Biochemical analysis of oral fluids for disease detection

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Abstract

The field of diagnostics using invasive blood testing represents the majority of diagnostic tests used as part of routine health monitoring. The relatively recent introduction of salivary diagnostics has lead to a major paradigm shift in diagnostic analyses. Additionally, in this era of big data, oral fluid testing has shown promising outcomes in a number of fields, particularly the areas of genomics, microbiomics, proteomics, metabolomics, and transcriptomics. Despite the analytical challenges involved in the interpretation of large datasets generated from biochemical studies involving bodily fluids, including saliva, many studies have identified novel oral biomarkers for diagnosing oral and systemic diseases. In this regard, oral biofluids, including saliva, gingival crevicular fluid (GCF), peri-implant crevicular fluid (PICF), dentinal tubular fluid (DTF), are now attracting increasing attention due to their important attributes, such as noninvasive sampling, easy handling, low cost, and more accurate diagnosis of oral diseases.

Recently, the utilization of salivary diagnostics to evaluate systemic diseases and monitor general health has increased in popularity among clinicians. Saliva contains a wide range of protein, DNA and RNA biomarkers, which assist in the diagnosis of multiple diseases and conditions, including cancer, cardiovascular diseases (CVD), auto-immune and degenerative diseases, respiratory infections, oral diseases, and microbial (viral, bacterial and fungal) diseases. Moreover, due to its noninvasive nature and ease-of-adoption by children, it is now being used in mass screening programs, oral health-related studies and clinical trials in support of the development of therapeutic agents. The recent advent of highly sensitive technologies, such as next-generation sequencing, mass spectrometry, highly sensitives ELISAs, and homogeneous immunoassays, suggests that even small quantities of salivary biomarkers are able to be assayed accurately, providing opportunities for the development of many future diagnostic applications (including emerging technologies, such as point-of-care and rapid molecular technologies). The present article explores the omics and biochemical compositions of various oral biofluids with important value in diagnostics and monitoring.

1. Introduction

The first common-place application of saliva as a biofluid dates back to ancient Chinese times. Many centuries ago, Chinese culture used the ability of an individual under extreme duress to produce what we now know to be the "*golden*" biofluid, saliva. In those early times, individuals suspected of a crime had rice placed into the mouth for a specified period while being questioned about the alleged crime they had committed. If the rice was

ejected from the mouth and was dry after the allotted time, the subject was believed to be guilty. Wet rice, on the other hand, indicated that the subject was not guilty of the alleged crime. The premise was that guilty people become stressed, causing lower salivary flow and potentially dry mouth. However, innocent people have no reason to become stressed, and therefore they have a normal salivary flow, and consequently, expel wet rice [1].

Modern day salivary diagnostics have achieved numerous advances since those early times and now include a wide array of diagnostic and prognostic tests based on oral specimens, including direct to consumer options, ancestry testing, assays for drugs of abuse, risk assessment tools and many others [2]. Despite the rapid emergence of saliva testing in recent years, the "modern day" history of saliva spans a relatively short time period of only 30 years to the late 1980s and early 1990s. This period was when the US FDA approvals were first issued and when the first significant clinical validations of saliva tests occurred [3]. In the present paper, we would like to highlight the strides in saliva tests in the last three decades, describe some of the key areas where saliva has impacted diagnostics, and provide published market resources that indicate an "exploding" market for salivary diagnostics.

1.1 Recent milestones for saliva

The in vitro diagnostics market is likely to expand from $64.02 billion USD (2017) to $87.93 billion by the year 2023, with a compound annual growth rate [CAGR] of 5.2% per annum, as reported by marketsandmarkets.com. These large numbers comprise multiple market sectors, including three major areas: laboratory-based testing, point-of-care and molecular testing. Despite the growth in the salivary diagnostics market, particularly over the last 5 to 7 years, with major inroads by companies such as 23andMe [who reportedly have processed 10 million saliva specimens in recent years for risk assessment testing] and ancestry.com [who have reported the processing of 12 million specimens for ancestry testing], we believe that salivary diagnostics still represents 1% or less of the total in vitro diagnostic [IVD] market, as the majority of tests are performed on blood, serum, tissue and urine specimens. The growth in salivary diagnostics is documented to significantly outpace the growth of testing of other bodily fluids and specimens in the future, mainly due to the positive attributes of saliva [noninvasiveness, patient-friendliness, simplicity, nonbiohazardous nature and ease of shipment] [4]. These features, coupled with the emergence of multiple

technologies with increased sensitivity to detect inherently lower levels of biomarkers in saliva relative to alternate biofluids, has led to an explosive growth in the development of saliva tools and diagnostics. Improved technologies include next-generation sequencing, mass spectrometry, microarrays and biosensor-based technologies, among others [5]. Modern history is charted by a number of key milestones that are summarized in Table 1 below, beginning with the first FDA 510(k) approval for a saliva collection device [OraSure by Epitope] in 1992.

Table 1 Chronological listing of recent saliva-based collection/testing milestones.

Year	Milestone	Company
1992	First FDA [510(k)] Approval for saliva collection OraSure® Device	Epitope, Inc.
1994	First FDA Approval for an oral fluid HIV test OraSure Device and Organon Teknika ELISA	Epitope, Inc.
1995	Second FDA [510(k)] Approval for saliva collection Saliva-Sampler™	Saliva Diagnostic Systems
2001	First FDA Approval for Oral Fluid Drug Testing System Intercept	OraSure Technologies
2003	First FDA Approval for saliva hormone assay	Salimetrics
2008	Adoption of saliva by consumer genetics company 23 and Me, OraGene Saliva DNA Device	DNA Genotek
2010	First Rapid Oral HIV Test to receive FDA Approval OraQuick Advance HIV 1/2	OraSure Technologies
2011	FDA [510(k)] Approval for the first saliva genomic assay [OraGene plus the eSensor Warfarin Sensitivity Test, Genmark Diagnostics]	DNA Genotek
2012	First Rapid Oral HIV Test to receive OTC Approval for home use [OraQuick In Home HIV Test]	OraSure Technologies
2012	Second Oral Fluid HIV Test to receive FDA Approval [DPP HIV 1/2 Test]	Chembio Diagnostic Systems
2015	Adoption of saliva by ancestry.com	ancestry.com
2015	First FDA Approval for a direct-to-consumer test by 23 and Me [OraGene Device]	DNA Genotek
2017	FDA Approval for the multiple risk assessment tests by 23 and Me [Oragene Device]	DNA Genotek

1.2 Saliva—A high growth area

1.2.1 Current opportunities

Benchmarks for any industry are usually reported in the form of marketing surveillance reports written by companies that specialize in marketing intelligence and market research. For instance, as reported earlier the global in vitro diagnostic [IVD] market was estimated to be worth $64.02 billion in 2017 by one of these companies. A number of similar companies provide additional reports, and each competes for customers in various industries and charge a significant fee to provide access to statistical reports and data, which are typically compiled through direct interviews with key players in the chosen industry.

A search of the literature reveals that up until very recently, no reports focused on the market for salivary diagnostics. However, a key indication of the rapid emergence of saliva tests is the publication of multiple reports outlining global market statistics focusing specifically on saliva testing or market sectors where saliva testing plays a key role. The interesting feature is that these reports cover a range of different market sectors where saliva testing features prominently. Examples include the consumer genetics market, oral fluid drug testing, point-of-care testing, use of cell-free DNA, hormone testing and rapid HIV screening. Some of these sectors are described in detail below.

The first major report focusing specifically on saliva collection tools and diagnostic platforms was published by Market Research Future in 2016 and estimated that the size of the market for these tools was $1.35 billion in 2015 and that the market would grow to $2.63 billion by the year 2022, representing approximately 100% growth in 7 years. This trend may be realistic, as advances in saliva-based diagnostics and research are achieved daily.

A very interesting report emerged in 2017 from Market Research Future [6], highlighting a large and rapidly expanding market for nutrigenomics. Nutrigenomics is a study of the interaction between genetics and nutrition that affects human health, and aims to devise a personalized diet to improve or maintain good health; this understanding of personalized diet and improving health, leads to personalized medicine [7]. Since nutrigenomics studies involve patient-centric genetics, saliva is the preferred specimen, and the report forecasts that $10.3 billion (approximately 60%) of the total nutrigenomics market worth $17.3 billion by the year 2023 will involve salivary testing [6]. The discussion section of the report states that "saliva testing is an easy and noninvasive approach of testing and thus accounts for a major share of the market" [6].

The fertility market is one area where saliva has been part of routine laboratory practice for quite a number of years. Since noncomplexed hormones may be assayed readily in oral fluid samples, saliva has become a popular choice for testing the levels of cortisol, testosterone, DHEA, progesterone and many other hormones using standard laboratory ELISAs [8]. Saliva is also used in ovulation prediction kits and fertility monitors, and thus as the fertility market grows, the adaptation of saliva tests will also increase [9]. A key report in this area by MarketsandMarkets.com, highlights a 7.2% compound annual growth rate for this market from $411.8 million in 2018 to $583.1 million in 2023 [10].

Tests for drugs of abuse in criminal justice, forensics, workplaces and roadside venues have been a strong area for saliva testing and are forecast to continue to display solid growth as more sensitive devices are developed to meet the stringent standards set by the Substance Abuse Mental Health Services Administration [11]. This agency is the division of the federal government responsible for the safety of federal employees and sets the standards for new drug testing devices entering the market. A report by Energias Market Research predicts an annual growth rate of 8.8% per annum with a market value of $4.924 billion in 2016 increasing to $8.90 billion by the year 2023 [12]. Point-of-care [POC] testing is generally an area that is growing significantly faster than the laboratory testing market as the need for faster turn-around of results and diagnostic solutions closer to the patient increases [5]. Driven by cost factors and the need for less invasive tests, the saliva proportion of the POC market is attracting a growing number of companies who are developing assays using wearables, lateral flow devices, biosensors and cell-phone based technologies to name a few. Research and Markets published a report in 2017 estimating a growth of over $14 billion from 2017 ($23.71 billion) to 2022 ($38.13 billion) at a CAGR of 10% per annum [13]. Two of the key growth sectors for saliva tests within the POC area are the HIV and drugs of abuse testing markets.

