Review

Zhijie Huang, Xiaoxia Yang, Yisheng Huang, Zhengming Tang, Yuanxin Chen, Hongyu Liu, Mingshu Huang, Ling Qing, Li Li, Qin Wang, Zhuye Jie, Xin Jin* and Bo Jia*

Saliva – a new opportunity for fluid biopsy

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Abstract: Saliva is a complex biological fluid with a variety of biomolecules, such as DNA, RNA, proteins, metabolites and microbiota, which can be used for the screening and diagnosis of many diseases. In addition, saliva has the characteristics of simple collection, non-invasive and convenient storage, which gives it the potential to replace blood as a new main body of fluid biopsy, and it is an excellent biological diagnostic fluid. This review integrates recent studies and summarizes the research contents of salivaomics and the research progress of saliva in early diagnosis of oral and systemic diseases. This review aims to explore the value and prospect of saliva diagnosis in clinical application.

Keywords: biomarkers; oral diseases; salivaomics; salivary diagnosis; systemic diseases.

Introduction

Traditionally, doctors usually need to issue a large number of tests, such as blood, urine and fecal examinations, to assist in the diagnosing and determining the type of disease. For some more complex diseases, the diagnosis needs to be identified by pathologists through the diseased tissues obtained by surgery, puncture or extraction, which is also the gold standard for diagnosing most tumor diseases [1, 2]. However, tissue biopsy also has many disadvantages. First of all, it needs to cut or extract part of the tissue from the patient, which will cause some trauma to the patient. In addition, it may be necessary for some diseases to dynamically detect the progress and evolution, while repeated tissue biopsies are unrealistic. Finally, the differential diagnosis of pathological sections is a great challenge to the ability of pathologists, which usually requires numerous and rigorous training to achieve a higher level [3–5] (Tables 1–3).

Fluid biopsy detects and analyses various biological body fluids instead of tissue biopsy. Currently, most of the fluid biopsies take blood as the main test object, and most diseases tested are tumors. High-throughput technology is used to detect circulating tumor cells, circulating free DNA, circulating tumor DNA and exosomes in peripheral blood, and to evaluate genomics, transcriptome and proteomics to provide an auxiliary diagnosis for diseases [108, 109]. However, blood samples are usually collected by venipuncture or other invasive methods, which will cause pain and inconvenience to patients [110]. Meanwhile, the operation difficulty and cost of the blood testing platform are on the high side. The storage and transportation of blood samples also need considerable conditions, limiting the promotion of blood-based fluid biopsy [111, 112]. Some studies have shown that other body fluids, such as saliva, cerebrospinal fluid, and pleural fluid, may contain more biological information than blood and contain many reliable markers, which can be used to detect various diseases [113, 114]. It suggests that non-blood derived fluid biopsy has considerable application value, broadening the concept of fluid biopsy. In the non-blood derived body fluids, saliva has the advantages of non-invasive and cost-effective, but also contains a wide range of proteins, DNA, RNA, various

Zhijie Huang and Xiaoxia Yang contributed equally to this work.

***Corresponding authors: Xin Jin**, BGI Genomics, BGI-Shenzhen, Shenzhen, P.R. China; and School of Medicine, South China University of Technology, Guangzhou, P.R. China, E-mail: jinxin@genomics.cn; and **Bo Jia**, Department of Oral Surgery, Stomatological Hospital, Southern Medical University, South Jiangnan Road 366, Guangzhou, 510280, P.R. China, E-mail: dentist-jia@163.com
Zhijie Huang, Yisheng Huang, Zhengming Tang, Yuanxin Chen, Hongyu Liu, Mingshu Huang, Ling Qing, Li Li and Qin Wang, Department of Oral Surgery, Stomatological Hospital, Southern Medical University, Guangzhou, P.R. China. https://orcid.org/0000-0002-7312-4982 (Z. Huang)
Xiaoxia Yang, Department of Endodontics, Stomatological Hospital, Southern Medical University, Guangzhou, P.R. China
Zhuye Jie, BGI Genomics, BGI-Shenzhen, Shenzhen, P.R. China; Shenzhen Key Laboratory of Human Commensal Microorganisms and Health Research, BGI-Shenzhen, Shenzhen, P.R. China; and Laboratory of Genomics and Molecular Biomedicine, Department of Biology, University of Copenhagen, Copenhagen, Denmark

Table 1: Part of salivary biomarkers for oral disease.

