Universidad Europea

TRABAJO DE FIN DE GRADO

Grado en Odontología

SALIVA METABOLOME: A PROMISING DIAGNOSTIC TOOL CONCERNING ORAL AND SYSTEMIC DISEASES

Madrid, curso 2020/2021

Número identificativo 241

Abstract

Introduction: Salivary Metabolomics is a non-invasive, fast technique with a great potential for the diagnostic and prevention of oral and systemic disorders. New technologies such as H-NMR spectrometry, and capillary electrophoresis mass spectrometry (CE-MS) are able to identify and quantify biomarkers that will help diagnose and monitor the general health, leading to an individualized metabolic profile. It is a promising diagnostic tool that will help recognize metabolic patterns of oral and systemic diseases to lead to an early prevention.

Objectives: The aim of this research is to review the literature regarding Salivary Metabolomics and its impact on the future of diagnostic and personalized medicine.

Methodology: The present study was carried out by an extensive literature review, using PubMed, Medline, Wiley Online Library, and the online resources provided by the Biblioteca Crai de la Universidad Europea de Madrid.

Discussion: Metabolomics makes possible the study metabolic pathways that influence human organisms thanks to a salivary sample. A comprehensive review of the oral and systemic diseases' biomarkers was conducted.

Conclusions: Future studies with a combination of technologies, a universal collection protocol, and medical staff 's education will bring salivary diagnostics to become one of the most used tools for preventing and monitoring general health.

Keywords: Saliva Metabolomics, Saliva Biomarkers, Saliva and oral diseases, Metabolomics and oral diseases

Resumen

Introducción: La Metabolómica Salival es una técnica no invasiva, rápida y con un gran potencial para el diagnóstico y prevención de trastornos orales y sistémicos. Nuevas tecnologías como la espectrometría H-NMR, y la electroforesis capilar acoplada a la espectrometría de masa (CE-MS), son capaces de identificar y cuantificar biomarcadores que ayudarán a diagnosticar y monitorizar la salud general, dando lugar a un perfil metabólico individualizado. Es una herramienta de diagnóstico prometedora que ayudará a reconocer los patrones metabólicos de las enfermedades orales y sistémicas y conducirá a la prevención temprana.

Objetivos: El objetivo de esta investigación es revisar la literatura relativa a la Metabolómica Salival y su impacto en el futuro de la medicina diagnóstica y personalizada.

Metodología: El presente estudio se ha realizado mediante una amplia revisión bibliográfica, utilizando PubMed, Medline, Wiley Online Library, y los recursos online proporcionados por la Biblioteca Crai de la Universidad Europea de Madrid.

Discusión: La metabolómica permite estudiar las vías metabólicas que influyen en el organismo humano gracias a una muestra salival. En este estudio se ha realizado una revisión exhaustiva de los biomarcadores de enfermedades orales y sistémicas.

Conclusiones: Estudios futuros con una combinación de tecnologías, un protocolo de recogida universal y la formación del personal médico harán que el diagnóstico salival se convierta en una de las herramientas más utilizadas para la prevención y el seguimiento de la salud general.

Palabras clave: Metabolómica salival, Biomarcadores salivales, Saliva y enfermedades orales, Metabolómica y enfermedades orales.

Abbreviations

BC	Breast cancer
BCAA	Branched-chain amino acids
CE-MS	Capillary electrophoresis mass spectrometry
CE-TOF-MS	Capillary Electrophoresis Time-flight-mass Spectrometry
EHMN	Edinburgh Human Metabolic Network
FAS	Fatty acid synthase
GAgP	Generalized Aggressive Periodontitis
GC-MS	Gas Chromatography-Mass Spectrometry
GCP	Generalized Chronic Periodontitis
HC	Healthy Control
HILIC	Hydrophilic Interaction Chromatography
HNSCC	Head and Neck Squamous Cell Carcinoma
HPLC-MS	High performance liquid chromatography- mass Spectrometry
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	Liquid Chromatography-Mass Spectrometry
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OBO	Open Biomedical Ontologies
OLK	Oral Leukoplakia
OSCC	Oral Squamous Cell Carcinoma
PD	Periodontal Disease

POC	Point of Care
pSS	Primary Sjogren's syndrome
RLPC	Reversed-Phase Chromatography
SALO	Saliva Ontology
SS	Sjogren's syndrome
sSS	Secondary Sjogren's syndrome
TCA Cycle	Tricarboxylic acid cycle
UPLC-QTOF MS	Ultra Performance liquid chromatographyquadrupole/time-of-flight mass spectrometry

Acknowledgments

This thesis involved the behind-scene support and work of so many people to whom I am grateful.

My tutor guided me with a dedication that made all the difference. For her generosity, her ferocious eye for details, and words of encouragement, I am deeply thankful. This thesis would not have come together without her.

This journey has been made possible by my family, my parents and siblings. Their support over the years has led me to this very moment.

My family never doubted me, even when I would not have given myself a chance. Their strength, love and grace have been the most precious gift and I'm forever grateful.

To Josef, he lifts me up with his humor and wit. His shoulders have always been a safe place where to rest.

To my sister Gaia whose example of courage and resilience is forever going to be what I aspire for.

To my best friend, Irene. Thank you for choosing me as your sister.

Finally, the people I dedicate these years of sacrifices and joys are my grandparents. You taught me to keep my head high, dream big, and you gave me the spark to light my own star. I will be eternally yours.

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Introduction

Diseases are often detected in their advanced stages because of a lack of proper techniques that provide specificity and early detection. The more advanced the disease is, the more invasive the diagnosis will be. The increasing need to provide early and non-invasive diagnosis opened doors to Salivary Diagnosis. It became a rising diagnostic and preventing tool in the past decade.

The study of metabolomics applied to the saliva is the most recent advancement in salivary diagnosis. It is the future for a fast, non-invasive, and still sensitive diagnostic [(1)]. By analyzing metabolites present in the saliva, using analysis techniques such as mass spectrometry (MS), it has been possible to identify biomarkers that will help diagnose oral and systemic diseases, from periodontitis, oral squamous cell carcinoma, and breast cancer among them (Figure 1) [(2)].

With the objective to gather and collect all the data from different studies based on saliva sample and to facilitate the research and faster diagnosis, the University of California (UCLA) created a data bank, known as The Salivaomics Knowledge Base and the Open Biomedical Ontologies (OBO) developed a new Saliva Ontology (SALO) [(3)]. Its purpose is to make accessible salivary data both for clinical diagnosis and research by organizing a new vocabulary to integrate disparate data among different experts, chemists, biologists, dentists [(4)].

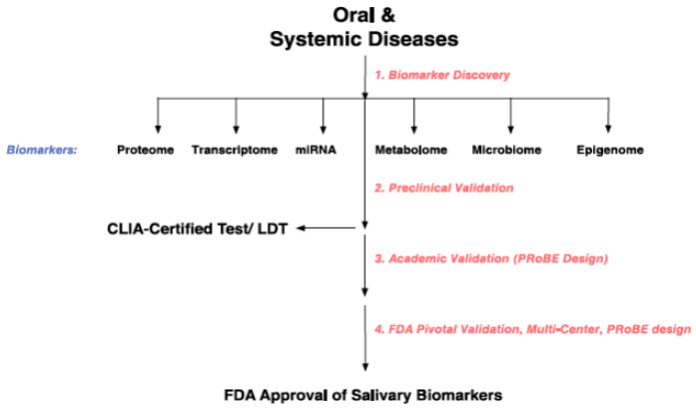

Figure 1. A schematic overview of the development of salivary biomarkers. (Dawes *et al.* 2019) [(8)]

The simplicity of sample collection is one of the most attractive prospects of the use of saliva as a useful and novel tool to monitor patients with both oral and systemic diseases. Metabolomics studies consider variables such as sex, habits, nutritional changes, age, and diurnal cycle, diseases and point the attention on the variabilities of the subjects, making evident the strength and the sensitivity of the techniques used to detect metabolites (Figure 2) [5,6].

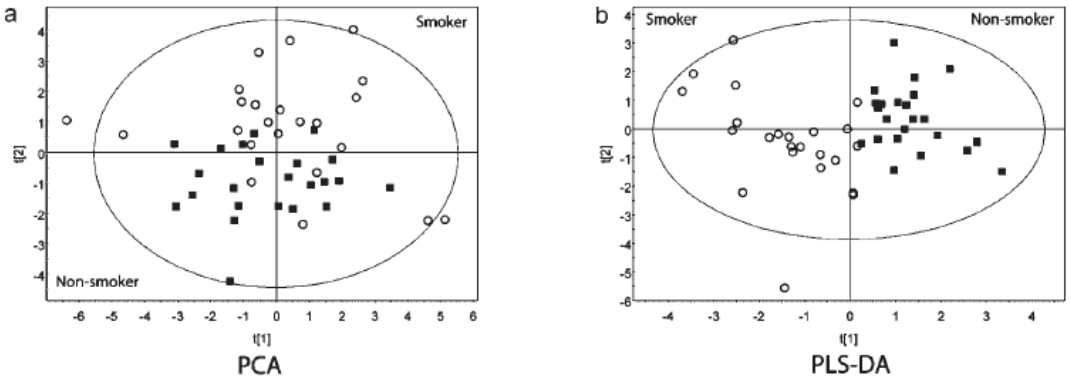

Figure 2. PCA and PLS-DA are comparing the saliva metabolites of smokers vs. nonsmokers. (Takeda *et al.* 2009) [7]

In many cases, specific components such as urea in the salivary pH or lactate are biomarkers of dental caries and future progression of the periodontitis. Many studies showed how Saliva Metabolomics made it possible to identify specific metabolites for diabetes type I in young children and breast cancer [7]. Moreover, it has been proved that the saliva metabolome can give an insight into the early diagnosis of oral cancer and recognize metabolite patterns of other forms of cancer [8]. This thesis will describe the diagnostic impact of the Saliva Metabolomics approach and its biomarkers in oral and systemic diseases.

1.1 Metabolomics

The term Metabolomics was first introduced by Roger Williams in the late 1940s. By testing biological fluids, Williams recognized specific patterns in them, considered distinctive for each individual. However, only in the 1960s, the progress in gas and liquid chromatography technology made it possible by analyzing simultaneously various components. What first was considered by Williams as a titanic task, is now made more approachable to many. It was possible to recognize what was referred to as *metabolic profiles.* Firstly, introduced by Hornings *et al.* [9], they stated that gas chromatography-mass spectrometry coupled with mass spectrometry is able to describe metabolic patterns and enabling the distinction between pathological and normal states for pharmacological and medical resources [9].

Steve Oliver in 1998 referred to Metabolomics as the set of metabolites in a biological cell, tissue, organs or organism, which are the end products of cellular processes [10,11]**.** Metabolites are molecules that are transformed during metabolism and are able to provide important information regarding any biochemical pathways. Moreover, Metabolomics is the quantitative and comprehensive study of dynamic metabolic processes in response to physiological and/or genetic alterations. The analysis of the metabolomics is the study of the final products of the previous "omics." While Genomics, Transcriptomic and Proteomics focuses on the identification, regulation and synthesis of a genetic product, the Metabolomics study the products of the metabolic reactions that take place inside the cells [12]. Thanks to Metabolomics, it is possible now to study the metabolic response produced by genetic and environmental changes (Figure 3) [13]. Thus, Metabolomics, combined with other "omics" studies, helps to create a complete and comprehensive map of what is happening in a specific moment inside the cells.

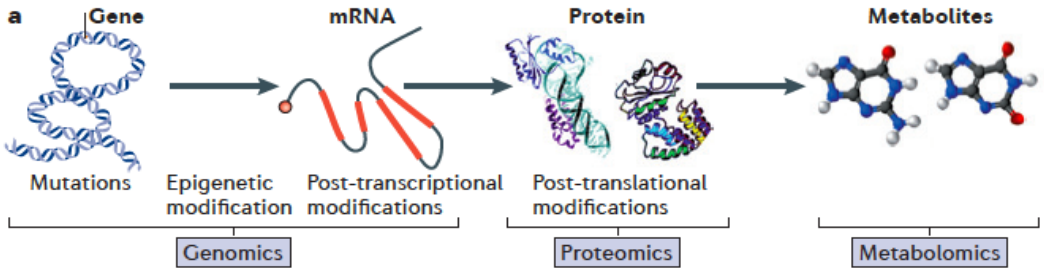

Figure 3. Metabolomics reveals the pathway from genotype to phenotype, representing the end-product of the biochemical activity. (Patti *et al.* 2012) [12]

Clinical sampling comprises a wide range of biological fluids, tissues, and cells. Urine, blood, plasma, primary cells, kidney tissue, liver tissue are some of the samples studied in metabolomics. The choice depends on what is the purpose of the study, where the biofluids are used to investigate biomarkers, tissues are collected to study specific pathophysiological mechanisms [14,15].

1.2 Targeted and Untargeted Metabolomics

Metabolomics has seen exponential growth in the past decade, thanks to introducing new techniques focusing on metabolomics profiling [16]. Every metabolic study is a comparative one, so two types of samples will be collected: one that will be studied, and another one that will be used as a control sample [14]. The research can be either based on *a priori hypothesis* or targeted metabolomics and *non-a priori*/untargeted metabolomics [13,15]. Targeted metabolomics aims to quantify one or more metabolites. This quantification can be done in any sample from tissues to blood and other body fluids. The targeted approach is often used for pharmacological and therapeutic reasons because it is based on a precise hypothesis and focused on a particular biochemical question. Thus, it is limited to the identification of a specific metabolite in a sample.

In fact, the major drawback of this approach is that the high specificity for determined chemical classes does not allow the discovery of new metabolites [14].

The untargeted metabolomics was firstly proposed to connect genotype to the phenotype for a single metabolite. Firstly, metabolites are extracted and then separated used different chromatographic approaches (gas or liquid chromatography) and then processed with NMR (nuclear magnetic resonance) or MS/MS (mass spectrometry) to obtain putative identification (Figure 4) [13,14]. The untargeted metabolomics research found an increasing number of endogenous metabolites that are still unknown, and more are expected to be found.

A new data bank called *The Human Metabolome Database* was explicitly created for a meta-analysis of all the untargeted metabolomics studies. It is now possible to link protein and gene functions to biochemical processes, identify them, and therapeutically target them. Together with *The Human Metabolome Database*, the *METLIN* is one of the most used databases. It contains more than 45,000 metabolites that have been examined with mass spectrometry. If combined, both databases are able to give both identification and interpretation of the metabolites [13]. Other database used for metabolome identifications worth to mention are: *MetabodID, MassBank, Chenomox NMRSuite, Metscape, Kyoto Encyclopedia of Genes and Genomes* (KEGG), *Edinburgh Human Metabolic Network* (EHMN) [5,14].

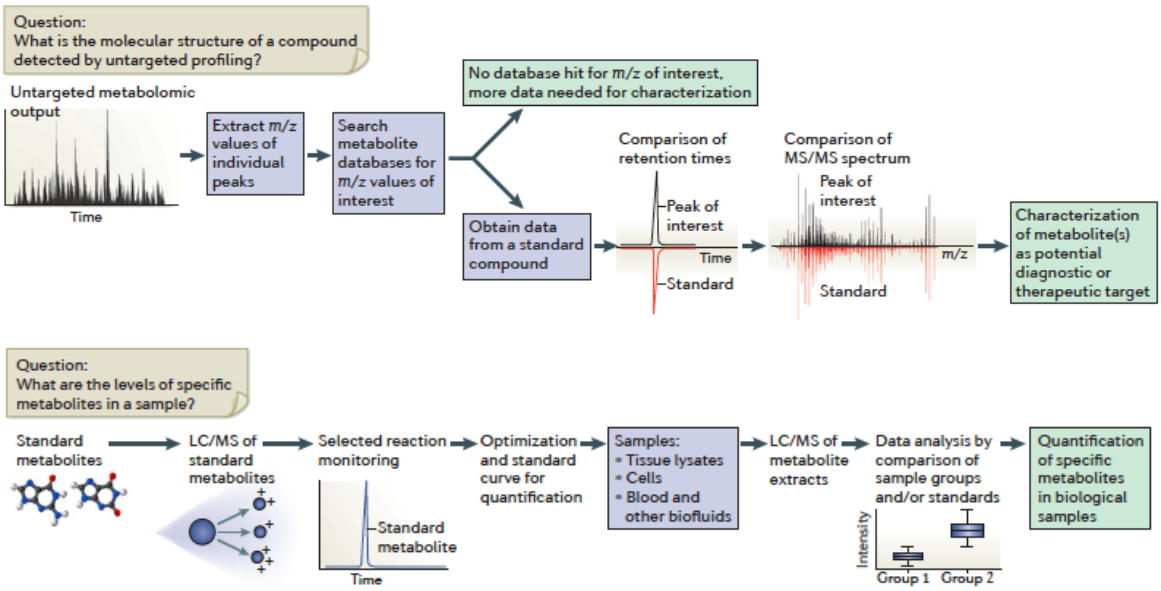

Figure 4. Untargeted and Targeted metabolomic workflow. (Patty *et al.* 2012) [11]

With new developments on the screening methods, it is possible to see as a mutation of a single gene can cause a domino effect of metabolite reactions, questioning the past hypothesis that one gene influences only one function in the cellular processes [13].

1.3 Human Saliva

Human saliva is such an attractive prospect for the future of diagnosis because it contains a large variety of enzymes, hormones, proteins. Secreted by salivary glands, where the submandibular gland produces 65-70% of the total amount [5]. The primary salivary glands are the parotid, the submandibular, and the sublingual glands (Figure 5), which affect the composition of saliva by providing different contents [17,18].

The parotid gland secretes more watery liquid than the others. The submandibular secretes fluid similar to the parotid but containing amylase, making the saliva more mucous. The sublingual gland instead contains mostly mucous cells [18]. Saliva is

composed of 99% water with electrolytes, organic and inorganic, mRNA, 0.3% of proteins, enzymes, and hormones, with a pH of 6.0 to 7.0.

The function of saliva is to protect and lubricate the oral cavity, together with mastication and speech. Most importantly, saliva has a protection function over the oral cavity. A healthy individual will make more or less 0.5L to 1.5L per day; loss of saliva production can significantly alter an individual's health, from a higher prevalence of caries to the start of periodontitis. Moreover, many constituents reach the oral cavity thanks to the cells by transcellular passive and active transport or paracellular routes within the salivary glands or even intraoral bleeding and gingival crevicular fluid [(3)]. Other components instead can originate from bronchial and nasal secretions.

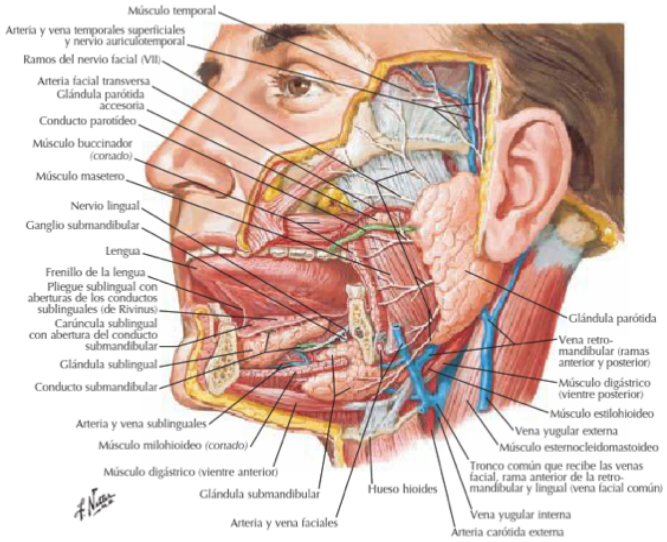

Figure 5. Anatomy of the Salivary Glands. (Netter 2014)[(17)]

1.4 Saliva as diagnostic tool

As mentioned previously, the composition of saliva represents the best resource pool to investigate oral and systemic diseases. Therefore, saliva can be considered a surrogate to plasma. In fact, most components present in the blood pass through the

salivary glands and gingival sulcus. In addition, hormonal changes, habits, and metabolic disorders affect the composition of saliva [3]. It has been referred to as the "mirror of the body" [1] the analysis of saliva can help the current medical investigation to monitor and diagnose systemic diseases in their early stages. To achieve this goal is not necessary any trained personnel, making the screening work faster and easy to perform chair-side. It has been demonstrated that patients and young children are more willing to take saliva samples than blood's, since it doesn't require the use of needles [19]. It is indeed easy to use, inexpensive and requires less manipulation compared to blood samples. Moreover, there is less risk for cross-contamination and it provides accurate diagnostic values [20]. It is important to take in consideration when analyzing saliva some parameters such as gender, age, habits, type of saliva collected (stimulated and unstimulated), and diet. As for the collection and the analysis method, they can influence and present different sensitivity to identify and quantify metabolites [21]. Many studies have demonstrated that unstimulated saliva contains more components due to the less watery content regarding the type of saliva. At the same time, a combination of analytical technologies such as NMR and Liquid Chromatography-Mass Spectrometry (LC-MS/MS) has been recommended to identify a broader range of metabolites. The main disadvantage is that currently, the database's size is relatively small for identification and interpretation. However, the direction of salivary diagnosis is going towards a personalized form of medicine. It is possible to identify biomarkers that explain the individual variations of metabolic pathways and the therapy's response [11].

All these characteristics make salivary diagnosis a promising candidate to monitor and identify biomarkers for oral and systemic diseases, and therefore defined saliva

as one of the most important biofluids [5]. There are many fields of application of saliva diagnostic, in medicine it is used to determine the early detection of various

cancers such as pancreatic cancer, breast cancer and oral. It has been proved an efficient tool for autoimmune disorders and infectious diseases.

In the last years, emerging technologies have challenged the gold standard of Salivaomics, mass spectrometry, and chromatography, among others, and introduced Point-Of-Care (POC) diagnostic, RNA sequencing, liquid biopsy, and electrochemical detection. These new technologies integrated with the traditional ones are to flourish and to introduce population-based screening programs [22].

Objectives

This thesis's main objective is to describe and review the analysis and study of Saliva Metabolome and its use to find out biomarkers for oral and systemic diseases. For this purpose, the following objectives were proposed:

- To understand and to evaluate saliva as a diagnostic tool.
- To study metabolomics and its developments in medicine and science.
- To study the different techniques of data analysis of saliva metabolomics.
- To review and analyze the literature regarding saliva metabolomics, highlighting the most important results.
- To identify and to determine biomarkers present in the saliva metabolomics for oral and systemic disease.

Methodology

The bibliographic research for this study was carried out using the following databases: PubMed, Medline, Wiley Online Library, and the online resources provided by the Biblioteca Crai de la Universidad Europea de Madrid. Additionally, Google Scholar was used to finding additional articles. Regarding the library search, it was conducted by an advance research using different keywords like “saliva metabolomics,” “metabolomics,” “targeted metabolomics”, “untargeted metabolomics”, “saliva diagnosis,” “saliva and oral diseases,” “saliva biomarkers,” “metabolomics and oral diseases.” Moreover, the data collection was performed by adding a time frame from 2010 and 2020.

Fifty-two articles have been studied and examined, with the limit of the publishing year of a maximum of the past 10 years, i.e., between 2010 and 2020. However, to comprehend better the beginning of Saliva Metabolomics an exception was made for one of the articles that was dated 2009 and one article dated 1978, which they were considered important for the history and development of Metabolomics. Two books were additionally consulted, through the online library of the Universidad Europea de Madrid. The entire literature was collected using a program called Mendeley and then integrated into the thesis. To write this study, it was made a comprehensive review of the literature on Saliva Metabolomics.

Keywords: Saliva Metabolomics – Saliva Biomarkers - Saliva and oral diseases - Metabolomics and oral diseases

Results and Discussion

1. Oral Diseases

1.1 Dental Caries

Dental caries is a process of demineralization of the tooth surface which is caused by a change in the oral microflora and sugar metabolism (Figure 6) [23]. The study of salivary metabolomics regarding dental caries is critical in preventive dentistry and assessing risk factors in children. The most important biomarkers found on the reviewed articles are outlined in Table 3.

To identify a putative metabolic profile of dental caries, some authors focused on identifying metabolic variations such as the TCA cycle (Tricarboxylic acid cycle) in the supragingival plaque, glycolysis, and on the hormonal changes in the different dentition stages [25,26].

Fidalgo *et al.* [25] conducted a study on children with the objective to investigate the metabolic changes according to the different dentitions correlated to the hormonal age changes. They observed that the subjects with cavities presented lower *phenylalanine, propionate,* and *saccharides* levels compared to the no caries group. Moreover, lower *saccharide* levels were observed, which may be explained by the bacterial metabolism that uses saccharide as an energetic reservoir and higher colonization of cariogenic bacteria. *Lactate, fatty acid, butyrate,* and *acetate* were instead increased.

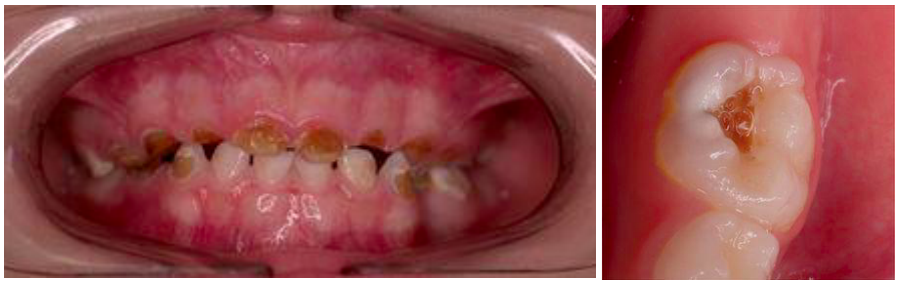

Figure 6. Dental caries (Pizarro *et al.* 2014) [24]

The significant presence of these metabolites lowers the pH and increases the susceptibility for the supragingival plaque by attracting bacteria such as *S.mutans* and *Lactobacillus*. It was found that after dental treatment, the levels of *S.mutans* decreased in children [26]*.* Since the oral microorganisms influence the production of the sugar fermentation and acetic acid, the saliva acts as a counterpart by using regulatory systems such as bicarbonate/carbonic acid buffer. For this reason, Fidalgo *et al.* [26] expected to find different variables of salivary metabolites on dental caries before and after restorative treatment. The authors analyzed *acetate, n-butyrate, fatty acids,* and *propionates* concentrations before and after dental treatments. What was found were decreased levels of *acetate, n-butyrate* and *fatty acids*. These findings strengthen the hypothesis that these components are correlated to the oral microorganism's presence.

In addition, *saccharide* concentrations were reduced after dental treatment, followed by a reduction of bacterial microorganisms. However, the authors noted as the decrease of rough surface areas reduces the bacterial adherence and maintenance of metabolites that promote it, causing demineralization due to the *sucrose*. Moreover, the lipid concentration was higher in the children more susceptible to caries [25,26]. In fact, salivary lipids are as well involved in the mechanisms of demineralization of the tooth surface, and high concentrations of lipids can be found in dental plaque which low pH favors the spread of *Lactobacillus*. This mechanism is fundamental in the prevention of caries since the presence of the dental plaque is essential in the promotion of cariogenic microorganisms. Said so, its periodical removal is critical in the dental practice in high-risk patients, both adults and children. Moreover, high concentration levels of *pentose-phosphate* pathway were found

where *Streptococcus* and *Actinomyces* were present, indicating a similar metabolic profile. This finding points out the fact that specific bacteria influence specific pathways which changes affect the demineralization of the tooth surface and the plaque bacteria influence the formation of dental caries (Figure 7) [27].

Takahashi *et al.* [27] observed that supragingival plaque yield all the metabolites of the central carbon metabolism. Moreover, when glucose rinsed the profile changed, it was possible to observe an increase in the levels of *pentose-phosphate*. Meanwhile in the TCA cycle components such as *succinate, fumarate* and *malate*, *cis-aconitate* and *isocitrate* were decreased, which indicates that the two pathways function as complementary. As for the glycolysis, *3-phosphoglycerate* and *phosphoenol-pyruvate* concentrations were decreased. *N-acetylate* sugars related to glycoproteins were instead increased, because they were released by the bacterial enzymes that promote hydrolysis of glycoproteins linkage to carbohydrate moieties. It was found that the advance intake of carbohydrates, influenced by dietary and oral habits, changed the metabolic profile of the saliva. *Fucose*, *galactose*, *xylose* and *glucose* concentration increased since presumably acting as carbohydrate moieties, in particular *fucose* increase has been reported as well in patients with periodontal diseases [28].

However, the studies [25–28] showed that despite the enhancement of the oral health of the patients, the metabolic profile of the children who presented caries and had active caries lesions, were not the same as those children who never had them. This suggests that once the child develops caries, the oral environment has a permanent metabolic change which it will, later on make the child more susceptible to caries in adulthood. Furthermore, this study proved that a deeper look into the metabolic profile of the saliva is giving a better prevention plan to recover the oral health status of patients at risk. In conclusion, preventive treatments, such as periodical prophylaxis and managing the

dietary intake of carbohydrates, appears to be fundamental in fighting the cariogenic microorganisms that alter the homeostasis of the oral environment, and it is especially critical in highly susceptible subjects.