Probably the most revealing saliva statistics are gleaned from the reports produced by the two major consumer genetics companies, who may be the largest analyzers of saliva in the world [14]. For example, the ancestry.com business is regularly reported in the popular press and in public statements by the company. As of the summer of 2018, ancestry.com made claims of having 12 million customers, and rival 23andMe claimed to have reported results on 10 million saliva specimens. Many other companies have entered this market using saliva as the biospecimen of choice. These companies include My Heritage, National Geographic, Paternity Depot, Color

Genomics, Invitae, Helix and many others. Exponential growth in this market has been observed, since as recently as 2013, the total number of consumer genetic tests performed by all companies was significantly less than half a million. Independent sources verify that consumer genetics is generally one of the largest markets in the testing world. For instance, a marketing report by Global Industry Analysts, Inc. [15] suggests that the market will be $310 million by 2022, while a competitor to Global Industry Analysts, Kalorama Information, agreed with these statistics [15,16]. A number of other marketing reports are available, and the list provided here is not meant to be exhaustive. Instead those reports listed are meant to provide specific references for the evaluation and quantitation of the current or future market sizes of salivary diagnostics alongside other available tools. It also highlights a stronger commitment by academicians to experiment with biomarker development platforms that can be used with multiple specimen types, including saliva.

1.2.2 Future opportunities for saliva testing

In addition to existing markets, a number of exciting new areas have embraced saliva as a useful specimen. For instance, the discovery that most of microRNAs (miRNAs) detectable in serum and saliva are concentrated in exosomes [17] has led to some interesting studies on the development of methods for the isolation and quantification of exosomes [18]. While the exploration of exosomes is in its infancy, market reports have already emerged suggesting explosive growth in this area for both therapeutics and diagnostics. An example is the report from BCC Research predicting rapid growth in the total market for exosomes from $25 million in 2018 to $180 million in 2023, representing a staggering CAGR of 48% per year. Although the proportion that will be attributed to saliva still remains undetermined, anecdotal data suggests that saliva has a key future role in the diagnosis of diseases using exosomes.

One area where saliva is just beginning to penetrate the market in a significant manner is the bio-banking area [19]. Biobanks are repositories of various bodily fluids, tissues and other specimens for drug clinical trials, advances in regenerative medicines, future trials for diagnostics, epidemiological studies and other applications. Samples stored in a bio-banking environment may be incubated at frozen temperatures for the purpose of future genomic, transcriptomic, metabolomic, proteomic and potentially other applications [20–22]. A single specimen type may not be appropriate or ideal for all applications, and thus multiple samples may be required. The choice of specimen and methods used to collect and store the sample are very

important. The versatility, simple collection and noninvasive nature of saliva makes the collection using oral samples a strong choice for certain bio-banking applications. Many countries are now establishing a system of networked biobanks for current and future applications. Thus, the significant growth in this area is not surprising. The 10 largest biobanks worldwide include the Shanghai Zhangjiang Biobank, which has a storage capacity of 10 million human-derived samples, the US "All of Us" Biobank with a target size of 1 million specimens, and the International Agency for Research on Cancer (IARC)/WHO Biobank (IBB) with a total of 5.1 million archived specimens [23]. Five other established biobanks in the UK, Finland, China, Canada, Qatar and Estonia contribute another approximately 2 million specimens [23]. Currently, only a small proportion of bio-banked samples are derived from oral sources, but this proportion is expected to increase quite rapidly as the value of saliva is more completely understood. In addition to specimen collection, the bio-banking market sector includes cryogenic storage systems to maintain the integrity of samples, ice machines, freezers, automated instruments, reagents for the isolation of subcomponents of bodily fluids, stabilizing agents and others. The growth in this industry is expected to be rapid, increasing from a current value of $3.65 billion in 2017 to $8.55 billion in 2023 [24], suggesting ample opportunity for a significant increase in the application of saliva in this particular market sector.

One last indication of the increasing popularity of saliva is the emergence of Saliva Symposia around the world. Last year, the North American Saliva Symposium [NASS] celebrated its fifth event in Houston Texas in October, following a number of successful triennial events in the EU hosted by the organization Acta in the Netherlands [25]. The next meeting will take place in Las Vegas in 2020. The North American event has led to highly successful biannual meetings in Australia and the inaugural meeting in China in 2017. The very first meeting in India [Saliva Symposium India, SALSI] in 2019 was highly successful, with 500 participants, and a similar event is planned for 2020 in Bangalore, India. The first of these meetings Brazil and South Africa may occur in 2020 or 2021. In each case, the symposia bring together key opinion leaders from academia and the commercial sector for oral presentations, posters, round tables and networking opportunities, with the goal of connecting the various groups for collaborations to benefit humanity. These groups forge partnerships to develop the next generation of innovative salivary diagnostics.

The present article explores the omics and biochemical compositions of various oral biofluids for diagnostics and monitoring.

2. Biochemistry and importance of oral fluids

Oral diagnostic approaches are best described as noninvasive approaches used to analyze a variety of analytes and to detect or monitor diseases. These analytes may include pathogens, antibodies, drugs, proteins, metabolites, and nucleic acids [26,27]. Oral biofluids include whole saliva, fractionated saliva, GCF, PICF, and dentinal tubular fluid [2,28].

2.1 Saliva as a biofluid

In the last decade, interest in salivary diagnostics has increased due to a number of factors, including the noninvasive nature of oral fluids, simplicity, compliance issues, and ease of disposal and storage. Cost is another advantage in its favor. The ability to detect plasma biomarkers in saliva and distinguish between healthy and diseased patients has attracted a much broader interest from the scientific and clinical community. Salivary biocomponents include hormones, cytokines, antibodies, proteins/enzymes, growth factors, drugs, metabolites, tumor markers, nucleic acids, viruses, bacteria, and fungi [9,29], although the major component of saliva is water [>99%]. Due to the association between oral and general health, health professionals are increasingly considering salivary analyses to diagnose and monitor systemic diseases and oral health [30]. For example, the salivary matrix metalloproteinase-8 (MMP-8) level is regarded as a promising candidate to diagnose and predict the progression of periodontal disease [31].

In addition, a significant reduction in salivary MMP-8 levels has been reported after nonsurgical periodontal therapy [32]. Similar diagnostic value has been reported for the pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) that mediate osteoclastogenesis [33]. Salivary biomarkers are also associated with connective tissue degradation, inflammation, and alveolar bone turnover. The presence of MMP-8, osteoprotegerin (OPG), macrophage inflammatory protein-1 alpha, IL-1β, IL-8 and TNF-α reflects the severity of disease [32]. The utility of salivary biomarkers is no longer limited to caries and periodontal diseases, and saliva testing now includes the diagnosis and/or monitoring of systemic diseases, such as metabolic syndrome [34,35], diabetes [36–38], cardiovascular disease [39], renal disease [40,41], autoimmune diseases (Sjögren's syndrome, multiple sclerosis, and sarcoidosis) [42–45] metabolic bone disorders [46,47], genetic disorders (cystic fibrosis and ectodermal dysplasia), diseases of the adrenal cortex (primary hyperaldosteronism, Cushing's syndrome, and adrenogenital syndrome) [48,49], infectious diseases of bacterial, viral, or fungal origin [50],

and even malignancies (salivary gland tumors, oral squamous cell carcinoma, and breast, pancreatic, lung, and ovarian tumors) [40,50–53]. Many proteins and peptides are also present in human saliva, such as histatins [54], cathelicidins [55], defensins [21], statherin and neuropeptides [56]. Many viruses (RNA, DNA, and envelope/capsule proteins) have been isolated from human saliva for the early detection of viral infections. Recently, *Zuanazzi, D.*, et al. reported the presence of the Zika virus (ZIKV) polyprotein in lyophilized saliva samples. The authors also identified 423 (maternal), 607 (baby A), and 183 (baby B) unique ZIKV peptides from saliva using tandem mass spectrometry (MS/MS) [57]. Another study reported the detection of Oropouche virus [an arthropod-borne virus] in the saliva and urine of infected patients with the onset of symptoms [58]. This finding provides opportunities for the development of point-of-care technology in the field of salivaomics.

2.2 Collection of saliva

The most commonly collected oral fluid is expectorated whole saliva, which is a mixture of major and minor salivary gland secretions, along with a modest contribution of GCF. Two key methods for saliva collection have been developed: stimulated saliva and unstimulated whole mouth saliva (UWS) collection [4]. UWS is collected either by the passive drooling method into a preweighed and calibrated vial to ensure that the salivary flow rate can be estimated [59] or by one of a number of highly standardized saliva collection devices that collect a uniform specimen each time [4]. The use of UWS is preferred over stimulated saliva, since it avoids potential differences generated by various reflex stimuli. However, one of the limitations of UWS includes the low volume collected, particularly from noncompliant groups (children), geriatric patients and patients with diabetes suffering from xerostomia (low saliva production) [60]. Over time, various modifications to the saliva collection process have been implemented and have improved multiple potential issues with UWS collection, including avoiding contamination, obtaining a pure sample, and increasing the volume collected.

3. Gingival crevicular fluid (GCF)

Serum exudate (GCF) arises from the periodontal pockets (or sulcus), and this particular specimen is regarded as a promising oral biological fluid for the detection of periodontal disease. Although the composition of GCF is similar to normal serum (including immunoglobulins, proteins, peptides,

enzymes, tissue degradation products, local mediators, toxin cells, and microorganisms) [28], its volume fluctuates in patients with certain oral inflammatory conditions, such as gingivitis, chronic periodontitis, caries, orthodontic tooth movement, and external root resorption [61].