Disease	Methodology	Biomarkers	Sensitivity, %	Specificity, %	AUC	Ref
Periodontitis	Genome	Global 5-methylcytosine methylation	–	–	1.00	[6]
	Transcriptome	miRNA-155	86.7	78.6	0.861	[7]
		hsa-miR-125a-3p	–	–	1.00	[8]
	Proteome	MMP-9, S100A8	85.0	76.0	0.860	[9]
		IL-1β, MMP-8, ICTP	90.3	84.0	0.929	[10]
		IL-1β, IL-1ra, MMP-9	73.3	88.9	0.853	[11]
		Del-1, IL-17, LFA-1	–	–	0.893	[12]
		MUC4, MMP-7	–	–	0.931	[13]
		C3	–	–	0.910	[14]
		C3c	–	–	0.840	
		IL-1β	78.0	100.0	0.960	[15]
		PGE2	78.0	91.0	0.920	
		IL-6	78.0	75.9	0.830	[16]
		MMP-8	65.3	66.7	0.728	
		MIP-1α	66.3	67.6	0.723	
		ARPC5, CLUS	–	–	0.970	[17]
		DMTB1, RAP1A			0.950	
		DMTB1, MMP-9			0.940	
	Metabolome	Cadaverine, 5-oxoproline, histidine	–	–	0.881	[18]
	Microbiome	*Tannerella forsythia, Eikenella corrodens,* *Porphyromonas gingivalis, Peptostreptococcus* *anaerobius, Treponema denticola*	93.0	79.0	0.940	[19]
		Prevotella intermedia, Catonella morbi, Porphyromonas pasteri, Prevotella nanceiensis, *Haemophilus parainfluenzae*	–	–	0.973	[20]
Sjögren's syndrome	Transcriptome	miR-17-5p, let-7i-5p			0.969	[21]
		MNDA, FCGR3BL, GBP-2	96.0	73.0	0.860	[22]
	Proteome	Neutrophil elastase, calreticulin, tripartite Motif-containing protein 29	–	–	0.974	[23]
		α-Enolase, β2m, cathepsin D	92.0	88.0	0.940	[22]
		Soluble siglec-5	69.0	70.0	0.774	[24]
		TRIM29	–	–	0.880	[25]
Oral lichen planus	Transcriptome	miR-27b, miR-21, miR-125a	–	–	–	[26, 27]
	Proteome	IFN-γ, IL-33, IL-4, CPR, soluble CD14, soluble TLR2	–	–	–	[28–31]
Oral leukoplakia	Proteome	CD44	91.6	72.7	0.712	[32]
		S100A7	81.8	72.7	0.744	
		S100P	81.8	54.5	0.760	
		IL-6	–	–	–	[33]
OSCC	Transcriptome	miR-106b-5p, miR-423-5p, miR-193b-3p	97.4	94.2	0.980	[34]
		MAOB, NAB2	92.0	86.0	0.910	[35]
		IL-1B, OAZ1, SAT, IL-8	91.0	91.0	0.950	[36]
		CCL20	100.0	98.0	0.892	[37]
		miR-30c-5p	86.0	74.0	0.820	[38]
		miR-24-3p	64.4	80.0	0.738	[39]
		miR-512-3p	–	–	0.847	[40]
		miR-412-3p	–	–	0.871	
		miR-27b	85.7	100.0	0.964	[41]
		miR-136	88.8	100.0	0.968	
		IL-6	94.5	81.9	0.937	[42]
	Proteome	Cathepsin V, kallikrein5、ADAM9	90.0	99.1	0.938	[43]
		MMP-1, cathepsin V, kallikrein 5, ADAM9	85.0	93.3	0.963	
		AHSG, KRT6C, KLK1, AZGP1	78.0	73.5	0.824	[44]

Table 1: (continued)

