three saliva types.

Metabolite	WS (µM)	PS (µM)	SM/SL (µM)
β-Alanine	NQ	4.2 ± 2.1 [1.1-9.1]	1.9 ± 0.8 [1.1-3.4] (15)
1,7-Dimethylxanthine	NQ	4.6 ±4.7 [1.3-22.8]	NQ
2-Aminoadipate	226.5 ± 130.8 [77.2-530.3]	38.7 ± 35.7 [2.4-117.3]	17.3 ± 19.0 [2.0-92.8]
2-Hydroxy-3-methylvalerate	10.9 ± 7.3 [1.6-24.7]	NQ	NQ
2-Hydroxybutyrate	15.7 ± 10.9 [1.4-53.1]	7.9 ± 2.5 [4.3-12.8]	2.6 ± 1.3 [1.5-6.5] (17)
2-Hydroxyisovalerate	2.3 ± 0.9 [1.1-4.1] (16)	2.6 ± 1.2 [1.4-5.8] (16)	NQ
3-Hydroxybutyrate	6.6 ± 3.4 [1.9-14.5]	8.2 ± 4.4 [3.7-20.1]	4.3 ± 2.2 [1.8-10.7]
3-Hydroxyisobutyrate	NQ	2.4 ± 0.8 [1.2-4.1] (19)	NQ
3-Methylglutarate	18.9 ± 11.1 [1.6-38.0] (17)	NQ	NQ
3-Methylxanthine	NQ	5.0 ± 3.1 [1.7-14.4] (18)	2.9 ± 1.9 [1.3-7.9] (17)
3-Phenylpropionate	13.2 ± 10.2 [2.0-38.1]	NQ	NQ
4-Hydroxyphenylacetate	8.8 ± 5.6 [1.9-19.0]	NQ	NQ
4-Hydroxyphenyllactate	7.9 ± 6.3 [2.2-23.8] (19)	NQ	NQ
5-Aminopentanoate	168.8 ± 104.2 [34.0-386.3] (15)	26.2 ± 25.8 [1.6-82.2] (19)	NQ
Acetate	2667.2 ± 1123.6 [734.1-4322.8]	676.4 ± 730.6 [81.8-3145.0]	382.0 ± 329.4 [54.0-1370.4]
Alanine	61.1 ± 59.1 [9.4-212.6] (16)	81.6 ± 40.2 [41.5-219.1]	24.8 ± 15.4 [8.1-74.9]
Alloisoleucine	6.2 ± 3.3 [1.9-14.0] (17)	3.4 ± 1.4 [1.7-7.5]	NQ
AMP	NQ	9.4 ± 5.6 [3.7-26.2] (19)	4.0 ± 2.6 [1.1-12.7] (19)
Anserine	NQ	NQ	1.9 ± 0.7 [1.1-3.5] (18)
Arginine	NQ	27.4 ± 14.9 [5.9-56.3]	
Aspartate	29.3 ± 18.4 [9.8-76.7]	29.4 ± 18.6 [7.9-70.4]	7.6 ± 3.8 [1.8-18.7]
Betaine	2.1 ± 0.7 [1.1-3.6] (17)	7.6 ± 5.4 [1.5-17.7] (19)	2.4 ± 1.8 [1.2-7.9] (15)
Butyrate	30.1 ± 23.6 [3.2-77.3]	6.1 ± 4.1 [1.5-14.8] (19)	5.0 ± 3.8 [1.1-18.6]
Cadaverine	NQ	5.8 ± 5.1 [1.2-17.6] (16)	N.Q.
Caprylate	NQ	NQ	5.5 ± 3.05 [1.6-13.0]
Carnitine	5.6 ± 3.5 [2.1-15.3] (17)	NQ	4.1 ± 2.1 [1.7-10.6] (19)
Choline	7.5 ± 4.6 [2.6-17.2]	9.7 ± 5.8 [3.2-21.8]	3.4 ± 1.5 [1.3-6.9] (19)
Citrate	14.9 ± 8.9 [2.9-33.0] (18)	43.7 ± 25.4 [16.0-125.8]	28.8 ± 29.6 [5.9-146.9]
Creatine	15.7 ± 12.6 [3.4-49.4]	53.1 ± 21.5 [23.7-116.7]	19.1 ± 9.1 [6.4-48.1]
Creatinine	5.7 ± 2.7 [2.1-12.7] (18)	5.9 ± 2.8 [2.3-14.1]	4.0 ± 1.8 [2.1-9.6] (19)
Ethanolamine	28.4 ± 25.9 [4.0-115.2]	45.5 ± 48.9 [3.8-185.6]	8.7 ± 7.9 [1.5-36.6] (19)
Ethylene glycol	NQ	3.8 ± 2.9 [1.1-11.9] (16)	NQ
Formate	60.3 ± 54.7 [8.7-234.0]	27.2 ± 26.8 [6.8-106.7]	24.3 ± 19.6 [6.5-97.9]
Fucose	61.2 ± 73.4 [11.5-275.8]	16.0 ± 18.3 [1.5-57.9] (19)	6.6 ± 4.4 [2.4-16.3]
Fumarate	NQ	2.9 ± 1.0 [1.3-4.5]	NQ
Galactose	37.1 ± 44.3 [10.2-173.9]	NQ	NQ

Glucose	23.2 ± 28.9 [6.8-137.8]	256.7 ± 180.0 [81.8-697.8]	63.1 ± 47.1 [7.6-211.8]
Glutamate	109.9 ± 63.0 [30.6-250.1]	79.8 ± 54.9 [27.7-224.0]	23.8 ± 15.4 [8.6-71.2]
Glutamine	46.6 ± 35.7 [6.6-134.6]	72.5 ± 36.3 [9.2-151.4]	26.5 ± 14.7 [2.3-70.6]
Glycerol	107.1 ± 30.7 [30.1-152.0]	102.3 ± 29.3 [59.5-170.1]	53.5 ± 10.8 [23.9-72.5]
Glycine	133.3 ± 104.0 [12.1-306.6]	83.1 ± 64.4 [3.6-218.8]	22.4 ± 17.5 [4.5-76.5]
Glycolate	NQ	NQ	12.2 ± 12.3 [1.8-55.4]
Histidine	24.3 ± 13.8 [4.1-49.9]	23.2 ± 17.4 [7.8-70.7]	7.6 ± 4.8 [2.9-21.1]
Homoserine	NQ	47.6 ± 32.3 [11.3-158.4] (18)	
Hypoxanthine	4.9 ± 4.5 [1.4-18.1] (19)	16.8 ± 12.0 [2.4-48.2]	5.1 ± 2.4 [1.2-9.5] (18)
Indole-3-lacate	2.7 ± 1.2 [1.2-5.1] (17)	NQ	NQ
Isobutyrate	21.2 ± 11.5 [3.7-47.6]	NQ	NQ
Isocaproate	9.2 ± 6.0 [1.8-22.7]	NQ	NQ
Isoleucine	10.4 ± 12.2 [1.2-44.2] (19)	5.5 ± 3.5 [2.0-13.4]	NQ
Isovalerate	9.5 ± 6.6 [1.1-21.1] (17)	NQ	NQ
Lactate	156.3 ± 121.5 [23.7-517.5]	798.7 ± 335.6 [408.8-1683.9]	187.3 ± 97.0 [84.0-444.5]
Lactose	30.5 ± 24.1 [3.8-115.4]	NQ	26.3 ± 13.6 [3.4-55.5]
Leucine	20.3 ± 16.1 [3.5-56.4]	14.5 ± 7.0 [6.3-30.7]	5.5 ± 2.1 [2.1-9.5]
Lysine	73.5 ± 44.2 [17.6-164.8]	31.4 ± 23.4 [10.1-97.5]	8.3 ± 7.9 [2.3-37.9]
Malonate	7.6 ± 5.1 [1.9-19.0]	6.8 ± 5.6 [1.4-19.1] (15)	NQ
Maltose	7.8 ± 12.9 [1.2-52.8] (18)	404.5 ± 336.3 [103.5-1587.9]	95.4 ± 85.6 [16.4-420.4]
Methionine	NQ	NQ	2.5 ± 0.8 [1.2-4.2] (19)
N-Acetylglucosamine	52.9 ± 42.5 [2.2-141.5]	NQ	14.2 ± 12.7 [1.4-40.8]
O-Phosphocholine	NQ	10.4 ± 4.0 [4.7-20.5]	
O-Phosphoethanolamine	34.5 ± 18.9 [6.5-67.6]	130.2 ± 49.6 [45.7-251.2]	59.2 ± 24.4 [24.8-123.8]
Phenylacetate	18.0 ± 12.0 [4.2-46.2]	NQ	2.1 ± 0.7 [1.1-3.3] (15)
Phenylalanine	23.5 ± 13.1 [6.4-48.6]	14.3 ± 9.5 [5.0-41.1]	5.4 ± 2.7 [2.0-12.7]
Proline	152.1 ± 138.1 [29.1-446.8]	55.1 ± 44.1 [10.8-156.0]	11.4 ± 15.5 [2.9-74.4]
Propionate	313.8 ± 167.7 [64.7-627.9]	48.6 ± 49.6 [4.4-200.8]	28.1 ± 32.5 [4.3-151.7]
Putrescine	45.7 ± 29.7 [8.5-96.4]	8.2 ± 7.3 [1.2-27.3] (19)	NQ
Pyroglutamate	16.8 ± 16.5 [2.9-70.5]	NQ	7.6 ± 3.1 [3.3-14.7]
Pyruvate	21.7 ± 12.7 [4.0-52.8]	35.7 ± 55.6 [1.4-232.0]	5.9 ± 3.2 [2.1-14.2]
Sarcosine	4.6 ± 2.5 [1.4-10] (19)	4.5 ± 2.8 [1.4-10.1] (18)	NQ
sn-Glycero-3-phosphocholine	NQ	5.0 ± 3.3 [2.2-13.7] (17)	NQ
Succinate	17.8 ± 8.2 [8.5-39.2]	13.2 ± 6.1 [3.8-23.3]	5.6 ± 2.3 [2.2-11.8]
Taurine	50.9 ± 30.3 [2.8-132.0]	169.6 ± 90.5 [35.6-342.2] (18)	65.3 ± 39.4 [3.3-146.9]
Trimethylamine	NQ	8.6 ± 4.6 [1.4-16.5] (15)	
Threonine	8.1 ± 7.8 [2.4-31.5]	NQ	8.5 ± 4.4 [2.9-22.3]
Tyrosine	43.6 ± 24.6 [10.5-93.5]	34.0 ± 19.8 [14.4-90.9]	12.1 ± 6.1 [3.0-30.8]
Uracil	6.3 ± 5.6 [1.7-19.8]	6.7 ± 5.3 [1.7-20.5]	NQ
Uridine	NQ	4.1 ± 2.3 [1.2-8.3] (17)	NQ
Valine	16.2 ± 16.0 [2.9-59.2]	15.2 ± 9.1 [3.0-38.0]	4.7 ± 2.7 [1.1-13.1] (19)
Xanthine	6.3 ± 5.0 [1.3-22.2]	11.7 ± 11.3 [1.8-43.9]	7.6 ± 7.6 [1.7-27.1] (19)
Xanthosine	NQ	2.8 ± 1.1 [1.4-4.8] (18)	NQ

a) In square brackets are indicated concentration range.

b) When the metabolite was not found in all the 20 subjects, the number of donors in which it was identified is indicated in round brackets. Only the metabolites present in at least 15 subjects (≥ 75% occurrence) have been considered.

c) The acronym NQ means that the metabolite in that type of saliva was not quantified.

The study allowed the quantification of 58 ± 2, 57 ± 2, and 48 ± 2 (mean ± SD) metabolites in WS, PS, and SM/SL samples, respectively.

Most of the metabolites quantified in this study (Table IV.2) were discussed in the publication Meleti et al "Metabolic Profiles of Whole, Parotid, and Submandibular/Sublingual Saliva" (presented forward).

Some additional data is reported in Figure IV.2. The heatmap of the metabolites derived from oral mucosa cells evidences the differences among the three salivary subtypes. PS shows a higher concentration of the metabolites: a possible explanation is that parotid saliva sampling takes a long time and, consequently, causes greater stress on the mucous membranes and epithelial tissue. This hypothesis is supported by results showing that a higher concentration of metabolites derived from cell membrane phospholipids, such as O-phosphocholine, O-phosphoethanolamine, ethanolamine, and glycerol, have been detected (Mayr et al, 2015).

Duarte et al. have investigated the metabolites alterations derived from the oral and microbial metabolisms at different temperatures and duration of storage (Duarte et al, 2020). Based on those data, we can state that the concentration of metabolites was preserved even in the PS collection time in our sampling conditions.