An analysis of the host response in GCF using new diagnostic approaches has been very interesting. GCF has been reported to contain more than 90 different biomarkers associated with the early diagnosis and prognosis of periodontal disease (PD) [62]. PD is an oral inflammatory disease affecting the periodontal tissues, in which oral microorganisms initiate a cascade of host-mediated inflammatory events that induce the production of biomarkers through a network of cells, mediators, and tissues. Thus, these molecules represent attractive markers of the onset and severity of PD [63]. The association of humoral immune response indicators with active PD is equivocal. Indicators of increased polymorphonuclear leukocyte (PMN) activity, lysosomal β-glucuronidase, prostaglandin-E_2, lysosomal collagenase, cytoplasmic enzymes and aspartate aminotransferase have all been linked to clinical attachment loss (loss of tooth-supporting tissues in patients with periodontal disease) [62,64].

Evidence is available to justify the inclusion of a GCF-based method for the acute inflammatory response in PD clinical trials [65], while its potential application also appears highly promising in the management of periodontal lesions (pre-, during, and posttreatment). In particular, GCF may provide a quantitative assessment of the levels of inflammatory protein markers (host response), and a solid indication of the efficacy of a treatment modality in reducing bone loss and clinical attachment loss and improving overall periodontal outcomes. Further research is required to recognize the true potential of salivary and GCF biomarkers in the identification of current and prospective PD activity [28]. Advances in proteomics and in-depth analyses of the GCF proteome may support the development of prognostic and diagnostic indicators, which in turn might lead to the identification of novel biomarkers of health or diseased periodontium. Interestingly, a recent study in the field of GCF proteomic analysis has revealed the presence of novel protein biomarkers, vitamin D binding protein (DBP) and sero-transferrin (Tf), which predict pubertal growth, suggesting their potential utility in orthodontics in the future [66].

3.1 Methods of GCF collection

Two main methods for GCF collection have been developed: the intra- and extra-cervical approaches [64]. The intracervical methodology is more

precise and the most commonly employed approach. It involves the use of a strip that is gently inserted into the gingival crevice (or sulcus) and either placed at the entrance of the crevice or inserted completely into the base of the pocket. In the extra-crevicular approach, the strips are placed on the gingival crevices to prevent trauma and gingival bleeding. Regardless of the approach used, the methods are further stratified into three basic strategies [64,67].

3.1.1 Gingival washing method

In this method, the gingival crevice is first perfused with a fixed volume of isotonic (Hanks' balanced salt) solution. The diluted GCF fluid is collected (by a paper strip/points) and constitutes a mixture of soluble plasma proteins and cells. The main advantage of this method is that all cells within the gingival crevicular region are harvested. However, not all fluid is able to be recovered from the crevices, and thus the precise calculation the volume and composition of GCF necessary to obtain a precise dilution factor is nearly impossible [62].

3.1.2 Capillary tubing or micropipettes

In this technique, the collection site is first isolated (dried) and micropipettes are inserted at the entry point of the gingival crevices (an extra-crevicular approach). GCF is obtained through a mechanism termed "capillary action." The volume of the collected fluid is able to be accurately determined from the internal diameter and calculation of the distance that the GCF migrates. Although this technique collects an undiluted "native GCF sample, which might appear ideal, it is associated with specific issues, such as operator dependence and a complex operation." Therefore, this method is used less frequently [62].

3.1.3 Absorbent filter paper strips

A periopaper strip is an extra-crevicular approach in which the paper strip is placed at the entrance of the crevice, while GCF migrates up the strip by capillary action. This approach is commonly used, fairly convenient, can be applied at a specific site, and is the least traumatic method [62]. The GCF-absorbed periopaper is transferred to a Periotron device to quantify the GCF volume [61]. This device should be precalibrated using a standard fluid volume pipetted onto a periopaper to develop a comparison curve against a test curve representing the fluid volume obtained freshly and directly from the GCF crevices. An increased GCF volume is a useful

indicator of periodontitis. Additionally, the GCF volume has also been used to concentrate the concentration of a biomarker within the GCF sample [61,62].

4. Peri-implant crevicular fluid (PICF)

Peri-implantitis is an inflammatory condition around an infected dental implant site caused by overwhelming microbial contamination. PICF is broadly characterized into two types: peri-implant mucositis and peri-implantitis [68,69]. The former is characterized by bleeding and suppuration, with no evidence of bone loss; meanwhile, the latter is a progressive, irreversible disease of the bone and soft tissues around osseo-integrated implants [69]. Peri-implantitis is characterized by bone loss, pocket formation, and suppuration. If left untreated, both conditions will lead to implant failure and are usually diagnosed in symptomatic phases, when conservative options are limited for the dentists [69]. The early detection of peri-implant destruction and monitoring bone loss progression is pivotal. An evaluation of potential biomarkers and enzymes in PICF has revealed promising results in differentiating healthy sites from peri-implantitis or inflamed tissues [70,71]. A recent meta-analysis identified increased expression of inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, IL-17, TNF-α, VEGF, and PGE2). The levels of some enzymes, such as MMPs (MMP-1, -3, -8, and -13), MPO and cathepsin-k, were increased in PICF from patients with peri-implantitis and mucositis [68]. Moreover, additional biomarkers for peri-implant bone-loss (in peri-implantitis) include increased expression of RANK, OPG/RANKL, and sclerostin compared to patients with mucositis and healthy subjects [68].

4.1 Methods of PICF collection

PICF collection involves the use of periopaper strips introduced into the peri-implant crevicular site, the fluid will migrate up the paper strip through capillary action. The strip is then tested for the presence of host-derived inflammatory proteins and enzymes [68]. One clinical trial reported the collection of peri-implant crevicular fluid from titanium and zirconia abutments for the analysis of MMP-8 levels [72]. This study improved our understanding of the roles of the host and microbiome in the remodeling and/or inflammation around dental implants for the early diagnosis of peri-implantitis. The collection of fluid is presented below in Fig. 1 [72].

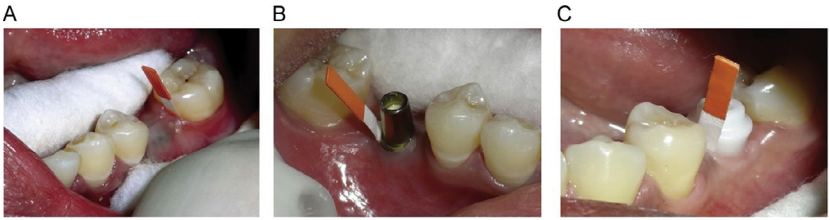

Fig. 1 Extraction of GCF and peri-implant crevicular fluid using a filter paper. (A) Extraction of GCF around the adjacent tooth, (B) around a titanium abutment, and (C) around a zirconia abutment. *Adapted with permission from Y. Kumar, V. Jain, S.S. Chauhan, V. Bharate, D. Koli, M. Kumar, Influence of different forms and materials (zirconia or titanium) of abutments in peri-implant soft-tissue healing using matrix metalloproteinase-8: a randomized pilot study, J. Prosthet. Dent. 118 (2017) 475–480. https://doi.org/10.1016/j.prosdent.2016.11.017.*

5. Dentinal tubular fluid (DTF)

Dentinal tubules are minute, wavy canals within the dentin layer of the tooth that contain the cytoplasmic processes of odontoblasts and extend radially, establishing communication between dentin and pulp. Injuries to the pulp-dentin complex expose the dentin layer of the tooth, producing a direct physical injury in the dentin-forming cellular structures (odontoblastic processes and cell bodies), causing the separation (disruption) of tight junctions between the odontoblasts and pushing them centrally into the pulp [73]. The outward flow of dentinal fluid plays a pivotal role in protecting the pulp from microbial invasion when the pulp-dentin complex is injured [73,74]. Pathophysiological mechanisms include inflammation, outflow of dentinal fluid containing plasma proteins (albumin, IgG, and fibrinogen), temporary blockage of tubules by protein molecules, subsequent dentinal sclerosis and reactionary dentine formation to close the avenues of direct pulp exposure [73]. With the onset of the inflammatory cascade (postinjury), the dentinal fluid exudate will mainly contain polymorphonuclear leucocytes (PMNs), outwardly migrating macrophages, and B-cells, and T-cells [75]. Therefore, the characterization of DTF will provide estimates of the extent of dento-pulpal injury [76], degree of pulpal inflammation [74], the ongoing healing process, and/or efficacy of dental restoration [73,75,77].

Very few studies have attempted to characterize the biochemical composition of the dentinal tubular fluid (DTF). According to Ozok et al., the DTF composition is an important parameter for determining the rate

of formation and progression of dentinal caries, while the DTF flow rate does not correlate with the degree of demineralization [76]. As shown in the study by Knutsson et al., the amount and quality of proteins present in DTF represent the composition of plasma proteins (in particular, albumin, IgG, and fibrinogen) in the pulpal interstitial fluid [78]. Meanwhile, *Geraldeli S* et al. measured the levels of chemokines and cytokines, and postulated that cytokines are locally produced in the pulp and not derived from serum, suggesting the active transport of cytokines from serum to the dentinal fluid [77]. In a comparison sound, carious, and restored (with amalgam) molars, the latter expressed TNF-α at the highest levels. Varying quantities of interleukins (IL-1B, IL-6, IL-8, IL-10, and IL-12) were expressed in a general amalgam-restored molar. Another potential biomarker for carious lesions is MMP-9 (matrix metalloprotinase-9; neutrophil gelatinase), which is associated with neutrophil-related degradation of the pulp tissue. Its levels increase with caries progression. As shown in the study by Ballal et al., MMP-9 levels are increased in both deeper and shallow lesions; although a weak correlation was observed, their levels increase in deep carious lesions [79]. Moreover, Zehnder et al. reported a correlation between higher MMP-9 levels and the degree of pulpal inflammation, which has clinical significance in improving the diagnostic ability to distinguish between reversible and irreversible pulpitis [74]. However, Pinto et al. introduces a fairly new concept of an association between carious lesions, the dentinal fluid and the tooth biofilm. The demineralized lesion facilitates the pathway for DTF to be actively transported into the proximal natural enamel caries and alters the microbial composition of the affected tooth biofilm [80].