Disease	Methodology	Biomarkers	Sensitivity, %	Specificity, %	AUC	Ref
		KLK5, uPA	94.2	89.5	0.950	[45]
		PRDX-2, ZAG	100.0	98.77	0.999	[46]
		IL-6, IL-8, TNF-α, MCP-1, HCC-1, PF-4	95.4	88.0	0.980	[47]
		M2BP, MRP14, CD59, catalase, profilin	90.0	83.0	0.930	[48]
		IL8, IL1β	–	–	0.817	[49]
		SLC3A2, S100A2, IL1RN	83.3	83.3	0.890	[50]
		MMP1, KNG1, ANXA2, HSPA5	87.5	80.5	0.910	[51]
		CFAH, AFAM, GELS, SAMP, VTDB	93.4	96.5	0.979	[52]
		Naa10p, CEA	92.5	85.0	0.944	[53]
		Transferrin	–	–	0.950	[54]
		Chemerin	100.0	100.0	1.000	[55]
		MMP-9	100.0	100.0	1.000	
		CFH	–	–	0.661	[56]
		FGA	–	–	0.740	
		SERPINA1	–	–	0.740	
		MMP-1	78.0	88.1	0.898	[57]
		THBS2	55.1	89.4	0.756	[58]
	Metabolome	Ornithine, *o*-hydroxybenzoate, R5F	–	–	0.869	[59]
		627 common peaks	–	–	0.991	[60]
		Indole-3-acetate, ethanolamine phosphate	–	–	0.856	[61]
		Choline, betaine, pipecolinic acid, L-carnitine	100.0	96.7	0.997	[62]
		L-phenylalanine, L-leucine	92.3	91.7	0.871	[63]
		Propionylcholine, acetylphenylalanine, sphinganine, phytosphingosine, S-carboxymethyl-L-cysteine	100.0	96.7	0.997	[64]
	Microbiome	*Actinobacteria, Fusobacterium, Moraxella, Bacillus, Veillonella*	–	100.0	0.957	[65]

metabolites and microflora, which can be used as molecular biomarkers for early detection, monitoring of diseases, and guiding individual and precision therapy [115, 116].

In this article, we review the research related with salivary diagnostics, including the content of salivaomics, the techniques and processes related to saliva detection samples, and the studies of saliva detection in various diseases (Figures 1 and 2).

Saliva

Saliva is mainly secreted by three major glands in the oral cavity (parotid gland, submandibular gland and sublingual gland), and the minor salivary glands distributed in the oral mucosa also secreted. Additionally, gingival crevicular fluid, oral mucosal exudate, leukocytes, epithelial cells and many microorganisms are involved in the composition of saliva [110, 117]. The acini of the salivary glands are highly permeable and surrounded by rich capillaries. Small molecules in the blood circulation can infiltrate into the acini and eventually become a part of saliva. Therefore, the electrolyte composition of saliva is similar to that of plasma ultrafiltrate [118]. Saliva is a foamy, slightly cloudy, milky hypertonic solution, slightly acidic (pH=6.6–7.1), odorless, with a specific gravity of 1.004–1.009, 94–99% composed of water, and also contains a small portion of organic compound (such as mucin, globulin, salivary amylase, uric acid, lysozyme, lactic acid, lactoferrin, cortisol and cytokines) and some inorganic compound (such as sodium, potassium, calcium, chloride, thiocyanate, bicarbonate and phosphate) [110]. The saliva secreted by the gland is different from each other. The parotid gland secretes serous saliva, while the sublingual gland produces mucinous saliva. The submandibular gland is a mixed gland, but mainly secretes mucinous saliva. Healthy adults can produce 1.0–1.5 L saliva per day, with an average flow rate of 0.3–0.5 mL per min [119]. Saliva secretion is affected by many factors. Smell, taste, age, mental state, oral hygiene, drugs and body exercise can stimulate saliva secretion [120]. Saliva has many functions: 1. Saliva can wash away food residues and microbes in the mouth, thus helping to clean the mouth; 2.

Table 2: Part of salivary biomarkers for malignant disease.