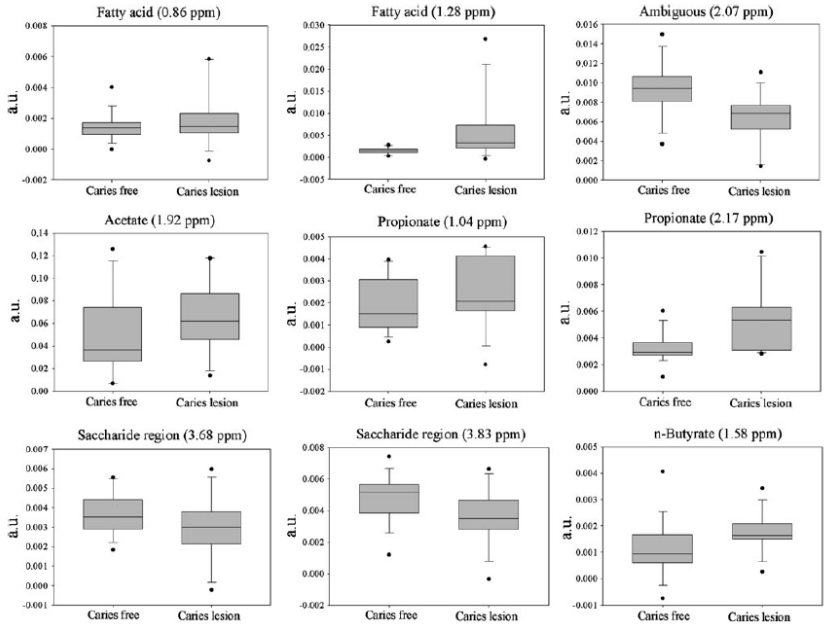

Figure 7. Box plots for dental caries metabolites (Fidalgo *et al.* 2013) [25]

Saliva Metabolites relevant to the identification of Dental Caries		
Metabolites	**Author**	**Method**
Phenylalanine Propionate Saccharides Lactate Fatty acid Acetate Butyrate	Fidalgo *et al.* [25]	NMR spectroscopy [25]
Cis-aconitate Isocitrate Pentose-phosphate Malate Fumarate Succinate 3-phosphoglycerate Phosphoenol-pyruvate	Takahashi *et al.* [27]	CE-MS [27]
Fucose Galactose Xylose Glucose N-acetylated sugars Lactate Acetate Hypoxanthine	Pereira et al. [28]	NMR spectroscopy [28]

Table 1. Saliva Metabolites identified as dental caries biomarkers.

1.2 Periodontitis

Periodontal disease is caused by multifactorial factors between the host and the oral bacteria in the subgingival areas. It is one of the most common oral diseases after caries, and its diagnosis is very relevant in everyday practice. This disease's early stage is manifested by gingival bleeding, redness of the gums, dental pockets, and increased gingival crevicular fluid. In advanced stages, periodontitis can cause loss of teeth, progressive bone loss and destruction of tissues (Figure 8) [29,30]. There are two clinical forms of Periodontitis, Chronic and Aggressive that can be either Localized or Generalized. In both representations, there are signs of inflammation associated with the presence of calculus, heavy or moderate, and similar risk factors. Therefore, it is considered Chronic Periodontitis when there is a superimposition on localized and generalized aggressive periodontitis [29]. If particularly severe, it can affect the quality of life and can lead to systemic diseases like arteriosclerosis, coronary artery disease [31]. To these days, periodontitis is diagnosed by the dentist by probing, assessing the level of attachment loss, and radiographs, and unfortunately, it is only recognized in its late stages.

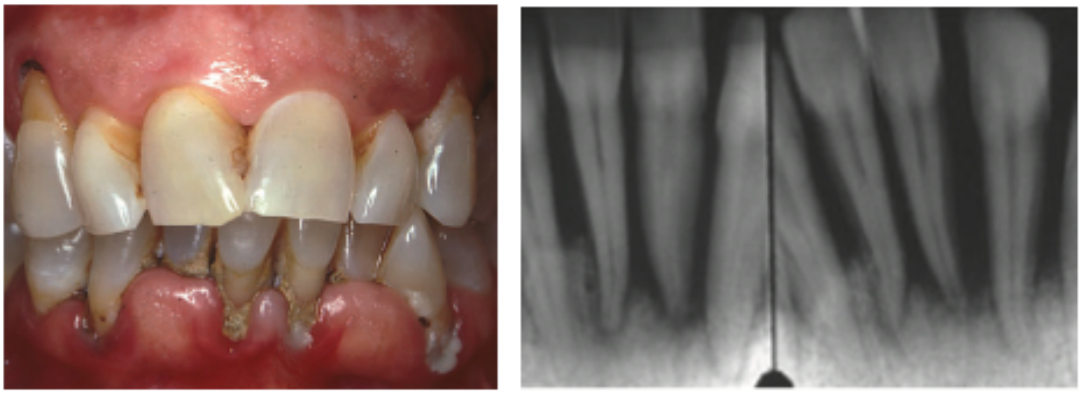

Figure 8. Clinical and radiological findings of Periodontitis (Armitage *et al.* 2010) [29]

For this reason, it is crucial to obtain a diagnostic method that could screen and monitor periodontitis in its early stages. Since periodontal diseases varies according to age, habits, for the salivary diagnosis, distinctions were made between age, sex, habits, and number of missing teeth. Periodontal analysis of probing depth and clinical attachment level are also necessary to determine the degree of the periodontal severity.

The results obtained from these studies [30–33] have been summarized in Table 2. Liebsch *et al.* [31], demonstrated that Periodontitis is prevalent in middle age patients, while the percentage of missing teeth influences the presence of Periodontitis in older subjects. By using two analysis methods ultra-high-performance liquid chromatography and tandem mass spectrometry for the identification of untargeted metabolites, the authors found that the tooth loss produced an increase in the *protease activity*, which leads to lower protein reduction and amino acid liberation. As for the metabolites, *phenolic acid metabolites* were repetitive in all subjects with the disease in all ages. In comparison, *phenylacetate* was especially prominent in younger patients. During the early stages of the disease, the inflammation causes an increase of pro-inflammatory mediators that originated from the *arachidonic acid*. This process does not only cause gingival inflammation but as well bone resorption. *Pro-inflammatory* PGE_2 was also found in patients with periodontitis and associated with stimulation of bone loss. More metabolites associated with periodontal inflammation are *5-Oxoproline, Phenylalanine catabolites, 5-aminovalerate*.

To gain insight into the molecular level, Generalized Aggressive Periodontitis (GAgP) and Generalized Chronic Periodontitis (GCP) several studies were performed [32,33]. The clinical distinction of these two forms of periodontitis is based on intraoral examination and radiological findings, which could lead to error. The aim of these

studies [32,33] was to identify discriminative biomarkers by finding metabolites that will differentiate patients with periodontitis and healthy individuals.

The authors found that what influenced most the saliva metabolomes were the products of bacteria and there was no significant distinction between the two forms of Periodontitis. Despite not being able to distinguish the metabolic profile of GAgP and GCP, the authors found different concentrations of metabolites that discriminated between the healthy control group and the group with periodontitis. In GCP patients the levels of *isoleucine*, *valine*, *tyrosine*, *proline*, and *phenylalanine* were higher than in the HC. While *N-acetyl* group, *pyruvate* and *lactate* were lower in the GCP's, as for the GAgP's with the addition of *sarcosine*. Higher concentrations of *formate, phenylalanine* and *tyrosine* were also found in the GAgP's. As the disease progresses, the conditions in the mouth in terms of pH, redox potential, oxygen, and macromolecules of the individual, which are associated with the bacterial composition, change the metabolic profile of the saliva. For further clarification, the presence *of P. gingivalis* and *Fusobacteria* produce an increase in *butyric acid*. Once this metabolite is released, it can cause a reduction in the functions of the epithelial cells and defense. Thus, *butyric acid* is associated with gingival inflammation. *Isovaleric acid* was observed to influence the progression of the disease. The increased combination of *butyric acid* and *isovaleric acid* produces a decrease of *lactic acid bacteria*, which represents the healthy microbial component of the oral environment. By decreasing it, more Gram-Negative bacteria will indeed proliferate. As a matter of fact, the metabolic profile of a patient with Periodontitis presents lower levels of *lactic acid* [32]. The reduced concentration of lactate is connected to the periodontal bacteria that convert *acetate* and *propionate*. The up-regulation of the metabolic activities of *protease*, *glycosidase* and *lipase* are the responsible for the tissue degradation and they are the

perfect reservoir for bacterial proliferation like *P.Gingivalis* and *A.actinomycetemcomitans* which are the main periodontal bacterial species [33].

Another study that focused on the diagnosis of periodontitis through saliva metabolomics was conducted by Kuboniwa *et al.* [30]. In order to improve the results and collect the most metabolites, the authors removed the supragingival plaque before collecting the saliva. (Figure 9). This clinical passage resulted in an improvement of the findings.

The authors found sixty-three metabolites, of which eight of them were able to link as biomarkers for periodontal inflammation. The most significant metabolites found were: *Ornithine, 5-Oxoproline, Valine, Proline, Spermidine, Hydrocinnamate, Histidine, Cadaverine*. *Proline* and *Histidine* were furthermore associated with plaque presence (Figure 10). Oral polyamine, histidine and tryptophan were also identified as correlated to gingival inflammation [34]. *Cadaverine, 5-Oxoproline and Histidine* were associated with moderate or severe periodontitis, because they are associated with dental biofilm. The accumulation of plaque enhances the production of *cadaverine* from *lysine* which prevents the oral epithelium to stop the proinflammatory bacteria.

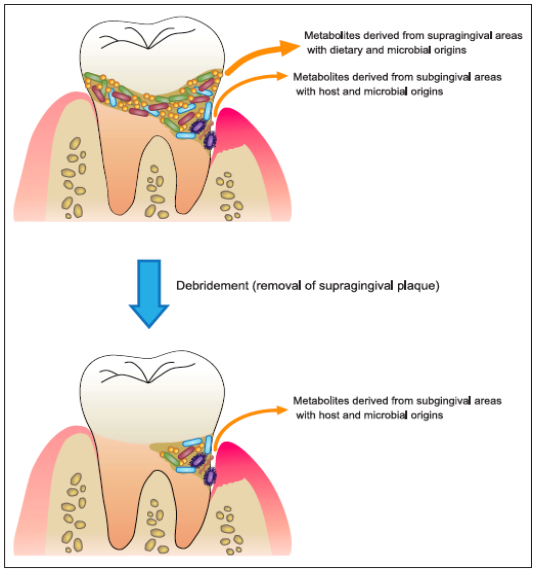

Figure 9. This model represents how the debridement improve the detection of metabolites in the saliva for the identification of periodontitis biomarkers. (Kuboniwa *et.al.* 2016)[30]

Metabolite	VIP
Ornithine	2.570
5-Oxoproline	1.994
Valine	1.993
Proline	1.354
Spermidine	1.152
Hydrocinnamate	1.079
Histidine	1.040
Cadaverine	1.000

VIP, variable importance in projection.

Figure 10. Metabolites associated to PD based on VIP values (Kuboniwa *et.al.* 2016) [30]

Saliva Metabolites relevant to the identification of Periodontitis		
Metabolite	***Author***	***Method***
Butyric Acid Isovaleric acid Lactic Acid	Rzeznik *et al.* [32]	NMR spectroscopy [32]
Phenolic acid metabolites Phenylacetate Proinflammatory PGE_2 5-Oxoproline Butyric Acid Isovaleric acid Lactic Acid	Liebsch *et al.* [31]	Nontargeted UHPLC-MS/MS [31]
Proline Histidine Cadaverine Spermidine Hydrocinnamate Phenolic acid metabolites Phenylacetate	Romano *et al.* [33]	NMR spectroscopy [33]
Polyamine Tryptophan Proline Histidine Cadaverine Spermidine Hydrocinnamate	Sugimoto *et al.* [34]	CE-TOF-MS [34]
5-aminovalerate Phenylalanine catabolites Ornithine Proinflammatory PGE_2 5-Oxoproline	Kuboniwa *et al.* [30]	GC-MS [30]

Table 2. Saliva metabolites biomarkers for Periodontitis.

1.3 Oral Cancer

Oral cancer is a widespread cancer that has a multifactorial origin and an overall survival of 5 years since its diagnosis. It is usually malignant and mainly located in the tongue and on the floor of the mouth (Figure 11). At its early stages, it can be asymptomatic and therefore difficult to diagnose. However, when the lesions reach a more significant size, it becomes painful, and it can be followed by ear pain, difficulty to speech, and also to hear. It can be common as well to find patients that developed cervical lymphadenopathy [35].

For this reason, a prompt diagnosis is essential to reduce its morbidity. Nowadays, the most common diagnosis method is a biopsy, still an invasive technique that must be performed by specifically trained dentists and doctors. Saliva presents an excellent opportunity to detect oral cancer in its early stages, improving the rate of survival [35].

To study the biomarkers of Oral Cancer that are present in the saliva, a review of the most recent articles [34,35,37–42] has been performed and the most significant findings have been summarized in Table 1.

The metabolomics of Oral cancer has shown altered cellular pathways, which are common to most types of cancers. Therefore, most of the drugs used in chemotherapy are based on targeting those specific metabolomic pathways.

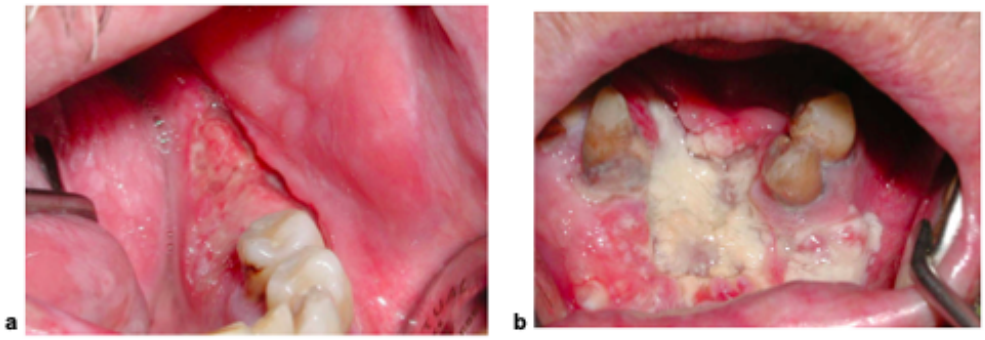

Figure 11. (a) OSCC on the gingiva; (b) Oral cancer on the floor of the mouth (Began *et al*.2010) [35]

The main metabolic pathway alterations were identified as: glycolysis, altered choline phospholipid metabolism, TCA cycle (tricarboxylic acid), antioxidant mechanism, increased influx of amino acids, lipolysis, glutaminolysis.

One of the main alterations is detectable in glycolysis and what's called the "Warburg effect", which causes the tumor to import glucose that it's needed for the glycolysis that serves as energy for the cancer cells. Moreover, glucose works together with glutamine, which is an amino acid that will provide the necessary energy to the tumor cell when glycolysis is not enough. Glucose metabolism through glycolysis will later produce precursors of amino acids, lipids, and nucleic acids to serve as the means of tumor cell proliferation. In fact, higher activity of the *glucose* and *glutamine* levels was detected in the OSCC groups [38,41].

Glycolysis produces *pyruvate acid* that supplies oxygen and *lactic acid*, which acid environment promotes the metastasis, and ultimately indicates the stage of the tumor. It is essential to mention as well that glucose metabolism produces the precursor of amino acids and lipids that are needed for cell proliferation [41].

More in detail, what it was found by using HPLC-MS, was a significantly lower concentration of *phenylalanine*, *GABA*, *valine,* and higher levels of *n-eicosanoic acid* and *lactic acid* in the OSCC group [42]. In fact, since cancer cells have increased glycolysis, it creates continuous production of *pyruvate* that will be converted into acetyl CoA, which will serve for fatty acid synthesis and start the TCA cycle. Indeed, increased levels of fatty acids have been observed as early biomarkers of cancer progression [41]. Moreover, higher concentration of *lactic acid*, *phenylalanine*, and *valine* are considered some of the best markers, providing as well as discriminative diagnostic between OSCC and Oral Leukoplakia (OLK) (Figure 12) [38,39,42].

These metabolites present high sensitivity and positive predictive value [39]. While lactic acid is produced in big quantities, lower levels of pyruvate are detected in patients with OSCC, which means that the TCA cycle production of energy will be impaired. *Branched-chain amino acids* (BCAA) will act as energy sources for the TCA cycle and were found in lower concentrations in cancer cells. It has been observed as well that the more advanced the tumor, the lower the levels of BCAA will be. It has been concluded that BCAAs play an important role in the energy production of the cancer cells, since their intermediates are used in the TCA cycle (Figure 13) [34]. Among the BCAA, higher levels of *taurine, valine, cadaverine, tryptophan, isoleucine, 2-oxoisovaleric acid, and leucine* were found as discriminatory biomarkers in cancer patients [40].

As part of the TCA cycle's cascade reaction, in patients with OSCC the salivary levels of GABA are decreased due to the increased metabolic demand of intermediates, BCAA, for the TCA cycle, *glutamate* is now used to reinforce the TCA cycle, leading to an increase consume of GABA, thus its lower concentration in the saliva [42]. The decreased *GABA* levels are an important marker to signal tumor cells for proliferation and are associated with lung and colon cancer cells.

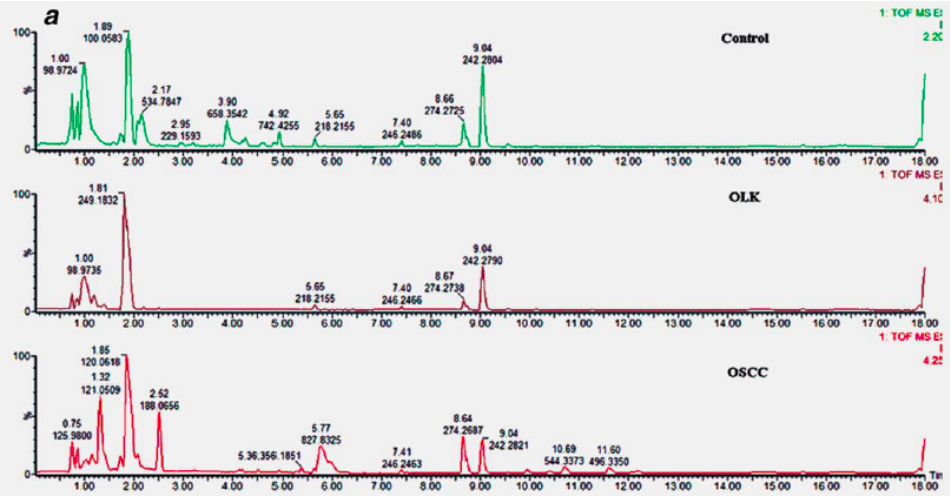

Figure 12. (a) UPLC-QTOGMS models based on peaks of intensity of the control group and the OLK, OSCC group saliva samples (Wei *et al.* 2011) [42]

Decreased levels of *urea* are identified as potential biomarkers, due to its presence which is possibly connected to the presence of *Helicobacter pylori* and poor nutrition, a characteristic of OSCC. Furthermore, the lower concentration of *3-hydroxybuyric acid* and higher lipids levels in the OSCC group are relevant as they both act in the metabolism of cell proliferation, which requires higher levels of lipids, while *3-hydroxybuyric acid* is increased in ketosis. This metabolite has also been associated with ovarian cancer, gastric, and pancreatic cancer [(30)].

P*yrroline hydroxycarboxylic acid*, *choline*, and *amino acids* were also identified as discriminative metabolites in the diagnosis between HC and OSCC groups. Higher concentrations of *polyamine* are related to higher proliferation and metastasis. Furthermore, *glutathione, polyamine* and choline were detected in patients with head and neck cancer and OSCC. Polyamines were found in higher concentrations where cancer had higher growth and significant proliferation, which could indicate discrimination of the type of cancer and its malignancy.

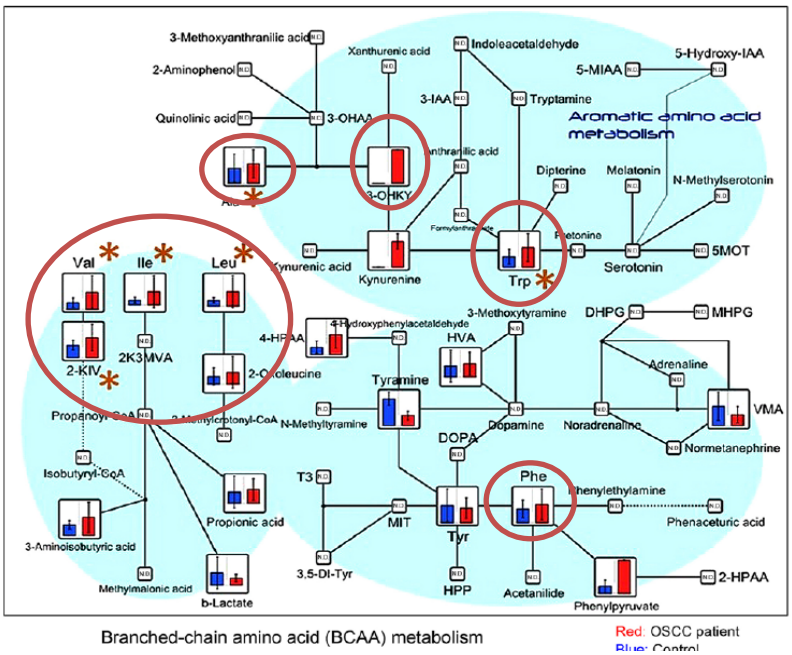

Figure 13. Metabolomic map of BCAA metabolism. Circled are the most relevant biomarkers for OSCC (Ohshima *et al. 2017)* [(40)]

They are also found in patients with periodontitis. More metabolites identified: *choline, histidine, pyrroline hydroxycarboxylic acid, glutamic acid, carnitine, alanine, piperidine* [39].

Choline metabolism is another relevant variation in the metabolomics of oral cancer. Several studies reported the association of higher levels of arachidonic acid and choline in Head and Neck Squamous Cell Carcinoma (HNSCC), and OSCC suggesting that the *choline phospholipid metabolism*, is correlated to the recurrence of HNSCC, and OSCC and their progression (Figure 14) [34,37–40].

Choline is especially important for the prognosis and to predict the treatment outcome. Its abnormal metabolism is a common characteristic associated with tumor progression. It enhances the membrane synthesis and degradation, which helps the metastasis [43]. If the levels significantly increase, the prognosis will be poor, and the treatment response too. In a study conducted by Chen *et al.* [38] more than thirty-five metabolites were identified. In particular, among the choline-containing metabolites, the most over expressed were: *phosphocholine* and *glycerophosphocholine*.

Choline is metabolized by *phosphocholine* and oxidized by *betaine*, which are both present in high concentration in the OSCC group. Thus, a higher concentration of betaine was also detected, with high concentration of *pipecolinic acid* and lower of *L-carnitine* [43]. *Betaine*'s concentration is proportional to the size of the malignant transformation of the cancer [34].

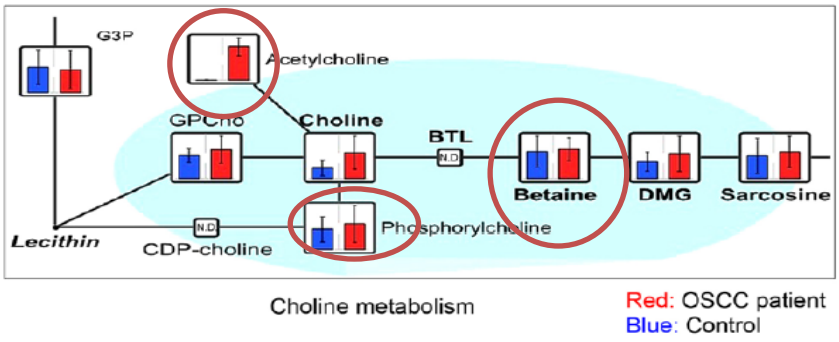

Figure 14. Metabolomic map of choline metabolism. (Ohshima *et al. 2017)* [40]

The *pipecolinic acid* is increased in OSCC patients because it is produced during the degradation of *lysine* which metabolism is upregulated in OSCC cells (Figure 15). Moreover, *L-carnitine* is essential for the transport of fat to the mitochondria of muscle cells and it's essential in fatty acid metabolisms. Its concentration is reduced in OSCC patients because of the downregulated fatty acid metabolism (Figure 15) [43].

Furthermore, it was possible to identify more metabolites, significant in the diagnosis of oral cancer: *propionylcholine N-acetyl-L-phenylalanine, S-carboxymethyl-L-cysteine, sphinganine* [39].

Indole-3-acetate and *ethanolamine phosphate* are increased in patients suffering from OSCC compared with Oral Lichen Planus patients control group [23,27].

Moreover, it reported that in the HNSCC was possible to find *fucose* and *1,2-propanedionol*, as highly unregulated metabolites.

Fucose, glycine, methanol, proline are downregulated and combined together gave higher discrimination for HNSCC detection [26]. It is also important to mention the rule of *fucosylation of glycoproteins* in detecting oral cancer.

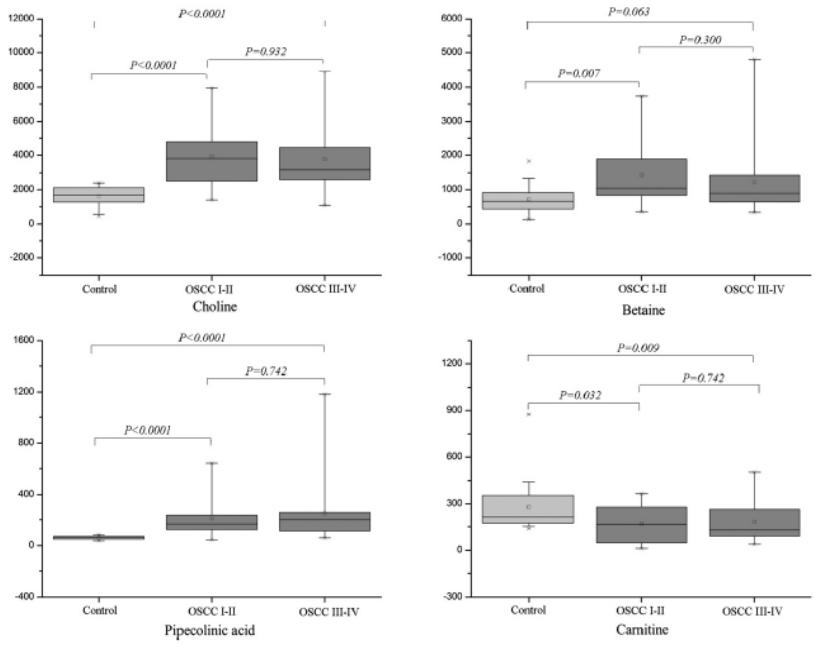

Figure 15. Biomarkers distinguishing HC and OSCC group. (Wang *et al.* 2014) [43]

In fact, it has been reported that tumor cells modulate the concentration of *fucose* by increasing it so it would make the tumor cell itself less recognizable. It is also associated with the stage of oral cancer and the level of nicotine consumed, since smoking is an enzymatic inducer and it enhances glycosylation which is responsible for the proliferation of cancer cells in OSCC [44].

Furthermore, *sialic acid*, present in the saliva, was identified as an essential biomarker in the oral cancer patient and as a pre-cancer marker. In fact, *salivary sialic acid* plays a role in the irregular glycosylation of proteins in cancer progress [23].

More studies found markers for cancers to be *cancer antigen-125, cytokeratin, tissue polypeptide antigen* [39]. Elevated levels of *transferrin* in patients with oral cancer and a positive correlation between the quantity of transferrin found and the size and stage of the tumor. In conclusion, considering the factors that impact metabolites in the saliva, such as if the subject is a smoker or not, if the saliva is unstimulated or stimulated, its collection, and its analysis techniques, the storage conditions, suggested a universal protocol and highlighted the advantages of using new and multiple technologies [27,28].

Saliva Metabolites relevant to the identification of Oral Cancer		
Metabolite	**Author**	**Method**
Taurine Piperidine Choline Betaine Pipecolinic acid L-carnitine Propionylcholine N-acetyl-L-phenylalanine S-carboxymethyl-L-cysteine Sphinganine	Mikkonnen *et al.* [38]	NMR spectroscopy [38] HILC with TOF-MS [38]
Phenylalanine GABA Valine *N*-eicosanoic acid Lactic acid	Wei *et al.* [42]	HLPC-MS [42]
Pyrroline Hydroxycarboxylic acid Choline Glutathione Polyamine Taurine Lactate	Chen *et al.* [38]	CE-TOF-MS [38]
Leucine Isoleucine Choline Histidine Pyrroline Hydroxycarboxylic acid Glutamic acid Carnitine Alanine Piperidine Taurine	Sugimoto *et al.* [34]	CE-TOF-MS [34]
Taurine Valine Choline Cadaverine Tryptophan Isoleucine Leucine Urea 3- hydroxybuyric acid	Ohshima *et al.* [40]	CE-MS [40]
Choline Pipecolinic acid L-carnitine Betaine	Wang et al. [43]	HILIC-UPLC-MS [43]

Table 3. Saliva metabolites biomarkers for Oral Cancer.