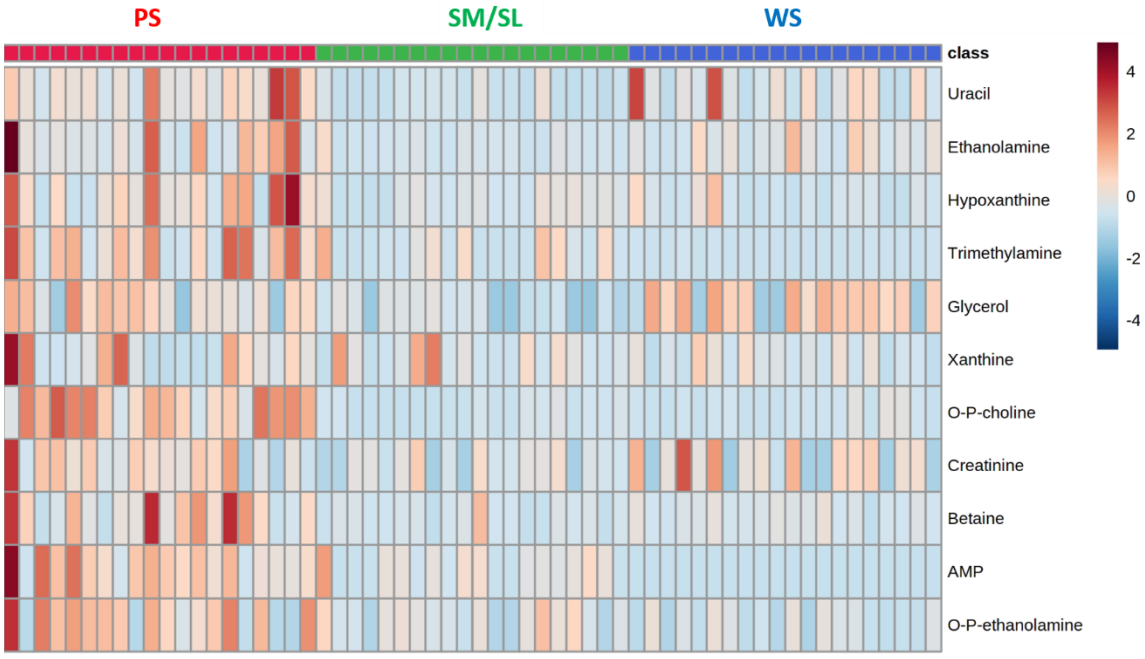

Figure IV.2. Heatmap analysis of selected metabolites: PS (red), SM/SL (green), and WS (blue) samples. The brightness of each colour corresponds to the magnitude of the difference when compared with the average value.

To further stress the diagnostic and prognostic importance of a reliable and reproducible qualitative and quantitative metabolic analysis of the various type of saliva it is worth to outline that among the metabolites quantified in salivary samples, a good number have been indicated as candidates for the diagnosis of inflammatory processes (Kuboniwa et al, 2016), or markers of aging (Teruya et

al, 2021). Sakanaka and co-workers (Sakanaka et al, 2017) reported a gradual decrease of WS ethanolamine levels with the increasing of periodontal inflammation severity and, in parallel, meta transcriptomics study suggested its involvement in the transition of the commensal microbial community to a dysbiotic microflora (Duran-Pinedo et al, 2015). Furthermore, it has been proposed that the purine degradation metabolites (hypoxanthine and xanthine) were increased in the gingival fluid of subjects with periodontal disease (Barnes et al, 2009).

In summary, recognizing that mapping the human salivary metabolome of each salivary gland is essential to understand most of the physiologic oral metabolic pathways, to translate these findings in the research of salivary biomarkers for oral and systemic diseases, we believe that the protocol we have developed has appropriate characteristics to become a standard protocol for the qualitative and quantitative analysis of the metabolome of salivary glands PS and SM/SL and the WS.

Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva.

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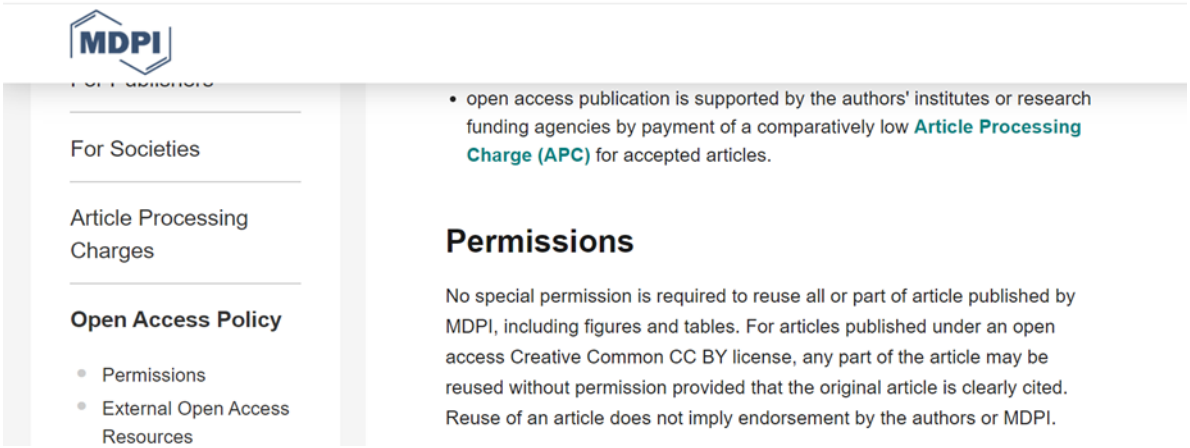

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Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

2. Results

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

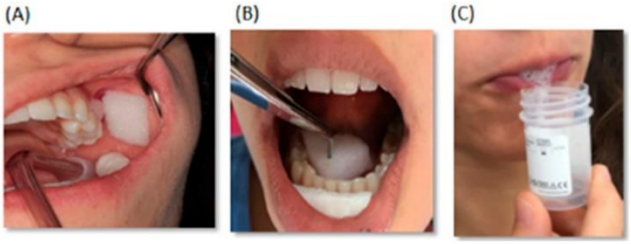

Figure 1. Experimental set-up used for parotid (**A**), submandibular/sublingual (**B**), and whole saliva (**C**) collection.

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

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

2.1. Metabolite Content of Saliva Subtypes: Emerging Differences

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

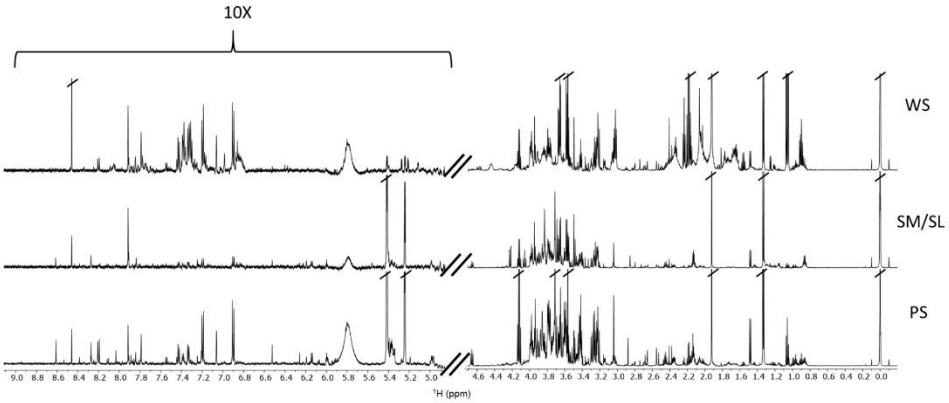

Figure 2. ^{1}H-NMR spectra of unstimulated WS, PS, and SM/SL saliva from the same participant, acquired at 25 °C. The left region of the spectra shows the vertical scale increased by a factor of 10.

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

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

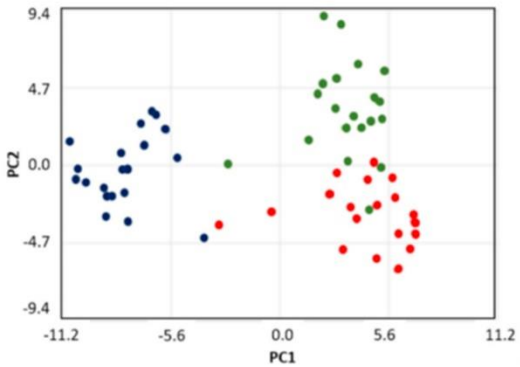

Figure 3. PCA score plot of ^{1}H-NMR spectra of whole saliva (blue), parotid saliva (red), and submandibular/sublingual saliva (green) samples. The contribution of the three principal components of the total variance are PC1 = 62.6%, PC2 = 6.8%, and PC3 = 5.1%.

2.2. Salivary Metabolites

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

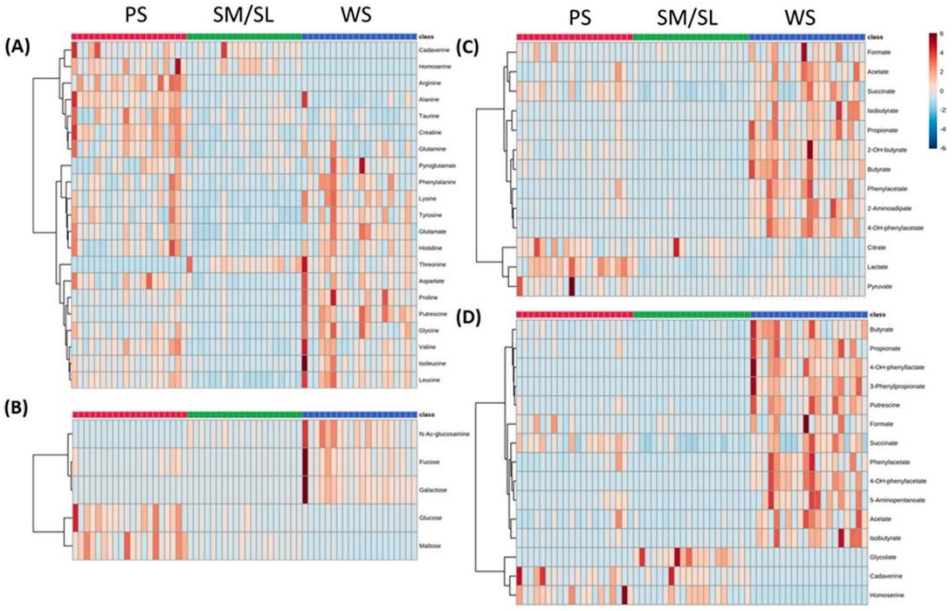

Figure 4. Heatmap analysis of metabolites grouped according to the categories: (**A**) amino acids, (**B**) carbohydrates, (**C**) organic acids, and (**D**) selected prokaryotic metabolites. PS (red), SMS (green), and WS (blue) samples.

2.3. Classes of Metabolites

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

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

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

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

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

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

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

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

2.4. Salivary Cell Count

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

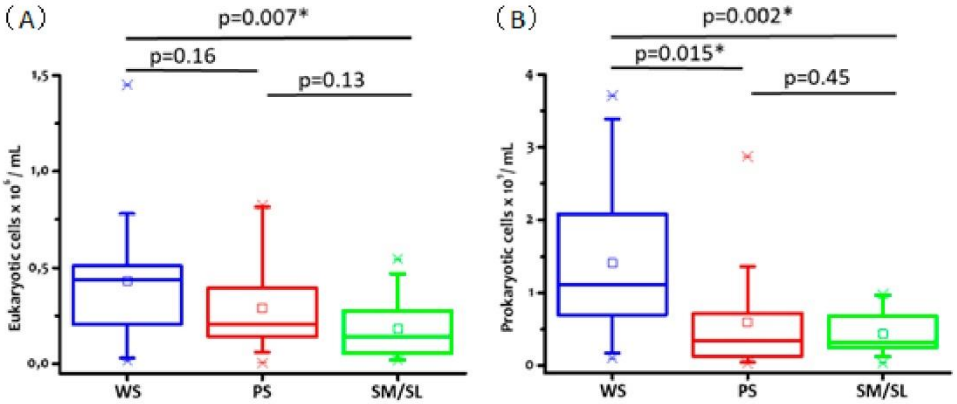

Figure 5. Eukaryotic (**A**) and prokaryotic (**B**) cell count distributions obtained with the three salivary types of all the subjects of the study. The significance level of the independent t-tests is set at 0.05 (* $p < 0.05$).

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

3. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Materials and Methods

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

4.1. Subjects of the Study

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

4.2. Saliva Collection

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

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

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

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

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

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

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

4.4. Cell Counting

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

4.5. Statistical Analysis

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

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

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

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3. WHOLE SALIVA BIOMARKERS IN EARLY DIAGNOSIS OF ORAL INFLAMMATION

Periodontal diseases are among the most common chronic inflammatory diseases (Pihlstrom et al, 2005). In a recent study carried out on a Northern Italy urban adult population (Aimetti et al, 2015), the estimated prevalence of severe periodontitis was 34.94% and the moderate one of 40.78%.

The onset and development of these pathologies are mainly due to an abundant and varied bacterial flora normally present in the oral cavity (Hill, 1987; Kumar, 2005; Colombo et al, 2009). The symbiotic bacteria consortium on oral surfaces produces a biofilm known as dental plaque (Kilian et al, 2016), which propitiates a wide range of inflammatory conditions affecting the periodontal tissues (gingiva, periodontal ligament, alveolar bone, cement), necessary as teeth support structures.