5.1 Method of DTF collection

Dentinal fluid is collected after proper cavity preparation, rubber dam isolation, and ensuring a dry field to prevent the percolation of oral fluids. The membrane is folded to form a cone and the blunt end of the cone is pressed at the base of the cavity for 30–60 s [81]. Afterward, it is immediately transferred to microcentrifuge tubes placed on ice and then stored in a freezer at −20 °C. Two types of membranes are typically used: (i) the conventional PVDF (polyvinylidene fluoride) membrane and (ii) the cellulose membrane [81]. Zahnder et al. compared the effectiveness of these two membranes in a clinical trial and confirmed that a cellulose membrane with a large pore-size (12–15 μm) absorbed more water and possessed the ability to yield higher amounts of target molecules (in DTF) when sampled from the exposed dentin compared to conventional PVDF membranes [74,81].

6. Analytical techniques

The pivotal role of saliva is well recognized in biomedical research due to several prominent characteristics, such as the diverse composition, non-invasive collection, high potential for an alternate diagnosis, and ease of monitoring and investigating disease pathogenesis. Numerous functions have been associated with saliva, such as conversation, food tasting, swallowing, oral digestion, tissue lubrication, antibacterial and antiviral activities, nurturing dental integrity, and maintaining the homeostasis of oral and general health. In the recent decade, the utility of saliva in biomedical investigations such as the diagnosis and monitoring of systemic diseases has received increasing attention. A survey of the literature revealed the successful use of human saliva to diagnose several systemic diseases. Several researchers have documented the successful use of human saliva in diagnosing many diseases, such as cancers (oral, lung, ovarian, breast, and pancreatic tumors) [82–87], autoimmunological disorders (Sjögren's syndrome, celiac or coeliac disorder, and Hashimoto thyroiditis) [42,88,89], infectious diseases [90–93], endocrinological diseases [48,94–97] and gastrointestinal tract disorders [98,99]. Furthermore, numerous applications of salivary fluid have been reported, including toxicological diagnostics [100,101], neurology [99], psychiatry [102,103] and forensic science [104]. Therefore, the widespread usefulness has endorsed saliva as an outstanding biofluid for immunological, biochemical and toxicological analyses.

The wide variety of biomolecules identified in saliva provides information about many organs and systems, and the identification of potential biomarkers might provide important opportunities in several disciplines of omics [105]. The identification and validation of multiple salivary biomarkers is necessary to ensure the correct disease diagnosis. Therefore, the most important challenge in a saliva analysis is to address the diversity and complexity of the salivary samples being analyzed. The term "*metabolites*" encompasses a wide range of bio-molecules present at various concentrations and possessing different physio-chemical features, such as the molecular mass, structure and polarity index. Approximately 853 salivary metabolites have been identified recently [106]. Considering the instrumentation, saliva is an easy sample to collect using a noninvasive method, and samples can be repetitively obtained. Thus, saliva is simple to analyze compared to plasma, urine or cerebrospinal fluid. Out D et al. analyzed in adult saliva samples ($n = 6900$) and Granger et al. analyzed saliva samples from

children ($n = 2178$) and documented the stability, generalizability and reproducibility of the method [107,108]. Nevertheless, saliva is not as frequently employed in diagnostic investigations as other biofluids, despite its established applicability to biomarker discovery [105], even for malignancies not associated to the pulmonary system [109]. A variety of methodologies have been exploited to study saliva, ranging from the more commonly applied techniques such as ELISA, gas chromatography–mass spectrometry (GC–MS), and liquid chromatography-mass spectrometry (LC-MS) to more sophisticated technologies such as tandem mass spectrometry (LC-MS/MS) and ^{1}H NMR spectroscopy. The utility of a selection of these more common methodologies in the diagnosis of several malignancies will be discussed below.

6.1 Radio immune assay (RIA)

6.1.1 Periodontal diseases

Periodontal diseases are globally prevalent oral diseases. Touitou et al. measured the circadian hormonal profile of two biomarkers (melatonin and cortisol) and some other steroids in the saliva secretions and urine from healthy prepubertal boys using RIA. A significant correlation between salivary cortisol and melatonin levels was observed [110]. *Christodoulides* et al. described the use of the lab-on-a-chip assay (LOC) methodology in patients with periodontitis and evaluated the levels of C-reactive protein (CRP), interleukin-1 (IL-1β), and matrix metalloproteinase-8 (MMP-8) in whole saliva. The LOC outcome was compared with ELISAs and revealed the possible value of these biomarkers in diagnosing rigorousness and magnitude of periodontitis [111].

6.2 Liquid chromatography-mass spectrometry (LC-MS/MS)

Recently, LC-MS/MS has become the technique of choice to analyze oral fluids from patients with many diseases due to the increased specificity and sensitivity compared to conventional immunoassays, including RIA [112].

6.2.1 Dental caries

Dental caries is among the major chronic diseases in children and poses significant health concerns worldwide [113–115]. Saliva plays a vital function in the occurrence and development of caries; thus, the use of salivary diagnostics has great clinical value. In the United States, only 10% of late adolescents and young adults have been reported to be either caries-free or caries-resistant, while >90% of individuals suffer from dental caries [116].

6.2.2 Periodontal diseases

Periodontal diseases are globally prevalent oral diseases. Gonçalves et al. determined and compared the profile of proteins in the USWS between healthy individuals and patients with periodontitis using two complementary techniques: 2D-GE and HPLC [117]. Protein spots of interest were characterized using MALDI-TOF-TOF. Data were subsequently analyzed using an ESI-Q-TOF method. The identified α-amylase variants appeared to be generated through hydrolysis by cysteine proteases under inflammatory conditions [117]. Salazar MG et al. precipitated salivary proteins from 20 patients with chronic periodontitis with trichloroacetic acid (TCA) and then performed an LC-MS/MS analysis [118]. In the search for disease-specific biomarkers, 344 human protein groups were identified, indicating that a label-free proteomic analysis of USWS potentially represents a powerful tool to describe the status of periodontal disease and to differentiate between healthy subjects and individuals with periodontitis [118].

6.2.3 Proliferative verrucous leukoplakia

Flores IL et al. reported the profile of the salivary proteome in patients with an infrequent variant of oral leukoplakia called proliferative verrucous leukoplakia (PVL) [119]. Two hundred eighty-three proteins were detected in USWS from 30 study subjects using an LC-MS/MS-based approach. Dipeptidyl peptidase 1 (DPP1) and angiotensinogen (AGT) were recognized as biomarkers of the etiology of PVL. Furthermore, DPPI and AGT may have an important function in the development of PVL [119].

6.2.4 Oral leukoplakia

Oral leukoplakia (OL) is a commonly occurring malignant disorder of the oral cavity that may lead to oral carcinoma. Camisasca et al. investigated the salivary biomarkers of leukoplakia in USWS using a two-dimensional (2D)-gel electrophoresis (GE) and mass spectrometry (MS) approach and compared the proteomic outcomes of individuals diagnosed with or without OL [120]. Based on the results, keratin-10 (CK10) represents a remarkable OL biomarker in patients with oral carcinogenesis and can be used to obtain a better understanding of the progression of cancer in the oral mucosa [120].

6.2.5 Oral squamous cell carcinoma

Patients with oral squamous cell carcinoma (OSCC) are commonly diagnosed at the advanced stage of malignancy and thus show a poor prognosis.

Timely diagnosis and early treatment are very important to achieve a superior outcome. Several molecules, such as corticosteroids (particularly glucocorticoids), glycosylation-related molecules, oxidative stress-related molecules, and inorganic molecules, have been reported to correlate with the OSCC diagnosis [121]. HPLC and commercial colorimetric methods are among the main tools used to analyze these biomarkers, but several other techniques have been reported to date [121]. Lee LT et al. used Luminex Bead-based Multiplex assays to discover diagnostic biomarkers in human saliva and plasma responsible for tumor progression in patients with oral squamous cell carcinoma and reported that IL-1β, MIP-1β, IFN-γ, TNF-α, IL-6, IL-8, and eotaxin are plausible salivary biomarkers [87]. Thus, the identified salivary biomarkers potentially play a valuable role as a complementary adjunct in the early detection of oral OSCC [121].

6.3 Enzyme-linked immunosorbent assay (ELISA)

The quantitation of biomarkers is a highly objective evaluation modality used to diagnose OSCC. In addition to other techniques, ELISA has been a widely used approach to determine biomarker expression [122–125]. Ebersole JL et al. showed the discriminatory diagnostic capacity of USWS analytes to distinguish subjects with chronic periodontitis from healthy volunteers using immunoassays based on Luminex multianalyte profiling (xMAP) technology and ELISA [126]. The study suggested the potential utility of salivary analytes for clinicians to identify and monitor the periodontal health of patients [126]. Gonçalves et al. compared the salivary concentrations of TGF-β1, IL-10, and soluble HLA-G between patients with OSCC using ELISAs and identified IL-10 as a probable biomarker [122].

6.3.1 Nuclear magnetic resonance spectroscopy (NMR)

Biochemical analyses are a growing area of investigation and an indispensable part of the diagnosis and monitoring of human diseases, ranging from basic to clinical applications. The recent decade has witnessed an ever-increasing interest in the identification of metabolites as key biomarkers for the diagnosis, therapy, and monitoring of several diseases. The rapidity and inexpensiveness of high-resolution proton nuclear magnetic resonance spectroscopy (^{1}H NMR) have facilitated the wide use of this technique to explore pathological metabolic processes [127]. ^{1}H NMR spectroscopy generates valuable information about the structure and composition of low-molecular-mass metabolites in biological fluids [128]. The key benefits of ^{1}H NMR spectroscopy are its high reproducibility, quantitative nature,

and unbiased metabolite detection [128–131]. Metabolic profiling of plasma is relatively well established compared to the profiling of saliva using ^{1}H NMR spectroscopy, for which it is considered underutilized [127]. ^{1}H NMR-based metabolomics of urine and plasma samples is frequently studied, due to the availability of validated and published protocols, including the collection procedure, storage, preparation, and analysis of plasma or urinary biofluids [132–134].