2. Systemic Diseases

2.1 Sjogren's syndrome

Sjogren's syndrome (SS) is a systemic autoimmune disease that affects exocrine glands, salivary and lacrimal glands. It presents two distinctive forms: primary and secondary. The primary *Sjogren's syndrome* (pSS) is characterized by dry eyes, xerostomia (dry mouth), and absence of correlated autoimmune diseases. Moreover, it is also characteristic to find in pSS's patient inflammation of the salivary and lacrimal glands and exocrine glands. In dental practice, it is common to see higher frothy saliva, dry mucosa due to hyposalivation, and chronic erythematous candidiasis (Figure 16) [45]. The secondary SS (sSS) is associated with other autoimmune disorders and represents the primary diagnosis for systemic lupus erythematosus, among others. Many hormonal changes, genetic and immunological factors have been associated with *Sjogren's syndrome*. Thus, its biomarkers are related to celiac disease, breast cancer, and other forms of cancer [41]. Several studies [46,47] yield data that showed a discriminative metabolic profile in people affected by SS.

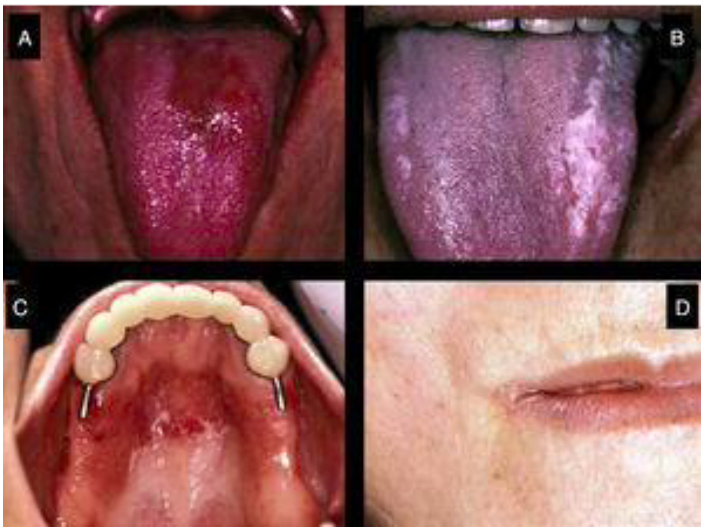

Figure 16. Oral involvement of pSS's patients. (Lopez-Pintor *et al.* 2015) [48]

Mikkonen *et al*. [46] identified twenty-four metabolites, from which *choline*, *taurine*, *alanine*, and *glycine* were significantly higher in pSS patients. *Butyrate*, *proline,* and *phenylalanine* were instead slightly higher than in the HC. These amino acids, especially *choline* and *taurine*, play an essential role as neurotransmitters and are associated with *muscarinic-M3*, which regulates the salivary flow [46]. Furthermore, *choline*'s metabolism is associated with oxidative stress and malignant cellular transformations. It was the only common metabolite to OSCC and SS.

Similar results were obtained by Herrala *et al.* [47] and Kageyama *et al.* [49], which respectively detected twenty-one and forty-one metabolites. Higher concentrations of *taurine* were also found. It regulates the oxidative stress and regulates the final salivary composition. *Choline* and *taurine* may be considered biomarkers of SS when monitoring its progression and tissue damage. Decreased levels of *glycine* and *alanine* concentrations were also found relevant to the SS metabolic profile. While *alanine* is also associated with patients with diabetes, since it is associated with the alanine aminotransferase which is correlated to the pathogenesis of diabetes [47]. Decreased

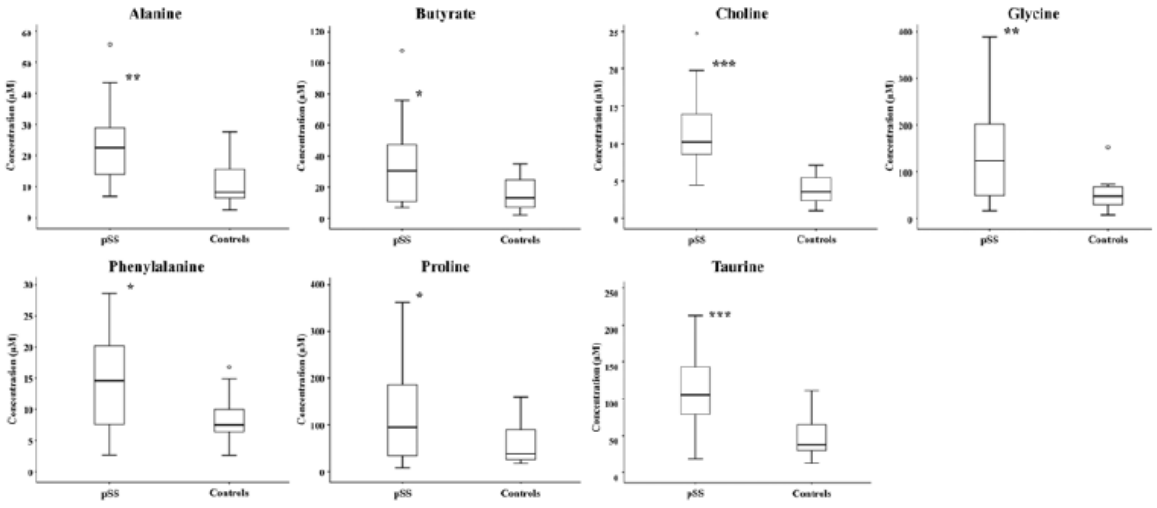

Figure 17. Box plots of metabolites concentrations in HC and pSS patients (Mikkonen *et al.* 2013) [46]

levels of *tyrosine*, *uric acid* and *fucose* were quantified in the SS group compared to the HC (Figure 17) [49].

Overall, patients suffering from SS presented a limited and less diverse production of salivary metabolites in comparison with healthy patients, as reflection of the damage suffered by the salivary glands [49].

Saliva Metabolites relevant to the identification of Sjogren's syndrome		
Metabolite	**Author**	**Method**
Choline Phenylalanine Glycine Taurine Alanine *Butyrate*	Mikkonen et al. [46]	HNMR spectroscopy [46]
Choline Taurine Alanine Glycine	Herrala *et al.* [47]	NMR spectroscopy [47]
Glycine Tyrosine Uric acid Fucose	Kageyama *et al.* [49]	GC-MS [49]

Table 4. Displaying the biomarkers relevant to the identification of Sjogren's syndrome.

2.2 Breast Cancer

Breast cancer (BC) is mainly affecting women of all ages. From an early age, young women with familiarity with BC get screened with mammography and additional clinical examinations. It accounts for 11.7% of all cancer, and it is the fifth leading cause of cancer death [50]. As mentioned in the previous section on Oral Cancer, metabolic changes are significant in cancer patients and the study of salivary metabolomics has been useful to identify biomarkers for the diagnosis of breast cancer.

In the study conducted by Zhong e*t al.* [23]*,* eighteen biomarkers were identified. The authors identified as potential biomarkers: *glycerol phospholipid*, *fatty amide*, *amino acids* and their derivates (*acetyl phenylalanine, histidine, phenylalanine, citrulline, histidine*), *choline, glyceroglycolipid, sphingolipid, saccharic acid* derivates *(4-Hydroxyphenylpyruvic acid),* and *denzene pyruvic acid* derivates (Figure 18).

Significant changes were detected in the phospholipid metabolism. Downregulated concentrations of *Lysophosphatidylcholine* have been found in cancer patients. It controls cell divisions and inflammation, and it can be considered as a biomarker for malignant cancer.

Lower concentrations of *phytosphingosine* were associated with an abnormal metabolism of *sphingolipid* and *palmitic amide*, which is involved in fatty acid metabolism. *Fatty acid synthase* (FAS) is the only enzyme that is able to anew fatty acid synthesis, which is largely expressed in cancer. The detection of high levels of this component has always been the focus for specific targeted treatment, since the high mortality rate of patients who present high concentrations of FAS. While *palmitic amide* is decreased in BC, since used by the FAS to promote cell proliferation.

The up-regulated were *phenylalanine*, *citrulline* and *histidine*, signaling malignant cell proliferation in patients BC. While *acetylphenylalamine* was the only amino acid derivate to present lower concentrations. This abnormality in the amino acid metabolism reflects a disrupted *phenylalanine* metabolism in BC. Moreover, *choline metabolites*, such as *propionylcholine*, are indicators of malignancy for different cancers. This may suggest a redundancy of these components as the early biomarkers signaling cancer.

Specific to breast cancer was the *monoacylglycerol* lipase's higher concentration, expressing an extremely aggressive form of breast cancer, and *N-acetylneuraminic acid* which concentration is higher in cancer cells since it is related to abnormal glycolipid metabolism. Significant levels of *putrescine, spermine* were also found, and the overexpression of *peptide Pro-Pro-Gly* is a further indication of tumor metastasis in breast cancer. This component is especially important because it inhibits the *matrix metalloproteinase-2,* which regulates the cell division [34].

Takayama *et al*. [51] focused on examining the concentration of salivary polyamines to determine if the concentration of *polyamines* was higher in cancer patients, as predicted.

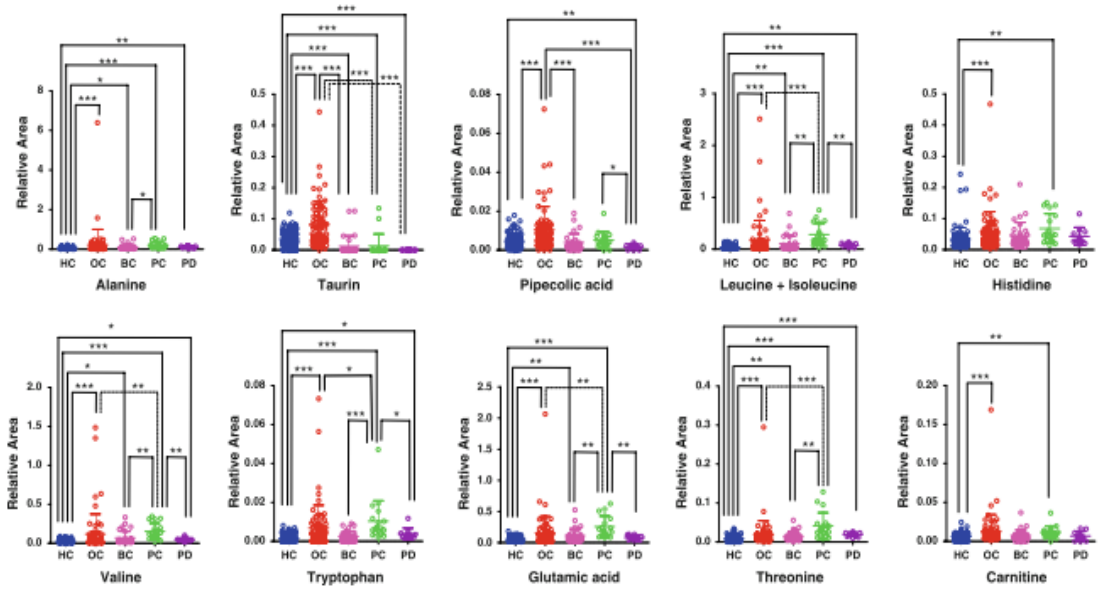

Figure 18. Dot plots of metabolites for HC (blue), OC (red), BC (pink), PC (green), PD (purple) (Sugimoto *et al.* 2010) [34]

Twelve metabolites were found to be relevant to the research as breast cancer biomarkers, and their concentration levels were correlated with the cancer stage. Therefore, an early diagnosis of breast cancer can be made by detecting *salivary polyamines*. In fact, there was a significant difference between a healthy person's concentration and BC stage I. Furthermore, it was interesting to observe that after breast surgery, the levels dropped and were similar to those of the HC [51]. It is safe to say that with further studies and support of mammography, the detection of salivary polyamines would be considered soon as a tool of a preventive diagnosis.

Saliva Metabolites relevant to the identification of Breast Cancer		
Metabolites	**Authors**	**Method**
Citrulline Histidine N-acetylneuraminic 4-Hydroxyphenylpyruvic acid Acetylphenylalanine Propionylcholine Phytosphingosine Lysophosphatidylcholine Monoacyglycerol	Zhong *et al.* [52]	HILIC – RPLC [52]
Putrescine Spermine Peptide Pro-Pro-Gly	Sugimoto *et al.* [34]	CE-TOF-MS [34]
Cadaverine Spermine Putrescine N^1-acetyl-spermidine N^8-acetyl-spermidine N^1N^{12}-diacetyl-spermine N^1N^8-diacetyl-spermine N^1-acetyl-putrescine Ornithine Diaminopropane Spermidine N^1-acetyl-spermine	Takayama *et al.* [51]	UPL-MS/MS [51]

Table 5. Saliva metabolites biomarkers for Breast Cancer.

2.3 Pancreatic Cancer

Pancreatic Cancer affects more and more people nowadays. Due to its malignancy, it has been predicted that it will soon surpass breast cancer and it will be the second leading cause of death [47]. If prevention is not done, the diagnosis is usually made when the stage is advanced, and the survival rate drops at 5% [53]. Sugimoto *et al.* [34] found forty-eight pancreatic cancer biomarkers, of which eight were specific markers of Pancreatic cancer (PC): *isoleucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine, leucine*, and *aspartic acid*. Higher levels of *tryptophan* were found as indicators of cancer development in both oral and pancreatic cancer. Amino acid metabolism was reported to be abnormal, decreased levels of *leucine*, *isoleucine*, *valine* and *alanine* were observed in the samples for pancreatic cancer. Important to mention was the *choline* metabolism, which in this case as well, it affected the synthesis and degradation of the membrane, enhancing the proliferation of malignant cells. While choline aberrant metabolism is common in oral cancer, it was pancreatic cancer-specific to the detection of decreased *phosphocholine* and *glycerophosphocholine*. Most recently, Asai *et al.* [54] published a study to examine *salivary polyamines* in pancreatic cancer patients, seen it is well known in other forms of cancer [51]. Their data revealed a significant increase of *spermine*, *N_1-acetylspermidine*, *N_1-acetylspermine*, *2-aminobutanoate* in the groups with pancreatic cancer. *N_1-acetylspermidine* was mostly elevated in Stage III, and *N_1-acetylspermine* was very pronounced in both Stage III and Stage IVb, and present in chronic pancreatic patients. In contrast, higher concentrations of *alanine* were present in the control group.

The study was able to show the significant difference in salivary polyamines concentration in PC patients compared to healthy individuals.

Saliva Metabolites relevant to the identification of Pancreatic Cancer		
Metabolite	**Author**	**Method**
Isoleucine Tryptophan Valine Glutamic acid Phenylalanine Glutamine Leucine Aspartic acid	Sugimoto *et al.* (34)	CE-TOF-MS (34)
Spermine N_1-acetylspermidine N_1-acetylspermine 2-aminobutanoate	Asai *et al.* (54)	CE-TOF-MS (54)

Table 6. Saliva metabolites biomarkers for PC.

Conclusions

Saliva is a complex biofluid, rich in contents and extremely important for the homeostasis of the oral environment, but as well it reflects the conditions of the body. As a diagnostic fluid, saliva is easier to process than blood. Its analytes are stable, and it is viable to multiple sampling avoiding pain and anxiety associated with other diagnostic methods. Thanks to the rich pool of information that saliva offers, it is possible to examine the smallest molecules present and recover from its metabolic pathways that will explain diagnostic and therapeutic questions. The study of Salivary Metabolomics is focalized on finding those answers. Thanks to extensive article research, it was explained how for different conditions, from Oral Cancer, Head and Neck cancer, to Breast cancer, among others, it possible to identify specific metabolic profiles. Moreover, several salivary metabolomics were found in common with oral diseases and systemic conditions, proving how the saliva diagnostic is a fundamental tool to understand the deep connection between the composition of saliva and the environmental, genetic, malignant transformations that happen in any part of the body. The advantages of metabolomics as diagnostic tool are a great sensitivity.

Furthermore, it is not only important in diagnostic, but as well effective prevention. Based on the findings, it is possible to recognize specific biomarkers that are warning signs of abnormal metabolic processes, as for an example for pancreatic cancer, which is often diagnosed too late.

A combined approach with a study of salivary metabolomics in subjects that present risk factors could profoundly affect the outcome of some of these diseases. It

was recurrent in all the articles mentioned that a standard protocol and the use of multiple analysis techniques can give broader results and identify more metabolites. Therefore, with the help of data banks such as *The Human Metabolome Database,* it will be more accessible to everyone connecting the metabolomes to their metabolic pathways. The education and training of dentists, assistants are essential for the successful outcome of this new diagnosis technique.

Responsibility

The present thesis reviews the literature on Salivary Metabolomics, looking into its diagnostic potential for oral and systemic diseases. The salivary diagnostic is the future of a new approach for treatment and prevention. It is non-invasive, which significantly helps both the patient and the clinician, and highly accessible since it doesn't require any training. The sampling is simple, fast, and non-traumatic for the patient. It can be easily performed on children, and it can be collected in the dental office. The study of Salivary metabolomics is its flourishing development, incremented by continuous scientific development. New biomarkers are found that can help prevent numerous cancers and improve the quality of life significantly. This will benefit the population, making prevention more accessible, fast to reach, and inexpensive, improving the efficiency and the cost reduction. On a bigger picture, the amount of information collected on a single sample of saliva can open doors to pharmaceutical, new medical frontiers for a more individualized approach. Therefore, the present research touches on a topic that has a larger purpose of benefiting not the single individual but the larger community.

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Annexes

Article 1

Appl Biochem Biotechnol (2012) 168:1718–1727
DOI 10.1007/s12010-012-9891-5

Saliva Metabolomics Opens Door to Biomarker Discovery, Disease Diagnosis, and Treatment

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Received: 10 June 2012 / Accepted: 31 August 2012 /
Published online: 13 September 2012

Abstract Metabolomics is the systematic study of the unique chemical fingerprints of low molecular weight endogenous metabolites or metabolite profiles in a biological sample. Metabolites that are important indicators of physiological or pathological states can provide information for the identification of early and differential markers for disease and help to understand its occurrence and progression. Analysis of these key biomarkers has become an important role to monitor the state of biological organisms and is a widely used diagnostic tool for disease. Metabolomic analyses are propelling the field of medical diagnostics forward at unprecedented rates because of its ability to reliably identify metabolites that are at the metabolic level in concentration. These advancements have benefited biomarker research to the point where saliva is now recognized as an excellent diagnostic medium for the detection of disease. Saliva contains a large array of metabolites, many of which can be informative for the detection of diseases. Salivary diagnostics offer an easy, inexpensive, safe, and noninvasive approach for disease detection. Discovery of salivary biomarkers that could be used to scrutinize health and disease surveillance has addressed its diagnostic value for clinical applications. Availability of emerging metabolomic techniques gives optimism that saliva can eventually be placed as a biomedium for clinical diagnostics. Comprehensive salivary metabolome will be an important resource for researchers who are studying metabolite chemistry, especially in the fields of salivary diagnostics, and will be helpful for analyzing and hence identifying corresponding disease-related salivary biomarkers. This review presents an overview of the value of saliva as a credible diagnostic tool, the discovery of salivary biomarkers, and the development of salivary diagnostics now and in the future. In particular, proof of principle has been demonstrated for salivary biomarker research.

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Article 2

Research

A Section 508–conformant HTML version of this article is available at https://doi.org/10.1289/EHP1011.

The Saliva Exposome for Monitoring of Individuals' Health Trajectories

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Background: There is increasing evidence that environmental, rather than genetic, factors are the major causes of most chronic diseases. By measuring entire classes of chemicals in archived biospecimens, exposome-wide association studies (EWAS) are being conducted to investigate associations between a myriad of exposures received during life and chronic diseases.

Objectives: Because the intraindividual variability in biomarker levels, arising from changes in environmental exposures from conception onwards, leads to attenuation of exposure–disease associations, we posit that saliva can be collected repeatedly in longitudinal studies to reduce exposure–measurement errors in EWAS.

Methods: From the literature and an open-source saliva–metabolome database, we obtained concentrations of 1,233 chemicals that had been detected in saliva. We connected salivary metabolites with human metabolic pathways and PubMed Medical Subject Heading (MeSH) terms, and performed pathway enrichment and pathway topology analyses.

Results: One hundred ninety-six salivary metabolites were mapped into 49 metabolic pathways and connected with human metabolic diseases, central nervous system diseases, and neoplasms. We found that the saliva exposome represents at least 14 metabolic pathways, including amino acid metabolism, TCA cycle, gluconeogenesis, glutathione metabolism, pantothenate and CoA biosynthesis, and butanoate metabolism.

Conclusions: Saliva contains molecular information worthy of interrogation via EWAS. The simplicity of specimen collection suggests that saliva offers a practical alternative to blood for measurements that can be used to characterize individual exposomes. https://doi.org/10.1289/EHP1011

Introduction

Because genetic factors typically account for only about 18% of chronic disease risks, it is reasonable to infer that nongenetic factors (i.e., exposures) are major causes of chronic diseases (Rappaport 2016). Given the myriad exposures from both exogenous and endogenous sources that an individual experiences during life [the "exposome" (Wild 2005)], investigators are performing exposome-wide association studies (EWAS) that interrogate levels of chemicals in biospecimens to discover causes of chronic diseases (Patel et al. 2010; Rappaport 2012; Wild et al. 2013). By measuring entire classes of chemicals (e.g., small molecules, protein modifications, antigens) in archived biospecimens from incident disease cases and matched controls, EWAS can pinpoint discriminating features that then generate hypotheses for targeted follow-up studies (Rappaport 2011; Rappaport et al. 2014). For example, Hazen and coworkers employed this avenue to implicate joint microbial/human metabolism of the nutrient choline as a potentially major cause of coronary heart disease (Wang et al. 2011; Tang et al. 2013; Koeth et al. 2013).

An important challenge to designing EWAS is the intraindividual variability in levels of circulating molecules arising from changes in diet, lifestyle factors, and sources of pollutants during decades of life that precede disease onset. This within-person variability in biomarker levels leads to exposure measurement errors that attenuate causal signals and obscure disease associations (Lin et al. 2005; Sampson et al. 2013). One way to circumvent such measurement errors is to perform longitudinal studies with repeated biospecimens, collected from subjects during critical stages of life (Rappaport 2011; Robinson and Vrijeheid 2015). The most logical approach for doing this relies on prospective cohorts that archived blood or other biospecimens repeatedly from the same subjects. However, such cohorts are rare and repeated collection of blood, the main archival specimen, is difficult to perform (Hansen et al. 2007; Randell et al. 2016).

Saliva (also referred as oral fluid) is a natural filtrate of blood that contains omic features (small molecules, metals, proteins, and DNA) worthy of interrogation via EWAS. Because collection is "stress-free," repeated specimens of saliva are routinely obtained for determination of steroid hormones in psychobiological studies (Hjortskov et al. 2004; Kajantie and Phillips 2006; Hunter et al. 2011). Sampling of saliva is straightforward and protocols are available that allow subjects to collect their own samples and ship them to a laboratory or repository.

Metabolomics is recognized as a powerful top-down approach for detecting small molecules in biological matrices (Nicholson and Wilson 2003; German et al. 2005). These small molecules can be either substrates or end products of cellular metabolism and can originate from exogenous sources via inhalation, ingestion and dermal absorption, or from endogenous processes including human and microbial metabolism. Adductomics is another top-down technique that employs modifications of blood proteins like hemoglobin or human serum albumin (HSA) to characterize exposures to reactive electrophiles that are inherently toxic but cannot be measured directly in biospecimens (Rubino et al. 2009; Li et al. 2011; Carlsson et al. 2014; Grigoryan et al. 2016). Because blood is in equilibrium with the tissues and saliva is in equilibrium with blood, both blood and saliva represent dynamic reservoirs of small molecules that are present in the body at a given time. Given the potential utility of saliva as a biospecimen for EWAS, we will evaluate the linkages between salivary metabolites and human metabolic pathways, as well as those between these pathways and chronic diseases. We will also consider methods for collection and analysis of saliva via untargeted metabolomics and adductomics.

Methods

Saliva Metabolome

Salivary metabolites ($n = 1,233$) were obtained from the saliva metabolome database (http://www.salivametabolome.ca/) that was recently integrated into the Human Metabolome Database

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The authors declare they have no actual or potential competing financial interests.

Received 23 August 2016; Revised 8 November 2016; Accepted 18 November 2016; Published 20 July 2017.

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Article 3

ORAL DISEASES

Oral Diseases (2011) 17, 345–354 doi:10.1111/j.1601-0825.2010.01773.x

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INVITED MEDICAL REVIEW

Saliva: diagnostics and therapeutic perspectives

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For the past two decades, salivary diagnostic approaches have been developed to monitor oral diseases such as periodontal diseases and to assess caries risk. Recently, the combination of emerging biotechnologies and salivary diagnostics has extended the range of saliva-based diagnostics from the oral cavity to the whole physiologic system as most compounds found in blood are also present in saliva. Accordingly, saliva can reflect the physiologic state of the body, including emotional, endocrinal, nutritional and metabolic variations and acts as a source for the monitoring of oral and also systemic health. This review presents an update on the status of saliva diagnostics and delves into their applications to the discovery of biomarkers for cancer detection and therapeutic applications. Translating scientific findings of nucleic acids, proteins and metabolites in body fluids to clinical applications is a cumbersome and challenging journey. Our research group is pursuing the biology of salivary analytes and the development of technologies for detection of distinct biomarkers with high sensitivity and specificity. The avenue of saliva diagnostics incorporating transcriptomic, proteomic and metabolomic findings will enable us to connect salivary molecular analytes to monitor therapies, therapeutic outcomes, and finally disease progression in cancer.

Oral Diseases (2011) **17**, 345–354

Keywords: saliva diagnostics; biomarker; transcriptome; proteome; therapeutic perspectives

Introduction

Human saliva is a clear, slightly acidic (pH = 6.0–7.0) biologic fluid containing a mixture of secretions from multiple salivary glands, including the parotid, submandibular, sublingual and other minor glands beneath the oral mucosa as well as gingival crevice fluid. This complex oral fluid serves the execution of multiple physiologic functions such as oral digestion, food swallowing and tasting, tissue lubrication, maintenance of tooth integrity, antibacterial and antiviral protection (Mandel, 1987). In addition to the important role of maintaining the homeostasis of the oral cavity system, the oral fluid is a perfect medium to be explored for health and disease surveillance.

Just as is the case with blood, saliva is a complex fluid containing a variety of enzymes, hormones, antibodies, antimicrobial constituents, and cytokines (Zelles *et al*, 1995; Rehak *et al*, 2000). Many of these enter saliva from the blood by passing through cells by transcellular, passive intracellular diffusion and active transport, or paracellular routes by extracellular ultra filtration within the salivary glands or through the gingival sulcus (Drobitch and Svensson, 1992; Haeckel and Hanecke, 1993; Jusko and Milsap, 1993). So, most compounds found in blood are also present in saliva. Accordingly saliva can reflect the physiologic state of the body, including emotional, endocrinal, nutritional and metabolic variations. Consequently, this fluid provides a source for the monitoring of oral and also systemic health. This is the basis of our vision to develop disease diagnostics and promote human health surveillance by analysis of saliva. In this article, we will review the current status of saliva proteomics and transcriptomics and their applications to the discovery of biomarkers for cancer detection and therapeutic applications.

Saliva diagnostics

For the past two decades, salivary diagnostic approaches have been developed to monitor oral diseases such as periodontal diseases (Kornman *et al*, 1997; Socransky *et al*, 2000) and to assess caries risk (Baughan *et al*, 2000). Recently, the combination of emerging biotechnologies and salivary diagnostics has extended the range of saliva-based diagnostics from the oral cavity to the whole physiologic system. Large numbers of medically valuable analytes in saliva have been gradually unveiled that represent biomarkers for different diseases including cancer (Boyle *et al*, 1994; Li *et al*, 2004a; Zhang *et al*, 2010), autoimmune (Streckfus *et al*, 2001; Hu *et al*, 2007b), viral (Chaita *et al*, 1995;

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Received 28 September 2010; revised 1 October 2010; accepted 4 October 2010

Article 4

Salivaomics

David T.W. Wong, DMD, DMSc

ABSTRACT

Background. The ability to monitor health and wellness, as well as detect oral and systemic illnesses early through noninvasive means, are highly desirable goals in health care promotion and delivery. Saliva is an emerging medium to be explored for health and disease surveillance, as well as for personalized medicine. A major mandate is to demonstrate clinicians' ability to use saliva to detect and monitor systemic diseases.

Methods. To realize the translational and clinical vision of salivary diagnostics, two prerequisites are essential. The first is the need to develop and optimize diagnostic tools tailored to saliva. The second is the need to substantiate the scientific underpinnings of salivary biomarkers reflecting systemic diseases.

Results. The author describes five diagnostic alphabets (proteome, transcriptome, microRNA, metabolome and microbiome) and point-of-care technology platforms that are in place to advance the translational and clinical path. For mechanistic studies (that is, basic science studies), animal models are in place to elucidate the scientific mechanisms of systemic diseases reflected in saliva.

Conclusions. Significant advancements have been made in the development of salivary diagnostic tools. The translation of the scientific mechanisms of systemic diseases reflected in saliva is in progress.