The cause of periodontitis is multifactorial and includes genetic, epigenetic, hormonal changes, lifestyle, and environmental factors (Page & Kornman, 1997; Tatakis & Kumar, 2005; Kilian et al, 2006).

The early phase of periodontal disease is gingivitis, characterized by a non-specific bacterial plaque accumulation at the gum line of the teeth. The release of toxins by the plaque affects the gingiva and triggers an inflammatory host response. The progression of this condition can drive to periodontitis, which is distinguished by tissue breakdown, bone destruction, pocket formation, and gingival recession, with consequent tooth loss (Barnes, 2011), Figure IV.3.

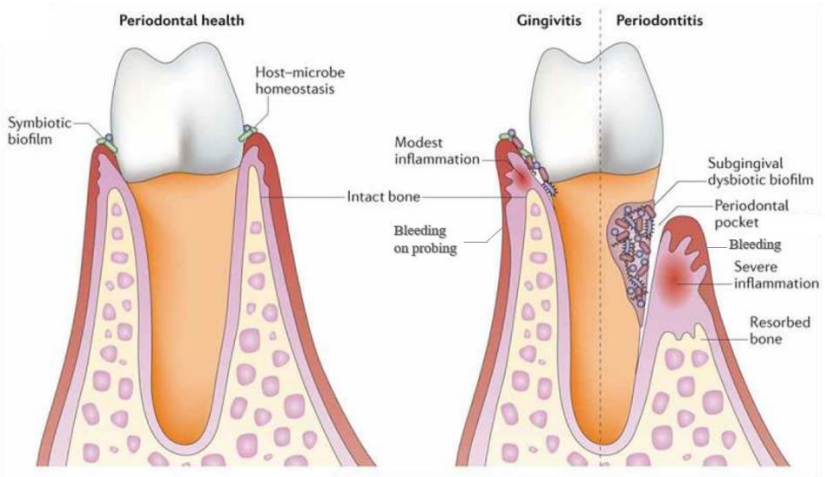


Figure IV.3. Progression of host-microbe symbiosis from health to gingivitis and to periodontitis, associated with a dysbiotic condition (from Hajishengallis, 2015).

Periodontitis (chronic or aggressive) onset and propagation are infection-driven inflammatory processes. The accumulation of dental plaque initiates an inflammatory host-response that causes

a dysbiosis of the commensal oral microbiota, leading to a hyper- or hypo responsiveness and/or a lack of inflammatory responses regulation: this leads to severe consequences in the periodontium of susceptible individuals (Könönen et al, 2019; Loos et al, 2020; Suárez et al, 2020).

The persistence of the inflammatory state and consequent pocket formation create a favourable environment for bacterial survival and spreading, promoting the shift to a dysbiotic microflora (Van Dyke et al, 2020), Figure IV.4.

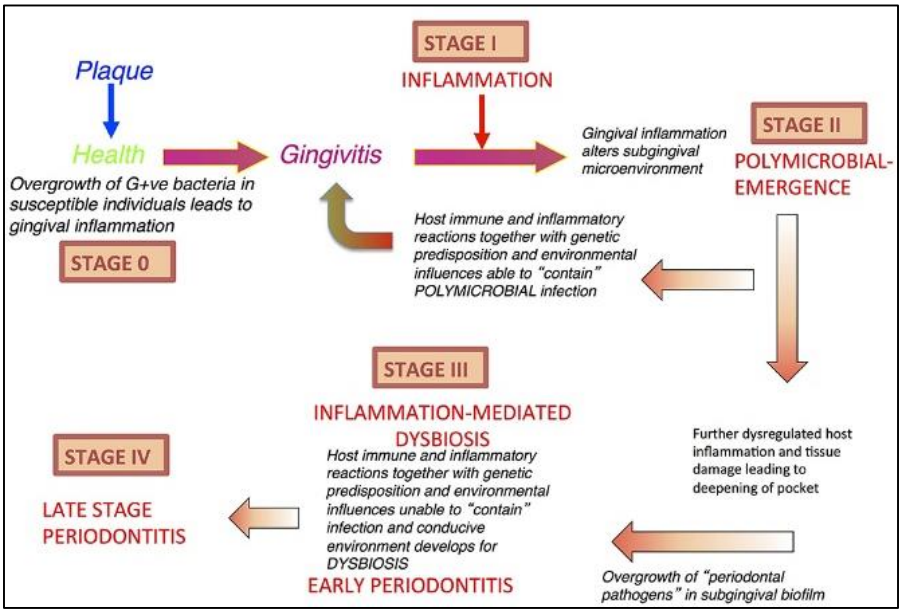


Figure IV.4. Proposed model of the 5 stages that drive inflammation-mediated dysbiosis to plaque-associated periodontitis (from Van Dyke et al, 2020).

The homeostasis imbalance between host and oral microflora generates a self-feeding cycle of dysbiosis and inflammation escalation, Figure IV.5.

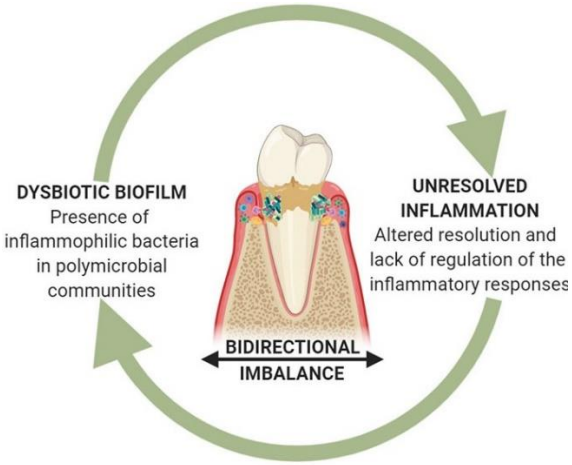


Figure IV.5. Bidirectional imbalance in periodontal diseases (Suárez et al, 2020).

In a dysbiotic biofilm, a variety of Gram-negative anaerobic species is present such as *Pophyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Peptostreptococcus micros*, *Prevotella intermedia*, *Treponema forsythia* (Niederman et al, 1997; Paster et al, 2000).

The colonization and proliferation of *Porphyromonas gingivalis* in the periodontal gingival pockets are associated with chronic periodontitis. This anaerobe bacterium releases toxic factors such as lipopolysaccharide (a bacterial endotoxin that induces the host immune reaction), gingipains (a cysteine protease that affects the composition of oral biofilm and promotes colonization), fimbriae/pili (filamentous structures which promote bacterial adhesion to periodontal surfaces) (Mysak et al, 2014). In addition, those events are associated with the changes in the oral cavity metabolome (Gawron et al, 2019).

Metabolomics approach

Salivary metabolomics is particularly useful for oral diseases diagnosis, from gingivitis to periodontitis (Zhang CZ et al, 2016; Javaid et al, 2016). This approach is primarily exploited to understand the complex pathogenesis of such oral diseases (Mikkonen et al, 2016; Kouznetsova et al, 2021; Tsuchida, 2020).

Due to the variation of saliva composition in response to pathological processes (bacterial metabolism, host-induced inflammatory response), there is a growing interest to analyse human saliva for early diagnosis of oral diseases (Lee & Wong, 2009; Gardner et al, Jan 2020; Liebsch et al, 2019).

Gingivitis can present different levels of severity, from early signs of inflammation, such as redness or swelling, to bleeding upon gentle probing (Klukowska et al, 2015). During the clinical assessment, bleeding on probing is the primary parameter to set the thresholds for gingivitis.

FMPS and FMBS assessed the periodontal health status of our cohort of healthy subjects to evaluate plaque and bleeding indexes (section III. Material and Methods). The obtained indexes were associated with their respective salivary metabolomes to identify specific metabolites that could be related to early stages of gingival inflammation (pre-clinical stage of gingivitis) and, therefore, suited to develop a salivary-based screening test for gingivitis.

Results

The low scores index of FMBS and FMPS reflect the good health condition of the participants. FMPS ranged from 2.8 to 24.8%, with a mean value of 13.5 ± 7.1 %, and FMBS index from 0 to 11.4% with a mean value of 3.8 ± 2.8% (Table III.3, section Materials and Methods).

The FMPS index did not correlate with the metabolites identified in WS or with the number of prokaryotic cells (data not shown). Conversely, the correlation between WS's number of bacterial cells and the bleeding index FMBS is r= 0.62 p<0.01. This value was considered significant since this association is calculated only for the aerobic bacteria population. Note that the count of prokaryotic cells in WS access only the aerobic bacterial colonies since the anaerobic colonies are resident in subgingival plaque (Mayanagi et al, 2004).

The cohort was divided into two subgroups: FMBS < 4% (n=12) and FMBS > 4% (n=8), with respect to FMBS mean value.

Partial Least Squares-Discriminant Analysis (PLS-DA) was performed on the whole saliva metabolite database (Table IV.2). The score plot of PLS-DA indicates the separation in two clusters, corresponding to the two groups of FMBS index, Figure IV.6.

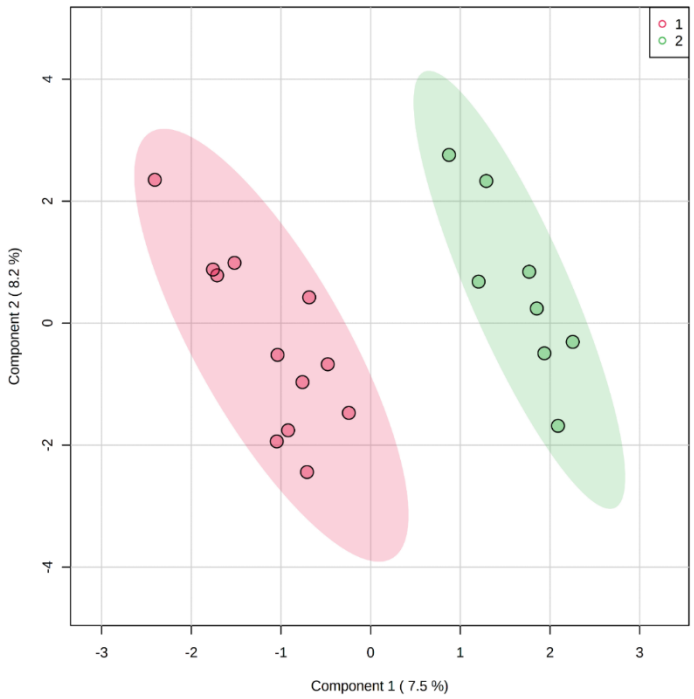
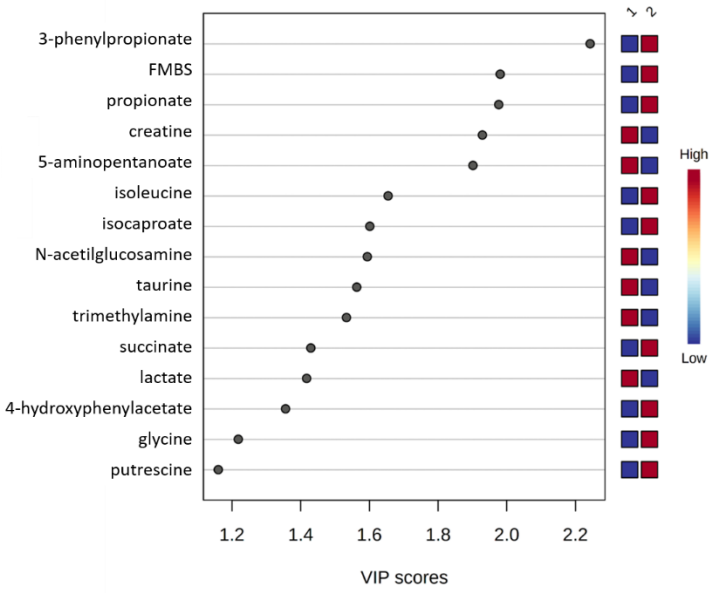


Figure IV.6. Score plot of Partial Least Squares-Discriminant Analysis (PLS-DA).
In red FMBS <4%, green FMBS >4%

PLS-DA VIP scores plot (Figure IV.7A) highlights the metabolites that mainly contribute to the separation in two clusters. These findings are also visualized in the volcano plot (Figure IV.7B), in which metabolites with a fold change greater than 1.0 and p-value < 0.05 are considered significant in the clustering.

A.



B.