However, in the case of saliva profiling using ^{1}H NMR spectroscopy, studies have reported high variability in the analyses [106,127,135–139]. Aimetti M et al. analyzed samples from patients with periodontal diseases (PD) using ^{1}H NMR and showed that the metabonomic analysis of USWS exhibits a generalized chronic periodontitis signature [140]. Using ^{1}H NMR, Fidalgo et al. investigated the variations in salivary metabolites in children during the developmental period commencing from primary dentition through a transition period with both primary and permanent (mixed) dentitions until reaching the permanent teeth [141]. The ^{1}H NMR data and PCA-LR were capable of categorizing the saliva of children with or without caries and identified markers of caries activity [141]. Sjögren's syndrome (SS) is classified as an autoimmune disorder with a complex and partially known pathogenesis. Mikkonen et al. investigated the practicality of ^{1}H NMR spectroscopy and analyzed the metabolic profile of WSMS among individuals with a well-defined primary Sjögren's syndrome (pSS) [142]. The authors tracked the levels of neurotransmitter amino acids (NAAs) and other metabolites in human saliva linked to pathogenesis of pSS and neural destruction of salivary glands [142]. In a cohort of 130 subjects, Villaescusa AG et al. were the first investigators to identify potential salivary biomarkers of glioblastoma, a fatal, neuro-endocrine tumor of the brain, using ^{1}H NMR technology [131]. However, the study did not reveal a significant relationship between glioblastoma and periodontitis disease [131].

7. Omics of oral fluids

Saliva is becoming the diagnostic fluid of choice due to its easy availability and noninvasive collection [54,56,121]. Advances in technology have resulted in a variety of omics fields, which in turn, have changed the way researchers approach experimental investigations [143]. The chapter will discuss the omics of saliva with reference to the macromolecules present in this fluid. Proteomics, genomics, transcriptomics, metabolomics,

glycomics and microbiomics studies of saliva will be discussed with respect to the approaches used and respective experimental platforms. Glycomics and microbiomics are discussed briefly, as researchers in both of these fields perform similar analyses with proteomics and molecular techniques, and the only difference lies in sample preparation methods, which contribute to the total protein and nucleic acid contents of saliva.

Saliva has attracted increasing interest as a diagnostic tool, mainly due to its noninvasive collection compared to the collection of blood and other body fluids. The composition of saliva makes it a fluid of choice [144]. It is a mixture that is mainly composed of water (~ 99%), proteins, lipids, carbohydrates, salts, urea, uric acid, creatinine and amino acids [54,56,121]. The presence of metabolites, DNA, RNA, microflora, bacterial metabolites, gingival fluid, serum components, exfoliated epithelial cells and leukocytes not only contributes to the detection of changes in the body but also to the continuous monitoring of oral and systematic disorders [30,145,146]. The term "Omics" refers to a large biological dataset that is generated using advanced technologies and informatics tools. Since 1950, consistent advances in the methods used to study macromolecules such as DNA, RNA and proteins have been achieved, resulting in the development of disciplines such as genomics, transcriptomics and proteomics [147]. In addition to these main fields of the central dogma, omics is now part of many other biological science disciplines, such as epigenomics, metabolomics, drugomics, and microbiomics. The integration of interdisciplinary data from different omics fields has become a much more informative approach (multiomics) with more strength than weaknesses in terms of future precision medicine applications [147]. Salivaomics is defined as the study of biomolecules that constitute the salivary secretions using the omics approaches [148]. A variety of these studies has been conducted to analyze the changes in saliva in patients with oral or systematic disorders and showed that proteins, DNA, RNA, hormones, ions and other salivary factors are altered and identified. Infectious diseases of viral, bacterial, and parasitic origin may produce identifiable changes in the salivary composition, serving as a potential indicator of disease severity and pathogenesis [148]. This chapter will mainly cover proteomics, genomics, transcriptomics, metabolomics, and very briefly glycomics and microbiomics analyses that lead to the identification and characterization of the components (biomarkers) of saliva. The biomarkers identified by any omics analysis are discriminatory and provide useful information about various disorders. Additionally, the development of microfluidic and mini-diagnostic technologies with mobile phone

applications facilitates an increasingly accurate disease diagnosis that is readily available at the convenience of the patient's home or a doctor's office. For example, in pediatrics, a drop of saliva has been utilized to diagnose and monitor diabetes, inflammation, infection, developmental conditions and other metabolic conditions in neonates and children of different ages [34,37,149].

We are experiencing the era of omics, which creates tremendous biological datasets. When these approaches are integrated, they will help us explore multiple molecular pathways in depth in health and diseased states [150–152]. Omics has continued to generate big data, which are being analyzed using advanced machine learning approaches to identify biomarkers, potentially leading to a paradigm shift in medical diagnosis and therapeutics.

7.1 Proteomics of saliva

The proteomic platform represents a different approach to the analysis of a biological sample. The technologies and approaches applied in proteomics are undergoing rapid improvements, based on the new avenues that are now being explored, for example, in cell biology [150]. The properties that are able to be explored using proteomics include protein abundance, posttranslational modifications, isoform identification, subcellular localization, and protein-protein interactions [150]. The bottom-up platform in which the digested sample is analyzed has almost completely overshadowed the top-down methodology in which intact protein samples are investigated. The bottom-up approach is applied to analyze a complex mixture of proteins, as it is much more sensitive. However, this method has some limitations, such as the redundancy of peptide sequences that causes an ambiguous determination of their origin. Multiple factors that should be considered when employing a particular proteomics strategy, as shown in Fig. 2. Sample preparation is one of these factors that must be considered when using shotgun proteomics or a gel-based analysis (2D electrophoresis), MALDI-TOF or SELDI platforms, or a solution-based top-down method with initial chromatographic/capillary electrophoretic separation step followed by mass spectrometry [153]. The other aspect to be considered is the technique to be utilized for the proteomics analysis. These techniques include conventional techniques, advanced techniques, high-throughput techniques and bioinformatics analyses. The quantitative or qualitative analysis is performed using bioinformatics tools after mass spectrometry. Likewise, when sequencing a protein using the Edman degradation method, an online database is

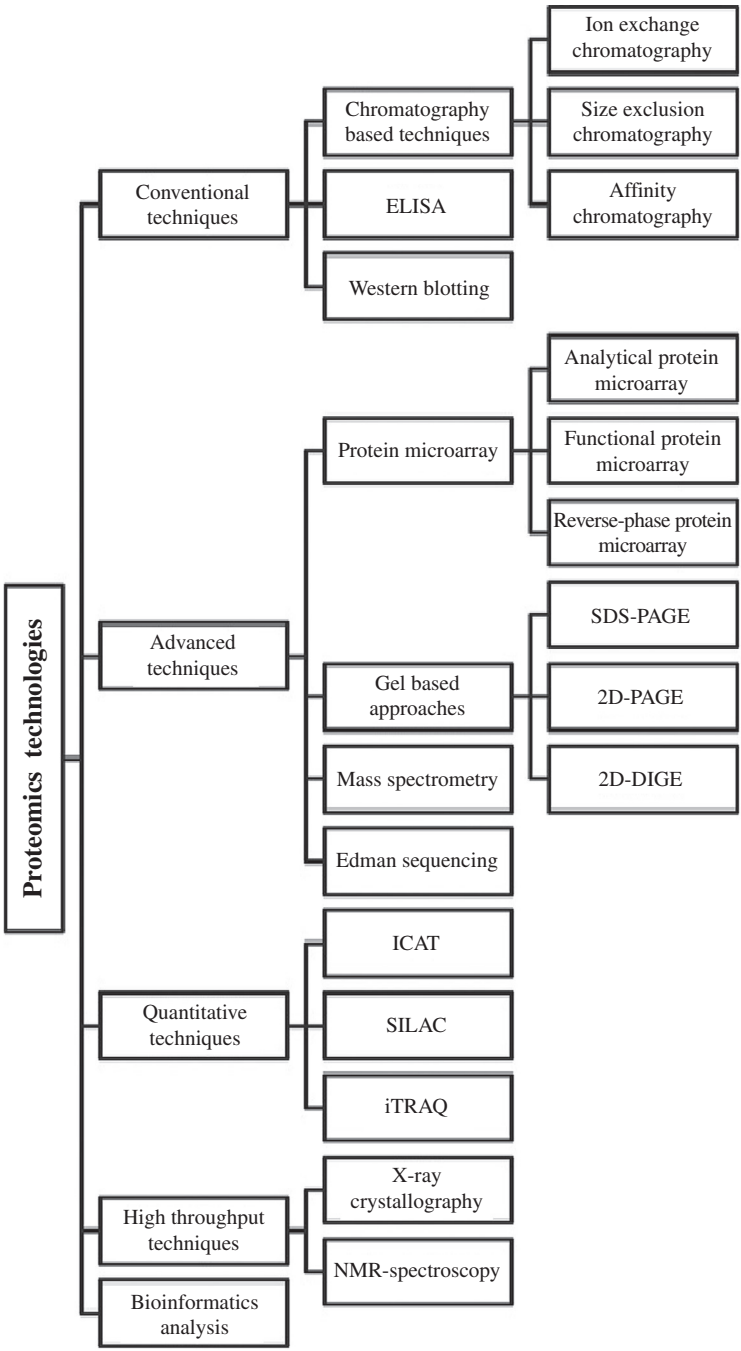

Fig. 2 An overview of proteomics techniques. *Adapted and modified from B. Aslam, M. Basit, M.A. Nisar, M. Khurshid, M.H. Rasool, Proteomics: technologies and their applications, J. Chromatogr. Sci. 55 (2017) 182–196. https://doi.org/10.1093/chromsci/bmw167.*

searched and various tools are employed. Therefore, bioinformatics is an essential computational component that is required in proteomics studies and other omics fields [147].