Clinical Implications. On the scientific credentialing of salivary biomarkers for the detection of systemic diseases, salivary diagnostics will have an effect on access to care, health disparities and global health. Dentistry can advance into the realm of primary health care with integration of chairside screening for medical conditions.

Key Words. Saliva; biomarkers; proteome; transcriptome; metabolome; microbiome; early detection; systemic diseases; point-of-care technology; personalized medicine.

JADA 2012;143(10 suppl):19S-24S.

The science of saliva has advanced substantially in the past decade. Both basic and translational sciences have progressed as a result of the balanced portfolio of research invested in by the National Institute of Dental and Craniofacial Research (NIDCR), Bethesda, Md. Much of this support was designated for the ambitious, visionary campaign to decipher and catalog the human salivary proteome and to apply that knowledge to the development of saliva-based point-of-care technologies. After a decade of scientific advancements, the incipient maturation of these basic and translational outcomes is leading to the development of clinical tests that benefit patients. In this article, I present the current status of saliva-based innovation and its application to the detection of oral and systemic diseases.

It is important to define a few ideas and terms. The first is the term "salivary diagnostics," which has become a catchall for the entire field of salivary science. Although we expect that clinicians eventually will use saliva to detect diseases, this application is not the most compelling at this time. As of March 2012, no salivary test exists to diagnose a single oral or systemic disease. Not even the U.S. Food and Drug Administration (FDA)–approved OraQuick ADVANCE Rapid HIV-1/2 Antibody Test (OraSure Technologies, Bethlehem, Pa.) is diagnostic for human immunodeficiency virus (HIV) infection; it claims only to

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Research Article

NMR IN BIOMEDICINE

Received: 5 September 2008, Revised: 7 January 2009, Accepted: 7 January 2009, Published online in Wiley InterScience: 3 March 2009

(www.interscience.wiley.com) DOI:10.1002/nbm.1369

Understanding the human salivary metabolome

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Saliva is a readily accessible biofluid that is important for the overall health, aiding in the chewing, swallowing, and tasting of food as well as the regulation mouth flora. As a first step to determining and understanding the human saliva metabolome, we have measured salivary metabolite concentrations under a variety of conditions in a healthy population with reasonably good oral hygiene. Using ^{1}H NMR spectroscopy, metabolite concentrations were measured in resting (basal) and stimulated saliva from the same subject and compared in a cohort of healthy male non-smoking subjects ($n = 62$). Almost all metabolites were higher in the unstimulated saliva when compared to the stimulated saliva. Comparison of the salivary metabolite profile of male smokers and non-smokers ($n = 46$) revealed citrate, lactate, pyruvate, and sucrose to be higher and formate to be lower in concentration in smokers compared with non-smokers ($p < 0.05$). Gender differences were also investigated ($n = 40$), and acetate, formate, glycine, lactate, methanol, propionate, propylene glycol, pyruvate, succinate, and taurine were significantly higher in concentration in male saliva compared to female saliva ($p < 0.05$). These results show that differences between male and female, stimulated and unstimulated, as well as smoking status may be observed in the salivary metabolome. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: NMR; metabolomics; metabonomics; saliva; targeted profiling; smoking

INTRODUCTION

Saliva is a complex biological fluid that is produced in, and secreted from, the salivary glands. It consists of 99% water with electrolytes, mucus, proteins, and small molecular weight metabolites making up the rest of the components (1,2). It is produced by three pairs of major salivary glands (parotid, submandibular, and sublingual) as well as several minor glands. Saliva is critical for preserving and maintaining the health of oral tissues, as it has a variety of functions including digestion and lubrication.

At rest without stimulation, a small, continuous salivary flow, termed basal unstimulated secretion, covers, moisturizes, and lubricates the oral tissues (1). The submandibular gland contributes 65–70% of the total saliva volume, with 20 and 8% due to the parotid and sublingual glands, respectively (1,3). Stimulated saliva is produced primarily by the parotid glands, is released upon smell, taste, mechanical, or pharmacological stimulus (1) and contributes to most of the daily salivary production. Varying physiological, pathological, and environmental factors may cause changes in the amount and composition of both stimulated and unstimulated saliva (1,3).

Saliva is an extremely important biofluid, as it is known that loss of salivary gland function, in cases such as radiation-induced xerostomia or Sjögren's syndrome, has been shown to have a profound effect on quality of life. Xerostomia impairs the ability to taste, chew, and swallow food, and alters oral microbial flora leading to the development of dental caries (4). It also causes problems with speech, and can be greatly demoralizing to patients (4). An understanding of the composition of this fluid may help to further understand the qualities of saliva, which potentially could aid those afflicted with low salivary production.

Advances in global profiling have impacted the number of salivary components that may be studied at one time. Protein profiling in saliva is becoming more advanced and sophisticated (5–7), and has even afforded a potential diagnostic for head and neck cancer (8). However, metabolite profiling of saliva remains largely under-explored despite demonstrated potential for biomarker detection in other biofluids such as urine and blood (9–12).

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Contract/grant sponsor: Alberta Cancer Board.

Abbreviations used: *DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; LDH, lactate dehydrogenase; PCA, principal components analysis; PLSDA, partial least squares discriminant analysis.*

Article 6

Advances

Getting to Know "The Known Unknowns": Heterogeneity in the Oral Microbiome

Advances in Dental Research
2018, Vol. 29(1) 66–70

DOI: 10.1177/0022034517735293
journals.sagepub.com/home/adr

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Abstract

Technological advances in DNA sequencing have provided unprecedented insights into the composition of the oral microbiome in health and disease, and RNA-sequencing and metabolomics-related technologies are beginning to yield information on the activities of these organisms. Importantly, progress in this area has brought the scientific community closer to an understanding of what constitutes a health-associated microbiome and is supporting the notion that the microbiota in healthy sites assumes an active role in promoting health and suppressing the acquisition, persistence, and activities of overt and opportunistic pathogens. It is also becoming clear that a significant impediment to developing a conclusive body of evidence that defines a healthy microbiome and the mechanisms by which beneficial bacteria promote health is that an inherent characteristic of the most abundant members of the oral flora, those that potentially play the greatest roles in health and disease, is intraspecies genomic diversity. In particular, individual isolates of abundant commensal and pathogenic streptococci show tremendous variability in gene content, and this variability manifests in tremendous phenotypic heterogeneity. Analysis of the consequences of this diversity has been complicated by the exquisite sensitivity these bacteria have evolved to environmental inputs, inducing rapid and substantial fluctuations in behaviors, and often only within subpopulations of the organisms. Thus, the conditions under which the oral microbiota is studied can produce widely different results within and between species. Fortunately, continually diminishing costs and ongoing refinements in sequencing and metabolomics are making it practical to study the oral microbiome at a level that will create a sufficiently robust understanding of the functions of individual organisms and reveal the complex interrelationships of these microbes ("the known unknowns") in a way that researchers will be able to engage in the rational design of reliable and economical risk assessments and preventive therapies.

Keywords: caries, microbiology, microbial ecology, microbial genetics, genomics, virulence

Introduction

It was not long after the discoveries of restriction enzymes that cleave DNA at specific sequences (Smith and Wilcox 1970) and the ability of DNA ligases to couple 2 strands of DNA that the first recombinant DNA molecules were created in 1972 (Jackson et al. 1972). The first report of a DNA molecule cloned from an oral bacterium was the entire cryptic plasmid (pVA381) from *Streptococcus mutans*, which was cloned onto the *Escherichia coli* plasmid vector pBR322 in 1981 by Roy Curtiss and friends (Hansen et al. 1981). In 1982, the Curtiss group also cloned the first genes from the chromosome of *Streptococcus mutans* and demonstrated that they could be expressed in *E. coli* (Holt et al. 1982; Jagusztyn-Krynicka et al. 1982). In the ensuing 30 y, major strides were made in molecular genetics and DNA-sequencing technologies that led to a much greater understanding of the virulence attributes of *S. mutans*, the most common human dental caries pathogen, how these factors contribute to disease, and how their production was regulated. In 2002, the entire genome sequence of *S. mutans* UA159 was completed (Ajdic et al. 2002) at a cost of more than 3 million U.S. dollars. Today, the cost to sequence an entire bacterial genome can be as low as $50. In 2013, Cornejo and coworkers sequenced nearly 60 genomes of isolates of *S. mutans* from around the globe and performed an analysis that demonstrated the remarkable genomic heterogeneity of this single species of oral streptococcus. While the core genome (i.e., genes present in all isolates) of *S. mutans* was shown to contain about 1,400 genes, the pangenome was estimated at about 3,400 genes and continues to grow because of lateral gene transfer. Viewed another way, each isolate of *S. mutans* carries about 600 genes that are not present in every other isolate of *S. mutans*. Work by Palmer and colleagues (2013) confirmed that the genomic heterogeneity of *S. mutans* manifests in tremendous phenotypic heterogeneity, with individual isolates displaying major differences in properties related to virulence: biofilm formation, acid and oxidative stress tolerance, genetic competence, and production of exopolysaccharides. Emerging genome-scale information and the knowledge that most streptococci can become naturally competent for DNA uptake provide evidence that a similar level of genomic and phenotypic heterogeneity is inherent in oral streptococci (Richards et al. 2014), the most abundant group of

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Article 7

International Journal of
Molecular Sciences

MDPI

Review

Metabolomic Studies of Oral Biofilm, Oral Cancer, and Beyond

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Academic Editors: Philip Newton Baker, Elizabeth McKenzie and Ting-Li (Morgan) Han
Received: 9 March 2016; Accepted: 25 May 2016; Published: 2 June 2016

Abstract: Oral diseases are known to be closely associated with oral biofilm metabolism, while cancer tissue is reported to possess specific metabolism such as the 'Warburg effect'. Metabolomics might be a useful method for clarifying the whole metabolic systems that operate in oral biofilm and oral cancer, however, technical limitations have hampered such research. Fortunately, metabolomics techniques have developed rapidly in the past decade, which has helped to solve these difficulties. *In vivo* metabolomic analyses of the oral biofilm have produced various findings. Some of these findings agreed with the *in vitro* results obtained in conventional metabolic studies using representative oral bacteria, while others differed markedly from them. Metabolomic analyses of oral cancer tissue not only revealed differences between metabolomic profiles of cancer and normal tissue, but have also suggested a specific metabolic system operates in oral cancer tissue. Saliva contains a variety of metabolites, some of which might be associated with oral or systemic disease; therefore, metabolomics analysis of saliva could be useful for identifying disease-specific biomarkers. Metabolomic analyses of the oral biofilm, oral cancer, and saliva could contribute to the development of accurate diagnostic, techniques, safe and effective treatments, and preventive strategies for oral and systemic diseases.

Keywords: metabolomics; oral biofilm; oral cancer; metabolism

1. Introduction

1.1. What Is Metabolomics?

Metabolomics is a relatively new form of omics research. Living cells contain many metabolites, which are derived from various metabolic activities. These metabolites are the final products of cellular biochemical processes, including gene transcription, mRNA translation, protein synthesis, and metabolic enzymatic reactions. The comprehensive identification and quantification of these metabolites is called "metabolomics". Metabolomics is essential to clarify cellular function.

In the past decade, metabolomics techniques have advanced markedly. Until recently, it was technically difficult to comprehensively and simultaneously analyze numerous metabolites, especially small ionic metabolites, such as phosphorylated sugars, carbonic acids, and amino acids, in the same sample. However, in the 2000s, new techniques, such as capillary electrophoresis (CE) and a time-of-flight mass spectrometer (TOFMS), were developed. CE consists of a capillary (diameter: 100 μm or less) and an electrode. The samples are injected into the capillary and a high voltage is applied to both ends of the capillary, causing small ionic metabolites in samples to move to the cathode or anode. The ionic radius and the electric charge of each metabolite are different, the movement velocities of each metabolites in the capillary is also different; therefore, the ionic metabolites in the sample can be separated in the capillary. The separation ability of CE is very excellent; the liquid chromatography cannot separate the metabolites having the same mass, while CE can separate

Article 8

JDR Centennial Series

Role of Saliva and Salivary Diagnostics in the Advancement of Oral Health

Journal of Dental Research
2019, Vol. 98(2) 133–141

DOI: 10.1177/0022034518816961
journals.sagepub.com/home/jdr

C. Dawes[1] and D.T.W. Wong[2]

Abstract

The objective of this article was to provide an account of some of the developments related to saliva over the first 100 years of the *Journal of Dental Research* and to outline some of the many biomarkers identified in saliva in the last few years. The first section covers findings in salivary physiology, biochemistry, calcium phosphate chemistry related to saliva, microbiology, and the role of saliva in maintaining oral health. The second section highlights salivary diagnostics, salivaomics, and saliva exosomics in the context of the emerging theme of personalized and precision medicine.

Keywords: parotid, submandibular/sublingual, exosomics, proteomics, salivaomics, transcriptomics

Salivary Physiology

Prior to the 20th century, little was known about human saliva physiology. However, it was known that saliva contained amylase, and the parasympathetic and sympathetic nerve supplies to most of the salivary glands had been determined, primarily by studies on animals. A few years before the first issue of the *Journal of Dental Research* in 1919, Carlson and Crittenden (1910) developed a collection device for parotid saliva (Fig. 1), which allowed the study of secretion from an individual salivary gland. However, it was not until 1955 that Schneyer developed a device for collection of submandibular and sublingual saliva, and an improved version was described by Truelove et al. (1967). The composition of secretions from minor salivary glands of the lips was first described by Dawes and Wood (1973). Veerman et al. (1996) collected and compared the compositions of stimulated parotid, submandibular, sublingual, and palatine secretions. A major stimulus for salivary physiology research, although primarily in animals, was the monograph by Burgen and Emmelin (1961).

Two pioneers who studied variations in flow rate and calcium and phosphate concentrations in human whole saliva were Becks and Wainwright, who published a series of articles in the *Journal of Dental Research* in the 1930s and 1940s. Their 1943 paper on the normal unstimulated flow rate of whole saliva is still widely quoted, and others have since confirmed their finding that about 10% of the population has an unstimulated salivary flow rate ≤0.1 mL/min, whereas the mean value in the population is 0.3 to 0.4 mL/min. However, flow rate is virtually zero during sleep (Schneyer et al. 1956).

An important study by Thaysen et al. (1954) on 3 young women showed that the concentrations of the main electrolytes (sodium, potassium, bicarbonate, and chloride) in parotid saliva elicited by beta-methyl-acetyl-choline were very dependent on flow rate. Since this is a key factor influencing saliva composition, development of a negative-feedback technique (Dawes 1967) for maintaining a constant stimulated flow rate, up to the physiologic limit of the gland, allowed study of the effects of other physiologic variables—such as flow rate itself, duration of stimulation, nature of the stimulus, circadian rhythms, previous stimulation, exercise, and stop-flow conditions—on human salivary composition.

Because of variation in nomenclature used in different branches of salivary research, a group of researchers in the field recommended a standard nomenclature (Atkinson et al. 1993), which seems to have been generally accepted.

The mechanisms by which salivary glands secrete electrolytes from plasma into saliva are rather complex, but a recent mathematical model (Vera-Sigüenza et al. 2018) appears to fit the theoretical processes and the experimental data quite well. Several neurotransmitters—including acetyl choline, norepinephrine, vasoactive intestinal peptide, substance P, and nitric oxide—act as transmitters in salivary secretion (Pedersen et al. 2018), but there is still much to be learned about the factors influencing the superior and inferior salivary nuclei in the pons and medulla. The mechanisms involved in secretion of protein by pancreatic cells (which also apply to salivary acini) were described by Jamieson and Palade (1967a, 1967b).

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Article 9

CLIN. CHEM. 24/10, 1663–1673 (1978)

review

Quantitative Metabolic Profiling Based on Gas Chromatography

Stephen C. Gates[1] and Charles C. Sweeley[2]

The quantitative metabolic profiles of volatilizable components of human biological fluids, particularly urinary organic acids, is reviewed, with emphasis on the use of gas-chromatography/mass spectrometer/computer systems. Various definitions of metabolic profiling are considered and techniques for obtaining such profiles are discussed. The role of computer processing of such data is examined, and statistical techniques for treating quantitative metabolic profiles are suggested.

The use of metabolic profiles has its beginnings in the late 1940s; however, *quantitative* metabolic profiles have only just barely become a reality. It may therefore seem premature to review a field that, as yet, has almost no history, especially since two excellent reviews of qualitative metabolic profiling have recently appeared (*1, 2*). The justification for this review is twofold: first, we have attempted to draw together concepts from several fields relevant to quantitative metabolic profiling and, second, it serves to introduce those not yet involved in the field to the potential uses and problems of this technique. The review is intended to be selective rather than exhaustive, and emphasizes gas chromatographic/mass spectrometric (GC-MS) techniques over other methods. More general uses of GC-MS techniques in the clinical laboratory have already been thoroughly discussed by Lawson (*3*) and Burlingame et al. (*4*).

Development of the Concept of Metabolic Profiling

The concept that individuals might have a "metabolic pattern" that would be reflected in the constitutents of their biological fluids was first developed and tested by Roger Williams and his associates during the late 1940s and early 1950s (*5*). Utilizing data from over 200 000 paper chromatograms, many run with techniques developed in his own laboratory for this purpose, Williams was able to show convincingly that the taste thresholds and the excretion patterns for a variety of substances varied greatly from individual to individual (Figure 1), but that these patterns were relatively constant for a given individual. He summarized his findings in 1951 as follows (*5*):

> It appears that each individual we have studied has whenever tested exhibited a characteristic pattern of measurements which is distinctive for that individual alone. While there are in every case day-to-day variations in saliva and urine compositions and in taste thresholds, certain items, at least, stand out as grossly distinctive and the patterns as a whole remain nearly constant.

Williams went on to use his methods to examine samples from a variety of subjects, including alcoholics, schizophrenics, and residents of mental hospitals, and produced what he considered to be very suggestive evidence that there were characteristic metabolic patterns associated with each of these groups (*5*).

The work of Williams and his group, however, was apparently not duplicated by others, to whom his task must have seem rather herculean, with but few promises of tangible results. Hence, his ideas about the utility of metabolic pattern analysis remained essentially dormant until the late 1960s, when gas chromatography and liquid chromatography was advanced sufficiently to permit such studies to be carried out with considerably less effort. Once these techniques became available, the rate of progress became extremely rapid. In 1970, for example, at least three different groups published papers describing multicomponent analyses of biological fluids and referred to the possibility of "considerable differences in excretion patterns of carbohydrates in disease" (*6*), "personal blood 'profiles' " (*7*), and a "characteristic excretion profile" of organic acids in urine of patients with phenylketonuria (*8*).

The phrase most often used to describe the chromatographic patterns observed in biological fluids has been "metabolic profile." This term was introduced by the Hornings in 1971 (*9, 10*); as originally defined, it meant "multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites" (*10*). Commenting on the potential usefulness of this type of technique, the Hornings suggested that "profiles may prove to be useful for characterizing both normal and pathologic states, for studies of drug metabolism, and for human developmental studies." This definition of metabolic profile has been adopted by some workers, essentially unchanged (*11*). Other workers have preferred just the term "profile" to mean the same thing (*12*). Johnson (*13*) has taken a more quantitative approach by defining a profile as

> . . . a vector of numerical values corresponding to measured characteristics or attributes of a given subject. In addition to clinical chemistry measurements, the profile may include measurements on demographic or physical variables such as age, weight, sex, exercise status, etc. Profile analysis is the study of several profiles for the purpose of characterizing the profiles of a given group of subject or comparing the profiles of a different group.

Several hospital laboratories have experimented with a related technique, "multiphasic screening" (reviewed in *14*), designed to measure multiple components of a single serum or urine sample. The principal difference between multiphasic and profile techniques has been one of technology: in multiphasic testing there are single tests for each of the components,

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Received May 3, 1978; accepted July 7, 1978.

ORIGINAL CONTRIBUTION

Metabolomic Characterization of Human Rectal Adenocarcinoma with Intact Tissue Magnetic Resonance Spectroscopy

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PURPOSE: This study was designed to test whether metabolic characterization of intact, unaltered human rectal adenocarcinoma specimens is possible using the high-resolution magic angle spinning proton (^{1}H) magnetic resonance spectroscopy technique.

METHODS: The study included 23 specimens from five patients referred for ultrasonographic staging of suspected rectal cancer. Multiple biopsies of macroscopically malignant rectal tumors and benign rectal mucosa were obtained from each patient for a total of 14 malignant and 9 benign samples. Unaltered tissue samples were spectroscopically analyzed. Metabolic profiles were established from the spectroscopy data and correlated with histopathologic findings.

RESULTS: Metabolomic profiles represented by principle components of metabolites measured from spectra differentiated between malignant and benign samples and correlated with the volume percent of cancer ($P = 0.0065$ and $P = 0.02$, respectively) and benign epithelium ($P = 0.0051$ and $P = 0.0255$, respectively), and with volume percent of stroma, and inflammation.

CONCLUSIONS: Magnetic resonance spectroscopy of rectal biopsies has the ability to metabolically characterize samples and differentiate between pathological features of interest. Future studies should determine its utility in *in vivo* applications for non-invasive pathologic evaluations of suspicious rectal lesions.

KEY WORDS: Rectal cancer; Metabolomics; Diagnosis; Magnetic resonance spectroscopy.

Rectal cancers account for approximately one-third of colorectal malignancies, and the estimated number of cases for 2008 is 40,740.[1] The expected overall five-year survival rate for patients with a diagnosis of rectal cancer is 65 percent in the United States, according to the National Cancer Institute.[2] Despite known prognostic factors and new and improved diagnostic methods, the severity of cancer stage is frequently underestimated.[3] Accurate diagnosis, staging, and management of the disease are crucial for achieving optimal outcomes. Currently, although histopathologic evaluation of biopsy samples can achieve most of these goals, radiologic tests such as computed tomography (CT), ultrasonography (US), positron emission tomography (PET), and magnetic resonance imaging (MRI) are unable to differentiate malignant from benign tissue with sufficient accuracy to be clinically useful for noninvasive diagnosis. The preoperative staging of the tumor is usually done according to the TNM system by using MRI, endorectal US, CT, or a combination of these tests to assess the tumor itself, whether it has spread to locoregional lymph nodes, and whether distant metastases are present.[4,5]

Supported by grants from the National Institutes of Health (CA115746, CA095624), and the Massachusetts General Hospital A. A. Martinos Center for Biomedical Imaging.

Read at the meeting of the International Society for Magnetic Resonance in Medicine, Toronto, Ontario, Canada, May 3 to 9, 2008.

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Dis Colon Rectum 2009; 52: 520–525
DOI: 10.1007/DCR.0b013e31819c9a2c

Received: 12 June 2017 | Accepted: 14 September 2017
DOI: 10.1002/mas.21548

REVIEW ARTICLE

WILEY MassSpectrometryReviews

Metabolomics toward personalized medicine

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Metabolomics, which is the metabolites profiling in biological matrices, is a key tool for biomarker discovery and personalized medicine and has great potential to elucidate the ultimate product of the genomic processes. Over the last decade, metabolomics studies have identified several relevant biomarkers involved in complex clinical phenotypes using diverse biological systems. Most diseases result in signature metabolic profiles that reflect the sums of external and internal cellular activities. Metabolomics has a major role in clinical practice as it represents >95% of the workload in clinical laboratories worldwide. Many of these metabolites require different analytical platforms, such as Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), and *Ultra* Performance Liquid Chromatography (UPLC), while many clinically relevant metabolites are still not routinely amenable to detection using currently available assays. Combining metabolomics with genomics, transcriptomics, and proteomics studies will result in a significantly improved understanding of the disease mechanisms and the pathophysiology of the target clinical phenotype. This comprehensive approach will represent a major step forward toward providing precision medical care, in which individual is accounted for variability in genes, environment, and personal lifestyle. In this review, we compare and evaluate the metabolomics strategies and studies that focus on the discovery of biomarkers that have "personalized" diagnostic, prognostic, and therapeutic value, validated for monitoring disease progression and responses to various management regimens.

KEYWORDS
biomarker discovery, cancer metabolomics, clinical metabolomics, inborn errors of metabolism, mass spectrometry, metabolomics

1 | INTRODUCTION

Combining high throughput experimental "OMIC based" techniques such as genomics, proteomics, and metabolomics with computational techniques such as bioinformatics and computer simulations enable us to understand specific targets in biological systems. Metabolomics relates to small molecule identification and quantification, and the resulting network interactions which represent the individual's functional genome. The entire qualitative collection of metabolites in a biological sample is called "metabolome," which is very dynamic.

Abbreviations: ADSL, adenylosuccinate lyase deficiency; ADA, adenosine deaminase; CSF, cerebrospinal fluid; DBS, dried blood spots; ESI, electrospray ionization; eGFR, estimated glomerular filtration rate; FT-ICR, fourier transform ion cyclotron resonance; FMLR, fast maximum likelihood reconstruction; GMAPS™, global metabolomic assisted pathway screen; GC-MS, gas chromatography-mass spectrometry; HMG-CoA, 3-hydroxy 3-methyl-glutaryl Coenzyme A; HbA1c, hemoglobin A1c; IPA, ingenuity pathway analysis; IEM, inborn errors of metabolism; LGPC, linoleoyl glycero phospho choline; MAS, magic angle spinning; MODY, maturity onset diabetes of the young; MS, mass spectrometry; NMR, nuclear magnetic resonance; NGS, next generation sequencing; OCT1, organic cation transporter 1; PCA, principal component analysis; PLS, partial least squares; REIMS, rapid evaporative ionization mass spectrometry; SRM, selected reaction monitoring; SIR, selected ion recording; SHGP, Saudi Human Genome Project; T2DM, Diabetes Mellitus Type 2; UPLC, ultra performance liquid chromatography; WES, whole exome sequencing.

Article 12

Current Oral Health Reports (2018) 5:242–249
https://doi.org/10.1007/s40496-018-0200-4

EPIDEMIOLOGY (M LAINE, SECTION EDITOR)

Saliva – A Promising Tool for Diagnosing Oral Diseases

David T. Wu[1] · Owen Tao[1] · Nathalie Trinh[1] · Mohammad A. Javaid[2] · Ahad S. Ahmed[3] · Robert Durand[4] · Simon D. Tran[1]

Published online: 25 October 2018

Abstract

Purpose of Review This review will present updates on the genome, transcriptome, proteome, metabolome, exosome, and microbiome of saliva and the wide range of biomarkers found within these salivary components. We will explore the effectiveness and potential of salivary diagnostics on specific diseases: dental caries and demineralization diseases, periodontal diseases and peri-implantitis, oral cancers, and Sjögren's syndrome.

Recent Findings Recent studies have shown the feasibility of saliva as a diagnostic tool, in addition to the emergence of many novel salivary biomarkers. Unfortunately, many of these studies do not explore further into the outcomes of patient-oriented health and do not demonstrate reliability in diverse patient populations.

Summary Salivary diagnostics is an emerging field that uses saliva, for its non-invasive, fast, and cost-effective collection properties, as a diagnostic tool. In the near future, we expect that salivary diagnostics will benefit from technological advances and further studies to be used as a routine chair-side test included in a comprehensive diagnostic and risk assessment tool.

Keywords Saliva · Diagnostics · Salivaomics · Dental caries · Periodontal disease · Oral cancer

Introduction

Human saliva is a complex biological fluid that is largely secreted by the three major salivary glands: the parotid, submandibular, and sublingual [1]. Saliva has multiple functions such as food digestion, the perception of taste, protection against biological, mechanical, and chemical factors, as well as having antibacterial, antifungal, and antiviral properties [1, 2]. Healthy individuals normally secrete 0.5 L to 1.5 L of saliva per day [3]. This colorless and odorless fluid, with pH 6.0 to 7.0, is composed of 99% water, 0.3% proteins, and 0.2% organic and inorganic substances [2, 4]. This composition includes substances such as urea, ammonia, uric acid, glucose, glycoprotein, peroxidase, Na^+, Cl^-, Ca^{2+}, K^+, and HCO_3^-, in addition to hundreds of microorganisms [4].

Oral diagnostics involves the use of these substances, mainly, antibodies, enzymes, growth factors, and/or hormones, which reflects the physiological state of the body and can be monitored for disease detection [2, 5]. Early disease detection is crucial in reducing the severity of the disease and saliva offers many advantages when compared to other diagnostic tools: saliva collection is fast, easy, non-invasive, and inexpensive [6••]. Saliva collection eliminates the need for trained medical personnel and can be performed at home, thereby minimizing both patient discomfort and the risks of injury [6••]. Saliva collection has minimal risks of contamination, compared to gingival crevicular fluid (GCF), while being more economical to sample, ship, and store than serum [2, 3]. A drawback, however, of using saliva is that the detected concentration of molecular biomarkers is diminished compared to both GCF and serum [3, 7]. And the low amounts of these analytes bring to question the accuracy and validity of the salivary diagnostics approach. In this review, we will present an update on the current state of salivary diagnostics and its potential as an alternative or complementary diagnostic tool.

This article is part of the Topical Collection on *Epidemiology*

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Article 13

FOCUS ON METABOLISM

PERSPECTIVES

INNOVATION

Metabolomics: the apogee of the omics trilogy

Gary J. Patti, Oscar Yanes and Gary Siuzdak

Abstract | Metabolites, the chemical entities that are transformed during metabolism, provide a functional readout of cellular biochemistry. With emerging technologies in mass spectrometry, thousands of metabolites can now be quantitatively measured from minimal amounts of biological material, which has thereby enabled systems-level analyses. By performing global metabolite profiling, also known as untargeted metabolomics, new discoveries linking cellular pathways to biological mechanism are being revealed and are shaping our understanding of cell biology, physiology and medicine.