Disease	Methodology	Biomarkers	Sensitivity, %	Specificity, %	AUC	Ref
Pancreatic cancer	Transcriptome	miR-3679-5p, miR-940	70.0	70.0	0.763	[66]
		KRAS, MBD3L2, ACRV1, DPM1	90.0	95.0	0.971	[67]
		has-miR-21	100.0	71.0	–	[68]
		has-miR-23a	100.0	86.0	–	
		has-miR-23b	100.0	86.0	–	
		has-miR-29c	100.0	57.0	–	
	Proteome	Cytokeratin-14, lactoperoxidase, cytokeratin-16, cytokeratin-17, peptidyl-prolylcis–transisomerase B	90.0	90.0	0.910	[69]
	Metabolome	Alanine, N1-cetylspermidine, 2-oxobutyrate, 2-hydroxybutyrate	–	–	0.887	[70]
		Leucine, isoleucine, valine, tryptophan, glutamic			0.930	[71]
		Acid, phenylalanine, glutamine, aspartic acid				
	Microbiome	*Neisseria elongate, Streptococcus mitis*	96.4	82.1	0.895	[72]
Gastric cancer	Transcriptome	SPINK7, PPL, SEMA4B, miR-140-5p, miR-301a	75.0	83.0	0.810	[73]
	Proteome	CSTB, TPI1, DMBT1	85.0	80.0	0.930	[74]
Breast cancer	Transcriptome, Proteome	CSTA, TPT1, IGF2BP1, GRM1, GRIK1, H6PD, MDM4, S100A8, CA6	83.0	97.0	0.920	[75]
	Proteome	CA125	62.5	59.4	0.686	[76]
		sFAS	72.5	61.1	0.676	
		BS-I, NPA	73.3	74.2	0.755	[77]
		VEGF, EGF	83.0	74.0	0.840	[78]
		15 free amino acid profile	88.2	85.7	0.916	[79]
		Sialic acid	80.0	93.0	0.950	[80]
	Metabolome	PG14:2	65.2	77.1	0.732	[81]
		Spermine	–	–	0.766	[82]
		LysoPC(18:1)	77.8	100.0	0.920	[83]
		LysoPC(22:6)	81.5	91.7	0.920	
		MG(0:0/14:0/0:0)	92.6	91.7	0.929	
Lung cancer	Genome	EGFR 19-del	–	–	0.940	[84]
		EGFR 21-L858R			0.960	
	Transcriptome	CCNI, EGFR, FGF19, FRS2, GREB1	93.7	82.8	0.925	[85]
	Proteome	Calprotectin, zinc α2-glycoprotein, haptoglobin hp2	88.5	92.3	0.900	[86]
		IL-10, CXCL10	60.6	80.8	0.701	[87]
	Metabolome	Catalase, triene conjugates, schiff bases, pH, sialic acids, alkaline phosphatase, chlorides	69.5	87.5	0.803	[88]
	Microbiome	*Capnocytophaga, Veillonella*	84.6/78.6	86.7/80.0	0.860/0.800	[89]
		Veillonella, Streptococcus	–	–	–	[90]

Various enzymes in saliva can decompose carbohydrates and lipids, thus promoting the digestive process; 3. In saliva, lysozyme, immunoglobulin and thiocyanate have antibacterial and bactericidal effects, which are important components of human non-specific immune system; 4. The inorganic compound in saliva play an essential role in preventing enamel demineralization and promoting remineralization, as same as maintaining the dynamic balance of enamel [121].

Saliva, as a part of the endocrine system, is closely related to the blood, and most of the components are permeated from the blood. Components in the blood can enter saliva by diffusion, active transport or extracellular ultrafiltration [122–124]. Small molecules can be passively diffused from the blood to acinar cells, which are affected by the size and charge of these molecules. For example, steroids are made up of fatty acids and have a smaller volume, making it easier for them to spread to the acini [125]. On the other hand, proteins in the blood can be actively transported by ligand-receptor binding. For instance, IgA secreted by B cells can be released into saliva by binding to receptors on the acini [126, 127]. In addition, biomolecules in the blood can enter saliva by ultrafiltration. Steroids can migrate through the gap between acini and ductal cells, and molecules smaller than 1900 Da (such as water, catecholamines, ions) can be

Table 3: Part of salivary biomarkers for systemic disease.