7.1.1 Gel electrophoresis-based proteomics

Polyacrylamide gel electrophoresis represents the basic method of protein characterization since it was developed and made available for protein analysis in the early 1960s. These electrophoretic techniques have now become much more advanced and robust. Saliva can be analyzed on a gel using common vertical gel electrophoresis protocols. Different buffer systems have been utilized for gel electrophoresis, including Tris-glycine SDS-PAGE, Bis-Tris SDS-PAGE and native PAGE. Gel-based investigations are exploited for uses as simple as standardizing the protocol for saliva collection and analyzing the levels of major components such as amylase in the saliva to an examination of the microvesicles present in the samples [154–156]. The bands from the gels could be excised and subjected to mass spectrometry for a further analysis of identity of the proteins [156]. These simple approaches have been used to apply gel electrophoresis to saliva proteomics studies. However, two-dimensional (2D) gel electrophoresis is the technique that is applied most saliva studies rather than simple gel electrophoresis. Two-dimensional gel electrophoresis separates the protein sample based on the isoelectric point and molecular mass of the components present in the sample that has been dissolved in the rehydrating buffer system used for 2D electrophoresis. The protein spots that appear on the gels are analyzed based on their presence or absence or difference in intensities during a comparison between the gels. The spots of interest are then identified using mass spectrometry techniques. The vast majority of studies employ simple 2D gels and DIGE (differential gel electrophoresis) to explore the saliva proteome for an analysis of differential protein expression and the identification of unique or signature proteins in patients with diverse disorders. In saliva from patients with oral cavity diseases, such as the saliva of patients suffering from chronic periodontitis, 2D gel electrophoresis was performed, followed by protein identification, and revealed the differences in immunoglobulin, amylase and cystatin levels [117]. Another study investigating patients with periodontitis diagnosed with type 2 diabetes used the same approach, except the isoelectric focusing step was performed with an IPG strip at pH 4–7 rather than pH 3–10. The results revealed the differential expression of several proteins, including plastin-2, actin-related protein 3, polymeric immunoglobulin receptor, leukocyte elastase inhibitor, immunoglobulin

J, carbonic anhydrases 6, and interleukin-1 receptor antagonist [157]. Other reports are based on only mass spectrometry and western blotting (discussed later) and highlight many other protein candidates that are differentially expressed in patients with periodontitis. Similar studies of patients with oral cancer have also been conducted with saliva samples, and 2D gels were run to investigate the differential expression of proteins (Fig. 3). The results revealed a list of proteins displaying increased or decreased levels, including interleukins, S100 calcium binding protein, p53 antibodies, albumin, endothelin-1, CA-125, transferrin, fibrin, keratin 36, cystatin A, myosin, actin, s100A7, catalase and other proteins [158–160]. The salivary glands are supplied with blood vessels that support and exchange material with the salivary gland tissues. Thus, molecules from different organ systems of

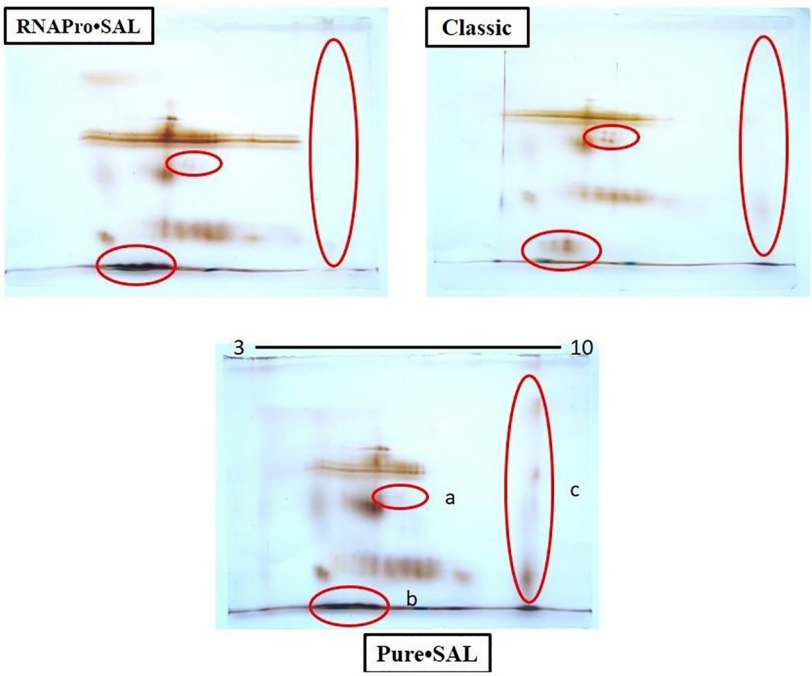

Fig. 3 A 2D gel map of saliva proteins separated with an IPG strip at pH 3–10 and 12.5% acrylamide gel. The diagram depicts the areas of proteins generally observed as showing differences between saliva samples from healthy subjects and patients with a disease. *Adapted from Z. Khurshid, S.F. Moin, R.S. Khan, M.A.S. Agwan, A.H. Alwadaani, M.S. Zafar, Human salivary protein extraction from RNAPro·SAL™, Pure·SAL™, and passive drooling method, Eur. J. Dent. 11 (3) (2017) 385–389. DOI: 10.4103/ejd.ejd_183_17.*

the body may be present in saliva. In addition to oral diseases, changes in salivary markers could be utilized to detect and diagnose disorders such as autoimmune diseases, cardiovascular diseases, diabetes and HIV [161]. A study was conducted with saliva samples and applying the 2D DIGE technique to identify lung cancer biomarkers, and three candidate biomarkers, namely, zinc alpha2-glycoprotein, haptoglobin and calprotectin, were validated for the detection of lung cancer [84]. A similar study was conducted to detect stomach cancer and validated triosephosphate isomerase (TPI1), cystatin B (CSTB), and deleted malignant brain tumors-1 protein (DMBT1) as candidate biomarkers for cancer from saliva samples using the same approach of 2D DIGE with tandem mass tags for quantitative analysis [162]. The purpose of utilizing gel electrophoresis is to reduce the complexity of the saliva samples and then identify the proteins and peptides. Zymograms are also gel-based has approaches that have been employed to analyze the levels of protein-degrading enzymes in saliva samples. For example, gelatin or collagen was used as the substrate and *co*-polymerized with the gel to assess the protease activity in saliva samples from patients with periodontitis [163]. The change in activity indicates the difference between the healthy and disease samples. This method is a simple tool to study the salivary proteases and has been applied to assess the activity of protease inhibitors in reverse zymography. Overall, gel-based decomplexation is one of the main and frequently practiced techniques to study saliva samples. It will remain the focus of analyses of saliva samples in the future due to the predicted advances and new modifications of the technique.

Western blotting has been utilized to detect and verify the identified candidate proteins that are differentially expressed in patients with different disorders, such as caries and lung cancer [84,164], or to analyze the stability and functions of components of saliva, such as mucins [165]. Western blotting involves separation on a normal SDS-PAGE gel, followed by the transfer of gel bands to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane through electro-blotting. Afterwards, the membrane is subjected to several steps of blocking and washing, treatment with primary (specific) and secondary antibodies, and finally detection and visualization. Western blotting is a reliable technique applied to proteomics studies to complement the results.

7.1.2 Mass spectrometry (MS)-based proteomics

The number of studies reporting a mass spectrometry analysis of saliva has increased, due to the continuous advances in the technique (Fig. 4). Mass spectrometry has been established as a main protein identification

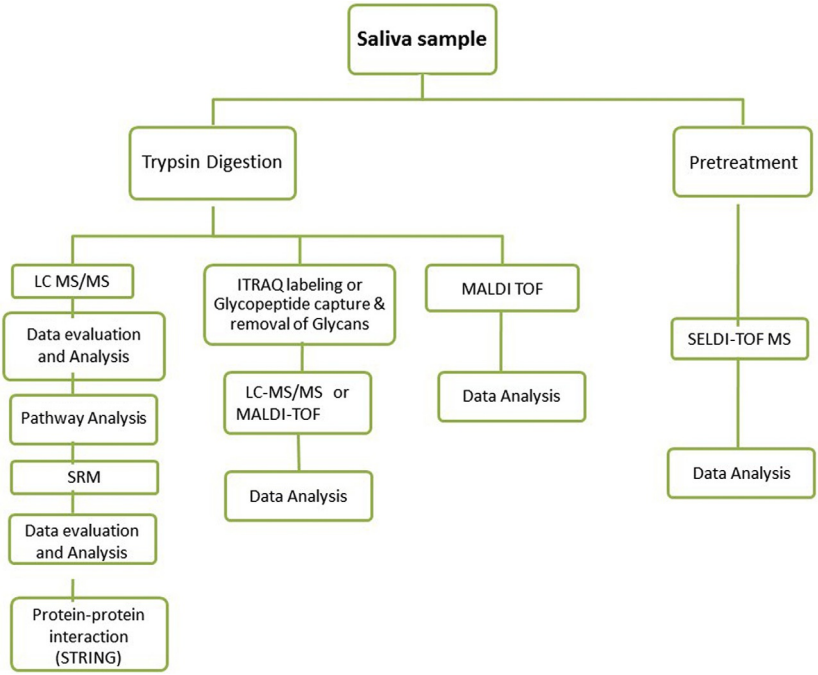

Fig. 4 An overview of mass spectrometry-based approaches adopted for saliva proteomics.