Metabolites are small molecules that are chemically transformed during metabolism and, as such, they provide a functional readout of cellular state. Unlike genes and proteins, the functions of which are subject to epigenetic regulation and post-translational modifications, respectively, metabolites serve as direct signatures of biochemical activity and are therefore easier to correlate with phenotype. In this context, metabolite profiling, or metabolomics, has become a powerful approach that has been widely adopted for clinical diagnostics.

The metabolome — typically defined as the collection of small molecules produced by cells — offers a window for interrogating how mechanistic biochemistry relates to cellular phenotype. With developments in mass spectrometry, it is now possible to rapidly measure thousands of metabolites simultaneously from only minimal amounts of sample[1]. In particular, recent innovations in instrumentation, bioinformatic tools and software enable the comprehensive analysis of cellular metabolites without bias. In many instances, these metabolites can be spatially localized within biological specimens with imaging mass spectrometry[2,3].

The application of these technologies has revealed system-wide alterations of unexpected metabolic pathways related to phenotypic perturbations. Moreover, many of the molecules detected are currently not included in databases and metabolite repositories, indicating the extent to which our picture of cellular metabolism is incomplete[4,5]. Nonetheless, the field of metabolomics has made remarkable progress within the past decade and has implemented new tools that have offered mechanistic insights by allowing for the correlation of biochemical changes with phenotype.

In this Innovation article, we first define and differentiate between the targeted and untargeted approaches to metabolomics. We then highlight the value of untargeted metabolomics in particular and outline a guide to performing such studies. Finally, we describe selected applications of untargeted metabolomics and discuss their potential in cell biology.

> “metabolites serve as direct signatures of biochemical activity”

Designing a metabolomic experiment

The first step in performing metabolomics is to determine the number of metabolites to be measured. In some instances, it may be of interest to examine a defined set of metabolites by using a targeted approach. In other cases, an untargeted or global approach may be taken in which as many metabolites as possible are measured and compared between samples without bias. Ultimately, the number and chemical composition of metabolites to be studied is a defining attribute of any metabolomic experiment and shapes experimental design with respect to sample preparation and choice of instrumentation.

Targeted metabolomics. This approach refers to a method in which a specified list of metabolites is measured, typically focusing on one or more related pathways of interest[6]. Targeted metabolomic approaches are commonly driven by a specific biochemical question or hypothesis that motivates the investigation of a particular pathway (FIG. 1a). This approach can be effective for pharmacokinetic studies of drug metabolism as well as for measuring the influence of therapeutics or genetic modifications on a specific enzyme[7]. Developments in mass spectrometry and nuclear magnetic resonance (NMR) offer distinct advantages for performing targeted metabolomic studies because of their specificity and quantitative reproducibility; however, there are many analytical tools available for measuring metabolites that could in principle be considered, such as ultraviolet-visible spectroscopy and flame ionization. Although the term ‘metabolomics’ was only recently coined, examples of targeted studies of metabolites date back to the earliest of scientific inquiries[8–12]. Therefore, there is a wealth of literature investigating optimal protocols for the sample preparation and analysis of specific classes of metabolites that has been discussed extensively elsewhere[13–17].

Not to diminish their significance, targeted approaches have undoubtedly played an important part in the development of the field of metabolomics. In particular, advances have been made in using triple quadrupole (QqQ) mass spectrometry to perform selected reaction monitoring experiments such that routine methods are now available for analysing most of the metabolites in central carbon metabolism, as well as amino acids and nucleotides at

Article 14

Alessandra Sussulini
Editor

Metabolomics: From Fundamentals to Clinical Applications

Springer

Article 15

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Dipartimento di Salute della Donna e del Bambino

SCUOLA DI DOTTORATO DI RICERCA IN :

Medicina dello Sviluppo e Scienze della Programmazione

INDIRIZZO: Ematooncologia, genetica, malattie rare e medicina predittiva.

CICLO: XXVII

LA METABOLOMICA: NUOVO APPROCCIO ALLA MEDICINA PREDITTIVA PER IL BAMBINO E IL NEONATO

Article 16

metabolites

MDPI

Review

Salivary Metabolomics: From Diagnostic Biomarker Discovery to Investigating Biological Function

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Received: 13 December 2019; Accepted: 24 January 2020; Published: 26 January 2020

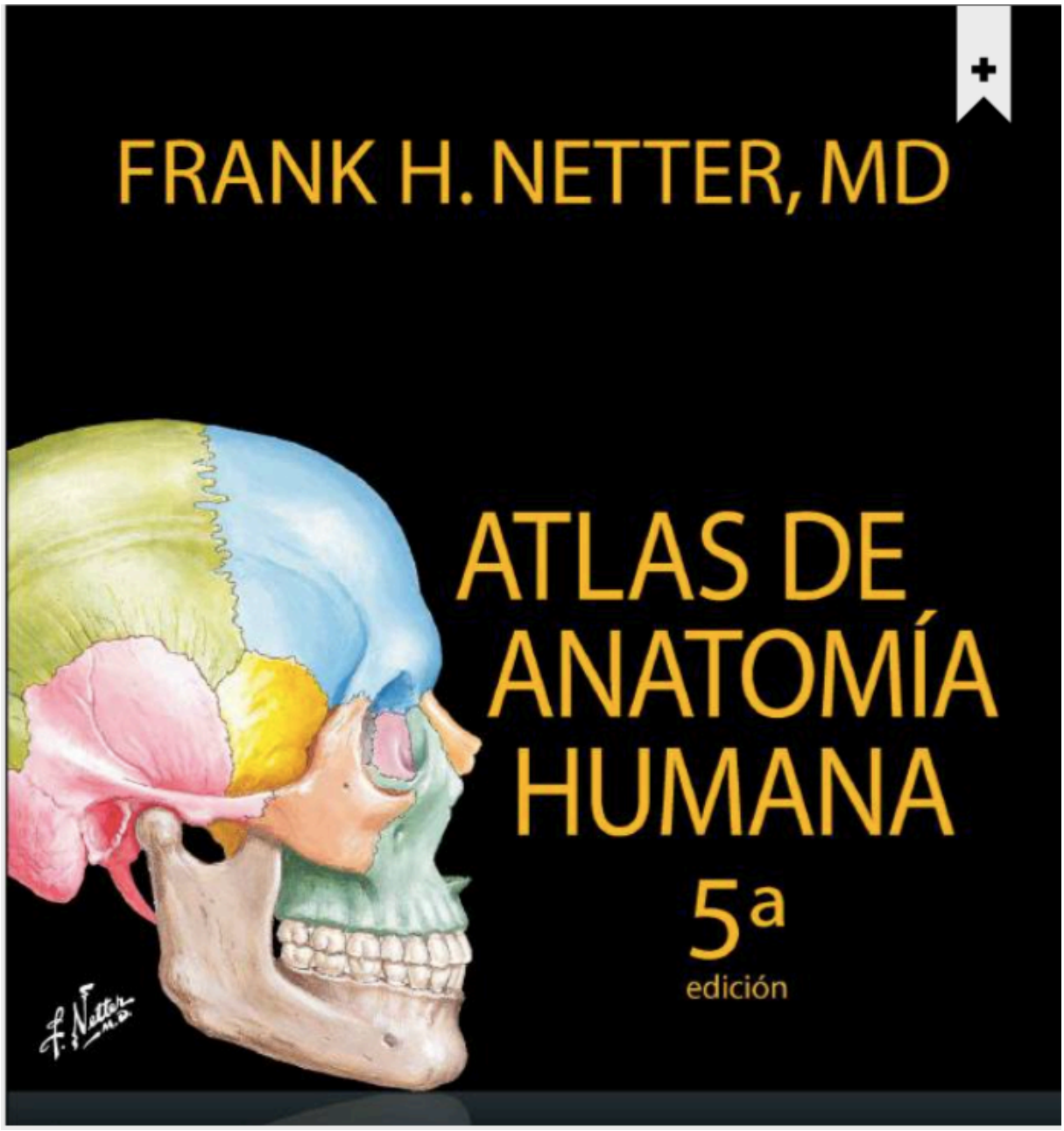
Abstract: Metabolomic profiling of biofluids, e.g., urine, plasma, has generated vast and ever-increasing amounts of knowledge over the last few decades. Paradoxically, metabolomic analysis of saliva, the most readily-available human biofluid, has lagged. This review explores the history of saliva-based metabolomics and summarizes current knowledge of salivary metabolomics. Current applications of salivary metabolomics have largely focused on diagnostic biomarker discovery and the diagnostic value of the current literature base is explored. There is also a small, albeit promising, literature base concerning the use of salivary metabolomics in monitoring athletic performance. Functional roles of salivary metabolites remain largely unexplored. Areas of emerging knowledge include the role of oral host–microbiome interactions in shaping the salivary metabolite profile and the potential roles of salivary metabolites in oral physiology, e.g., in taste perception. Discussion of future research directions describes the need to begin acquiring a greater knowledge of the function of salivary metabolites, a current research direction in the field of the gut metabolome. The role of saliva as an easily obtainable, information-rich fluid that could complement other gastrointestinal fluids in the exploration of the gut metabolome is emphasized.

Keywords: whole-mouth saliva; parotid saliva; gingival–crevicular fluid; submandibular/ sublingual fluid; oral microbiome; metabolic profiling; NMR; MS

1. Introduction

Saliva is a biological fluid produced in the oral cavity by three pairs of major glands and up to one thousand minor glands. It is important to make the distinction between the different fluids that may be described under the umbrella term "saliva". When referring to the fluid produced upon spitting or drooling, whole-mouth saliva (WMS) would be a more apt descriptor. This reflects the fact that WMS is essentially the net product of major and minor glands, eukaryotic cells (epithelial and leukocytic), bacteria, and gingival–crevicular fluid (GCF) [1]. GCF is a serum-derived filtrate that enters the mouth at the gingival margins of the teeth. While the non-glandular contributions to WMS compose only a fraction of the net fluid volume, their contributions to the net composition are significant. When referring to saliva produced by specific glands, descriptors are used to denote the gland of origin. Fluid produced by the parotid glands is termed parotid saliva (PS), and fluid produced by the submandibular gland and sublingual gland is typically described together as submandibular/sublingual (SM/SL) saliva. This is due to the fact that the submandibular and sublingual ducts share an opening to the oral cavity,

Citation 17 – Book 1:



Citation 18 - Book 2:

Saliva, Diagnostics, and Dentistry

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Adv Dent Res 23(4):353-359, 2011

Presented at the 3rd Fall Focused Symposium, November 12-13, 2010, Waterview Conference Center, Arlington, VA, sponsored by the American Association for Dental Research

ABSTRACT

Saliva, a scientific and clinical entity familiar to every oral health researcher and dental practitioner, has emerged as a translational and clinical commodity that has reached national visibility at the National Institutes of Health and the President's Office of Science and Technology. "Detecting dozens of diseases in a sample of saliva" was issued by President Obama as one of the 14 Grand Challenges for biomedical research in the 21st Century (National Economic Council, 2010). In addition, NIH's 2011 Government Performance Report Act (GPRA) listed 10 initiatives in the high-risk long-term category (Collins, 2011). The mandate is to determine the efficacy of using salivary diagnostics to monitor health and diagnose at least one systemic disease by 2013. The stage is set for the scientific community to capture these national and global opportunities to advance and substantiate the scientific foundation of salivary diagnostics to meet these goals. A specific calling is to the oral, dental, and craniofacial health community. Three areas will be highlighted in this paper: the concept of high-impact diagnostics, the role of dentists in diagnostics, and, finally, an infrastructure currently being developed in the United Kingdom—The UK Biobank—which will have an impact on the translational and clinical utilizations of saliva.

DOI: 10.1177/0022034511420432

HIGH-IMPACT DIAGNOSTICS

In vitro diagnostic (IVD) tests serve an important role in the practice of medicine today. The number of tests available to the clinician is staggering. High-impact diagnostics refers to diagnostics that will have greater impact on clinical decision-making, patient outcomes, and/or healthcare costs than others. Examples include tests for PSA, cholesterol, HIV viral load, breast cancer prognosis, HbA1c, and many others. Often, 'high impact' translates into high value and significant reimbursement levels (*e.g.*, OncoType DX® from Genomic Health, Inc., Redwood City, CA, USA). It is important to identify a high-impact diagnostic and move such a product from the research stage to the commercial marketplace. As salivary biomarkers continue to emerge as credible reflections of oral and systemic diseases, an appreciation of the translational and clinical path forward toward high-impact diagnostics will be prudent.

Assessing the Impact of a New Diagnostic Product

Many diagnostic products are developed every year, but few become high-impact products. Some do not perform well enough, some are not supported by sound clinical studies, while others might suffer from low reimbursement levels or inadequate physician education on the part of the diagnostics company. It is important that these issues be addressed early in the product development process, to maximize resource utilization and potential return on the R&D investment. A new product's impact upon the practice of medicine and dentistry depends upon how it will be used to make clinical decisions and the degree to which the new decision-making process affects clinical and economic outcomes. Diagnostic tests can be grouped based upon their intended uses during the progression of a particular disease. Fig. 1 shows different categories of need for diagnostic tests. Different products with different performance requirements might be needed for disease screening or risk stratification than for therapy selection or monitoring. It is important that unmet medical needs and user requirements be taken into consideration in the design of a new diagnostic test.

Key Words

molecular diagnostics, dentistry, saliva, biobanking, biomarkers, medical screening.

Article 20

JOURNAL OF ORAL BIOLOGY AND CRANIOFACIAL RESEARCH 6 (2016) 67–76

Available online at www.sciencedirect.com

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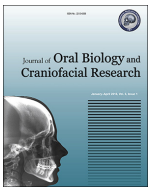
journal homepage: www.elsevier.com/locate/jobcr



Review Article

Saliva as a diagnostic tool for oral and systemic diseases

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ARTICLE INFO

Article history:
Received 14 July 2015
Accepted 8 August 2015
Available online 9 September 2015

Keywords:
Saliva
Diagnostics
Salivary

ABSTRACT

Early disease detection is not only vital to reduce disease severity and prevent complications, but also critical to increase success rate of therapy. Saliva has been studied extensively as a potential diagnostic tool over the last decade due to its ease and non-invasive accessibility along with its abundance of biomarkers, such as genetic material and proteins. This review will update the clinician on recent advances in salivary biomarkers to diagnose autoimmune diseases (Sjogren's syndrome, cystic fibrosis), cardiovascular diseases, diabetes, HIV, oral cancer, caries and periodontal diseases. Considering their accuracy, efficacy, ease of use and cost effectiveness, salivary diagnostic tests will be available in dental offices. It is expected that the advent of sensitive and specific salivary diagnostic tools and the establishment of defined guidelines and results following rigorous testing will allow salivary diagnostics to be used as chair-side tests for several oral and systemic diseases in the near future.

1. Introduction

Early diagnosis of diseases is crucial to prevent complications that could have a negative impact on a patient's quality of life. For instance, ovarian cancer, the fifth most common cancer and cause of death in females, has a 5-year-survival rate of 10% when detected at stage 4 in comparison to 93% if diagnosed at stage 1.[1] Similarly, type 2 diabetes, which affects 7% of the adult population, can be solely controlled by diet and change in lifestyle if the diagnosis is made earlier.[2] Furthermore, despite the regular screenings and check-ups, many diseases are undetected until a late phase where morbid symptoms become apparent. To overcome these challenges, researchers are unravelling biomarkers. These biomarkers include genetic material (e.g. DNA, RNA) and protein molecules that reflect the current physiological state of an individual and hence help scientists to better understand the underlying cause of a disease.[3] Over the years, studies have shown that alterations in human genetics can be detected by molecular diagnostics, and anomalies in nucleic acids and proteins present in the patient's body fluids such as blood, cerebrospinal fluid (CSF) and urine can be used as effective biomarkers for disease diagnosis.[4–6] However, many obstacles remain such as lack of

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http://dx.doi.org/10.1016/j.jobcr.2015.08.006

Article 21

Metabolomics (2013) 9:454–463
DOI 10.1007/s11306-012-0464-y

ORIGINAL ARTICLE

Physiological and environmental parameters associated with mass spectrometry-based salivary metabolomic profiles

Masahiro Sugimoto · Juri Saruta · Chisa Matsuki · Masahiro To · Hiromi Onuma · Miku Kaneko · Tomoyoshi Soga · Masaru Tomita · Keiichi Tsukinoki

Received: 13 July 2012 / Accepted: 17 September 2012 / Published online: 29 September 2012

Abstract Mass spectrometry (MS)-based metabolomic methods enable simultaneous profiling of hundreds of salivary metabolites, and may be useful to diagnose a wide range of diseases using saliva. However, few studies have evaluated the effects of physiological or environmental factors on salivary metabolomic profiles. Therefore, we used capillary electrophoresis-MS to analyze saliva metabolite profiles in 155 subjects with reasonable oral hygiene, and examined the effects of physiological and environmental factors on the metabolite profiles. Overall, 257 metabolites were identified and quantified. The global profiles and individual metabolites were evaluated by principle component analysis and univariate tests, respectively. Collection method, collection time, sex, body mass index, and smoking affected the global metabolite profiles. However, age also might contribute to the bias in sex and collection time. The profiles were relatively unaffected by other parameters, such as alcohol consumption and smoking, tooth brushing, or the use of medications or nutritional supplements. Temporomandibular joint disorders had relatively greater effects on salivary metabolites than other dental abnormalities (e.g., stomatitis, tooth alignment, and dental caries). These findings provide further insight into the diversity and stability of salivary metabolomic profiles, as well as the generalizability of disease-specific biomarkers.

Keywords Capillary electrophoresis · Metabolomics · Mass spectrometry · Oral hygiene · Saliva · Temporomandibular joint disorders

1 Introduction

Saliva is a readily accessible and informative biofluid that is ideal for the early detection of many diseases, including periodontitis, hepatitis, human immunodeficiency virus, viral hepatitis, and cancer (Lee et al. 2009). It can also be used to monitor the concentrations of various drugs, including marijuana, cocaine, and phencyclidine (Schramm et al. 1992). Advantages of using saliva as a diagnostic tool are that it is easy and noninvasive to collect, associated with less discomfort than venipuncture, and does not entail privacy issues, unlike urine collection (Lee and Wong 2009). Consequently, *omics* technologies, including transcriptomics and proteomics, have been used for high-throughput identification of disease-associated salivary biomarkers, to better understand the complex biology of the oral cavity, and facilitate the diagnosis of diseases in and remote from the oral cavity (Grant 2012; Spielmann and Wong 2011).

Electronic supplementary material The online version of this article (doi:10.1007/s11306-012-0464-y) contains supplementary material, which is available to authorized users.

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Article 22

J. Cell. Mol. Med. Vol 21, No 4, 2017 pp. 640-647

Emerging technologies for salivaomics in cancer detection

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Received: August 11, 2016; Accepted: September 13, 2016

Abstract

Salivary diagnostics has great potential to be used in the early detection and prevention of many cancerous diseases. If implemented with rigour and efficiency, it can result in improving patient survival times and achieving earlier diagnosis of disease. Recently, extraordinary efforts have been taken to develop non-invasive technologies that can be applied without complicated and expensive procedures. Saliva is a biofluid that has demonstrated excellent properties and can be used as a diagnostic fluid, since many of the biomarkers suggested for cancers can also be found in whole saliva, apart from blood or other body fluids. The currently accepted gold standard methods for biomarker development include chromatography, mass spectometry, gel electrophoresis, microarrays and polymerase chain reaction-based quantification. However, salivary diagnostics is a flourishing field with the rapid development of novel technologies associated with point-of-care diagnostics, RNA sequencing, electrochemical detection and liquid biopsy. Those technologies will help introduce population-based screening programs, thus enabling early detection, prognosis assessment and disease monitoring. The purpose of this review is to give a comprehensive update on the emerging diagnostic technologies and tools for the early detection of cancerous diseases based on saliva.

Keywords: salivary diagnostics • cancer • RNA-Sequencing • point-of-care • liquid biopsy

Introduction

Saliva is a complex fluid that is composed of water, cells, debris, organic and inorganic molecules that may reflect the physiological state of an individual condition, since many of the componenets of the saliva also play an important role in processes taking part in distal portions of the body [1]. Currently, approximately 40% of markers suggested for diseases such as cancer, cardiovascular disease and stroke can also be found in whole saliva [2]. A biomarker can be defined as a measurable and quantifiable biological parameter that can serve either as an indicator for health, disease status, environmental exposure or pharmacological responses to a therapeutic intervention [3]. Prognostic biomarkers are used as indicators of a benign or a malignant condition, whereas diagnostic biomarkers show the development of a cancer [4].

Siegel *et al.* reported that 1.7 million Americans are diagnosed with cancer every year and over 500,000 individuals do not survive the disease [5]. Hence, a lot of efforts have been done to advance the field of salivary diagnostic technology, which is likely to revolutionize the way cancerous diseases will be diagnosed in the future [6].

To successfully translate research on salivary biomarkers to the chairside, biomarker studies should follow the principles laid out in the prospective-specimen-collection, retrospective-blinded evaluation

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doi: 10.1111/jcmm.13007

Article 23

Untargeted saliva metabonomics study of breast cancer based on ultra performance liquid chromatography coupled to mass spectrometry with HILIC and RPLC separations

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Abstract

Breast cancer (BC) is not only the most frequently diagnosed cancer, but also the leading cause of cancer death among women worldwide. This study aimed to screen the potential salivary biomarkers for breast cancer diagnosis, staging, and biomarker discovery. For the first time, a UPLC-MS based method along with multivariate data analysis, was proposed for the global saliva metabonomics analysis and diagnosis of BC, which used both hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RPLC) separations and operated in both positive (ESI+) and negative (ESI-) ionization modes. On account of different polarities of endogenous metabolites, this method was established to overcome the boundedness of a single chromatographic approach. As a result, 18 potential metabolites for diagnosing BC were identified. A nonparametric Mann-Whitney U test, heat map, and the receiver operating characteristic (ROC) were exploited to analyze the data with the purpose of evaluating the predictive power of the 18 biomarkers. Significant differences ($P<0.05$) were disclosed in terms of the levels of the 18 potential biomarkers between BC patients and healthy controls (HC). Among the 18 biomarkers, three up-regulated metabolites, LysoPC (18:1), LysoPC (22:6) and MG (0:0/14:0/0:0) displayed the area under the curve (AUC) values of 0.920, 0.920 and 0.929, respectively, indicating the high accuracy of this method to predict BC. In this study, an integrated metabonomics analysis in human saliva for identifying potential biomarkers to diagnose and stage BC was successfully eastablished, which was non-invasive, reliable, low-cost, and simple. The HILIC was demonstrated to be essential for a comprehensive saliva metabonomics profiling as well as RPLC separation. This saliva metabonomics technique may provide new insight into the discovery and identification of diagnostic biomarkers for BC.

La caries dental: una enfermedad que se puede prevenir

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Introducción

La caries es una enfermedad *infecciosa* producida por bacterias productoras de ácido, que se *transmiten* al niño fundamentalmente en el primer año de vida[1]. Es una de las enfermedades crónicas de mayor prevalencia en la infancia, *extendida* por todo el mundo sin distinción de raza o género. Por otra parte, y al igual que la mayoría de las enfermedades crónicas más frecuentes en la actualidad, es *multifactorial* y está muy relacionada con estilos de vida, fundamentalmente hábitos de alimentación e higiene oral insuficiente, la alimentación nocturna del niño, el alto consumo de azúcares, la colonización bacteriana precoz y el bajo nivel socioeconómico de los padres[2].
Los últimos estudios epidemiológicos realizados en niños españoles en edad preescolar indican que, independientemente de la comunidad autónoma, casi el 20% a los 3 años tiene caries y el 40% a los 5 años[3].
El mejor enfoque terapéutico es, por tanto, la prevención y la determinación de los factores que aumentan el riesgo de enfermar se ha convertido en uno de los pilares fundamentales de este enfoque[4].

Puntos clave

- La caries dental es una *enfermedad transmisible* y la mayoría de los niños adquieren las bacterias cariogénicas de manera vertical de la saliva de sus madres o cuidadores. Por ello, se recomienda a los padres evitar compartir utensilios con el bebé, limpiar el chupete con su saliva, enfriar la comida soplando sobre ella o dar besos en la boca.
- La caries dental es una *enfermedad multifactorial*, condicionada por elementos como son las características del huésped, la presencia de bacterias y el sustrato (carbohidratos refinados). La combinación de todos estos factores y su *frecuencia* en el tiempo son los que determinan conjuntamente la sensibilidad a la caries dental y la evolución de esta.
- Las características del alimento pueden influir en el *potencial cariogénico* de este: concentración de sacarosa, consistencia, aclaramiento oral, combinación de alimentos, secuencia y frecuencia de ingestión y pH de los alimentos.
- Se deben limpiar los dientes del niño con pasta dental con flúor lo más pronto posible y para minimizar el riesgo de fluorosis dental es importante usar un barrillo de pasta en niños menores de 3 años y, a partir de los 3 años y hasta los 6, una cantidad similar a un guisante. Se pueden usar pastas de bajo contenido en flúor (500 ppm), pero solo existe evidencia científica de efecto preventivo a partir de concentraciones de 1.000 ppm de flúor en adelante.

Concepto actual de la caries dental

La caries en la infancia presenta graves repercusiones en la salud general del niño, como dolor intenso, infecciones faciales, hospitalizaciones y visitas a urgencias, alto coste de tratamiento y disminución en la calidad de vida en relación con la salud.
La caries dental es una patología multifactorial que como tal cuenta con unos factores causales, una patogénesis, sus manifestaciones clínicas y una serie de factores de riesgo predisponentes. Se considera una infección bacteriana caracterizada por la destrucción de los tejidos calcificados del diente, debido a la acción de los microorganismos que integran la placa dental. Es una enfermedad transmisible y la mayoría de los niños adquieren las bacterias cariogénicas de manera vertical de la saliva de sus madres o cuidadores[1,2].
La caries se manifiesta con lesiones normalmente progresivas, que si no se tratan, aumentarán de tamaño, progresando hacia la pulpa dentaria, dando como resultado inflamación, dolor y finalmente, necrosis y pérdida de vitalidad del diente (fig. 1). Pero a su vez, la caries no es un proceso simple y unidireccional de desmineralización, sino que puede ser cíclico, alternando periodos de desmineralización con periodos de remineralización, lo que posibilita la reparación y prevención[5].

Article 25

Metabolomics (2013) 9:657–666
DOI 10.1007/s11306-012-0484-7

ORIGINAL ARTICLE

Salivary metabolite signatures of children with and without dental caries lesions

Tatiana K. S. Fidalgo · Liana B. Freitas-Fernandes · Renata Angeli · Adriane M. S. Muniz · Elicardo Gonsalves · Raquel Santos · Jurandir Nadal · Fabio C. L. Almeida · Ana P. Valente · Ivete P. R. Souza

Received: 20 August 2012 / Accepted: 20 November 2012 / Published online: 5 December 2012

Abstract A metabolomic approach was used to analyze endogenous metabolites and to correlate with a specific biological state. The analysis of salivary metabolites is a growing area of investigation with potential for basic and clinical applications. Analyses of children's saliva in different dentitions and with or without caries could potentially reveal a specific profile related to oral disease risk. Nuclear Magnetic Resonance (NMR) is well suited for mixture analysis followed by Principal Component Analysis combined with Linear Regression (PCA-LR) statistics and was used to identify differences in the salivary metabolites. The classificatory analysis was performed using PCA-LR based on 1,000 cross-validation bootstrap runs from both classifiers in order to increase the data information from a small sample size. The PCA-LR presented a statistically good classificatory performance for children with and without caries with an accuracy of 90.11 % ($P < 0.001$), 89.61 % sensitivity ($P < 0.001$), and 90.82 % specificity ($P < 0.001$). Children with caries lesions presented higher levels of several metabolites, including lactate, fatty acid, acetate and n-butyrate. Saliva from subjects with different dentition stages was also analyzed. Although the salivary samples were poorly classified, permanent dentition presented increased levels of acetate, saccharides and propionate. The NMR data and PCA-LR were able to classify saliva from children with or without caries, with performance indexes comparable to the partial least-squares regression discriminant analysis (PLS-DA) results also performed. Our data also showed similar salivary metabolite profiles for healthy subjects despite the differences in their oral hygiene habits, socioeconomic status and food intake.

Keywords Children · Saliva · Dental caries · Metabolomic profile · NMR

Electronic supplementary material The online version of this article (doi:10.1007/s11306-012-0484-7) contains supplementary material, which is available to authorized users.