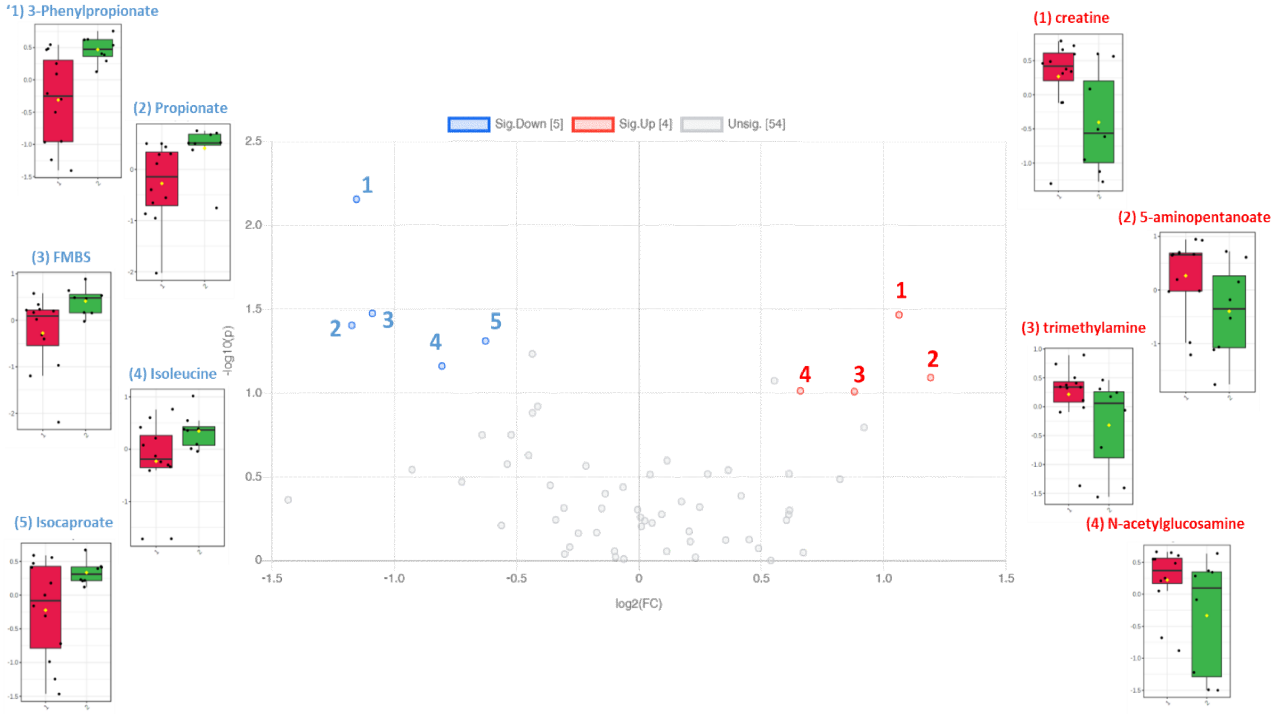


Figure IV.7. (A) VIP scores plot. Boxes on the right side are the heatmap, high concentration (red), and low concentration (blue). FMBS <4% (1), FMBS >4% (2). (B) Volcano plot. Combination of fold change (FC) analysis and t-tests for all the metabolites identified. FMBS <4% (red), FMBS >4% (green)

The critical metabolites for cluster separation are present with higher concentrations in the subjects with higher FMBS index. They are short-chain fatty acids (SCFAs) related to bacterial metabolism: isocaproate, propionate, and 3-phenylpropionate.

SCFAs are end-products of bacterial metabolism and are associated with periodontal inflammation and bleeding on probing (Rzenik et al, 2017). Several anaerobic periodontal bacteria produce SCFAs with concentrations in the millimolar range, that can stimulate gingival inflammatory response and bleeding: microorganism colonization stimulates the vascular system for the unceasing request of blood nutrients (Niederman et al, 1997).

SCFAs inhibit leukocyte apoptosis and cell division in the gingival epithelium at the cellular level, making oral mucosa repair much more difficult (Quiqiang et al, 2012).

Propionate has an inhibitory effect on gingival fibroblasts growth and adhesion when exceeding a concentration of 4 mM (Jeng et al, 1999): being a population of healthy subjects, the mean concentration of propionate in our study is 404.2 ± 164.8 μM for the participants with FMBS > 4%.

Other studies have identified propionate as a discriminative metabolite for oral hygiene status (Klukowska et al, 2015) since its concentration significantly decreases with periodontal treatments (García-Villaescusa et al, 2018).

Moreover, previous publications, investigating alterations in salivary metabolome due to periodontal inflammation, have shown an increase in isocaproate (García-Villaescusa et al, 2018) and 3-phenylpropionate, matching our findings (Liebesch et al, 2019; Kuboniwa et al, 2016).

Succinate belongs to the SCFAs class. Saccharolytic bacteria can degrade food-derived carbohydrates through the Embden-Meyerhof-Panas pathway to produce succinic and other organic acids, using oxygen via NADH oxidase. These processes create acidic and anaerobic conditions: a favourable environment for bacterial colonization (Takahashi, 2005).

SCFAs pathways are closely related to the β-branched amino acids (BCAA), such as isoleucine and leucine, since the catabolism of BCAA is an energy source for oral bacteria (Liebsh et al, 2019). Leucine is converted by *Eubacterium* species, through Stickland reaction, into isocaproic or isovaleric acid, and by *P. gingivalis* and *P. intermedia*, through deamination/ decarboxylation reactions, in isobutyric acid (Takahashi et al, 2000), Figure IV.8. Furthermore, numerous oral bacteria (*S. sanguinis, S. mitis, Streptococcus gordonii, Lactobacillus*, and *Actinomyces* species) convert arginine into putrescine, exploiting the arginine deiminase system. This process, by generating alkaline molecules seems to counterbalance the acidic environment generated by sugar

metabolism (Liu et al, 2012). Putrescine also contributes to the putrid odour of halitosis conditions (Jo et al, 2021).

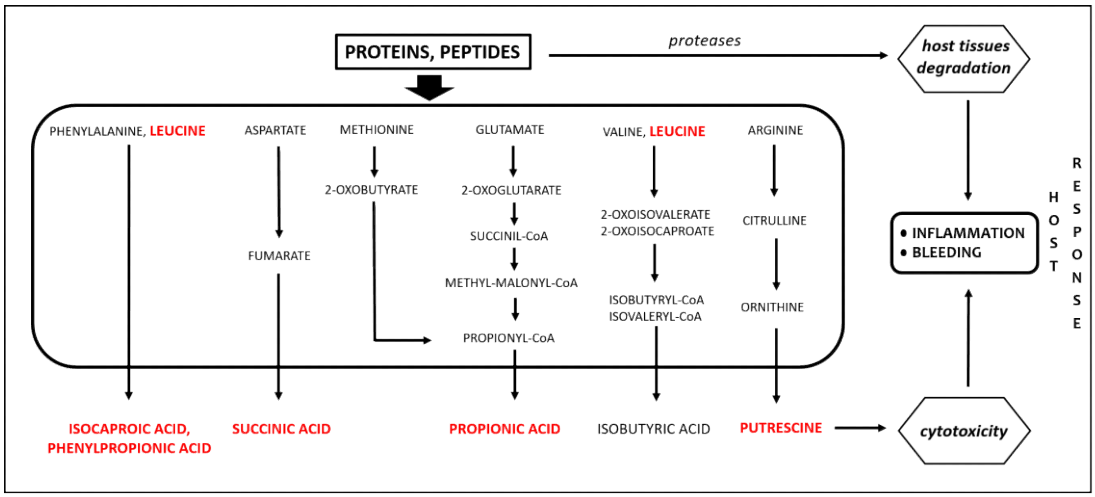


Figure IV.8. Oral microbiota metabolism of proteins, peptides, and amino acids and the effects on the host response. In red the metabolites identified that mainly contributed to the separation in two clusters (Adapted from Takahashi, 2005).

In 2015 (Takahashi, 2015), Takahashi summarized the current knowledge of oral microbiome metabolism and reported the changes in metabolic pathways related to periodontal diseases development. Our findings are in agreement with Takahashi's results.

Salivary glycoproteins are degraded into sugars and proteins by bacterial and human glycosidases. Proteins can be broken down into peptides and amino acids by bacterial and human proteases. Sugars are metabolized to acids by saccharolytic bacteria (*Streptococcus, Actinomyces, Lactobacillus*, etc.), while proteolytic bacteria metabolize amino acids in SCFAs and ammonia with the production of ATP (Kilian et al, 2016).

Takahashi (Takahashi, 2005) proposed that these metabolic end-products could stimulate an important gingival inflammatory response and inflammatory cytokine release, impairing host cell functions and subsequently disturbing the host defence.

Together with a direct degradation of host tissues by bacterial proteases, these effects have the potential to induce a notable host response, consisting of oral inflammation and, consequently, gingival bleeding.

After identifying the salivary metabolites associated with the early stages of gingival inflammation, to develop a preventive periodontitis screening model for each WS metabolite we created a ROC

curve from the database (Table IV.2, section Results). A metabolite panel was generated using the ones that have the most significant AUC values and T-test values (Table IV.3). All these metabolites correlate with oral inflammation.

Table IV.3. AUC and T-test values of the WS metabolites that resulted the most predictive in the ROC curve model

	AUC	T-test
3-Phenylpropionate	0.85	0.00698
FMBS	0.77	0.03351
Isobutyrate	0.71	0.28688
Isocaproate	0.72	0.04893
Isoleucine	0.74	0.06912
Leucine	0.77	0.05849
Propionate	0.86	0.03949
Valine	0.73	0.33922

Using the metabolites identified in Table IV.3 together with the FMBS index, we designed a model to distinguish the subjects prone to developing gingivitis. The final ROC curve in Figure IV.9A shows an AUC of 0.96. The ROC curve without including FMBS value (Figure IV.9B) determines an AUC of 0.93: a very efficient model to distinguish the two populations, the one with FMBS index >4% from the one with FMBS index <4%.

The concentration of these metabolites is strongly correlated with periodontal inflammation, and our model could be considered an early diagnostic tool for the discrimination of subjects more prone to periodontal diseases progression. This panel of metabolites is promising for developing a chairside point-of-care test for the primary diagnosis of gingivitis.

4. METABOLIC SALIVARY PROFILE OF ORAL DISEASES

Oral cancer is the 16th most common type of cancer, with 377,713 new cases and 177,757 deaths worldwide in 2020 (https://gco.iarc.fr/; Goldoni et al, 2021).

Around 90% of oral cancer cases are Oral Squamous Cell Carcinoma (OSCC), invasive epithelial neoplasia that initiates in the squamous cells. The tumours have different degrees of differentiation and the propensity of metastasis in the lymph node (Rivera, 2015; Sasahira & Kirita, 2018, https://gco.iarc.fr/). The remaining 10% includes salivary minor glands tumour, melanomas, and lymphomas (Dhanuthai et al, 2017).

OSCC pathogenesis is a multistage process: accumulation of genetic changes and epigenetic anomalies which lead to uncontrolled cell proliferation, invasion of adjacent tissues, and metastasis (Rivera & Venegas, 2014).

OSCC may affect all oral subsites: the most common are tongue, oral floor, and lower lip, with heterogeneous clinical manifestations: from mucosal ulcerations of variable size to nodules, erosion, exophytic lesions, and white and/or red patches Figure IV.10. Pain, burning sensation, and bleeding are reported as the main symptoms (Bagan et al, 2010). Due to the rich lymphatic vascularization of the oral cavity, OSCC can cause cervical lymph nodes and distant metastases (Sasahira et al, 2018).

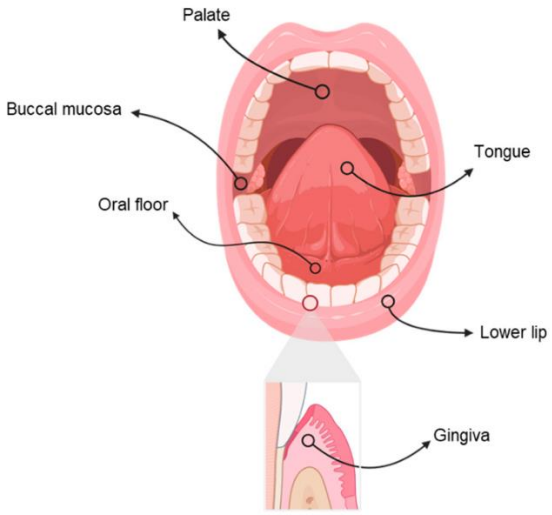


Figure IV.10. OSCC most common oral cavity subsites (Goldoni et al, 2021).

The risk factors of oral cancer include tobacco and alcohol consumption (with a synergistic effect), betel quid chewing, age, male gender, human papillomavirus (HPV), dietary habits, oral inflammation, and ultraviolet radiation (Rivera C, 2015; Montero & Patel, 2015; Panarese et al, 2019;

Kumar et al, 2016; Trimarchi et al, 2014; Lanzillotta et al, 2018; Rosenquist K, 2005; Gupta & Johnson, 2014).

From the 90s, epidemiological studies on the occurrence of oral cancer among relatives have highlighted the presence of a genetic predisposition (Foulkes et al, 1996; Jefferies et al, 1999).

Research groups are investigating the involvement of the oral microbiome, and the consequent mucosal inflammation, in disease development (Li et al, 2020; Singhvi et al, 2017).

Oral cancer is preceded by asymptomatic clinical manifestations, oral mucosa lesions, known as potentially malignant disorders (PMDs) - leukoplakia, erythroplakia, submucosal fibrosis, and lichen planus (Warnakulasuriya, 2020), Table IV.4).

Table IV.4. Definitions of various potentially malignant disorders (Warnakulasuriya, 2020).