technique after the development of a soft ionizing procedure for macromolecules, particularly proteins, during last few decades of the previous century. Most methods are being developed with mass spectrometry for saliva samples from patients with various conditions, including head and neck cancer, oral squamous cell carcinoma, breast cancer, gastric cancer and Sjögren's syndrome [166]. Liquid chromatography (LC) combined with mass spectrometry represents an effective method for proteomics known as the bottom-up approach (peptide masses identify proteins). The continuous development of mass spectrometers, such as linear ion trap, Orbitrap, quadruple time of flight, linear triple quadrupole Orbitrap with electrospray ionization (ESI), has significantly expanded sample throughput and the confidence of the identification. The LC step may be multidimensional, such as using a single microcapillary column filled with fused silica RP-C18 + SCX + RP-C18 in sequence. This type of LC coupled with MS has resulted in multidimensional protein identification technology called MudPIT. Saliva collected from the parotid glands from patients with Sjögren's syndrome has been used to detect differentially expressed proteins with MudPIT [167]. Again, if we start from the oral cavity, the saliva of patients with

periodontitis contained more than 100 proteins after an analysis using shotgun proteomics, with significant differences between healthy subjects and patients. Five proteins were validated to display a high predictive value for the disease by conducting additional experiments, including stable isotope labeling during multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) to reproducibly quantify proteins [168]. The shotgun mass spectrometry-based proteomics approach uses a procedure in which the whole sample is digested directly with trypsin and applied to a high-resolution (occasionally hybrid) mass spectrometer, such as a linear triple quadrupole Orbitrap mass spectrometer with a nanospray ion source. The mass spectrometer generates large amounts of MS and MS/MS data, which are then input into bioinformatics software for identification and further analysis. The shotgun methodology is useful for label-free quantification and is a fast procedure for proteomics studies. SRM MS is a recently developed methodology with high reproducibility to validate selected peptide biomarkers [169]. Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) has also been used to measure saliva samples. MALDI MS is also a soft ionizing technique where the sample is mixed with a matrix and laser energy is used to vaporize the sample crystalized with the matrix. MALDI MS is suitable for proteins with a high molecular weight, for instance, to study the alpha amylase and albumin levels in patients with oral cancer [170]. The technique has been applied in many other studies for the direct or indirect identification of proteins and peptides in saliva samples from patients with gastric cancer [171], lung diseases [172], Sjögren's syndrome [42] and hematopoietic stem cell transplantation [173]. Although MALDI MS has short analysis time, ease of use, high sensitivity and high throughput, but the development of this MS technique has been limited due to certain biases and the limited mass range window. Surface-enhanced laser desorption ionization mass spectrometry (SELDI MS) is a variation of MALDI that modifies the surface of the target plate or solid phase chip surface on which the sample is spotted with a certain functionality to ensure that a particular set of proteins is retained, and the remaining sample is washed away. The protein chip or the target plate is then placed in the mass spectrometer. SELDI is a good approach for specifically targeting a subset of proteins in complex samples. Saliva has been analyzed using SELDI MS to profile proteins and peptides with a low molecular mass (<10 kDa) [174,175]. For example, the saliva of patients undergoing orthodontic treatment showed differences in peaks ranging in size from 3 to 10 kDa [176,177].

Various parameters, such as the storage temperature, number of freeze-thaw cycles, sample type and chip surface, significantly affect the detection of proteins. The sample treatment, dilutions, matrix and delay in processing also affect protein profiling using SELDI MS [178–180]. One of the limitations of the SELDI MS analysis is the drift or noise that affects the reproducibility of the analysis, in addition to the inability to analyze high-molecular mass proteins (>100 kDa). Therefore, different factors affect the analysis of saliva and some other parameters also shape the experimental investigations. Several issues to be considered in the saliva SELDI MS analysis include the timing of saliva collection, addition of proteases inhibitors, addition of denaturing agents while using chip arrays (except CM 10) and the removal of glycoproteins from the sample. The implementation of these steps substantially improves the reproducibility and quality of SELDI MS [174]. Mass spectrometry is advancing daily in the field of proteomics and it might replace the decomplexation techniques over time to become the singular approach used in the field.

7.2 Genomics of saliva

The genome (complete DNA of an organism) involves the characterization of all the genes associated with producing proteins. It is studied by sequencing the DNA and using bioinformatics tools. Since the first genome sequence was published in the early 1980s, thousands of genomes have been sequenced from prokaryotes to eukaryotes. For molecular studies and an analysis of the DNA, blood and other tissue samples have been acquired using invasive procedures. Saliva has emerged as an alternate source to extract DNA for genetic and genomic studies. For oral cavity disorders, such as oral cancer lesions, a saliva sample becomes the best choice, as it not only contains the molecules but also the exfoliated cells that might facilitate the identification and screening of potential biomarkers. One of the early reports in which saliva was utilized as a tool to detect possible alterations in the salivary DNA identified mutations in exon 4, codon 63 of the p53 gene in 5 of 8 patients with oral cancer. Subsequent studies reported the heterogeneity of the p53 gene in patients with oral cancer, and IgG and IgA antibodies against p53 were present in saliva and serum samples [181–183]. Similarly, a previous study analyzed DNA extracted from saliva to detect polymorphisms in the Fc receptor gene and produced the same results as DNA obtained from a blood sample through the PCR amplification of specific alleles. A single nucleotide substitution in one receptor gene was

identified. The DNA isolated per mL of saliva was reported to be ~19.2 μg [184]. Afterward, the methods and technology started to be developed for the analysis of DNA extracted from saliva, such as fluorescence-based sequence detection using real time PCR to identify single nucleotide polymorphisms [185]. The DNA obtained from saliva was recently reported to serve as a good tool for investigating oral and systematic disorders, genome wide studies and forensic testing. The DNA yield from saliva ranges from 11 to 46 μg per mL with a human DNA percentage of 37–77%. Using commercially available kits, saliva DNA collection is an easy process that obtains a sufficient quality and quantity. Large genetic studies have been performed due to ease of handling of the sample and less or no hesitancy in participation from the population [186]. For example, a DNA self-collection kit was tested for the quality of DNA obtained from the saliva of more than 4000 subjects. Storage for even a few months did not significantly reduce the quality of DNA in the sample. A good average yield of 83 μg per mL was also obtained [187]. The genome-based studies targeting the methylation of DNA are considered highly informative about the gene expression pattern. This methylation pattern in turn is a marker for a particular abnormality in the body. Molecular biology techniques specifically designed to analyze DNA and RNA are being developed in a reasonably simple manner. PCR, real time PCR, and DNA sequencing with informatics tools are the main platforms for genomics. Starting from DNA isolation, bisulfite conversion, amplification and fragmentation of the genome, almost all the steps are performed with kits. Afterwards, bead chips and other arrays are applied for sequencing, methylation and epigenetic studies. Sanger sequencing is currently the gold standard for routine sequencing and has also been used to validate high-throughput sequencing data obtained from next-generation sequencing (NGS) instruments. The raw data from the sequencer is analyzed using computational tools. Genomics investigations have been conducted to determine the suitability of DNA extracted from saliva to identify epigenetic biomarkers of schizophrenia and bipolar disorder, and positive results showing alterations at the DNA level have been reported [188,189]. With the advances in molecular biology techniques, methylated DNA immunoprecipitation (MeDIP) has been used to isolate DNA fragments that are methylated with antibodies against 5-methyl cytosine. After isolation, the methylated DNA is subjected to DNA microarrays or next-generation sequencing to analyze the methylome of a particular DNA sample. This method has been used to extract DNA from blood and saliva, to compare the results and to confirm the utility of saliva as

an alternate to blood for DNA methylation studies. The data obtained from saliva displayed a similar pattern and quality to the data obtained from peripheral blood [190]. Based on these findings, saliva genomics has been established as an alternative to blood-derived genomics in almost all aspects. The minor differences observed are attributed to the cell composition. Blood cells and the cells in the oral cavity, specifically the bacterial cells, contribute to the total DNA content extracted from the respective samples. However, the use of collection kits for saliva and DNA extraction kits have significantly decreased the bacterial DNA content compared to other methods of saliva collection, such as swabs and mouthwashes. Thus, saliva genomics would be extensively used for saliva diagnostics in the near future.

7.3 Transcriptomics of saliva

The translation of an mRNA molecule results in protein production, and the single stranded mRNA is degraded soon after translation. The fragile nature of RNA makes it less likely to be present in fluids such as saliva. Moreover, the stabilizing agent used to detect RNA in saliva may exert some additional effects, leading to damage or degradation [191,192]. However, the construction of a cDNA library from pooled saliva samples collected from 10 healthy individuals revealed the presence of RNA in saliva [193]. Another study had reported the RNA profile using microarray technology [194]. These studies mentioned the presence of 117 and 185 molecules of mRNA, respectively. As mentioned above, the fragile nature of RNA molecules increases the difficulty of their detection. The property that has been exploited for mRNA capture and amplification is the poly A tail at the 3′-end. However, if the mRNA is degraded, then the capture of the poly A tail would be reduced and only a small percentage of the available RNA would be amplified, producing partial results. The presence of all exons in the mRNA was considered, and an all exon array-based approach was used to identify the salivary transcriptome of 851 mRNA molecules in 18 healthy subjects [195]. After the presence of mRNA was investigated in saliva, the differential expression of mRNA in patients with disorders such as oral cancer, in whom the saliva might be in direct contact with lesions, was conducted. The study reported at least 7 mRNA transcripts, namely, IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT, as potential biomarkers [196]. Importantly, analyses of mRNA, small noncoding RNA or microRNA (miRNA) and long noncoding RNA (lncRNA) samples were conducted using saliva, and number of potential RNA molecules as differentially

expressed in patients with oral cancer. One study reported 658 lncRNAs that were differentially expressed between saliva samples from healthy subjects and patients with the disease, of which 14 were validated by three independent datasets for significant expression. Moreover, 8 of those 14 lncRNAs were reported for the first time [197]. Alterations in miRNA expression are a common finding in patients with OSCC. These alterations were found to play critical role as either tumor suppressors or oncogenes. However, a comprehensive miRNA profile, even from exosomes derived from carcinoma cells, in vitro with a large number of specimens from patients with OSCC is lacking but might provide information about expression at specific stages of the disease [198,199]. Similar to the DNA analysis, RNA extraction, isolation, conversion, amplification, sequencing and other downstream experimental analyses are performed using kits and subsequently measured using microarrays, sequencing machines and PCR machines with computational tools required for the analysis of the data obtained. Systemic diseases, such as lung cancer, pancreatic cancer and diabetes, have been studied by analyzing the salivary transcriptome, resulting in mRNA biomarkers displaying significantly different expression between patients and healthy controls when measured in combination [86,200,201]. The transcriptome from saliva has been characterized in detail, with differences in sample collection and analytical procedures and approaches. The basis of the biomarker obtained from transcripts in saliva or any other body fluid is based on a combination of mRNA molecules rather than a single mRNA. If we consider saliva transcriptomics, the determination of the appropriate method for diagnosis, prognosis and therapeutics still requires large population-based studies using a set of approaches that is more likely to be the next phase of transcriptomics.