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1 Introduction

Among the biofluids, saliva is likely the easiest biofluid to collect and is very informative with regard to biological status. Saliva composition presents a potential source of novel diagnostic markers for both systemic and oral diseases because most components found in the blood are also present in saliva (Grootveld and Silwood 2005; Pfaffe et al. 2011; Ryan et al. 2011; Zhang et al. 2012). Salivary metabolite signatures have been identified for different

Article 26

Metabolomics
DOI 10.1007/s11306-014-0717-z

ORIGINAL ARTICLE

Longitudinal evaluation of salivary profile from children with dental caries before and after treatment

Tatiana K. S. Fidalgo · Liana B. Freitas-Fernandes · Fabio C. L. Almeida · Ana P. Valente · Ivete P. R. Souza

Received: 17 April 2014 / Accepted: 25 July 2014

Abstract Saliva is a biofluid largely used in metabolomics for the assessment of local and systemic diseases. Our group has previously demonstrated the salivary metabolomic signature of children with dental caries (Fidalgo et al. Metabolomics 9(3):657–666, 2013). The aim of the current study was investigation, using NMR spectroscopy, of the changes observed for metabolite markers for caries lesions before and after dental treatment. Saliva from children with and without dental caries before and after treatment was analyzed by NMR. Partial least squared discriminant analysis (PLS-DA) conducted on the spectroscopic data sets showed a clear separation of saliva metabolic profile of children with and without caries, and multilevel PLS-DA demonstrated difference before and after dental treatment. Our results demonstrate that organic acids are associated with disease activity because their reductions were observed after dental treatment. There was a demonstrated reduction here in the levels of acetate, propionate, fatty acid, butyrate and saccharides. We also observed a drop in the level of microorganisms upon dental treatment. The dental treatment therefore modified the properties of the oral cavity, leading to changes in the salivary profiles after treatment.

Keywords Saliva · Metabolomic profile · Children · NMR · Dental caries · Oral microorganisms

1 Introduction

Saliva has been shown to be an emerging and attractive choice of biofluid for the early detection of orally local and systemic disorders (Aimetti et al. 2012; Bertram et al. 2009; Fidalgo et al. 2013; Takeda et al. 2009). Some studies have suggested that NMR-detectable biomarkers in saliva could be used to identify systemic diseases such as cancer, diabetes mellitus, cardiovascular disease, and others (Bertram et al. 2009; Cuevas-Cordoba and Santiago-Garcia 2014; Grootveld and Silwood 2005; Ng et al. 2011; Sugimoto et al. 2010). However, it is important to consider the oral status when a systemic condition is evaluated using saliva as a biofluid. As the metabolite fingerprints from oral diseases can be erroneously associated with systemic disorders (Aimetti et al. 2012; Fidalgo et al. 2013; Silwood et al. 1999).

Saliva plays an important role in the maintenance of oral health through the action of low molecular weight compounds, ions, and protein balance (Aimetti et al. 2012; Fidalgo et al. 2013; Van Nieuw Amerongen et al. 2004). Regarding the oral flora, it is known that *Streptococcus mutans* can colonize the oral cavity beginning at pre-dentate periods and can be acquired subsequently by caregivers, especially mothers (Caufield et al. 1993). It was demonstrated that children with dental caries present increased counts of *S. mutans,* and after dental treatment of

Electronic supplementary material The online version of this article (doi:10.1007/s11306-014-0717-z) contains supplementary material, which is available to authorized users.

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Published online: 08 August 2014

RESEARCH REPORTS
Clinical

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J Dent Res 89(12):1383-1388, 2010

Metabolomics of Supragingival Plaque and Oral Bacteria

ABSTRACT

Dental caries is initiated by demineralization of the tooth surface through acid production by sugar metabolism of supragingival plaque microflora. To elucidate the sugar metabolic system, we used CE-MS to perform metabolomics of the central carbon metabolism, the EMP pathway, the pentose-phosphate pathway, and the TCA cycle in supragingival plaque and representative oral bacteria, *Streptococcus* and *Actinomyces*. Supragingival plaque contained all the targeted metabolites in the central carbon metabolism, except erythrose 4-phosphate in the pentose-phosphate pathway. After glucose rinse, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and pyruvate in the EMP pathway and 6-phosphogluconate, ribulose 5-phosphate, and sedoheptulose 7-phosphate in the pentose-phosphate pathway, and acetyl CoA were increased. Meanwhile, 3-phosphoglycerate and phosphoenolpyruvate in the EMP pathway and succinate, fumarate, and malate in the TCA cycle were decreased. These pathways and changes in metabolites observed in supragingival plaque were similar to the integration of metabolite profiles in *Streptococcus* and *Actinomyces*.

KEY WORDS: metabolomics, plaque, sugar metabolism, *Streptococcus*, *Actinomyces*.

DOI: 10.1177/0022034510377792

Received April 7, 2010; Last revision May 28, 2010; Accepted June 12, 2010

INTRODUCTION

Dental caries is initiated by demineralization of the tooth surface through acid production from sugar by supragingival plaque microflora. Biochemical mechanisms of sugar metabolism by plaque bacteria, such as *Streptococcus* and *Actinomyces*, have been investigated for many years, because it is essential to elucidate the metabolic regulation of bacterial acid production, not only to understand caries etiology from a bacterial perspective, but also to develop more effective and safer caries-preventive reagents and protocols.

Metabolomics (metabolome analysis) is the comprehensive identification and quantification of metabolites in biological systems, which is one of the most powerful approaches to metabolism research. In the 1960s, Minakami *et al.* (1965) succeeded in quantifying metabolic intermediates of the Embden-Meyerhof-Parnas (EMP) pathway in human red blood cells by a photometry-coupled enzymatic method using purified glycolytic enzymes. This method was then modified and developed by the authors' laboratory for oral bacteria, including oral *Streptococcus* (Iwami *et al.*, 1975, 1992, 2000; Yamada and Carlsson, 1975a; Iwami and Yamada, 1980, 1985; Abbe *et al.*, 1982; Hata *et al.*, 1990; Takahashi *et al.*, 1991) and *Actinomyces* (Takahashi and Yamada, 1992). Later, Conyers *et al.* (1976) developed thin-layer chromatography using radio-labeled metabolic substrates to quantify glycolytic intermediates, and Thompson and his colleagues (Thompson and Thomas, 1977; Thompson, 1978; Thompson and Chassy, 1983) adopted this method for *Streptococcus lactis*, a species similar to oral streptococci. Furthermore, Ugurbil *et al.* (1978) developed a nuclear magnetic resonance (NMR) method to quantify glycolytic intermediates in intact *Escherichia coli* cells using stable isotopes, and Thompson and Torchia (1984) applied this method to *Streptococcus lactis*. These studies clarified changes in the profile of glycolytic intermediates and enabled us to speculate on the regulatory mechanisms of bacterial glycolysis; however, these methods have long been limited mainly to the EMP pathway, because purified metabolic enzymes for the enzymatic method were not available to determine other metabolites, and both the thin-layer chromatography and NMR methods were not capable of separating and identifying other metabolites.

In the past two decades, metabolomics has developed rapidly, mainly because of the combination of chromatography or electrophoresis for separation with high-resolution and mass spectrometry (MS) for precise identification of biological molecules. Recently, capillary electrophoresis (CE) has been adopted for the separation of metabolites, because most metabolites are polar and ionic small molecules, such as phosphorylated sugars, carboxylic acids, amino acids, and nucleotides. CE-MS is thus suitable for separating and quantifying metabolites in terms of the central carbon metabolism, including the EMP pathway, the pentose-phosphate pathway, and the Krebs tricarboxylic

Article 28

Received: 21 January 2019 | Revised: 28 April 2019 | Accepted: 7 May 2019

DOI: 10.1111/odi.13117

ORIGINAL ARTICLE

ORAL DISEASES WILEY

Saliva NMR metabolomics: Analytical issues in pediatric oral health research

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Funding information
Fundação para a Ciência e a Tecnologia, Grant/Award Number: SFRH/BD/119509/2016,UID/CTM/50011/2019 and POCI-01-0145-FEDER-007679

Abstract

Objectives: Saliva metabolome is a promising diagnostic tool concerning oral and systemic diseases. We aimed at establishing a suitable protocol for saliva collection and gauging the relative impacts of gender, dentition stage, and caries on the saliva metabolome of a small children cohort.

Subjects and methods: A nuclear magnetic resonance-based metabolomics cross-sectional study of children saliva (n = 38) compared the effects of: (a) stimulation and unstimulation conditions, and (b) collection through passive drool and using an absorbing device. Multivariate and univariate statistical analyses were applied to evaluate such effects and those related to gender, dentition stage and caries.

Results: No significant differences were found between unstimulated and stimulated saliva, and the former was used for subsequent studies. Swab collection induced significant changes in sample composition, indicating passive drool as preferential. The impacts of gender and dentition stage were not significant compared to that of caries, which induced variations in the levels of 21 metabolites. These comprised amino acids and monosaccharides observed for the first time to our knowledge regarding children caries, suggesting protein hydrolysis and deglycosylation.

Conclusions: Unstimulated passive drool saliva metabolome may carry a caries signature.

KEYWORDS
biomarkers, dental caries, metabolome, metabolomics, pediatric dentistry, saliva

1 | INTRODUCTION

Dental caries is the most common chronic pediatric disease and affects 60%–90% of schoolchildren worldwide (Petersen, 2003). Afflicted children undergo dentition destruction, leading to feeding and sleeping disturbances, malocclusion, and pain, often requiring hospital admission (Anil & Anand, 2017). Early childhood caries (ECC) is a particularly severe form of caries with early onset and rapid progression in children younger than 71 months (American Academy of Pediatric Dentistry, 2016). Since ECC standard care frequently involves sedation or general anesthesia, it carries both financial and quality of life burdens, thus becoming a public health problem (Anil & Anand, 2017). Research has established the multifactorial nature of caries and its dependence on several factors (enamel defects, saliva characteristics microbial flora, diet/dental hygiene habits), although the most consistent risk predictor is still the previous occurrence of caries (Anil & Anand, 2017; Hart et al., 2011).

Ana Luísa Costa and Ana M. Gil contributed equally to this work.

Periodontology 2000

Periodontology 2000, Vol. 53, 2010, 70–88
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PERIODONTOLOGY 2000

Comparison of the microbiological features of chronic and aggressive periodontitis

GARY C. ARMITAGE

Any discussion of the comparative microbiology of chronic and aggressive forms of periodontitis must begin with the premise that our knowledge of the microbiota associated with these infections is incomplete. This does not mean that the field of oral microbiology has neglected the study of periodontal infections. On the contrary, the microbiota associated with periodontal health and disease has been intensely studied for well over a century by several generations of skilled scientists and clinicians (9, 15, 23, 42, 47, 48, 60, 65, 82, 101, 102, 105, 113, 114, 122, 123, 135, 145, 147, 149, 171, 175, 177, 181, 184, 206). The basic problem is that the oral microbiota is an enormously complex and dynamic entity that is profoundly affected by perpetually changing local environments and host-mediated selective pressures. In addition, the microorganisms live in hard-to-study biofilms comprising organized polymicrobial communities that are elegantly adapted to thriving and surviving in the multiple micro-ecosystems of the oral cavity.

Another complication in any discussion of periodontal microbiology is the educational bias regarding the nature of infectious diseases that healthcare professionals develop during their training. Most dentists and physicians were taught the classical features of infectious diseases in their basic courses in medical microbiology. The dominant paradigm taught in these courses is that an exogenous pathogen overcomes the innate and adaptive immune defenses of the host, replicates within the body, and causes disease through a variety of virulence factors. Diagnosis of these classical infections often involves submitting a clinical specimen obtained from the infected patient to a clinical laboratory for isolation and identification of the pathogen by growing it in pure culture on artificial medium. These cultures are then used to determine the sensitivity of the isolated pathogen to a panel of antibiotics. Treatment of the infection involves administration of an appropriate regimen of antibiotics that is intended to suppress or eliminate the pathogen. This model for infectious diseases is certainly not the only one that is taught at most medical or dental schools, as it is widely known that some infections can be caused by multiple bacteria (i.e. 'mixed' infections) and some diseases can be due to commensal opportunistic pathogens (82). Nevertheless, the one pathogen/one disease model dominates the thinking of most physicians and many dentists.

Unfortunately, this dominant paradigm does not apply in cases of periodontal infections. These infections are caused by an extremely diverse consortium of microorganisms that are part of the endogenous microbiota of most people (82, 157). Many individuals who are periodontally healthy carry or harbor some periodontal pathogens as part of their normal supragingival and subgingival microbiota (4, 26, 32, 35, 43, 61, 62, 77, 94, 119, 129, 143, 150, 153, 155, 179, 180, 189, 196, 200, 203, 207). When detectable at healthy sites, the pathogens are usually present in very low numbers and show a limited range of phylotypes. It is quite clear that many individuals can harbor potential pathogens for long periods of time without developing periodontal disease. Carriage of putative periodontal pathogens at low levels as part of the commensal or normal oral microbiota is probably beneficial, since it is likely that their

Article 30

Research Reports: Clinical

Prediction of Periodontal Inflammation via Metabolic Profiling of Saliva

Journal of Dental Research
1–6

DOI: 10.1177/0022034516661142
jdr.sagepub.com

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Abstract

Periodontal disease is characterized by chronic inflammation in subgingival areas, where a vast array of inflammation-associated metabolites are likely produced from tissue breakdown, increased vascular permeability, and microbial metabolism and then eventually show a steady flow into saliva. Thus, prolonged periodontal inflammation is a key feature of disease activity. Although salivary metabolomics has drawn attention for its potential use in diagnosis of periodontal disease, few authors have used that to investigate periodontal inflammation detection. In this pilot study, the authors explored the use of salivary metabolites to reflect periodontal inflammation severity with a recently proposed parameter—periodontal inflamed surface area (PISA)—used to quantify the periodontal inflammatory burden of individual patients with high accuracy. Following PISA determination, whole saliva samples were collected from 19 subjects before and after removal of supragingival plaque and calculus (debridement) with an ultrasonic scaler to assess the influence of the procedure on salivary metabolic profiles. Metabolic profiling of saliva was performed with gas chromatography coupled to time-of-flight mass spectrometry, followed by multivariate regression analysis with orthogonal projections to latent structures (OPLS) to investigate the relationship between PISA and salivary metabolic profiles. Sixty-three metabolites were identified. OPLS analysis showed that postdebridement saliva provided a more refined model for prediction of PISA than did predebridement samples, which indicated that debridement may improve detection of metabolites eluted from subgingival areas in saliva, thus more accurately reflecting the pathophysiology of periodontitis. Based on the variable importance in the projection values obtained via OPLS, 8 metabolites were identified as potential indicators of periodontal inflammation, of which the combination of cadaverine, 5-oxoproline, and histidine yielded satisfactory accuracy (area under the curve = 0.881) for diagnosis of periodontitis. The authors' findings identified potential biomarkers that may be useful for reflecting the severity of periodontal inflammation as part of monitoring disease activity in periodontitis patients.

Keywords: periodontal disease, diagnosis, dental plaque, deriodontium, biomarkers, metabolomics

Introduction

Chronic marginal periodontitis is driven by sustained periodontal inflammation caused by microbial communities that form in subgingival areas, where intricate interactions of microbial communities with the host induce subversion of host homeostasis, leading to destructive inflammation (Lamont and Hajishengallis 2015). Thus, prolonged periodontal inflammation is one of the key features of disease activity, and many studies have reported attempts to evaluate its status. Recently, a novel method termed *periodontal inflamed surface area* (PISA) was proposed for determining periodontal inflammatory status by calculating the amount of inflamed periodontal tissue (Nesse et al. 2008). PISA allows for quantitative determination of the burden of periodontal inflammation as a continuous variable for evaluation of periodontal inflammation in individual patients, with high accuracy.

Presently, periodontitis is diagnosed with radiography findings as well as clinical measurements of probing pocket depth (PPD), bleeding on probing, and clinical attachment level (CAL). However, those have limitations for detection of present disease activity, as these clinical signs are often indicators of a previous disease activity (Ji and Choi 2015). Additionally, clinicians find it difficult to decide the optimum maintenance interval for their patients with periodontitis, as resources for

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A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

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Article 31

Research Reports: Clinical

Journal of Dental Research
2019, Vol. 98(6) 642–651

DOI: 10.1177/0022034519842853
journals.sagepub.com/home/jdr

The Saliva Metabolome in Association to Oral Health Status

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Abstract

Periodontitis is one of the most prevalent oral diseases worldwide and is caused by multifactorial interactions between host and oral bacteria. Altered cellular metabolism of host and microbes releases a number of intermediary end products known as metabolites. There is an increasing interest in identifying metabolites from oral fluids such as saliva to widen the understanding of the complex pathogenesis of periodontitis. It is believed that some metabolites might serve as indicators toward early detection and screening of periodontitis and perhaps even for monitoring its prognosis in the future. Because contemporary periodontal screening methods are deficient, there is an urgent need for novel approaches in periodontal screening procedures. To this end, we associated oral parameters (clinical attachment level, periodontal probing depth, supragingival plaque, supragingival calculus, number of missing teeth, and removable denture) with a large set of salivary metabolites ($n = 284$) obtained by mass spectrometry among a subsample ($n = 909$) of nondiabetic participants from the Study of Health in Pomerania (SHIP-Trend-0). Linear regression analyses were performed in age-stratified groups and adjusted for potential confounders. A multifaceted image of associated metabolites ($n = 107$) was revealed with considerable differences according to age groups. In the young (20 to 39 y) and middle-aged (40 to 59 y) groups, metabolites were predominantly associated with periodontal variables, whereas among the older subjects (≥60 y), tooth loss was strongly associated with metabolite levels. Metabolites associated with periodontal variables were clearly linked to tissue destruction, host defense mechanisms, and bacterial metabolism. Across all age groups, the bacterial metabolite phenylacetate was significantly associated with periodontal variables. Our results revealed alterations of the salivary metabolome in association with age and oral health status. Among our comprehensive panel of metabolites, periodontitis was significantly associated with the bacterial metabolite phenylacetate, a promising substance for further biomarker research.

Keywords: periodontitis, metabolomics, biomarkers, metabolism, inflammation, bacteria

Introduction

Periodontitis is an infectious inflammation of the periodontium mainly induced by pathogenic bacteria and individual host immune reaction (Lamont and Hajishengallis 2015). It is regarded as the second-most prevalent dental disease worldwide after dental decay and one of the most prevalent human diseases (Kassebaum et al. 2014). The early phase of disease (gingivitis) is characterized by gingival reddening, bleeding, and swelling, as well as increased production of gingival crevicular fluid and pocket formation (Lang et al. 2015). As the disease progresses, further periodontal tissue destruction and advanced attachment loss occur, leading to mobile teeth and finally tooth loss, if left untreated (Pihlstrom et al. 2005). Severe periodontitis directly affects quality of life in terms of reduced functional capacity, such as chewing, biting, or speaking, and reduced dental aesthetics. In addition, chronic periodontitis is associated with widespread systemic diseases, including cardiovascular problems (e.g., arteriosclerosis, coronary artery diseases, and stroke; Pietiainen et al. 2018).

Currently there are no valid screening tests that detect periodontitis among affected subjects and predict prospective periodontal tissue destruction. Usually, dentists identify periodontitis by visual inspection, periodontal probing, and inspection of dental radiographs. Unfortunately, a complete regular

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A supplemental appendix to this article is available online.

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Article 32

PLOS ONE

RESEARCH ARTICLE

Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using ^{1}H nuclear magnetic resonance (NMR) spectroscopy

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Citation: Rzeznik M, Triba MN, Levy P, Jungo S, Botosoa E, Duchemann B, et al. (2017) Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. PLoS ONE 12(8): e0182767. https://doi.org/10.1371/journal.pone.0182767

Editor: Stefano Tiziani, University of Texas at Austin, UNITED STATES

Received: December 20, 2016

Accepted: July 24, 2017

Published: August 24, 2017

Data Availability Statement: NMR data are available on metabolight platform as EMTBLS524.

Funding: The study has been partly supported by a grant from Institut Français de Recherche Odontologique (IFRO, 2013). There was no additional external funding received for this study.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Periodontitis is characterized by the loss of the supporting tissues of the teeth in an inflammatory-infectious context. The diagnosis relies on clinical and X-ray examination. Unfortunately, clinical signs of tissue destruction occur late in the disease progression. Therefore, it is mandatory to identify reliable biomarkers to facilitate a better and earlier management of this disease. To this end, saliva represents a promising fluid for identification of biomarkers as metabolomic fingerprints. The present study used high-resolution ^{1}H-nuclear magnetic resonance (NMR) spectroscopy coupled with multivariate statistical analysis to identify the metabolic signature of active periodontitis. The metabolome of stimulated saliva of 26 patients with generalized periodontitis (18 chronic and 8 aggressive) was compared to that of 25 healthy controls. Principal Components Analysis (PCA), performed with clinical variables, indicated that the patient population was homogeneous, demonstrating a strong correlation between the clinical and the radiological variables used to assess the loss of periodontal tissues and criteria of active disease. Orthogonal Projection to Latent Structure (OPLS) analysis showed that patients with periodontitis can be discriminated from controls on the basis of metabolite concentrations in saliva with satisfactory explained variance (R2X = 0.81 and R2Y = 0.61) and predictability (Q2Y = 0.49, CV-AUROC = 0.94). Interestingly, this discrimination was irrespective of the type of generalized periodontitis, *i.e.* chronic or aggressive. Among the main discriminating metabolites were short chain fatty acids as butyrate, observed in higher concentrations, and lactate, γ-amino-butyrate, methanol, and threonine observed in lower concentrations in periodontitis. The association of lactate, GABA,

Article 33

Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics.

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Journal of Periodontology, 21 Aug 2018, 89(12):1452-1460
DOI: 10.1002/jper.18-0097 PMID: 29877582

Abstract

Background

Recent findings about the differential gene expression signature of periodontal lesions have raised the hypothesis of distinctive biological phenotypes expressed by generalized chronic periodontitis (GCP) and generalized aggressive periodontitis (GAgP) patients. Therefore, this cross-sectional investigation was planned, primarily, to determine the ability of nuclear magnetic resonance (NMR) spectroscopic analysis of unstimulated whole saliva to discriminate GCP and GAgP disease-specific metabolomic fingerprint and, secondarily, to assess potential metabolites discriminating periodontitis patients from periodontally healthy individuals (HI).

Methods

NMR-metabolomics spectra were acquired from salivary samples of patients with a clinical diagnosis of GCP (n = 33) or GAgP (n = 28) and from HI (n = 39). The clustering of HI, GCP, and GAgP patients was achieved by using a combination of the Principal Component Analysis and Canonical Correlation Analysis on the NMR profiles.

Results

These analyses revealed a significant predictive accuracy discriminating HI from GCP, and discriminating HI from GAgP patients (both 81%). In contrast, the GAgP and GCP saliva samples seem to belong to the same metabolic space (60% predictive accuracy). Significantly lower levels ($P < 0.05$) of pyruvate, N-acetyl groups and lactate and higher levels ($P < 0.05$) of proline, phenylalanine, and tyrosine were found in GCP and GAgP patients compared with HI.

Conclusions

Within the limitations of this study, CGP and GAgP metabolomic profiles were not unequivocally discriminated through a NMR-based spectroscopic analysis of saliva.

Article 34

Metabolomics (2010) 6:78–95
DOI 10.1007/s11306-009-0178-y

ORIGINAL ARTICLE

Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles

Masahiro Sugimoto · David T. Wong · Akiyoshi Hirayama · Tomoyoshi Soga · Masaru Tomita

Received: 8 April 2009 / Accepted: 18 August 2009 / Published online: 10 September 2009

Abstract Saliva is a readily accessible and informative biofluid, making it ideal for the early detection of a wide range of diseases including cardiovascular, renal, and autoimmune diseases, viral and bacterial infections and, importantly, cancers. Saliva-based diagnostics, particularly those based on metabolomics technology, are emerging and offer a promising clinical strategy, characterizing the association between salivary analytes and a particular disease. Here, we conducted a comprehensive metabolite analysis of saliva samples obtained from 215 individuals (69 oral, 18 pancreatic and 30 breast cancer patients, 11 periodontal disease patients and 87 healthy controls) using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS). We identified 57 principal metabolites that can be used to accurately predict the probability of being affected by each individual disease. Although small but significant correlations were found between the known patient characteristics and the quantified metabolites, the profiles manifested relatively higher concentrations of most of the metabolites detected in all three cancers in comparison with those in people with periodontal disease and control subjects. This suggests that cancer-specific signatures are embedded in saliva metabolites. Multiple logistic regression models yielded high area under the receiver-operating characteristic curves (AUCs) to discriminate healthy controls from each disease. The AUCs were 0.865 for oral cancer, 0.973 for breast cancer, 0.993 for pancreatic cancer, and 0.969 for periodontal diseases. The accuracy of the models was also high, with cross-validation AUCs of 0.810, 0.881, 0.994, and 0.954, respectively. Quantitative information for these 57 metabolites and their combinations enable us to predict disease susceptibility. These metabolites are promising biomarkers for medical screening.

Keywords Salivary metabolome · Capillary electrophoresis-mass spectrometry · Oral cancer · Breast cancer · Pancreatic cancer

M. Sugimoto and D. T. Wong contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11306-009-0178-y) contains supplementary material, which is available to authorized users.

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1 Introduction

Saliva is an important biological fluid that provides various functions, including lubrication for speech, digestion of food, and protection from microorganisms. It is produced by multiple salivary glands; particularly the three major salivary glands parotid, submandibular and sublingual, and several minor glands. Saliva is comprised of 99% water with minerals, mucus, electrolytes, nucleic acids and proteins such as enzymes, enzyme inhibitors, growth factors, cytokines, immunoglobulins, and other glycoproteins (de Almeida Pdel et al. 2008). Saliva is a filtration of blood,

Article 35

Oral Oncology 46 (2010) 414–417

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Review

Oral cancer: Clinical features

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ARTICLE INFO

Article history:
Available online 18 April 2010

Keyword:
Oral cancer

SUMMARY

Oral squamous cell carcinoma (OSCC) is a well-known malignancy that accounts for more than 90% of all oral cancers. In this article we will perform a brief review of its clinical characteristics and the differential diagnosis. Regarding symptoms, pain is the most frequent presentation and the tongue and the floor of the mouth have the highest occurrence. OSCC in its initial stages shows an erytholeukoplastic area without symptoms but in advanced stages there are ulcers and lumps with irregular margins which are rigid to touch.

The different diagnosis should be established with other oral malignant diseases such as lymphomas, sarcomas and metastasis, which have rapid growth rates as opposed to the typical OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is a well-known malignancy which accounts for more than 90% of all oral cancers.[1]

The overall 5-year survival rate in OSCC has not significantly increased in the last few years. The overall and disease-free survival rates are 56% and 58%, respectively.[2] The most important task is to establish an early diagnosis at the first stages of the disease.[3]

Symptoms

Pain is a common symptom in oral cancer patients, representing 30–40% of their main complaints. There were 12 different descriptions of pain; pain was related to TNM staging in the tongue and the tongue/mouth floor.[4] Although pain is the main symptom, it usually arises only when the lesions have reached a remarkable size, and is the time when the patient requests medical assistance. Thus, early carcinomas often go unnoticed because they are asymptomatic.[5] In later and larger lesions, symptoms may vary from mild discomfort to severe pain, especially on the tongue. Other symptoms include ear pain, bleeding, mobility of teeth, problems in breathing, difficulty in speech, dysphagia and problems using prosthesis, trismus, and paraesthesia.[6]

In some locations, such as the tongue or the floor of the mouth, pain can arise early on. In the case of OSCC of the tongue, the tongue's movement against the teeth causing more discomfort. In contrast, carcinomas of the lip and buccal mucosa only show intense pain at advanced stages.[7]

Occasionally patients may present with cervical lymphadenopathy without any other symptoms. In terminal stages, patients may develop skin fistulas, bleeding, severe anaemia and cachexia.[8]

Jainkittivong et al.[9] found that swelling and/or pain were the first signs or symptoms in the 342 (52.6%) OSCC patients studied. Other authors reported that the main symptoms were ulceration and swelling[10], followed by pain, bleeding, decreased mobility of the tongue, dysphagia and paraesthesia. Gorsky et al.[11] reported a series of patients with OSCC of the tongue, finding that the main symptom was pain on the tongue (66.5%), while 29% had a lump on the tongue. Symptoms such as ear pain, voice changes, dysphagia, and cervical tumours were more common in tumours at the tongue base.

Location

OSCC may appear in any location, although there are certain areas in which it is more commonly found. The most common locations are the tongue and the floor of the mouth[12–16], mainly in Western countries; it occurs in over 50% of cases. Other areas of involvement are the buccal mucosa, retromolar area, gingiva, soft palate and, less frequently, the back of the tongue and hard palate. The lip is involved more frequently in some geographic areas.[6]

Hirata et al.[12] in their study of 478 carcinomas of the oral cavity performed between 1947 and 1970 found that, excluding the lip, 40% of tumours were located on the tongue and 33% on the floor of the mouth. Oliver et al.[13] in a review of 92 cases, found that the lateral and ventral surfaces of the tongue were the most frequent locations, followed by the floor of the mouth. The lateral

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doi:10.1016/j.oraloncology.2010.03.009

Article 36

Journal of
Clinical Medicine

MDPI

Review

Saliva and Oral Diseases

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Received: 29 December 2019; Accepted: 3 February 2020; Published: 8 February 2020

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Abstract: Saliva is a fascinating biological fluid which has all the features of a perfect diagnostic tool. In fact, its collection is rapid, simple, and noninvasive. Thanks to several transport mechanisms and its intimate contact with crevicular fluid, saliva contains hundreds of proteins deriving from plasma. Advances in analytical techniques have opened a new era—called "salivaomics"—that investigates the salivary proteome, transcriptome, microRNAs, metabolome, and microbiome. In recent years, researchers have tried to find salivary biomarkers for oral and systemic diseases with various protocols and technologies. The review aspires to provide an overall perspective of salivary biomarkers concerning oral diseases such as lichen planus, oral cancer, blistering diseases, and psoriasis. Saliva has proved to be a promising substrate for the early detection of oral diseases and the evaluation of therapeutic response. However, the wide variation in sampling, processing, and measuring of salivary elements still represents a limit for the application in clinical practice.