Condition	Definition
Leukoplakia (OLK)	- A white patch or plaque that cannot be characterized clinically or pathologically as any other disease. - A white patch or plaque that cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent except the use of tobacco. - White plaques of questionable risk having excluded (other) known diseases or disorders that do not caran ry increased risk for cancer. - A predominantly white patch or plaque that cannot be characterized clinically or pathologically as any other disorder; oral leukoplakia carries an increased risk of cancer development either in or close to the area of the leukoplakia or elsewhere in the oral cavity.
Erythroplakia	A fiery red patch that cannot be characterized clinically or pathologically as any other definable disease.
Proliferative Verrucous Leukoplakia (PVL)	Leukoplakias tend to spread and become multifocal. PVL is slow-growing, persistent, and irreversible, and in time areas become exophytic and warlike
Oral submucous fibrosis	Oral submucous fibrosis is a chronic, insidious disease that affects the lamina propria of the oral mucosa, and as the disease advances, it involves tissues deeper in the submucosa of the oral cavity with resulting loss of fibroelasticity
Oral lichen planus (OLP)	A chronic inflammatory disease associated with cell-mediated immunological dysfunction
Oral lichenoid lesion/ reaction	OLP-like lesions found in close proximity to any dental restoration

Oral leukoplakia and Oral lichen planus

Oral leukoplakia is a white lesion or plaque in the oral mucosa that cannot be brushed off and cannot be characterized clinically or histologically as any other definable lesion. Therefore, a process of exclusion establishes the diagnosis of the disease (Rethman et al, 2010; Warnakulasuriya et al, 2007).

Leukoplakia is often associated with tobacco smoking, but the lesions are from idiopathic causes in some cases.

The prevalence of leucoplakia in the US and European population is 2-3.6%, especially among men (Bewley & Farwell, 2017). The 2-3% per year of leukoplakia lesions progress to squamous cell carcinoma (Bewley & Farwell, 2017; Carrard & Van Der Waal, 2018).

The lesions are homogeneous, with a uniform area, and non-homogeneous, with nodular, exophytic, or papillary/verrucous surfaces. The prompt recognition is fundamental since the latter has a higher probability of neoplastic degeneration (Warnakulasuriya et al, 2007).

The non-homogeneous class includes the proliferative verrucous leukoplakia (PVL), a rare type but with a malignant transformation rate of 60%-100% (Munde & Karle, 2016).

Instead, oral lichen planus (OLP) is a chronic inflammatory disease and immunologically driven that usually affects women aged 30 to 60 years (Maymone et al, 2019). Different subtypes have been classified: reticular, papular, plaque, atrophic, erosive, and bullous. Erosive and atrophic types are the most invasive, causing severe pain that hamper speech and swallowing and the highest risk of malignant transformation (Olson et al, 2016). The rates of transformation of OLP into oral cancer are still a matter of debate, although, to date, the literature seems to agree on the 0% -12.5% range (Villa et al, 2011).

Most PMDs do not progress to oral cancer, but some present specific morphological alterations leading to malignant transformation. The neoplastic site originates as an epithelial dysplasia and is characterized by the accumulation of DNA mutations, followed by an altered proliferation of dysplastic squamous cells (Scully & Bagan, 2009). Dysplastic regions show irregular epithelial stratification, loss of polarity of basal cells, drop-shaped ridges, increased number of mitotic figures, abnormally superficial mitoses, and dyskeratosis, Figure IV.11. The dysplasia is categorized as mild, moderate, or severe based on the lesion severity and the depth to which it extends (Ranganathan & Kavitha, 2019), Figure IV.12.

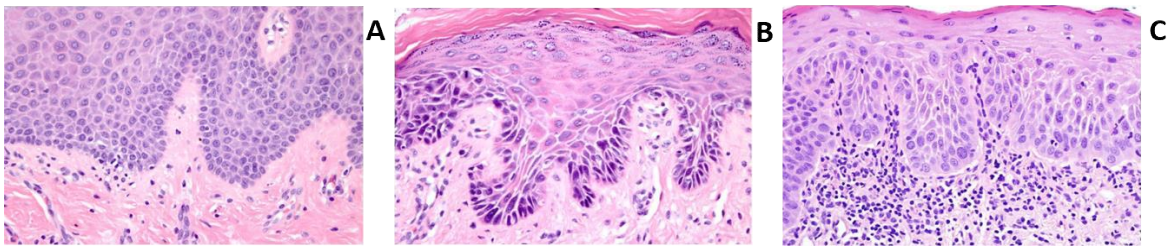


Figure IV.11. Histopathological coloration of oral mucosa tissues with no evidence of epithelial dysplasia (A), moderate dysplasia (B) and severe dysplasia (C), respectively (Adapted from Woo, 2019).

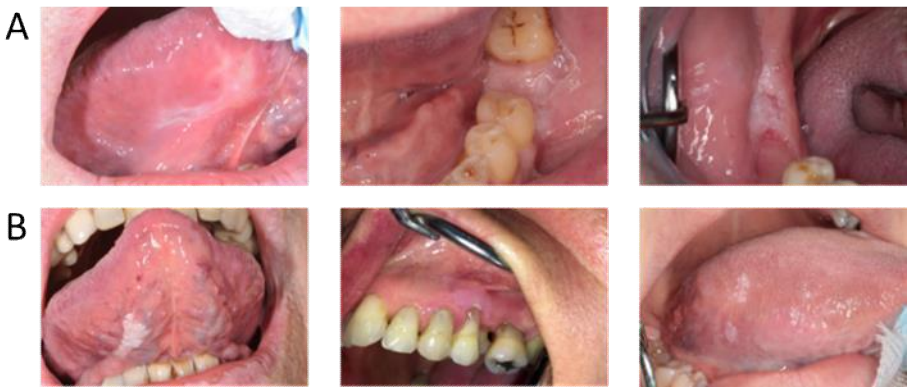


Figure IV.12. Photographs of the oral cavity of patients with leukoplastic lesions with no evidence of dyplasia (A) and patients with leukoplakia and dyplasia (B).

Oral cancer early diagnosis

Mainly because of the frequent late OSCC diagnosis, the overall 5-years survival rate is lower than 50% (https://gco.iarc.fr/).

Prognosis depends on the stage at diagnosis, defined through the Tumour-Node-Metastasis (TNM) staging system, which takes into account the size (T), involvement of regional lymph nodes (N), and presence or absence of distant metastases (M) (Edge & Compton, 2010). Early diagnosis (Stage I-II) is associated with a 5-year survival rate of approximately 80-90% (Silverman et al, 2010).

Therefore, early diagnosis and premalignant oral lesions treatment are essential for prognosis and survival rates improvement (Abati et al, 2020).

The current gold standard for OSCC diagnosis is histopathologic analysis, which, however, is an invasive procedure that requires qualified staff and equipment (Chakraborty et al, 2019).

Several adjunctive diagnostic approaches have been proposed. These include the use of visual diagnostic aids (e.g., detection of tissue autofluorescence), techniques based on mucosal scraping (exfoliative cytology), and staining methods (toluidine blue staining). However, none of those diagnostic approaches are associated with reliable diagnostic accuracy; their sensitivity and

specificity are usually low (Giovannacci et al, 2016). For these reasons, it is necessary to develop new screening methods that can be easily used as point-of-care by clinicians to early identify suspicious lesions (Abati et al, 2020).

Genomics, proteomics, and metabolomics have the potential to detect alterations in the saliva composition due to the disease, and these omics have been widely exploited in the search for potential saliva biomarkers for OSCC (Khurshid et al, 2018; Kaur et al, 2018; Nguyen et al, 2020).

Wei and colleagues demonstrated the efficiency of metabolomics in identifying pathological changes in saliva metabolic pathways of patients with OSCC and PMDs, enhancing the technique's applicability for diagnostic and prognostic purposes (Wei et al, 2011).

Saliva, produced and delivered in the same anatomical district of OSCC, is expected to contain molecules directly originated by neoplastic cells and tumour microenvironment.

Patil and colleagues in 2021 reviewed all the studies present in literature from 2005 to July 2020, which examine the saliva profiles of patients with oral cancer and PMDs (Patil & More, 2021). Using "Salivary Metabolomics," ''Oral Cancer", "Oral leukoplakia," and "Oral Lichen planus" as keywords, they assessed only nine studies that point to approximately 80 statistically significant metabolites belonging to different chemical classes, Table IV.5.

Table IV.5. Salivary metabolomics studies on oral cancer and PMDs (Patil & More, 2021).

	Authors	Study subjects	Detection method	Metabolomic findings
1	Almadori et al, 2007	OLP, OSCC	HPLC	Salivary glutathione was characteristically different among cancer and control groups indicative of oxidative stress in cancers.
2	Yan et al, 2008	OLK, OLP, OSCC	HPLS-MS	Metabolic profiling data differentiated between OSCC, OLP, and OLK.
3	Sugimoto et al, 2010	OSCC	CE-TOF-MS	28 differentially expressed metabolites were detected and used to predict oral cancer outcomes The salivary polyamine, ornithine, and putrescine levels were significantly higher than the other metabolites expressed.
4	Wei et al,2011	OSCC, OLK	UPLC-QTOF-MS	Valine, lactic acid, and phenylalanine in combination yielded satisfactory accuracy (0.89, 0.97), sensitivity (86.5% and 94.6%), specificity (82.4% and 84.4%) and positive predictive value (81.6% and 87.5%) in distinguishing OSCC from the controls or OLK, respectively.
5	Ishikawa et al, 2016	OSCC	CE-TOF-MS	Eighty-five tumour metabolites and 43 saliva metabolites showed significantly different concentrations between OSCC and controls, ($P<0.05$ adjusted by FDR); in total, 17 metabolites showed significantly higher average concentrations consistently in both saliva and tissues. Among the salivary metabolites, a combination of S-adenosylmethionine (SAM) and pipecolate showed statistical

				significance (p<0.0001) in distinguishing OSCC cases from controls.
6	Ohshima et al, 2017	OSCC	CE-TOF-MS	A total of 499 metabolites were detected as CE-MS peaks in the saliva tested from the two groups. A total of 25metabolites were revealed as potential markers to discriminate between patients with OSCC and healthy controls. Choline, metabolites in the branched-chain amino acids (BCAA) cycle, urea, and 3-hydroxybutyric acid were found to be significantly different among the OSCC cases and controls.
7	Lohavanichbutr et al, 2018	OSCC	NMR, LC-MS	Levels of two metabolites (glycine and proline) were significantly different between OSCC and control. Four metabolites, including glycine, proline, citrulline, and ornithine were associated with early-stage OSCC.
8	Sridharan et al, 2019	OLK	Q-TOF-LC MS	Characteristic overexpression of 1-methylhistidine, inositol1,3,4-triphosphate, d-glycerate- 2-phosphate,4-nitroquinoline- 1-oxide, 2 oxoarginine, norcocaine nitroxide, sphinganine-1- phosphate, and pseudouridine was seen in oral leukoplakia and OC. The downregulated metabolites in the study group are l-homocyst acid, ubiquinone, neuraminic acid, and estradiol valerate.
9	Ishikawa et al, 2019	OLP, OSCC	CE-TOF-MS	Fourteen metabolites were found to be significantly different between the OSCC and OLP groups. Among them,indole-3-acetate and ethanolamine phosphate were statistically significant.

HPLC-High Performance Liquid Chromatography; **NMR**-Nuclear Magnetic Resonance Spectroscopy; **Q-TOF-LC** Liquid Chromatography Surface-enhanced laser desorption/ionization time-of-flight; **CE-TOF-MS** Capillary Electrophoresis Surface-enhanced laser desorption/ionization time-of-flight; **LC–MS** Liquid Chromatography Mass Spectroscopy. **UPLC-QTOF-MS** Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

The review identified an excessive variety of protocols for the analyses, which produces inconsistent results. Therefore, an unambiguous description of the salivary metabolic profiles of oral lesions is still missing.

Identifying and validating promising diagnostic metabolite biomarkers of potentially malignant lesions can improve the development of saliva-based point-of-care for early diagnosis of risk patients, reducing the mortality rate of oral cancer.

This section aims to investigate the metabolic profiles of patients with leukoplakia and patients with OSCC, with a particular focus on the metabolic alterations that accompany the progressive malignant transformation of PMD lesions.

Results

Due to the pandemic emergency COVID-19, the salivary sample collection of the patients and the control group was prolonged and, for a period, suspended due to the infection risks associated with the collection protocol. The results present in this chapter are, for that reason, preliminary.

The total number of patients enrolled in this study was 45: 23 with oral leukoplakia (OLK) with different dysplasia degrees, 12 with oral lichen planus (OLP), and 11 with oral carcinoma together with a control group composed of 21 healthy volunteers (Table III.5).

Some collected saliva samples were very viscous, and some presented blood contamination. The final samples analyzed were 20 OLK, 11 OLP, and 9 OSCC carcinomas.

The OLK group was divided into two subgroups, patients with no evidence of dysplasia lesion, and patients with co-presence of dysplasia with different degrees of severity.