7.4 Metabolomics of saliva

In addition to macromolecules such as proteins, DNA and RNA, saliva contains a large number of small molecules or metabolites. Two types of studies have been conducted to characterize the salivary metabolome: studies involving a single analytical platform and studies involving a multi-platform analysis (Table 2). An investigation of saliva metabolites utilizing proton and carbon-13 nuclear magnetic resonance spectroscopy (H^1 and C^{13} NMR spectroscopy) resulted in the identification of >60 metabolites in saliva, as well as molecules of different origins, in 20 patients with no oral disease who were visiting the dental clinic for regular dental care. The components were organic acids and malodorous amines, and the concentrations of 9 of 11 the components monitored varied between subjects [136]. Using the

Table 2 Metabolites identified utilizing various analytical platforms.

Platform	Metabolites
NMR	Acetic acid, acetoacetic, butyric, citric, formic, glycolic, lactic, propionic and pyruvic acids, valeric acid, acetoacetic acid, L-alanine, D-glutamic acid, L-methionine, L-valine, L-threonine, L-tyrosine, L-leucine, and L-phenylalanine, L-proline, ethanol, ethanolamine, sorbitol, dimethylamine, and methylamine, putrescine with mono-, di- and trimethylamine, D-glucose, D-galactose, propylene glycol, caffeine, acetone, creatinine
GC/MS[a]	Hydroxyisobutyric acid, hydroxyisovaleric acid, formic acid, fumaric acid, phosphoric acid, malic acid, nicotinic acid, palmitic acid, phenylacetic acid, stearic acid, isopropyl alcohol
LC/MS/MS	Acylcarnitines, amino acids, biogenic amines, phosphatidylcholines, sphingomyelins, lysophosphatidylcholines and hexose/glucose
HPLC UV/FD	Vitamins, thiols and nucleotides
ICP MS	Aluminum, arsenic, barium, boron, calcium, cerium, cesium, chromium, cobalt, copper, gallium, germanium, hafnium, iron, lanthanum, lead, lithium, magnesium, manganese, molybdenum, neodymium, nickle, niobium, palladium, phosphorus, platinum, potassium, rubidium, selenium, sodium, strontium, tellurium, thallium, tin, titanium, tungsten, vanadium, yttrium, zinc, zirconium

[a]Metabolites detected by GC/MS alone.
Adapted from Valdinete Alves do Nascimento, João Hugo Abdalla Santos, Dana Cristina da Silva Monteiro, Karina Pinheiro Pessoa, Antonio José Leão Cardoso, Victor Costa de Souza, et al., Oropouche virus detection in saliva and urine, Mem. Inst. Oswaldo Cruz. 115 (2019) e190338. https://doi.org/10.1101/758839.

NMR spectroscopy technique, the nuclear spins of isotopic elements, such as hydrogen, carbon, and nitrogen, are measured. The measurements of the individual spins, couple spins and relative spins provide insights into the structure of molecule. Another study of healthy population analyzed under a variety of conditions using proton NMR identified at least 10 metabolites showing different levels under different conditions [137]. A similar study was conducted to investigate the relationship of the circadian cycle with the metabolome using blood and saliva as samples and GC or LC MS as the platform. Generally, the amino acid content in saliva and fatty acid content in plasma of approximately 15% of the total metabolites showed correlations with the circadian cycle [202]. Gas chromatography differs from liquid chromatography because the mobile phase is a carrier gas rather than a solvent that passes through the stationary phase (column) for the separation. The components eluted from the column enter the mass spectrometer for

detection. Liquid chromatography mass spectrometry with a time of flight analyzer has also been applied to saliva metabolomics, while optimizing the sample preparation protocol. One hundred eight different metabolites were identified in acidic and basic extracts of saliva samples, including amino acids, polyamines, lipids, antioxidants, vitamin B3, and ethyl-phosphate [203]. Capillary electrophoresis mass spectrometry was also applied to study the changed in the saliva metabolome in response to physiological and environmental factors [204]. Capillary electrophoresis separates the sample analytes based on charge as they migrate through an electrolyte solution in a fused silica capillary under an applied electric field. The masses of analytes eluting from the capillary are then detected using MS. A multiplatform analysis of saliva together with computer-assisted literature mining resulted in the identification of more than 700 metabolite species.

The alteration of metabolites in different disorders has been explored, and a number of small-molecule panels have been identified. Saliva and tumor tissues from patients with oral cancer were analyzed, and a combination of two metabolites, *S*-adenosylmethionine and pipecolate, resulted in the most significant discrimination between patients and healthy controls [205]. In patient patients with periodontal disease, the results obtained from three different platforms revealed increased levels of dipeptides, monosaccharides and fatty acids, indicating an expansion of the microflora in the oral cavity [206]. In a recent report on Alzheimer's disease metabolomics of saliva, a panel composed of amino-dihydroxybenzene, glucosylgalactosyl hydroxylysine and amino butyric acid molecules was validated to differentiate patients with Alzheimer's disease from subjects with a mild cognitive impairment. Meanwhile, a glucosylgalactosyl hydroxylysine and glutamine carnitine panel was used to discriminate between subjects with a mild cognitive impairment and cognitively normal individuals. The study adopted isotope labeling ultra-performance liquid chromatography (UPLC) and LC MS to generate the metabolite libraries [207]. An earlier study predicted the utility of cytidine and sphinganine-1-phophate as biomarkers in saliva for the conversion of a mild cognitive impairment to Alzheimer's disease [208]. A number of studies utilizing more than one platform of analysis with computational tools or an advanced single platform have explored the salivary metabolome in patients with a variety of ailments, such as aphthous ulcer [35], or even to evaluate sports performance [209]. Thus, panels of metabolite biomarkers identified from saliva may achieve the specificity required for their application as a true diagnostic fluid, if the validity and reliability are confirmed.

7.5 Glycomics and microbiomics of saliva

Saliva is rich in glycoproteins, and like all other contents of saliva, the composition and content of glycoproteins also change. Investigations of the N-linked (asparagine-linked) or O-linked (serine/threonine-linked) glycoproteins in saliva have been conducted. The glycoproteins are separated using affinity-based methods, such as lectin-based columns or hydrazide chemistry. After de-glycosylation and trypsin digestion, the proteins are identified using LC MS/MS. The glycomic studies conducted with saliva showed variations in both O-linked and N-linked glycoproteins between groups stratified by the time of day, gender and age [210,211]. A recent study was conducted to evaluate the N-/O-linked glycoproteins in patients with atrophic gastritis and gastric cancer and revealed alterations in fucosylated N-/O-linked glycans in saliva [212]. The list of glycomics analyses of saliva would follow the same pattern as already discussed in other omics fields.

The microbial flora of the oral cavity has even been explored. The microbiome studies utilize the 16S rRNA or DNA probe to identify the microbial species. Several studies have been conducted to investigate the changes in the microbiome in saliva. According to one study, the shared environment is the dominant factor affecting the composition of the saliva microbiome, rather than shared genetics [213]. Similarly, the oral flora was affected in patients with oral and systematic disorders, as the abundance of disease-associated taxa and health-associated taxa decrease or increase in disease or health conditions [43,214]. The presence of a high sugar level in saliva has been shown to significantly affect the oral microbiota, resulting in an overall reduction in the abundance and alterations in the composition bacterial species [36]. Obese individuals with health periodontium presented a low bacterial diversity. The genera of abundant bacteria include *Catonella*, *Granulicatella*, *Mogibacterium*, *Peptostreptococcus*, *Prevotella*, and *Solobacterium*, while the genera *Corynebacterium*, *Capnocytophaga*, *Haemophilus*, and *Staphylococcus* were less abundant [37,215]. A reduction in the relative abundance of the genus *Actinobacteria* has been observed in saliva samples from patients with polycystic ovarian syndrome [216]. Advances in molecular microbiology will continue to provide an exceptional opportunity to translate the vast diversity of the oral microbiome to the diagnosis of a number of conditions. Future studies will target large numbers of samples and use advanced computational tools to excavate the maximum information about the microbiome of the oral cavity and its correlations with the respective disorder.

8. Conclusions

The combination of different omics fields represents the future of salivary diagnostics. These fields have already transformed various approaches, such as risk evaluations, screening, and therapeutic management, for a variety of biomedical applications. Most probably, saliva omics will lead to the development of more individualized treatments for use at the initial stages of any disorder, minimizing the progression to advanced stages. Salivary diagnostics are predicted to be refined to assess various diagnostic biomarkers in the future. In the present article, we compiled a description of oral fluids, omics and the future uses of biomarkers detected in these fluids to diagnosis and maintain oral health.

Conflict of interest

All authors declare no conflict of interest.

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