Keywords: biomarkers; saliva; oral cancer; oral lichen planus; psoriasis; oral diseases

1. Introduction

Saliva is a biological fluid secreted by major and minor salivary glands. The major salivary glands are the parotid, submandibular, and sublingual glands. Minor salivary glands are widely disseminated throughout the entire oral cavity. Saliva provides lubrication; facilitates mastication, digestion, and taste; it has antimicrobial properties; and serves as buffer for acidic food. Moreover, saliva inhibits the demineralization of teeth and protects from caries [1]. The physiological secretion generates 0.75–1.5 L per day, with a decrease during the night [2]. Saliva contains 99% water and proteins for the remaining 1% (mucins, enzymes, immunoglobulins), electrolytes, lipids, and inorganic substances [3].

There are many advantages to employing saliva as a substrate for diagnostic analysis. Its sampling is fast, inexpensive, non-invasive, and well tolerated by children and people with disabilities; moreover, it is a safe procedure for healthcare providers [4]. Many serum substances enter saliva through passive diffusion, active transport, or extracellular ultrafiltration [5]. Obviously, compared with blood, levels of several analytes are lower, which was an obstacle until a few years ago [6]. Nowadays, highly sensitive molecular methods are available and can be used in the detection of many elements in saliva, despite their dimensions and concentrations [7].

In recent decades, enormous progress has been made in early diagnosis and screening for many diseases, especially for neoplastic conditions. However, some of these methods are invasive or expensive, and for certain conditions, accurate tests are still not available. This is the case for oral cancer, the sixth most common cancer worldwide, frequently diagnosed at an advanced stage with a 5 year survival rate of 50% [8].

In accordance with Biomarkers Definitions Working Group 2011, a biomarker is a characteristic that can be objectively measured and evaluated as indicator of normal biological or pathogenic

www.nature.com/scientificreports

SCIENTIFIC REPORTS

OPEN

Identification of salivary metabolomic biomarkers for oral cancer screening

Received: 24 May 2016
Accepted: 21 July 2016
Published: 19 August 2016

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The objective of this study was to explore salivary metabolite biomarkers by profiling both saliva and tumor tissue samples for oral cancer screening. Paired tumor and control tissues were obtained from oral cancer patients and whole unstimulated saliva samples were collected from patients and healthy controls. The comprehensive metabolomic analysis for profiling hydrophilic metabolites was conducted using capillary electrophoresis time-of-flight mass spectrometry. In total, 85 and 45 metabolites showed significant differences between tumor and matched control samples, and between salivary samples from oral cancer and controls, respectively ($P < 0.05$ correlated by false discovery rate); 17 metabolites showed consistent differences in both saliva and tissue-based comparisons. Of these, a combination of only two biomarkers yielded a high area under receiver operating characteristic curves (0.827; 95% confidence interval, 0.726–0.928, $P < 0.0001$) for discriminating oral cancers from controls. Various validation tests confirmed its high generalization ability. The demonstrated approach, integrating both saliva and tumor tissue metabolomics, helps eliminate pseudo-molecules that are coincidentally different between oral cancers and controls. These combined salivary metabolites could be the basis of a clinically feasible method of non-invasive oral cancer screening.

Oral cancer (OC) is defined as a malignant tumor of the oral cavity, and is the sixth most common cancer worldwide, with an annual incident of 400,000 new cases accounting for 4% of cancers in men and 2% of cancers in women[1–3]. More than 7000 cases of oral cancer are diagnosed each year in Japan alone. This number has been steadily increasing during recent decades, and the rate of increase is higher in Japan than in the United States and other Western countries. Conventional visual and tactile examination (CVTE) is still the most common way to detect OC because oral cancer occurs in areas that can be adequately visualized. However, accurate diagnosis of subtle symptoms of early OC and inflammatory lesions is still difficult[4], leading to diagnosis of OC in advanced stages[5–7] with low prognosis, despite advances in treatment, which have resulted in an overall 5-year survival rate of approximately 50%[1,6–9].

Few clinically established biomarkers for detecting OC currently exist and open biopsy is presently the only assured criteria to confirm a diagnosis of cancer. Although open biopsy is effective to diagnose OC, this method provides definitive drawbacks, such as invasiveness. Thus, novel, adjunctive screening aids (devices or tests) are desperately needed. For example, commercially available handheld wide-field devices that emit light in variable wavelengths that can result in reflectance and/or autofluorescence of the oral mucosa have been heavily marketed to the dental communities as an inexpensive and rapid way of improving CVTE[10]. However, there are limited data supporting their ability to increase diagnostic accuracy or assist the decision-making process for clinically evident lesions[11,12], and therefore more reliable, objective, and biologically relevant methods are necessary.

Molecular biomarkers are ideal for objective screening and diagnosis, enabling the early detection of OC[13–15]. Compared with biomarkers in blood[16], salivary biomarkers have obvious advantages; sampling is non-invasive, convenient and safe, thus facilitating frequent screening for oral cancers. This fluid is also clinically important as it filters the blood, possibly reflecting systemic physiological conditions. However, conventional tumor markers,

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Article 38

Metabolomics (2019) 15:22
https://doi.org/10.1007/s11306-019-1483-8

REVIEW ARTICLE

Metabolomics study of oral cancers

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Received: 8 November 2018 / Accepted: 29 January 2019 / Published online: 8 February 2019

Abstract

Background Oral cancer is one of the most frequently occurring cancers. Metabolic reprogramming is an important hallmark of cancer. Metabolomics characterizes all the small molecules in a biological sample, and a complete set of small molecules in such sample is referred as metabolome. Nuclear magnetic resonance spectroscopy and mass spectrometry are two widely used techniques in metabolomics studies. Increasing evidence demonstrates that metabolomics techniques can be used to explore the metabolic signatures in oral cancer. Elucidation of metabolic alterations in oral cancer is also important for the understanding of its pathological mechanisms.

Aim of review In this paper, we summarize the latest progress of metabolomics study in oral cancer and provide the suggestions for the future studies.

Key scientific concepts of review The metabolomics studies in saliva, serum, and tumor tissues revealed the existence of metabolic signatures in bio-fluids and tissues of oral cancer, and several tumor-specific metabolites identified in individual study could discriminate oral cancer from healthy controls or precancerous lesions, which are potential biomarkers for the screening or early diagnosis of oral cancer. Metabolomics study of oral cancers in the future should aim to establish a routine procedure with high sensitivity, profile intracellular metabolites to find out the metabolic characteristics of tumor cells, and investigate the mechanism behind metabolomic alterations and the metabolic response of cancer cells to chemotherapy.

Keywords Oral cancer · Oral squamous cell carcinoma · Metabolomics · Metabolome · Metabolites

1 Introduction

Oral cancer, a type of head and neck cancer, is the sixth most common cancer in the world, and over 90% of them are oral squamous cell carcinoma (OSCC) (Rai et al. 2018). Several oral lesions, such as lichen planus, leukoplakia, erythroplakia, and oral sub-mucous fibrosis are considered as oral potentially malignant disorders (Chen and Zhao 2017). The pathological mechanisms of oral cancer are not well understood, and thus the early diagnosis is critical for the improvement of patient's survival rates. Metabolic reprogramming has been demonstrated to be an important hallmark of cancer (Yu et al. 2017). Cancer cells are more likely to use glycolysis, even when sufficient oxygen is available. This phenomenon is known as aerobic glycolysis or the Warburg effect (Warburg 1956; Yu et al. 2017), which promotes tumorigenesis and cancer progression. In addition to the Warburg effect, cancer undergoes complex metabolic changes, including metabolic reprogramming of lipids and amino acids, such as glutaminolysis (Yang et al. 2017; Nakagawa et al. 2018; Sun et al. 2018). Whether oral cancer has special metabolic characteristics has not been well studied. Elucidation of metabolic alterations in oral cancer is of great importance for identifying novel biomarkers and understanding the development and progression of oral cancer.

Metabolomics aims to characterize all the small molecules in a sample, and a complete set of small molecule chemicals found within a such biological sample is referred as metabolome (Nicholson and Lindon 2008). Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are two common analytical techniques used for metabolomics (Markley et al. 2017; Rai et al. 2018). Nuclear magnetic resonance detects hydrogen atoms in metabolites

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Journal of
PERIODONTAL RESEARCH

J Periodont Res 2015

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12327

Review Article

Salivary metabolomics in the diagnosis of oral cancer and periodontal diseases

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Mikkonen JJW, Singh SP, Herrala M, Lappalainen R, Myllymaa S, Kullaa AM. Salivary metabolomics in the diagnosis of oral cancer and periodontal diseases. J Periodont Res 2015; doi: 10.1111/jre.12327 © 2015 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Metabolomics is a systemic study of metabolites, which are small molecules generated by the process of metabolism. The metabolic profile of saliva can provide an early outlook of the changes associated with a wide range of diseases, including oral cancer and periodontal diseases. It is possible to measure levels of disease-specific metabolites using different methods as presented in this study. However, many challenges exist including incomplete understanding of the complicated metabolic pathways of different oral diseases. The review concludes with the discussion on future perspectives of salivary metabolomics from a clinician point of view. Salivary metabolomics may afford a new research avenue to identify local and systemic disorders but also to aid in the design and modification of therapies. A MEDLINE search using keywords "salivary metabolomics" returned 23 results in total, of which seven were omitted for being reviews or letters to the editor. The rest of the articles were used for preparation of the review, 13 of these were published in the last 5 years.

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Key words: omic study; oral cancer; periodontal disease; saliva

Accepted for publication August 28, 2015

Metabolomics is a systemic study of metabolites, which are small molecules generated by the process of metabolism. Salivary metabolites are important in elucidating the pathways underlying different diseases or making it ideal for the early detection of a wide range of diseases, including oral cancer and periodontal diseases.

Oral cancer is the 15th most common cancer and accounts for ~2.1% of total malignant tumors worldwide. Incidence rates are high among males in south central Asia and among females in eastern and central Europe (1). Disease-free survival rates associated with oral cancers have not changed much over decades and it is still ~50% (2). Tobacco (both smoking and chewing) and alcohol use are regarded as the major etiological factors (3). High mortality and morbidity rates associated with oral cancers are primarily attributed to the late detection. Absence of definitive biomarkers and lack of simple, portable and accurate diagnostic platforms suitable for the screening of early stage cancers are the biggest hurdles in bringing down the mortality rates (4).

Periodontal diseases are a group of inflammatory diseases considered as a major cause of tooth loss (5). They are characterized by loss of connective tissue around the teeth followed by formation of periodontal pockets (6). The disease is mainly caused by gram-negative bacteria that colonize deeper in gingival sulcus and later in periodontal pockets (5). If left untreated this might lead to progressive bone degradation and tooth loss. Periodontal diseases are also shown associated with several systemic conditions such as diabetes, cardiovascular diseases and infant prematurity (7–9). Even though there has been considerable improvement in dental care, still severe periodontitis is found in 5–20% of the adult population, worldwide (10).

The fact that the earlier a disease or its associated symptoms are identified, the more likely the treatment is successful, supports the need of novel diagnostic approaches. As cancer is known to affect metabolic pathways and intermediate products, an objective monitoring of these changes can provide vital clues for early diagnosis. There have been considerable efforts

ONCOLOGY REPORTS 37: 2727-2734, 2017

Metabolomic analysis of the saliva of Japanese patients with oral squamous cell carcinoma

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Received November 30, 2016; Accepted February 1, 2017

DOI: 10.3892/or.2017.5561

Abstract. The aim of the present study was to characterize the metabolic systems in Japanese patients with oral squamous cell carcinoma (OSCC) using capillary electrophoresis-mass spectrometry (CE-MS) metabolome analysis of saliva samples. A previous study showed variations among ethnicities and tumor sites in the saliva metabolome of patients with OSCC using CE-MS. In the present study, saliva was obtained from 22 Japanese patients with OSCC and from 21 healthy controls who visited the Department of Dentistry, Oral and Maxillofacial Surgery, Tokyo Dental Collage Ichikawa General Hospital, Tokyo, Japan, and all samples were subject to comprehensive quantitative metabolome analysis using CE-MS. A total of 499 metabolites were detected as CE-MS peaks in the saliva tested from the two groups. A total of 25 metabolites were revealed as potential markers to discriminate between patients with OSCC and healthy controls: Choline, *p*-hydroxyphenylacetic acid, and 2-hydroxy-4-methylvaleric acid ($P<0.001$); valine, 3-phenyllactic acid, leucine, hexanoic acid, octanoic acid, terephthalic acid, γ-butyrobetaine, and 3-(4-hydroxyphenyl)propionic acid ($P<0.01$); and isoleucine, tryptophan, 3-phenylpropionic acid, 2-hydroxyvaleric acid, butyric acid, cadaverine, 2-oxoisovaleric acid, *N6,N6,N6*-trimethyllysine, taurine, glycolic acid, 3-hydroxybutyric acid, heptanoic acid, alanine, and urea ($P<0.05$, according to the Wilcoxon rank sum test). A previous study by Sugimoto and co-workers detected 24 discriminatory metabolites, 7 of which (taurine, valine, leucine, isoleucine, choline, cadaverine, and tryptophan) were also detected in the present study. In the present study, however, choline, metabolites in the branched chain amino acids (BCAA) cycle, urea, and 3-hydroxybutyric acid were also characterized. Choline and metabolites of the BCAA cycle have previously been reported in OSCC using metabolome analysis. To the best of our knowledge, no previous reports have identified urea and 3-hydroxybuyric acid in the metabolome of patients with OSCC. These findings suggest the usefulness of metabolites as salivary biomarkers for Japanese patients with OSCC. Further studies using larger patient cohorts should be conducted to validate these results.

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Key words: biomarker, capillary electrophoresis-mass spectrometry, metabolome, Japanese, oral squamous cell carcinoma, saliva

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignancy that affects ~300,000 individuals per year worldwide (1). OSCC is often associated with loss of eating and speech functions, disfigurement, and psychological distress. The primary treatment for OSCC is surgical intervention. Despite considerable advances in the treatment of OSCC over the past two decades, the overall disease outcomes have improved only modestly (2). Local tumor recurrence affects ~60% of patients, and metastasis develops in ~15-25% of patients (3). The prevention and management of OSCC will greatly benefit from the identification of molecular markers and targets indicative of the disease (4,5).

Over the course of the last 20 years, saliva has been used to evaluate periodontal disease and the risk of dental caries. It has recently been reported that biomarkers for various diseases, including cancer, may be identified in the saliva, indicating the potential value of saliva as a test sample instead of blood. Recently, salivary diagnosis using various biochemical analytical techniques for the detection of breast and pancreatic cancers has been developed (6).

Using two-dimensional electrophoresis for whole saliva, which can be easily sampled in a non-invasive manner, Katakura *et al* (7) successfully identified an enolase 1 that is characteristically expressed in the whole saliva of patients with oral cancer. Therefore, the research program of the present authors has continued to focus on salivary metabolomics in our conducting a metabolome analysis and attempting a simultaneous exhaustive search for low-molecular-weight markers for the identification of a plethora of metabolites. Sugimoto *et al* (6) reported 24 candidate metabolites from saliva samples that were able to serve as biomarkers for cancer patients of various races, geographic regions, and tumor types. This previous study used capillary electrophoresis-mass spectrometry (CE-MS), which is a combined method that has been adapted for the high-resolution separation of ionic compounds, and may be used for metabolome analysis.

Article 41

Metabolites **2013**, *3*, 552-574; doi:10.3390/metabo3030552

OPEN ACCESS

metabolites

ISSN 2218-1989

www.mdpi.com/journal/metabolites/

Review

A Review of Applications of Metabolomics in Cancer

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Received: 24 April 2013; in revised form: 17 May 2013 / Accepted: 24 June 2013 / Published: 5 July 2013

Abstract: Cancer is a devastating disease that alters the metabolism of a cell and the surrounding milieu. Metabolomics is a growing and powerful technology capable of detecting hundreds to thousands of metabolites in tissues and biofluids. The recent advances in metabolomics technologies have enabled a deeper investigation into the metabolism of cancer and a better understanding of how cancer cells use glycolysis, known as the "Warburg effect," advantageously to produce the amino acids, nucleotides and lipids necessary for tumor proliferation and vascularization. Currently, metabolomics research is being used to discover diagnostic cancer biomarkers in the clinic, to better understand its complex heterogeneous nature, to discover pathways involved in cancer that could be used for new targets and to monitor metabolic biomarkers during therapeutic intervention. These metabolomics approaches may also provide clues to personalized cancer treatments by providing useful information to the clinician about the cancer patient's response to medical interventions.

Keywords: cancer; metabolomics; metabonomics; personalized medicine; biomarker

1. Introduction

Metabolomics is the latest of the omics technologies that employs state of the art analytical instrumentation in conjunction with pattern recognition techniques to monitor and discover metabolic changes in subjects related to disease status or in response to a medical or external intervention. Global metabolomics alterations reflect changes due to environmental factors, genetic variation and regulation, changes in gut microflora, and altered kinetic activity or levels of enzymes. Therefore, metabolomics alterations represent changes in the phenotype and molecular physiology [1–3]. Metabolomics, like the other omic technologies, is currently being used for the identification of

UICC global cancer control

IJC International Journal of Cancer

Salivary metabolite signatures of oral cancer and leukoplakia

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Oral cancer, one of the six most common human cancers with an overall 5-year survival rate of <50%, is often not diagnosed until it has reached an advanced stage. The aim of the current study is to explore salivary metabolomics as a disease diagnostic and stratification tool for oral cancer and leukoplakia and evaluate the potential of salivary metabolome for detection of oral squamous cell carcinoma (OSCC). Saliva metabolite profiling for a group of 37 OSCC patients, 32 oral leukoplakia (OLK) patients and 34 healthy subjects was performed using ultraperformance liquid chromatography coupled with quadrupole/time-of-flight mass spectrometry in conjunction with multivariate statistical analysis. The OSCC, OLK and healthy control groups demonstrate characteristic salivary metabolic signatures. A panel of five salivary metabolites including γ-aminobutyric acid, phenylalanine, valine, *n*-eicosanoic acid and lactic acid were selected using OPLS-DA model with S-plot. The predictive power of each of the five salivary metabolites was evaluated by receiver operating characteristic curves for OSCC. 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. The utility of salivary metabolome diagnostics for oral cancer is successfully demonstrated in this study and these results suggest that metabolomics approach complements the clinical detection of OSCC and stratifies the two types of lesions, leading to an improved disease diagnosis and prognosis.

About 1.5 million new cancer cases are expected to be diagnosed in 2009 in the United States and >0.5 million Americans are expected to die of cancer this year, averaging about 1,500 deaths per day.[1] These numbers have been steadily increasing over the past 15 years, despite significant progress in cancer treatment. Oral cancer represents one of the six most common human cancers with a high morbidity rate and an overall 5-year survival rate of <50%.[2,3] Reports indicate an increasing worldwide incidence of oral cancer at an earlier age.[4–6] Over 90% of oral cancer is oral squamous cell carcinoma (OSCC) which arises from the oral mucosal lining.[7] A critical factor in the lack of prognostic improvement is the fact that a significant proportion of cancers initially are asymptomatic lesions and are not diagnosed or treated until

Key words: metabolomics, saliva, oral squamous cell carcinoma, ultraperformance liquid chromatography quadrupole-time of flight mass spectrometry, multivariate statistical analysis, receiver operating characteristics

Abbreviations:: OSCC: oral squamous cell carcinoma; OLK: oral leukoplakia; UPLC-QTOFMS: ultraperformance liquid chromatography-quadrupole/time-of-flight mass spectrometry; PCA: principal component analysis; OPLS-DA: orthogonal partial least squares-discriminant analysis; ROC: receiver operating characteristic; LR: logistic regression; VIP: variable importance in the projection; FDR: false discovery rate
Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Basic Research Program of China; **Grant number:** 2007CB914700; **Grant sponsor:** National Science and Technology Major Project; **Grant number:** 2009ZX10005-020; **Grant sponsor:** National Science Foundation of China; **Grant number:** 20775048

DOI: 10.1002/ijc.25881

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History: Received 20 Jun 2010; Accepted 8 Dec 2010; Online 28 Dec 2010

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Int. J. Cancer: **129**, 2207–2217 (2011)

Article 43

Clinica Chimica Acta 427 (2014) 79–85

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Investigation and identification of potential biomarkers in human saliva for the early diagnosis of oral squamous cell carcinoma

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ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form 17 September 2013
Accepted 3 October 2013
Available online 19 October 2013

Keywords:
Early diagnosis
Saliva
HILIC–UPLC–MS
Biomarker
Oral cancer

ABSTRACT

Background: Oral cancer is 1 of the 6 most common human cancers, with an annual incidence of >300,000 cases worldwide. This study aimed to investigate potential biomarkers in human saliva to facilitate the early diagnosis of oral squamous cell carcinoma (OSCC).
Methods: Unstimulated whole saliva obtained from OSCC patients (n = 30) and apparently healthy individuals (n = 30) were assayed with ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) in hydrophilic interaction chromatography mode. The data were analyzed using a nonparametric Mann–Whitney *U* test, logistic regression, and the receiver operating characteristic (ROC) to evaluate the predictive power of each of 4 biomarkers, or combinations of biomarkers, for OSCC screening.
Results: Four potential salivary biomarkers demonstrated significant differences ($P < 0.05$) in concentrations between patients at stages I–II and the healthy individuals. The area under the curve (AUC) values in control vs OSCC I–II mode based on choline, betaine, pipecolinic acid, and L-carnitine were 0.926, 0.759, 0.994, and 0.708, respectively. Four salivary biomarkers in combination yielded satisfactory accuracy (0.997), sensitivity (100%), and specificity (96.7%) in distinguishing OSCC I–II from control.
Conclusions: Salivary metabolite biomarkers for the early diagnosis of OSCC were verified in this study. The proposed approach is expected to be applied as a potential technique of preclinical screening of OSCC.

1. Introduction

Human saliva is a multi-component oral fluid, which has high potential for the early diagnosis of diseases. In recent years, there have been many studies of disease diagnosis using salivary biomarkers in lung cancer [1], breast cancer [2], pancreatic cancer [3], oral cancer [4], Sjögren's syndrome [5], etc. Over 90% of oral cancers are oral squamous cell carcinoma (OSCC), which is one of the six most common human cancers, with an annual incidence of over 300,000 cases worldwide [6,7]. OSCC occurs in the lips, oral cavity, and pharynx, and has a relatively high rate of related morbidity. The World Health Organization has reported that OSCC has one of the highest mortality rates among other malignancies, with a 5-year mortality rate of approximately 50% [8]. Therefore, the early detection or prevention of this disease and the screening of high risk populations with precancerous lesions will be the most effective strategy.

The increasing worldwide incidence of OSCC urgently demands the discovery of new biomarkers. Saliva is a noninvasive and stress-free alternative to plasma and serum, and is widely accepted as a potential medium for clinical diagnostics. It also has the advantages of being simple to collect, easy to store, and less expensive compared with blood sample collection [9–11]. Saliva is secreted primarily by three major glands, i.e., the parotid gland, submandibular gland, and sublingual gland [12,13]. In general, the flow rate of unstimulated saliva is 0.3 ml/min. Saliva contains approximately 99% water as well as minerals, nucleic acids, electrolytes, mucus, and proteins [14]. It is one of the most complex, versatile, and important body fluids, which reflects a large range of physiological needs and information. Therefore, saliva is also known as the "mirror of the body".

At present, the standard method for OSCC diagnosis and screening is time-consuming and requires extensive experience. Therefore, modern high-throughput metabolomics approaches have been used extensively to observe the altered expressions of various metabolites in a range of cancers, including OSCC, with varying degrees of sensitivity and specificity. Metabolomics is a new platform for studying systems biology, which facilitates high throughput screening processes in the pharmaceutical industry and in clinical diagnosis [15,16]. The major analytical techniques used for metabolomics investigations are based on ^{1}H nuclear magnetic resonance (NMR) spectroscopy [17–19], LC–MS [20–22], and GC–MS [23]. Principal component analysis and orthogonal partial least squares discriminant analysis are used most frequently to screen for biomarkers of disease [24]. Sugimoto et al. used capillary electrophoresis mass spectrometry to discriminate individuals with oral cancer from healthy control and 28 salivary metabolite biomarker

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0009-8981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2013.10.004

Article 44

National Journal of Maxillofacial Surgery
Official Publication of Maxillofacial Society of India
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Natl J Maxillofac Surg. 2015 Jul-Dec; 6(2): 176–179.
doi: 10.4103/0975-5950.183869: 10.4103/0975-5950.183869
PMCID: PMC4922228
PMID: 27390492

Fucose: A biomarker in grading of oral cancer

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Abstract

Introduction:

Early diagnosis of cancer helps a great deal in the management of oral cancer patients. Number of proteinous markers have been employed for this purpose. Majority of them are not specific. Recently conjugated oligosaccharide with proteins and lipids have gained considerable importance in the present postgenomics and postproteomic period in the diagnostic and prognostics of cancer cases.

Materials and Methods:

In this study, serum fucose levels were estimated in 50 control cases and 75 cases of oral cancer by the method of Dische and Shettles as adopted by Winzler.

Results:

Serum fucose levels were found to be significantly higher in oral cancer cases (46.63 ± 5.29 mg/dl) as compared to the control cases (7.22 ± 0.26 mg/dl). The stepwise elevated serum fucose levels were found to be correlated with the histopathological grading of oral cancer.

Conclusions:

Estimation of such fucose conjugated proteins is suggestive to be good biomarkers in the diagnosis of oral cancer cases as well as in assessing the prognosis of such cases.

Keywords: Fucosylation, oral cancer patients, prognosis, serum 6-deoxy-L-galactose

Introduction

Sjögren's syndrome – an update for dental practitioners

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VERIFIABLE CPD PAPER

IN BRIEF

- Outlines the oral, ocular and systemic manifestations of Sjögren's syndrome.
- Highlights that patients with Sjögren's syndrome have an increased risk of developing non-Hodgkin's lymphoma.
- Describes the dental aspects of care for patients with Sjögren's syndrome.
- Stresses the crucial role of the dental practitioner in the diagnosis of patients with Sjögren's syndrome.

PRACTICE

Sjögren's syndrome (SS), an autoimmune, multi-factorial disorder, affects around 5% of females and 0.5% of males in the general population. The dental practitioner has a key role in recognising the clinical features of this condition, organising referral for specialist care and managing the oral health of these patients. In this article, we summarise the clinical manifestations, diagnosis and management of SS relevant to dental practitioners.

INTRODUCTION

Sjögren's Syndrome (SS) is an autoimmune disorder which affects around 0.5% of adults, with prevalence increasing with age and a female to male ratio of 9:1.[1] The cardinal symptoms of SS is a dry mouth (xerostomia), together with dryness of the eyes (keratoconjunctivitis sicca).[1] Primary Sjögren's syndrome (PSS) is a systemic autoimmune disorder characterised by inflammation of the exocrine glands, such as lacrimal and salivary glands without any associated connective tissue disease. PSS ultimately results in hypofunction and dryness of the mucosal surfaces, particularly of the eyes and mouth.[2]

Secondary Sjögren's syndrome (SSS) has similar pathophysiology, signs and symptoms as PSS, but is associated with some forms of connective tissue disease such as rheumatoid arthritis and systemic lupus erythematosus. Primary biliary cirrhosis and other autoimmune conditions can also be associated with SSS.

It is important to recognise that as SS is a systemic disease; patients can often suffer from disabling fatigue, skin lesions, haematological problems such as anaemia, vulval dryness and gastrointestinal complaints.[3] Sjögren's Syndrome was originally reported by Henrik Sjögren in 1930.[4]

ORAL MANIFESTATIONS

The predominant effects of SS on the oral cavity are mainly consequent to hyposalivation. Recent evidence indicates that over half of the patients with PSS experienced an oral symptom as their first manifestation of the condition.[2]

However, PSS can also indirectly cause oral manifestations secondary to systemic involvement. An appropriate example is that of SS-induced thrombocytopenia manifesting as oral bruising or purpura. Significant thrombocytopenia at levels below 50×10^9 may also influence any planned dental treatment.

PSS patients are 44 times more likely to develop B cell lymphomas of the salivary gland.[5] The presence of any firm or discrete swelling involving major salivary glands or presenting intraorally should raise suspicion and may warrant further investigation.