The PLS-DA of the spectral binning of OLK, OSCC, and control samples (Figure IV.13 A) show a discrete clusterization; it is more evident in the 3D graph (Figure IV.13 B). The cases of OLP were excluded from this analysis since they present different etiological characteristics. It can be seen a net separation between controls and OSCC patients. As expected, the potentially malignant disorders are grouped in-between, progressing from healthy to neoplastic conditions.

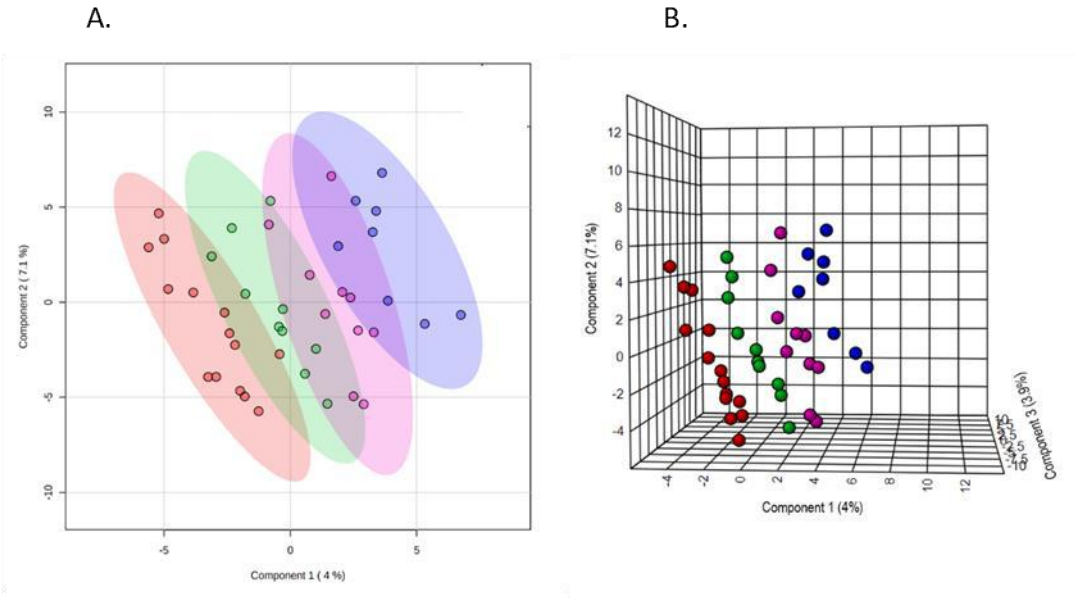


Figure IV.13. (A) Score plot of Partial Least Squares-Discriminant Analysis (PLS-DA) applied on the spectral binning. control group (red); OLK patients with no dysplasia (green); OLK patients with dysplasic lesions (magenta), and OSCC patients (blue). (B) 3D plot of PLS-DA.

The spectral analysis allowed the identification and quantification (above 1.1 μM) of 47 ± 5, 47 ± 7, 43 ± 6, and 48 ± 4 (mean ± SD) metabolites in lichen planus, leukoplakia, OSCC, and control group samples, respectively.

PLS-DA on the identified metabolites is illustrated in Figure IV.14.

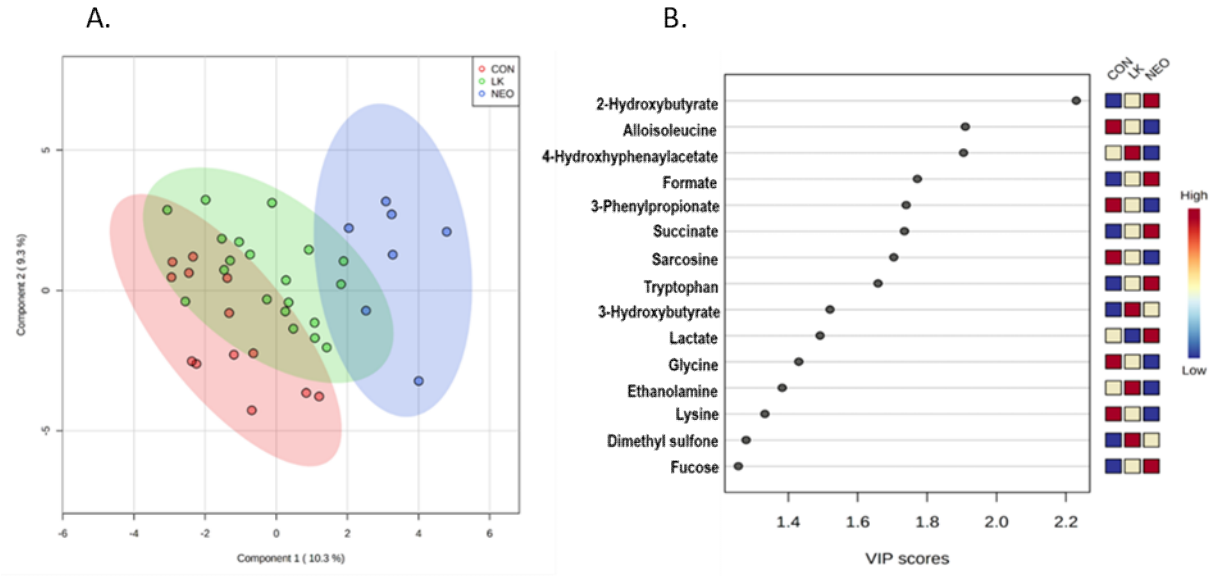


Figure IV.14. (A) Score plot of Partial Least Squares-Discriminant Analysis (PLS-DA) using the quantified metabolites: healthy subjects (red); OLK patients (green); OSCC patients (blue). (B) VIP scores plot. The boxes on the right side are the heatmap: the brightness of each colour corresponds to the magnitude of the difference when compared with the average value (high concentration in red and low concentration in blue).

The score plot confirms the cluster separation of Figure IV.13, suggesting different metabolomes for the three diverse types of saliva. The Variable Importance in Projection (VIP) scores (Figure IV.14 B) shows the metabolites that implicated in the clusters separation:

Tryptophan has a higher concentration in OSCC saliva, with a progressive decrease up to healthy controls. Tryptophan, an essential amino acid, has already been reported as a discriminant metabolite of patients with oral cancer (OC) and controls (Tankiewicz et al, 2006; Sugimoto et al 2010; Ohshima et al, 2017). Not only tryptophan but also its derivatives, such as kynurenine and indole, showed higher concentrations in oral cancer patients, indicating increased reactive oxygen species stress in tumour tissues (Tothova et al, 2015; Ishikawa et al, 2016).

The observation of a higher concentration of succinate in OSCC compared to controls agrees with the work of Lohavanichbutr and colleagues, who included succinate among the salivary metabolites differentiating OSCC by controls, although they identified two different concentration ranges depending on the technique used, NMR and LC-MS / MS (Lohavanichbutr et al, 2018).

Fucose is another metabolite present at higher concentration in OSCC than in control and leukoplakia. Our finding correlates with results by Mikkonen and colleagues who detected an increase of fucose level in the saliva of patients with head and neck cancer (Mikkonen et al, 2018)

and by Shah and colleagues that found a significantly increased serum fucose level in OSCC patients compared with PMDs or healthy controls (Shah et al, 2008).

Epithelial dysplasia is the alteration of the architectural and cytologic features of the mucosa; the progressive changes lead towards the acquisition of a malignant phenotype (Walsh et al, 2013). To investigate the leukoplakia more accurately, with and without dysplasia, and being in search of early biomarkers that may facilitate the diagnosis, we explored how the lesion's presence can impact the salivary metabolic compositions.

In the PLS-DA analysis (Figure IV.15), the samples with and without dysplasia are well separated. The group of subjects with leukoplakia but no dysplastic lesion is less dispersed, while patients with dysplasia show more significant variability due to the different grades of dysplasia.

VIP scores plot (Figure IV.15 B) highlights the metabolites that mainly contributed to this separation in two clusters. These findings are also visualized in the volcano plot (Figure IV.16), in which metabolites with a fold change greater than 1.0 and p-value < 0.1 are considered significant in the clustering.

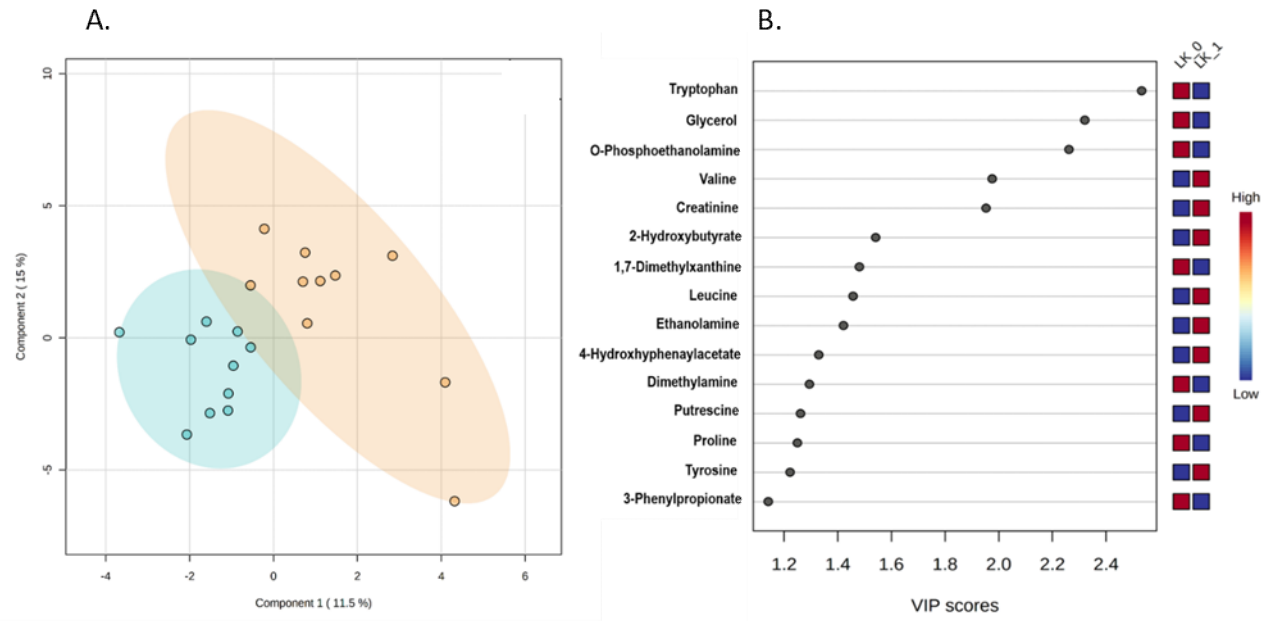


Figure IV.15. (A) Score plot of Partial Least Squares-Discriminant Analysis (PLS-DA): OLK patients with no dysplasia (cyan); OLK patients with dysplastic lesions (orange) (B) VIP scores plot. The boxes on the right side are the heatmap: each colour's brightness corresponds to the magnitude of the difference when compared with the average value (high concentration in red and low concentration in blue).

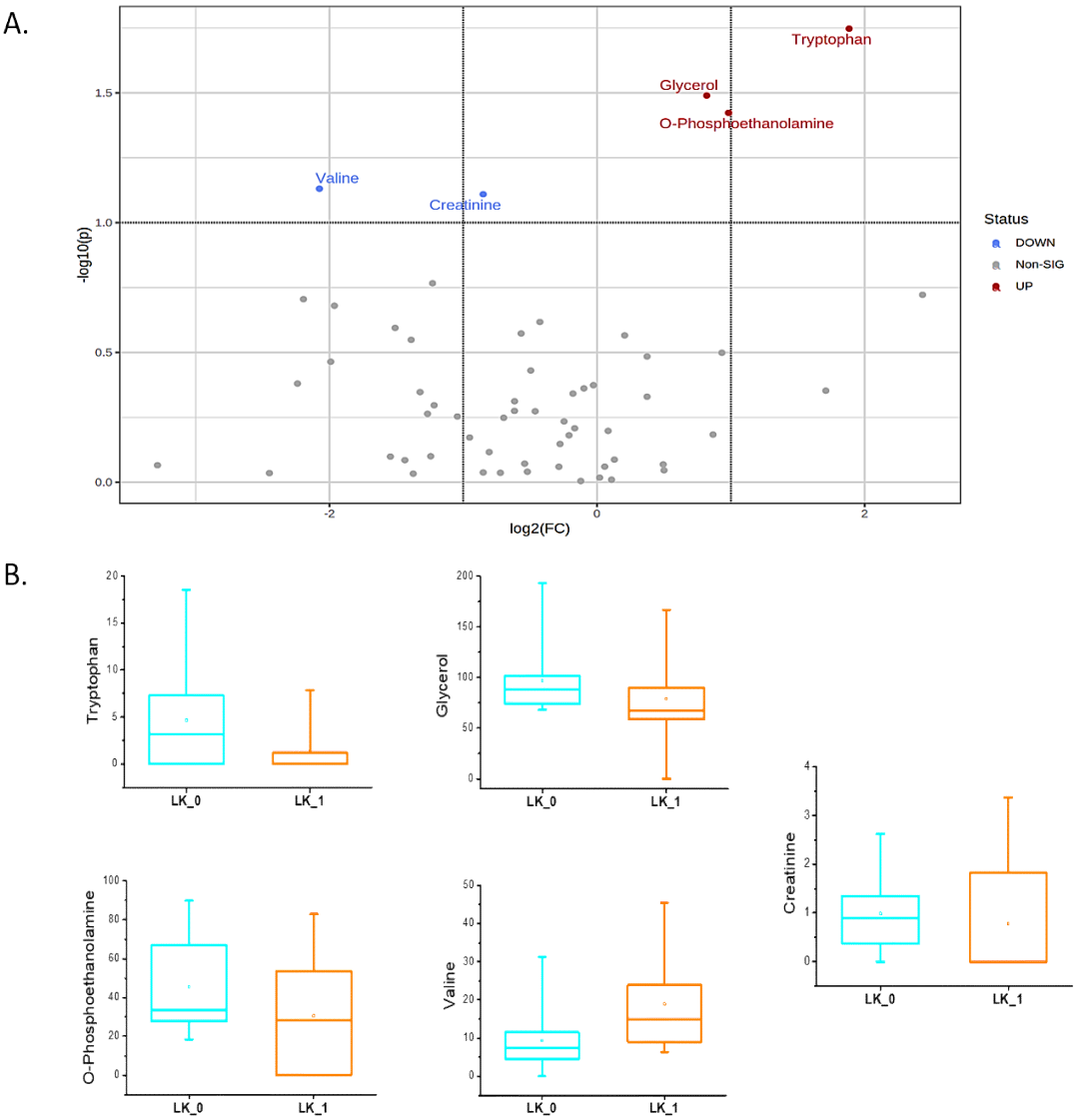


Figure IV.16. (A) Volcano plot. Combination of fold change (FC > 1.0) analysis and p-value (< 0.1) for all the metabolites identified. (B) Box plot of the concentrations of metabolites found relevant from the volcano plot (without dysplasia -LK_0 in cyan; with dysplasia - LK_1 in orange)