Lack of saliva causes difficulties in oral function. Patients may complain that their lips stick together and they have difficulties eating, speaking, chewing, swallowing and with denture retention.[6,7]

Saliva may be of a frothy consistency with an absence of saliva pooling in the floor of mouth. It may not be possible to express saliva from the parotid and submandibular ducts. The tongue may appear erythematous, dry and fissured. Alternatively the tongue may be coated and appear brown or black, a presentation appropriately termed black hairy tongue. The oral mucosa may appear dry and stick to the dental mirror or gloved finger, have a normal appearance or appear erythematous. Such dry erythematous mucosa is often uncomfortable to touch with patients complaining of soreness even in the absence of overt clinical infection.[8] Patients may also complain of dysguesia and halitosis. Oral candidosis occurs in individuals with SS more frequently than the general population. The most common clinical variant is that of chronic erythematous candidosis, although other types such as acute pseudomembranous candidosis may complicate SS.[9,10]

Patients with overt intraoral candidosis may be completely asymptomatic. They may simply seek medical attention after developing complications such as angular chelitis, which may cause soreness on mouth opening. Angular chelitis may be due to candidal or staphylococcal infection, however, it is important to exclude other predisposing factors such as anaemia.

Patients may present with new, recurrent and atypical patterns of dental

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Refereed Paper
Accepted 5 September 2012
DOI: 10.1038/sj.bdj.2012.890
©British Dental Journal 2012; 213: 353–357

Metabolomics : Open Access

Mikkonen et al., Metabolomics 2013, 3:3
http://dx.doi.org/10.4172/2153-0769.1000128

Research Article Open Access

Metabolic Profiling of Saliva in Patients with Primary Sjögren's syndrome

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Abstract

Objective: To investigate the feasibility of ^{1}H-NMR spectroscopy for metabolic profiling of human saliva samples and to determine whether the concentration of certain salivary metabolites, mainly representing small organic acids and amino acids, differ between patients with primary Sjögren´s syndrome (pSS) and healthy controls.

Methods: Stimulated whole-mouth saliva (SWMS) was collected from female pSS patients (n =15, all fulfilling the revised European Community proposed criteria). Salivary flow rate was immediately determined, samples were then frozen and subsequently analyzed by ^{1}H-NMR spectroscopy in comparison with samples collected from healthy individuals (n=15).

Results: From each sample, up to 24 metabolites could be identified and quantified. Choline and taurine concentrations were very significantly higher in the pSS patients compared to healthy controls ($p<0.001$), but their concentrations correlated negatively with salivary flow rate. Alanine and glycine concentrations were significantly higher ($p=0.004$, $p=0.007$, respectively), whereas butyrate ($p= 0.034$), phenylalanine ($p=0.026$) and proline ($p=0.032$) were only slightly higher in pSS saliva samples than in controls.

Conclusions: NMR spectroscopy has a potential for quantitative metabolic profiling of saliva samples. NMR spectroscopy is suitable for the analysis of NAAs in saliva and it can bypass the other methods, which are normally suitable for analysis of just one metabolite.

Keywords: Saliva; Metabolomics; NMR spectroscopy; Amino acid neurotransmitters; Biomarkers; Sjögren´s syndrome

Introduction

Sjögren´s syndrome (SS) is an autoimmune rheumatic disease which causes chronic inflammation in the exocrine glands. Salivary glands' hypofunction is a consequence of ductal and acinar cell destruction and causes lower salivary secretion [1]. Sjogren´s syndrome manifests in patients in two different forms: primary (pSS) and secondary (sSS). sSS usually is a consequence of some other rheumatic disease, for example systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). pSS is otherwise already a disease by itself [2]. SS occurs worldwide with similar prevalence (0.5-1.5%) and around 90% of the patients are female. Typical age of the patients is between 40 and 60 years, but the disease also exists in all age groups [1]. Patients who suffer from SS have often a serious malfunction of exocrine glands, sometimes it is called the ´sicca syndrome`. The main clinical features are xerostomia, ´dry mouth` and xerophthalmia, ´dry eyes` [3]. SS can affect also exocrine glands in esophagus, stomach, bowel, pancreas and bladder [4].

The pathogenesis of SS is complex and still partially unknown. Many factors, as genetic, hormonal, environmental, innate and adaptive immunity and the autonomic nervous system, have been thought to be involved in the pathogenesis of SS. The diagnostic criteria for SS has been presented by the American-European Consensus Group where the classification criteria includes the six main clinical findings; defining also separate diagnostic standards for pSS and sSS [5].

Recent advances in metabolic profiling techniques offer a powerful and promising approach to identify biomarkers associated with several disorders such as celiac disease [6,7], leukemia [8], breast cancer [9,10] and oral carcinomas [11]. A variety of different analytical techniques have been used in the metabolic profiling studies: currently the mass spectrometry (MS) has been utilized most frequently. Among MS techniques, especially two-dimensional gel electrophoresis (2D-GE) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) have mostly been applied to analyze SS saliva samples [12-16].

Instead, another powerful metabolic profiling technique, namely nuclear magnetic resonance (NMR) spectroscopy, has remained largely underexplored in saliva analysis. Although some salivary metabolites have been successfully identified and inter- and intra-subject variability has been investigated by using ^{1}H NMR [17-21] or ^{13}C NMR spectroscopy [22], well-designed studies aiming to biomarker identification associated to certain health disorder are very rare [23].

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Received October 23, 2013; **Accepted** November 26, 2013; **Published** November 29, 2013

Citation: Mikkonen JJW, Herrala M, Soininen P, Lappalainen R, et al. (2013) Metabolic Profiling of Saliva in Patients with Primary Sjögren's syndrome. Metabolomics 3: 128. doi:10.4172/2153-0769.1000128

Article 47

Journal of Oral Science, Vol. 63, No. 1, 22-26, 2021

Original article

Variability of salivary metabolite levels in patients with Sjögren's syndrome

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(Received December 4, 2019; Accepted April 21, 2020)

Abstract

Purpose: To investigate inter- and intra-individual variation in the levels and outputs (concentration multiplied by salivary flow rate) of salivary metabolites in patients with primary Sjögren's syndrome (pSS).
Methods: A total of 56 samples of stimulated saliva were collected from 14 female pSS patients during four laboratory visits within 20 weeks and analyzed using proton nuclear magnetic resonance spectroscopy. Single saliva samples from each of 15 controls were also analyzed.
Results: Among 21 quantified metabolites, choline was significantly elevated in the pSS patients at each time point ($P \leq 0.015$), taurine at the last three time points ($P \leq 0.013$), alanine at the last two time points ($P \leq 0.007$) and glycine at the last time point ($P = 0.005$). Inter-individual variation in metabolite concentrations was generally larger among the patients than among the controls, and significantly large variations were observed for glycine ($P \leq 0.007$, all time points), choline ($P \leq 0.033$, three last time points) and alanine ($P = 0.028$, baseline). Metabolite output analysis showed that choline had the lowest intra-patient variation.
Conclusion: In spite of considerable intra- and inter-individual variation, levels and outputs of specific metabolites in patients with pSS differ from those in controls, and may be potentially applicable as new biological markers for monitoring of the response to treatment.

Keywords; biological markers, hyposalivation, metabolomics, oral diagnosis, proton magnetic resonance spectroscopy

Introduction

Sjögren's syndrome (SS) is a systemic and slowly progressive autoimmune disease affecting mainly the salivary and lacrimal glands, although other exocrine glands may also be affected. There are two forms of SS: primary (pSS) and secondary (sSS). pSS is a discrete disease with all the typical symptoms, whereas sSS is associated with other forms of autoimmune disease that may constitute the primary diagnosis, for example systemic lupus erythematosus (SLE) [1]. There is evidence to suggest that the defective secretory processes that characterize SS are due to dysfunction of neural regulation [2]. Although the environmental factors responsible for SS development remain unknown, a recent study [3] has suggested that dysbiosis may play an important role. Typically, SS patients may suffer common symptoms of SS such as severe dry mouth and eyes for many years before being definitively diagnosed [4]. Primary SS may exhibit various clinical phenotypes with diverse outcomes. Patients with specific clinical symptoms such as purpura, peripheral nervous system involvement and salivary gland enlargement have an increased risk of lymphoma [5,6]. Therefore, it is important to develop new methods for earlier diagnosis of SS and to monitor patients' conditions, disease development and treatment responses. Currently there is no specific laboratory test for diagnosis of pSS.

Salivary metabolomics, the global analysis of low-molecular-weight metabolites, provides an alternative to the traditional single-biomarker approach for assessment of oral diseases. Metabolomics allows quantitative measurement of the oral defense system's multi-parametric metabolic responses to pathophysiological stimuli by revealing dynamic changes in salivary metabolites. In diagnostics based on salivary metabolites, a combination pattern of several biomarkers rather than only one may define a specific disease [7]. The specific metabolite profile mirrors the current state of any given individual's health, and can be useful for monitoring of patients with various diseases.

High-resolution nuclear magnetic resonance (NMR) spectroscopy is a powerful and reproducible metabolic profiling technique, and when combined with advanced multivariate analysis methodologies it has several advantages over classical biochemical assays [8]. Recently, techniques such as gas chromatography mass spectrometry (GC-MS), liquid-chromatography mass spectrometry (LC-MS) and two-dimensional gel electrophoresis have been frequently used to analyze saliva samples. However, the use of NMR spectroscopy in saliva research has been very limited [9,10]. Recently, Gardner et al. have suggested that a protocol 'gold standard' should be established for preparation of saliva samples for NMR analysis [11].

A number of previous studies have investigated the salivary metabolic profile of patients with pSS. So far, a total of 24 metabolites have been identified in samples of stimulated saliva [12], and some of them, including choline, butyrate, proline, taurine, alanine, phenylalanine and glycine, have been shown to have significantly higher concentrations in saliva from pSS patients than in that from controls. In particular, the concentrations of choline and taurine have been shown to be associated with changes in salivary flow rate [12].

The factors that influence the metabolic composition of saliva and contribute to variations in metabolic profiles include genetics, sex, age, diumal cycle, diet, hormone concentrations, drug effects, stress, oral health, oral microflora and oral hygiene [13]. Therefore, the metabolic profile of saliva shows considerable inter- and intra-individual variation and this can shed light on the physiological factors that might contribute to it.

The aim of the present study was to assess inter- and intra-individual variation of salivary metabolic profiles in patients with pSS in comparison with the salivary metabolome of control subjects using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The working hypothesis was that there would be differences in the inter- and intra-individual metabolomic profiles of pSS patients and that these differences would be detectable using quantitative ^{1}H-NMR spectroscopy.

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J-STAGE Advance Publication: October 29, 2020
Color figures can be viewed in the online issue at J-STAGE.
doi.org/10.2334/josnusd.19-0504
DN/JST.JSTAGE/josnusd/19-0504

Article 48

Reumatol Clin. 2015;11(6):387–394

Reumatología Clínica

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Review Article

Oral Involvement in Patients With Primary Sjögren's Syndrome. Multidisciplinary Care by Dentists and Rheumatologists☆

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ARTICLE INFO

Article history:
Received 22 December 2014
Accepted 27 March 2015
Available online 27 October 2015

Keywords:
Sjögren syndrome
Dry mouth
Xerostomia
Cavities
Saliva flow
Oral candida infection

ABSTRACT

Primary Sjögren's syndrome is a chronic systemic autoimmune disease that causes destruction of lacrimal and salivary glands. The most common and earliest symptoms are oral and ocular dryness. Dry mouth makes talking difficult, tasting and chewing properly, impairing quality of life of these patients. The most common oral signs and symptoms are hyposialia with or without xerostomia, tooth decay, fungal infections, traumatic oral lesions, dysphagia, dysgeusia, and inflammation of salivary glands. There are different therapeutic strategies, depending on the severity of each case, and the increase in the amount of saliva, to reduce the number of cavities and oral infections. It is particularly important to establish a close relationship between the dentist and the rheumatologist in order to make an early and correct diagnosis, promoting appropriate dietary and hygiene measures, as well as to treat and prevent potential oral complications.

Afectación oral en el paciente con síndrome de Sjögren primario. Manejo multidisciplinar entre odontólogos y reumatólogos

Palabras clave:
Síndrome de Sjögren
Sequedad oral
Xerostomía
Caries
Flujo salival
Candidiasis oral

RESUMEN

El síndrome de Sjögren primario (SSp) es una enfermedad autoinmune sistémica crónica, que cursa con destrucción del tejido glandular lagrimal y salival. Sus síntomas más frecuentes y tempranos son la sequedad oral y ocular. La sequedad oral dificulta que el paciente hable, deguste y mastique correctamente, lo que disminuye la calidad de vida del enfermo. Los signos y síntomas orales más frecuentes son la hiposialia con o sin xerostomía, la caries dental, las infecciones fúngicas, las lesiones orales traumáticas, la disfagia, la disgeusia y la inflamación de las glándulas salivales. Existen distintas estrategias terapéuticas en función de la gravedad de cada caso que aumentan la cantidad de saliva y disminuyen el número de caries e infecciones orales. Por ello, es de especial importancia establecer una relación cercana entre el dentista y el reumatólogo que permita hacer un diagnóstico temprano y correcto, fomentar las medidas dietéticas e higiénicas adecuadas, tratar y prevenir las posibles complicaciones orales.

Introduction

Primary Sjögren's syndrome (pSS) is a chronic, systemic, autoimmune, rheumatologic disease characterized by the presence of a lymphocytic inflammatory infiltrate in the salivary and lacrimal glands, which results in the destruction of the gland tissue. The most common and earliest symptoms of this disease are dry eyes and mouth, although extraglandular manifestations involving musculoskeletal, pulmonary, gastrointestinal, hematological, cutaneous, renal and neurological systems can also develop.[1,2]

Primary Sjögren's syndrome was described for the first time by the Swedish physician, Henrik Sjögren, who reported the cases of 19 women presenting with ocular dryness, the great majority of whom had rheumatoid arthritis.[3] There are 2 types of Sjögren's

☆ Please cite this article as: López-Pintor RM, Fernández Castro M, Hernández G. Afectación oral en el paciente con síndrome de Sjögren primario. Manejo multidisciplinar entre odontólogos y reumatólogos. Reumatol Clin. 2015;11:387–394.

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Article 49

Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12683

Metabolomics analysis of saliva from patients with primary Sjögren's syndrome

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Summary

The recent development of salivary proteomics has led to the identification of potential biomarkers for diagnosing patients with primary Sjögren's syndrome (pSS). Here we sought to identify differentially produced salivary metabolites from pSS patients and healthy controls (HCs) that might be used to characterize this disease. We obtained salivary samples from 12 female pSS patients (mean age 44.2 ± 13.01) and 21 age-matched female HCs. The metabolite profiles of saliva were analysed by gas chromatography-mass spectrometry. The total metabolite levels in each of the samples were calculated and compared across the study participants. A total of 88 metabolites were detected across the study samples, 41 of which were observed at reduced levels in the samples frompSS patients. Principal component analysis (PCA) revealed a loss in salivary metabolite diversity in the pSS patient samples compared to the HC samples. The reduced presence of glycine, tyrosine, uric acid and fucose, which may reflect salivary gland destruction due to chronic sialoadenitis, contributed to the loss of diversity. Comparative PCA of the pSS patients revealed the presence of two subpopulations based on their metabolite profiles, and these two subpopulations showed a significant difference in the prevalence of major salivary glanditis ($P = 0{\cdot}014$). In this study, we found that the salivary metabolite profile of pSS patients was less diverse than that of HCs and that the metabolite profiles in pSS patients were affected by the presence of major salivary glanditis.

Keywords: major salivary glanditis, metabolomics, saliva, Sjögren's syndrome

Introduction

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by salivary and lacrimal gland hypofunction. Mononuclear cell infiltration, followed by tissue destruction, results in the development of oral and ocular dryness. Changes in the quantity and quality of saliva are hallmarks of pSS and are presumed to reflect the disease pathogenesis in the salivary glands.

Whole saliva is a fluid produced by three pairs of major salivary glands, namely the parotid, submandibular and sublingual glands, and numerous minor salivary glands, all of which are located beneath the oral mucosa. Whole saliva is a complex fluid containing a variety of substances, including metabolites, proteins, mRNAs, DNAs, enzymes, hormones, antibodies, anti-microbial constituents and growth factors, and evidence suggests that changes in the levels of these substances are associated with the pathogenesis of various diseases [1]. The collection of saliva is a simple, non-invasive and low-cost procedure, and recent technical advances have shown that the analysis of saliva has the potential to monitor the status of various physiological systems [1].

Because the salivary glands are a major site of autoimmune destruction in pSS, changes in salivary components are assumed to reflect the pathogenesis of this disease. Several proteomic studies have shown differential protein expression in the saliva of pSS patients and healthy control subjects (HCs), which could lead to the development of diagnostic biomarkers [2–4]. In addition, transcriptome analysis of the saliva from pSS patients and HCs revealed differences in mRNA expression levels [3].

CA CANCER J CLIN 2018;

Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries

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DISCLOSURES: Lindsey A. Torre, Ahmedin Jemal, and Rebecca L. Siegel are employed by the American Cancer Society, which received a grant from Merck Inc for intramural research outside the submitted work; however, their salaries are solely funded through American Cancer Society funds. The remaining authors report no conflicts of interest.

doi: 10.3322/caac.21492. Available online at cacancerjournal.com

Abstract: This article provides a status report on the global burden of cancer worldwide using the GLOBOCAN 2018 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer, with a focus on geographic variability across 20 world regions. There will be an estimated 18.1 million new cancer cases (17.0 million excluding nonmelanoma skin cancer) and 9.6 million cancer deaths (9.5 million excluding nonmelanoma skin cancer) in 2018. In both sexes combined, lung cancer is the most commonly diagnosed cancer (11.6% of the total cases) and the leading cause of cancer death (18.4% of the total cancer deaths), closely followed by female breast cancer (11.6%), prostate cancer (7.1%), and colorectal cancer (6.1%) for incidence and colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) for mortality. Lung cancer is the most frequent cancer and the leading cause of cancer death among males, followed by prostate and colorectal cancer (for incidence) and liver and stomach cancer (for mortality). Among females, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death, followed by colorectal and lung cancer (for incidence), and vice versa (for mortality); cervical cancer ranks fourth for both incidence and mortality. The most frequently diagnosed cancer and the leading cause of cancer death, however, substantially vary across countries and within each county depending on the degree of economic development and associated social and life style factors. It is noteworthy that high-quality cancer registry data, the basis for planning and implementing evidence-based cancer control programs, are not available in most low- and middle income countries. The Global Initiative for Cancer Registry Development is an international partnership that supports better estimation, as well as the collection and use of local data, to prioritize and evaluate national cancer control efforts. **CA: Cancer J Clin. 2018;68:394-424. © 2018 American Cancer Society**

Keywords: cancer, epidemiology, incidence, survival

Introduction

Noncommunicable diseases (NCDs) are now responsible for the majority of global deaths,[1] and cancer is expected to rank as the leading cause of death and the single most important barrier to increasing life expectancy in every country of the world in the 21st century. According to estimates from the World Health Organization (WHO) in 2015, cancer is the first or second leading cause of death before age 70 years in 91 of 172 countries, and it ranks third or fourth in an additional 22 countries (Fig. 1).

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Clinica Chimica Acta 452 (2016) 18–26

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Diagnostic approach to breast cancer patients based on target metabolomics in saliva by liquid chromatography with tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 13 August 2015
Received in revised form 28 October 2015
Accepted 29 October 2015
Available online 30 October 2015

Keywords:
Polyamine
Saliva
Breast cancer patient
Discrimination equation
UPLC-ESI-MS/MS

ABSTRACT

Background: Breast cancer is one of the most fearful diseases due to its increasing worldwide prevalence. A number of screening tests has been employed including clinical examinations and mammography. However, another screening method, which is a simple, not embarrassing, and low cost, is highly desired. Based on these findings, we are currently investigating the determination of polyamines including their acetylated structures for the diagnosis of breast cancer patients. We established a diagnostic approach to breast cancer patients based on the ratios of polyamines in saliva by a UPLC-MS/MS analysis.
Methods: Twelve polyamines including their acetylated form were labeled with DBD-F, separated by a reversed-phase chromatography and detected by a Xevo TQ-S tandem mass spectrometer.
Results: Eight polyamines (e.g., SPM, CAD, Ac-SPM, N1-Ac-SPD, N8-Ac-SPD) strongly correlated with the cancer patients. A simple 1-order equation was developed for the discrimination of the breast cancer patients and healthy persons ($Y = 0.5X_{SPM} - 3X_{Ac\text{-}SPM} - 0.15X_{SPD} - 3.5X_{N8\text{-}Ac\text{-}SPD} + 0.5X_{N1\text{-}Ac\text{-}SPD} + 0.04X_{CAD}$). The concordance rate of the breast cancer patients and the healthy persons by the equation was 88% and 76% on the training set, respectively, whereas those on the validation set was both 88%. The score Y in the equation tended to correlate with the cancer stage of the patients and increased with the more serious conditions. The determination of polyamines in the saliva after the cancer patient operations was also performed to identify the concentration change before and after the surgical treatment. The discriminant analysis using 6 polyamines (i.e., N8-Ac-SPD, N1-Ac-SPD, CAD, DAc-SPD, PUT, and Ac-PUT), which were the most influenced molecules derived from the ROC analysis, was performed using the relative percentage. Both the sensitivity and specificity indicated nearly 80% from the ROC analysis result using the ratio of N8-Ac-SPD/(N1-Ac-SPD + N8-Ac-SPD).
Conclusion: The discrimination equation appears to be useful for the diagnosis of breast cancer patients. Furthermore, the ratio of N8-Ac-SPD/(N1-Ac-SPD + N8-Ac-SPD) may be adopted as an index of the health status after the surgical treatment.

1. Introduction

Breast cancer is one of the most fearful diseases due to its increasing worldwide prevalence, not only in European-Americans, but also in Asian countries [1]. Its prevalence is due to not only intrinsic genetic specificity, but also modern lifestyles. Most types of breast cancers are easy to diagnose by microscopic analysis of the biopsy. While screening techniques are useful in determining the possibility of cancer, further testing is necessary to confirm the disease state. A number of screening tests have been employed including clinical examinations and mammography. However, women struggle against these tests because of the embarrassment, waste of time, and high expense. Consequently, another screening method, which is a simple, not embarrassing, and low cost, is highly desired. There are many different tumor markers, which are indicative of a particular disease process, and they are used in oncology to help detect the presence of cancer. Several tumor markers, such as CEA and CA15-3, are known to increase in breast cancer patients [2]. However, the elevated level of the tumor markers can also be due to other causes. These markers are high-molecular mass substances such as glycoproteins and enzymes.

As relatively small molecules, polyamines have been significantly associated with rapid tumor growth due to their biosynthesis and accumulation [3]. Therefore, the concentrations of the polyamines,

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http://dx.doi.org/10.1016/j.cca.2015.10.032
0009-8981/

Article 52

Untargeted saliva metabonomics study of breast cancer based on ultra performance liquid chromatography coupled to mass spectrometry with HILIC and RPLC separations

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Abstract

Breast cancer (BC) is not only the most frequently diagnosed cancer, but also the leading cause of cancer death among women worldwide. This study aimed to screen the potential salivary biomarkers for breast cancer diagnosis, staging, and biomarker discovery. For the first time, a UPLC-MS based method along with multivariate data analysis, was proposed for the global saliva metabonomics analysis and diagnosis of BC, which used both hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RPLC) separations and operated in both positive (ESI+) and negative (ESI-) ionization modes. On account of different polarities of endogenous metabolites, this method was established to overcome the boundedness of a single chromatographic approach. As a result, 18 potential metabolites for diagnosing BC were identified. A nonparametric Mann-Whitney U test, heat map, and the receiver operating characteristic (ROC) were exploited to analyze the data with the purpose of evaluating the predictive power of the 18 biomarkers. Significant differences ($P<0.05$) were disclosed in terms of the levels of the 18 potential biomarkers between BC patients and healthy controls (HC). Among the 18 biomarkers, three up-regulated metabolites, LysoPC (18:1), LysoPC (22:6) and MG (0:0/14:0/0:0) displayed the area under the curve (AUC) values of 0.920, 0.920 and 0.929, respectively, indicating the high accuracy of this method to predict BC. In this study, an integrated metabonomics analysis in human saliva for identifying potential biomarkers to diagnose and stage BC was successfully eastablished, which was non-invasive, reliable, low-cost, and simple. The HILIC was demonstrated to be essential for a comprehensive saliva metabonomics profiling as well as RPLC separation. This saliva metabonomics technique may provide new insight into the discovery and identification of diagnostic biomarkers for BC.

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JORMAS-695; No. of Pages 5
ARTICLE IN PRESS

J Stomatol Oral Maxillofac Surg xxx (2018) xxx–xxx

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Review

Interest of studying the saliva metabolome, transcriptome and microbiome in screening for pancreatic cancer

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ARTICLE INFO

Article history:
Received 22 January 2018
Accepted 22 April 2019

Keywords:
Biomarkers
Saliva
Screening
Pancreatic neoplasm

ABSTRACT

Introduction: Pancreatic cancer is a public health problem because its mortality rate is close to its incidence rate. If it were possible to detect this cancer before the onset of symptoms, 5-year survival could reach 75%. Numerous studies have attempted to accelerate the diagnosis to improve survival. Saliva presents interesting characteristics as a fluid for screening and diagnosis. Its many components provide a promising source of constitutive biomarkers with a specific signature of the disease. The aim of this work was to determine the interest of studying the metabolome, the transcriptome and the microbiome of saliva in screening for pancreatic cancer.
Materials and methods: A review of the literature was conducted using the PubMed search engine. The last search was conducted in July 2017.
Results: Nine references, all original studies, published between 2010 and 2017 were included.
Discussion: Different combinations of metabolites, RNA and bacteria were found. Analysis of the saliva transcriptome and metabolome seems to be the most promising avenue.
Conclusion: The identification of an early salivary signature of pancreatic cancer is still in its infancy and the results obtained here must be confirmed in larger prospective multicentre studies.

1. Introduction

Worldwide, about 200,000 people every year develop pancreatic cancer and 98% of these people die from this disease [1]. Median survival is 6 months [2]. When the diagnosis is made at an early stage, 5-year survival is greater than 20% and drops to 5% when the diagnosis is made at an advanced stage [3,4]. Pancreatic cancer is thus a public health problem because of its mortality rate which is close to its incidence rate [5]. In 2030, pancreatic cancer could become the second leading cause of death from cancer in the United States [6].

The symptoms that reveal this cancer are non-specific: epigastric abdominal pain and weight loss [6]. The clinical manifestations often occur at an advanced loco-regional or even metastatic stage [1,3,4]. At the loco-regional stage, only 15 to 20% of cancers are resecable at the time of the diagnosis [5,7,8]. Frequently, the treatment can thus be regarded as palliative radiochemotherapy [3,8].

If it were possible to detect the disease before the onset of symptoms, 5-year survival could reach 75% [3]. The disease could take 5 years to progress from the first tumour cells to the metastatic stage [3]. This window of opportunity could be propitious to early detection and perhaps curative treatment of the disease [3]. Several methods exist to detect pancreatic cancer early, but they are generally invasive and given the small number of patients concerned, they have neither the sensitivity nor specificity necessary to set up more generalised screening [3,6]. Currently, only the serum tumour antigen 19-9 [CA19-9] has shown any value, but only for the follow-up of patients undergoing treatment [3]. The discovery of non-invasive biomarkers would thus bring hope [3].

Saliva, a biological fluid that contributes to the lubrication of the oral cavity for speech and digestion, protects against micro-organisms and plays a role in social life, could be used as a non-invasive means of

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https://doi.org/10.1016/j.jormas.2019.04.013
2468-7855/

Please cite this article in press as: Sturque J, et al. Interest of studying the saliva metabolome, transcriptome and microbiome in screening for pancreatic cancer. J Stomatol Oral Maxillofac Surg (2019), https://doi.org/10.1016/j.jormas.2019.04.013

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cancers

MDPI

Article

Elevated Polyamines in Saliva of Pancreatic Cancer

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Received: 10 January 2018; Accepted: 2 February 2018; Published: 5 February 2018

Abstract: Detection of pancreatic cancer (PC) at a resectable stage is still difficult because of the lack of accurate detection tests. The development of accurate biomarkers in low or non-invasive biofluids is essential to enable frequent tests, which would help increase the opportunity of PC detection in early stages. Polyamines have been reported as possible biomarkers in urine and saliva samples in various cancers. Here, we analyzed salivary metabolites, including polyamines, using capillary electrophoresis-mass spectrometry. Salivary samples were collected from patients with PC (n = 39), those with chronic pancreatitis (CP, n = 14), and controls (C, n = 26). Polyamines, such as spermine, N_1-acetylspermidine, and N_1-acetylspermine, showed a significant difference between patients with PC and those with C, and the combination of four metabolites including N_1-acetylspermidine showed high accuracy in discriminating PC from the other two groups. These data show the potential of saliva as a source for tests screening for PC.

Keywords: pancreatic cancer; saliva; metabolomics; polyamines

1. Introduction

Pancreatic cancer (PC) remains one of the cancers with the worst prognoses, and its five-year survival rate is still under 5% [1]. The high mortality rate of PC is caused by the lack of early specific symptoms [2]. The delay in diagnosis may also increase this rate [2]. Recent diagnostic imaging technologies, such as positron emission tomography (PET)-CT, magnetic resonance imaging (MRI), computed tomography (CT), and endoscopic ultrasonography (EUS), have helped to improve the diagnosis of PC. However, 30% of patients present with a locally advanced tumor, 50% present with