Among the most impacting metabolites in clustering we find glycerol, o-phosphoethanolamine, and ethanolamine, which are metabolites deriving from cell membrane phospholipids: this could be correlated to the altered cellular morphology underling the development of dysplastic regions (Mayr et al, 2015). Ishikawa and colleagues had previously reported a significant difference in O-phosphoethanolamine concentration between patients with OSCC and healthy controls (Ishikawa et al, 2017).

It is known that all the branched-chain amino acids, and tryptophan, are building blocks for protein synthesis and are used to produce energy in the rapid cell proliferation and growth of tumours (Ananieva & Wilkinson, 2018).

In a recent review, Assad and colleagues reported the most significant salivary metabolites for the early detection of oral cancer (Assad et al, 2020). Valine is frequently reported as a discriminant metabolite, which is more concentrated in patients than healthy people. Our data show that valine is present in higher concentrations in the lesions associated with dysplasia, Figure IV16B, thus suggesting a progress towards tumour transformation. The metabolomic signatures of dysplasic lesions affecting other tissues have already been partially studied (Lal et al, 2018; Macioszek et al, 2021).

Regarding the metabolic study of oral cavity precancerous lesions, with and without dysplasia, to date, there is a single study that compares serum metabolites in patients with Esophageal Squamous Cell Carcinoma and Esophageal Squamous Dysplasia (Zhang S et al, 2020).

Summarizing, though due to the limited time available, the results presented derive from preliminary analysis of experiments carried out on saliva samples, it is worth noting, that they agree with results obtained from serum, in particular showing a progressive decrease of glycerol from leukoplakia without dysplasia to those with dysplasia. This finding confirms that saliva is a convenient biofluid for the diagnosis of oral pathologies.

In that perspective, it will be interesting to study the differences between the saliva metabolic profile of patients with different dysplasia severity degrees and healthy subjects to identify biomarker for the presence of morphological changes precursors of the malignant transformation.

In conclusion, we foresee that progressing in these studies will allow correlating metabolomic data with subjects' clinical and epidemiological features.

V. CONCLUSIONS AND FUTURE PERSPECTIVES

Albeit blood remains the gold standard for clinical investigations, saliva is gaining increased attention: it does not coagulate, sampling is non-invasive and relatively stress-free facilitating multiple collections; it does not require highly skilled staff and sample handling is safer compared to blood (Javaid et al, 2016).

Saliva, a heterogeneous biofluid containing a large number and variety of biomolecules, is a very suitable tool for diagnostic applications (Pfaffe et al, 2011; Goldoni et al, 2021).

In the past decades, many studies on saliva, based on the various *omics* (genome, RNA, proteins, metabolites, and microbiome) have explored its diagnostic potential in medicine and dentistry (Castagnola et al, 2017; Pappa et al, 2019). Saliva-based diagnostic tests are being recognized as promising tools to ease population screening, risk assessment, prognosis and diagnosis determination, therapy response (Pfaffe et al, 2011).

To date, it is recognized that the metabolomics approach to analyse saliva may support the identification of diagnostic biomarkers (Fuhrer et al, 2015).

In order to confirm that potentiality, it is necessary to obtain high-quality and reproducible data and therefore to improve and standardize saliva collection, handling, and processing protocols (Cuevas-Córdoba & Santiago-García, 2014). Timing of collection, stimulus, intrinsic inter-individual variability, and the confounding factors from the environment and daily activities, are factors that must be taken into consideration as they impact saliva composition (Gardner et al, Jun 2020; Goldoni et al, 2021).

Indeed, the translation of salivary metabolomics findings into diagnostic aids and clinical recommendations is hampered by the absence of validated protocols that must assure good reproducibility and reliable comparison of the results.

^{1}H-NMR spectroscopy is not considered the technique of choice for salivary metabolomics because: a) the frequent low concentration of the metabolites often exceeds the detection limit of the technique; b) while for urine and plasma validated guidelines for collection, storage, preparation, and ^{1}H-NMR analyses are available, for saliva a consensus on equivalent protocols has not been reached, yet (Gardner et al, Jan 2020).

In the past years, a few studies have investigated the possible alterations introduced during saliva collection as a consequence of the sampling method, the addition of antibacterial agents, the preparatory steps prior to storage, and the storage conditions (Duarte et al, 2020; Gardner et al, 2018).

The present study proposes a protocol for saliva collection and sample preparation for ^{1}H-NMR studies that provides good reproducibility and improves metabolites' identification and quantification.

The use of an ultracentrifugation step, essential to eliminate the broad NMR peaks generated by the macromolecules, with the addition of a freeze-drying procedure, overtakes the limited NMR sensibility, increasing the efficiency in metabolites identification by~30%, with the quantification of low concentration (1.1 µM) metabolites.

Following the complete procedure, we analysed the metabolic composition of the unstimulated parotid (PS), submandibular/sublingual (SM/SL), and whole (WS) saliva in a cohort of young and healthy subjects and we identified: 58 ± 2 in WS, 57 ± 2 in PS, and 48 ± 2 in SM/SL (mean ± SD) metabolites.

These data resulted essential for the progression of the project. In fact, a detailed analysis of the human salivary metabolome is necessary to interpret the dynamic metabolic status in physiological conditions and to prevent the misinterpretation when analysing saliva from patients with a specific pathological state in search of disease biomarkers.

We found that a considerable contribution to the saliva metabolome derives from bacterial metabolism.

WS, the saliva directly in contact with the oral cavity, showed a high number of prokaryotic cells and, consequently, a significant number of bacterial metabolites (amino acids, and organic acids).

Bacterial glucosidases metabolize saliva glycoproteins, generating monosaccharides such as fucose, N-acetylglucosamine, and galactose. These sugars are converted into lactic, formic, acetic, succinic, propionic, and other organic acids, called short-chain fatty acids (SCFAs), through the glycolytic pathways. Note that SCFA metabolites are present at a sensibly lower concentration in glandular saliva (PS, SM/SL) as compared to WS, a fact that might be back to their reduced bacterial contamination.

Since host-induced inflammatory processes and dysbiotic bacterial metabolism take part and influence the metabolic pathways of WS, they were investigated in search of diagnostic biomarkers for periodontal diseases: infection-driven inflammatory processes (Gardner et al, 2020a; Liebsch et al, 2019; Miller et al, 2010).

To date, in clinical practice, periodontal disease diagnosis can assess only the advanced stage, but not the disease evolution because these pathologies do not have a linear progression as they are characterized by progression/remission periods (Ko et al, 2021; Nomura et al, 2017).

For this pathology, we are interested in salivary biomarkers that may allow an early-stage periodontal diagnosis favouring an easy, safe, and non-invasive scheme to plan appropriate treatment strategies. Transferring scientific findings to clinical practice is relevant also for developing rapid, low-cost, and accurate point-of-care technologies (Cuevas-Córdoba & Santiago-García, 2014).

In our study, among the various clinical parameters accessed by dentists, we evaluated the level of periodontal bleeding (FMBS - "Full Mouth Bleeding Score") because it detects the possible presence of inflammation linked to bacterial microflora dysbiosis. The homeostasis imbalance between host and oral microflora generates an inflammation that could lead to severe periodontal diseases (Van Dyke et al, 2020).

The selected cohort was composed of healthy subjects. Though their clinical parameters were characterized by low scores, the evaluation of the periodontal bleeding and the index of oral inflammation, were correlated to the salivary metabolic composition. In WS, we found 7 metabolites, 3-phenylpropionate, isobutyrate, isocaproate, isoleucine, leucine, propionate, and valine, linked to oral inflammation, and all of them strongly correlated to bacterial metabolic pathways. The panel composed of those 7 metabolites was used as an early diagnostic tool to discriminate subjects more prone to periodontal disease progression. This result represents the first relevant step to develop a point-of-care for primary diagnosis of early periodontal disease inflammation.

Early diagnosis is essential in more severe oral diseases: e.g. oral cancer (OSCC), whose overall 5-years survival rate is lower than 50% (https://gco.iarc.fr/) due to the diagnostic delay. Therefore, early diagnosis and premalignant oral lesions treatment are essential factors for survival rates improvement.

There is an increase in advertising campaigns that encourage self-screening as well as in the request for medical checks in case of suspicious oral lesions. Indeed, the breakthrough would be to device a point-of-care that dentists can easily use to detect potentially malignant oral lesions before degeneration (Abati et al, 2020).

Although there are studies pointing to some metabolites as promising biomarker for early oral cancer detection (Hyvärinen et al, 2021), investigations on premalignant lesions as a risk factor are still very few.

We have studied the WS metabolome of OSCC and leukoplakia (OLK) patients, particularly the metabolic profiles of the OLK patients with and without epithelial tissue dysplasia. The early

identification and grading of dysplasia in OLK lesions could be a precious risk assessment tool for progression to cancer.

The results presented are still preliminary because of the COVID-19 epidemiological emergency. Despite this, it has been possible to identify a progressive modification of the metabolic profiles, from healthy subjects to patients with OSCC, passing through patients with OLKs regularly ordered from the ones without to those with dysplasia, which have a profile similar to the neoplastic patients: some metabolites present in OLKs with dysplasia are the same as those identified in the literature in the saliva of patients with oral cancer (valine), while others indicate the presence of an altered cellular morphology (o-phosphoethanolamine, glycerol).

We plan to study the differences between the metabolic profiles of patients with different dysplasia severity degrees and compare those profiles with ones of healthy subjects to search for metabolites that may indicate the presence of morphological changes precursors to the malignant phenotype degeneration.

FUTURE PERSPECTIVES

The combination of salivary biomarkers associated with conventional oral examination may become an effective strategy for early detection and treatments of oral precancerous lesions.

Multi-Omics study of saliva from other oral and systemic diseases and the development of high sensitivity biosensors and point-of-care will allow an earlier identification and more effective management of the disease progression (Ko et al, 2021) and will improve the personalized approach of precision medicine.

It is also recognized that metabolomics studies of saliva will modernize oral microbiology, expanding our understanding of the microbial species of the oral cavity and their role in many disease-related dysbiotic changes (Gardner et al, Jan 2020).

Finally, we envisage as an interesting development to apply the approaches we discussed on saliva samples collected at several standpoints before and after treatment or at different stages of the disease: the objective is to obtain a dynamic description of the metabolic activity ongoing in the oral cavity.

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CURRICULUM VITAE

Informazioni personali

Nome/ Cognome	Eleonora Quartieri
Data di nascita	23.01.1993
E-mail	Eleonora.quartieri@unipr.it

Istruzione e formazione

Date	**01/11/2018 - In corso**
Titolo della qualifica rilasciata	Dottorato di Ricerca in Medicina Molecolare
Principali tematiche	*Metabolomics in Biomedicine* *Analisi chimiche e biochimiche ed utilizzo di tecnica di Risonanza Nucleare Magnetica (1H-NMR) per la caratterizzazione metabolomica di vari biofluidi (saliva, siero) a scopi diagnostici e clinici.*
Nome e tipo d'organizzazione erogatrice dell'istruzione e formazione	Dipartimento di Medicina e Chirurgia, Università di Parma in collaborazione con AUSL-IRCCS Reggio Emilia
Livello nella classificazione nazionale o internazionale	Dottorato di ricerca, PhD

Date	**25/10/2018**
Titolo della qualifica rilasciata	Laurea in Chimica e Tecnologia Farmaceutiche
Principali tematiche	*Metal ions, dopamine and oxidative stress in Parkinson's disease*
Livello nella classificazione nazionale o internazionale	Laurea specialistica a ciclo unico

Date	**07/2012**
Titolo della qualifica rilasciata	Diploma di maturità scientifica
Nome e tipo d'organizzazione erogatrice dell'istruzione e formazione	Liceo Scientifico “L. Da Vinci” – Villafranca in Lunigiana (MS)
Livello nella classificazione nazionale o internazionale	Diploma di scuola secondaria superiore

Esperienze professionali

Date	**10/2019-10/2020**
Lavoro o posizione ricoperti	Tutor studenti

Principali attività e responsabilità	*Attività di supporto per il recupero del debito formativo in Chimica Generale per gli studenti iscritti al I° Anno Accademico del Corso di Laurea in Infermieristica dell'Università di Parma presso le sedi di Parma e Piacenza.*
Nome e indirizzo del datore di lavoro	Università di Parma, Dipartimento di Medicina e Chirurgia.
Date	**01/2017-01/2018**
Lavoro o posizione ricoperti	Tirocinio / Collaboratrice in farmacia
Principali attività e responsabilità	*Tirocinio con mansioni di assistenza al banco, nelle preparazioni magistrali, nella distribuzione per conto. Archiviazioni di farmaci e della documentazione relativa. Esperienza lavorativa come magazziniera, addetta all'archiviazione farmaci e parafarmaci e collaboratrice nella gestione degli ordini informatici d'acquisto presso il sito ufficiale della farmacia.*
Nome e indirizzo del datore di lavoro	Farmacia Giannotti Corso Europa Unita 1, 54016 Licciana Nardi (MS) (Italia) www.farmaciaforyou.com

Partecipazioni a congressi

- 1° Research Day del Dipartimento di Medicina e Chirurgia dell'Università di Parma, Castello di Rivalta (PC) 1 Ottobre 2021
POSTER: Metabolomics in Biomedicine
E. Quartieri, E. Ferrari, M. Gallo, A. Spisni, T.A. Pertinhez

- 2° Convegno virtuale in Medicina Trasfusionale-SIMTI, ONLINE 22-24 Settembre 2021
1. POSTER: PRP da aferesi: una valutazione in vitro del potenziale osteogenico
L. Merolle, S. Pulcini, C. Marraccini, B. Iotti, P. Berni, D. Schiroli, **E. Quartieri**, R. Sala, T.A. Pertinhez, R. Baricchi
2. POSTER: Comparazione di due metodologie per la preparazione di emazie concentrate nella terapia trasfusionale della beta-talassemia maggiore
D. Schiroli, T. Fasano, R. Chicchi, **E. Quartieri**, E. Di Bartolomeo, L. Merolle, C. Marraccini, R. Biguzzi, R. Baricchi

- 32a Riunione Nazionale "A. Castellani" dei Dottorandi di Ricerca in Discipline Biochimiche,
Brallo di Pregola (PV) 13-16 Settembre 2021
COMUNICAZIONE ORALE: Metabolic characterization of whole, parotid and submandibular/sublingual saliva: a valuable tool for diagnostics
E. Quartieri, M. Pezzi, R. Antonelli, M.V. Viani, B. Ghezzi, G. Setti, T. Ciociola, E. Ferrari, E. Casali, M. Gallo, A. Spisni, M. Meleti, T.A. Pertinhez

- Young Scientist Program Webinar series per 20th IUPAB Congress - 45th Annual Meeting of SBBf - 49th Annual Meeting of SBBq, ONLINE 04 agosto 2021

COMUNICAZIONE ORALE: Metabolic characterization of whole, parotid and submandibular/sublingual saliva: a valuable tool for diagnostics
E. Quartieri, M. Pezzi, R. Antonelli, M. V. Viani, B. Ghezzi, G. Setti, T. Ciociola, E. Ferrari, E. Casali, M. Gallo, M. Meleti, T.A. Pertinhez

- 17° Annual Conference of the Metabolomics Society- METABOLOMICS 2021, ONLINE, 22-24 Giugno 2021

POSTER: Signature of salivary metabolites associated to gingival host-microbial interactions
E. Quartieri, E. Ferrari, M. Gallo, R. Antonelli, A. Spisni, M. Meleti, T. A. Pertinhez

- 21° Annual NATA Symposium on Patient Blood Management, Haemostasis and Thrombosis, ONLINE 12-16 Aprile 2021

POSTER: Can plasmapheresis procedure be optimised? The role of plasma unit weight
C. Marraccini, **E. Quartieri**, S. Pulcini, G. Molinari, L. Merolle, P.G. Rossi, R. Baricchi, T.A. Pertinhez

- 28° Congresso Nazionale del Collegio dei Docenti Universitari di discipline Odontostomatologiche, ONLINE 12-14 Aprile 2021

POSTER: Identification of salivary metabolites associated to gingival bleeding
R. Antonelli, M.E. Pezzi, M.V. Viani, G. Setti, E. Ferrari, T.A. Pertinhez, **E. Quartieri**, M. Meleti.

- 27° Congresso Nazionale del Collegio dei Docenti Universitari di discipline Odontostomatologiche, ONLINE Settembre 2020.

1.POSTER: Amino acids evaluation in whole, parotid and submandibular/sublingual saliva
R. Antonelli, **E. Quartieri**, E. Ferrari, M. Pezzi, B. Ghezzi, M. Meleti, T.A. Pertinhez. *Menzione d'onore*
2.POSTER: Salivary cytokines for the diagnosis of oral cancer: a review
M.E. Pezzi, E. Ferrari, R. Antonelli, **E. Quartieri**, G. Setti, M.V. Viani, P. Vescovi, T.A. Pertinhez, M. Meleti

- Advances in NMR and MS based metabolomics; Lucca 20-22 Novembre 2019

POSTER: Metabolomic profiling of human saliva
E. Quartieri, R. Antonelli, M.E. Pezzi, M.V. Viani, B. Ghezzi, T. Ciociola, E. Ferrari, E. Casali, M. Meleti, T.A. Pertinhez.

- XIX Congresso Nazionale SIdEM, Rimini 23-26 Ottobre 2019

1. COMUNICAZIONE ORALE: L'uso delle scienze "omiche" nello studio delle dislipidemie
E. Quartieri, L. Merolle, C. Marraccini, L. Scarano, E. Di Bartolomeo, S. Bergamini, R. Baricchi, T. A. Pertinhez.

2.POSTER**:** Potenziale riparativo dei derivati piastrinici sul tessuto osseo: uno studio in vitro
S. Pulcini, C. Marraccini, P. Berni, **E. Quartieri**, D. Mori, T.A. Pertinhez, R. Sala, R. Baricchi.

- XV National and III International Congress of the Italian Society of Oral Pathology and Medicine (SIPMO), Bari 17-19 Ottobre 2019
1. POSTER: Characterization of Bacterial Metabolites in Parotid, Submandibular/Sublingual and Whole Saliva of Healthy Subjects. Proceedings 2019, 35, 71; doi:10.3390/proceedings2019035071
M.E. Pezzi, R. Antonelli, M.V. Viani, E. Casali, T.A. Pertinhez, **E. Quartieri**, P. Vescovi, M. Meleti.
2. POSTER: Salivary Metabolic Analysis in Healthy Subjects and Perspectives for Patients with Oral Cancer: Pilot Study and Systematic Review. Proceedings 2019, 35, 44
R. Antonelli, M.E. Pezzi, M.V. Viani T.A. Pertinhez, **E. Quartieri**, B. Ghezzi, G. Setti, P. Vescovi, M. Meleti.

- 15°Annual Conference of the Metabolomics Society METABOLOMICS2019, The Hague (The Netherland) 23-27 Luglio 2019
POSTER: Omics approach to study dyslipidemia
E. Quartieri, L. Merolle, C. Marraccini, E. Di Bartolomeo, L. Scarano, S. Bergamini, R. Baricchi, T. A. Pertinhez,

Pubblicazioni

1. **Quartieri E**, Casali E, Ferrari E, Ghezzi B, Gallo M, Spisni A, Meleti M, Pertinhez TA. Sample optimization for saliva 1H NMR metabolic profiling. Analitical Biochemistry. Vol 640, 2022, 114412, ISSN 0003-2697, https://doi.org/10.1016/j.ab.2021.114412.

2. Schiroli D, Merolle L, **Quartieri E**, Chicchi R, Fasano T, De Luca T, Molinari G, Pulcini S, Pertinhez TA, Di Bartolomeo E, Biguzzi R, Baricchi R, Marraccini C. Comparison of Two Alternative Procedures to Obtain Packed Red Blood Cells for β-Thalassemia Major Transfusion Therapy. Biomolecules. 2021 Nov 4;11(11):1638. doi: 10.3390/biom11111638.

3. Pulcini S, Merolle L, Marraccini C, **Quartieri E**, Mori D, Schiroli D, Berni P, Iotti B, Di Bartolomeo E, Baricchi R, Sala R, Pertinhez TA. Apheresis Platelet Rich-Plasma for Regenerative Medicine: An In Vitro Study on Osteogenic Potential. Int J Mol Sci. 2021 Aug 16;22(16):8764. doi: 10.3390/ijms22168764.

4. **Quartieri E**, Marraccini C, Merolle L, Pulcini S, Buzzi M, Guardi M, Schiroli D, Baricchi R, Pertinhez TA. Metabolomics comparison of cord and peripheral blood-derived serum eye drops for the treatment of dry eye disease. Transfus Apher Sci. 2021 Aug;60(4):103155. doi: 10.1016/j.transci.2021.103155.

5. Schiroli D, Marraccini C, Zanetti E, Ragazzi M, Gianoncelli A, **Quartieri E**, Gasparini E, Iotti S, Baricchi R, Merolle L. Imbalance of Mg Homeostasis as a Potential Biomarker in Colon Cancer. Diagnostics (Basel). 2021 Apr 20;11(4):727. doi: 10.3390/diagnostics11040727.

6. Meleti M, **Quartieri E**, Antonelli R, Pezzi ME, Ghezzi B, Viani MV, Setti G, Casali E, Ferrari E, Ciociola T, Spisni A, Pertinhez TA. Metabolic Profiles of Whole, Parotid and Submandibular/Sublingual Saliva. Metabolites. 2020 Aug 6;10(8):318. doi: 10.3390/metabo10080318.

7. Merolle L, Marraccini C, Latorrata A, **Quartieri E**, Farioli D, Scarano L, Fasano T, Bergamini S, Bellei E, Monari E, Tomasi A, Di Bartolomeo E, Baricchi R, Pertinhez TA. Heparin-induced lipoprotein precipitation apheresis in dyslipidemic patients: A multiparametric assessment. J Clin Apher. 2020 Jun;35(3):146-153. doi: 10.1002/jca.21770.

8. Bacchella C, Gentili S, Bellotti D, **Quartieri E**, Draghi S, Baratto MC, Remelli M, Valensin D, Monzani E, Nicolis S, Casella L, Tegoni M, Dell'Acqua S. Binding and Reactivity of Copper to R1 and R3 Fragments of tau Protein. Inorg Chem. 2020 Jan 6;59(1):274-286. doi: 10.1021/acs.inorgchem.9b02266.

Capacità e competenze personali

Madrelingua(e)	italiano
Altra(e) lingua(e)	inglese
Autovalutazione Livello europeo (*) inglese	Comprensione= Ascolto B2 (avanzato), Lettura B2 (avanzato) Parlato = Interazione B2 (avanzato), Produzione B2 (avanzato) Scritto= B2 (avanzato)
Capacità e competenze sociali	Spirito di gruppo; Buona capacità di adeguarsi ad ambienti multiculturali;
Capacità e competenze organizzative	Possiedo una forte attitudine all' organizzazione e alla pianificazione e sono abituata a ragionare per obiettivi. Buona esperienza nella gestione di progetti.
Capacità e competenze tecniche	Gestione e avviamento esperimenti di laboratorio. Utilizzo autonomo di strumenti di analisi chimica e biochimica; Chimica analitica, Chimica farmaceutica, Biochimica, Biologia cellulare. Spettroscopia 1H-NMR, potenziometria, spettrofotometria, colture cellulari, basi di citofluorimetria.

Capacità e competenze informatiche	Ottimo utilizzo pacchetto Office e altri programmi analisi dati di laboratorio: Microsoft Office, GraphpadPrism, MestreNova, Origin.
Capacità e competenze sportive	Ho praticato pallavolo a livelli agonistici e possiedo il patentino dal allievo-allenatore.
Altre capacità e competenze	
Patente	Automobilistica (Patente B)

Autorizzo il trattamento dei miei dati personali ai sensi del D.Lgs. 30 giugno 2003, n. 196 "Codice in materia di protezione dei dati personali" e per gli adempimenti previsti dal D.Lgs. 14 marzo 2013, n.33 riguardante gli obblighi di pubblicità, trasparenza e diffusione di informazioni da parte delle pubbliche amministrazioni.

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