Disease	Methodology	Biomarkers	Sensitivity, %	Specificity, %	AUC	Ref
Acute myocardial infarction	Proteome	CPR	–	–	–	[91]
		CK-MB	–	–	–	[92]
		Tn1	–	–	–	[93]
Diabetes	Proteome	S100A9, PRP-1/PRP3	–	–	–	[94]
	Metabolome	$1,5\text{-AG}_0$	63.5	60.6	0.657	[95]
		$1,5\text{-AG}_{120}$	62.2	60.4	0.660	
Rheumatoid arthritis	Proteome	Calgranulin A, calgranulin B, apolipoprotein A-1, 6-phosphogluconate dehydrogenase, peroxiredoxin 5, epidermal fatty acid-binding protein, GRP78/BiP, 14-3-3 proteins	–	–	–	[96]
Alzheimer's disease	Proteome	Lactoferrin, amyloid-β 42, P-tau	–	–	–	[97–99]
	Microbiome	*Porphyromonas gingivalis, Veillonella parvula*	–	–	–	[100]
Parkinson's disease	Transcriptome	miR-153	81.8	71.4	0.790	[101]
		miR-223	72.7	71.4	0.770	
	Proteome	α-Synuclein, DJ-1	–	–	–	[102]
		Heme oxygenase-1	75.0	70.0	0.760	[103]
Stress	Proteome	α-Amylase, cortisol	–	–	–	[104]
Depression	Proteome	Cortisol, s-IgA	–	–	–	[105, 106]
Anxiety	Proteome	Cortisol, s-IgA	–	–	–	[106, 107]

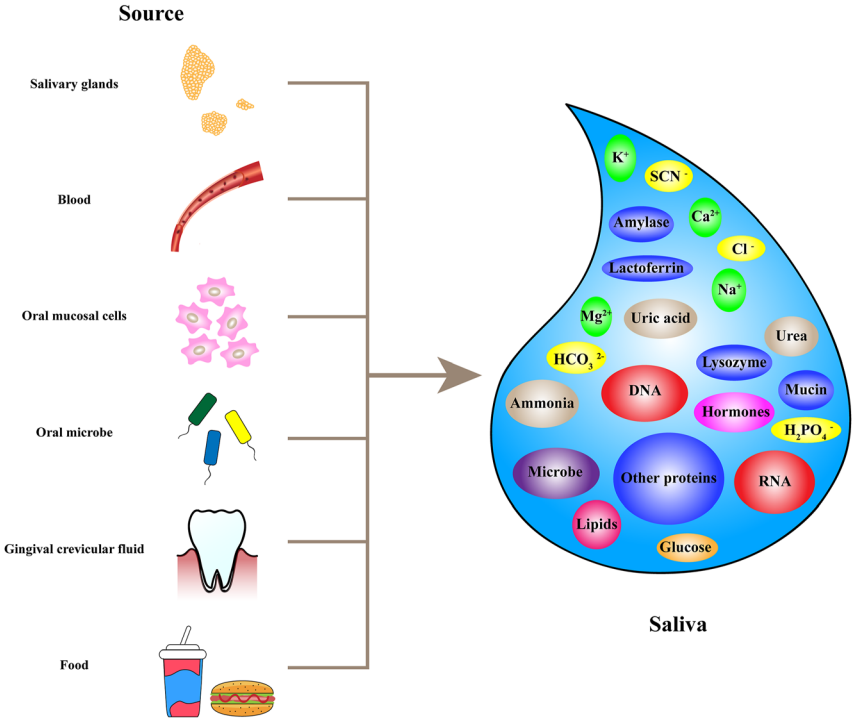

Figure 1: The source and composition of saliva. Saliva is mainly secreted by salivary glands. Blood, oral mucosa cells, gingival crevicular fluid and food are also the importance source of saliva, providing rich informative biomolecular. Saliva is a kind of biological fluid containing DNA, RNA, proteins, inorganic substance, and metabolite.

transferred through gap junctions between secretory units [128]. Some studies have shown that disease-related biomolecules in the blood can eventually enter saliva [110, 129, 130]. Therefore, saliva, like blood, can be used as a "mirror" to reflect the physiological and pathological state of the body. Moreover, unlike blood, saliva collection is a non-invasive operation, its transportation and preservation are also quite convenient, and the risk of

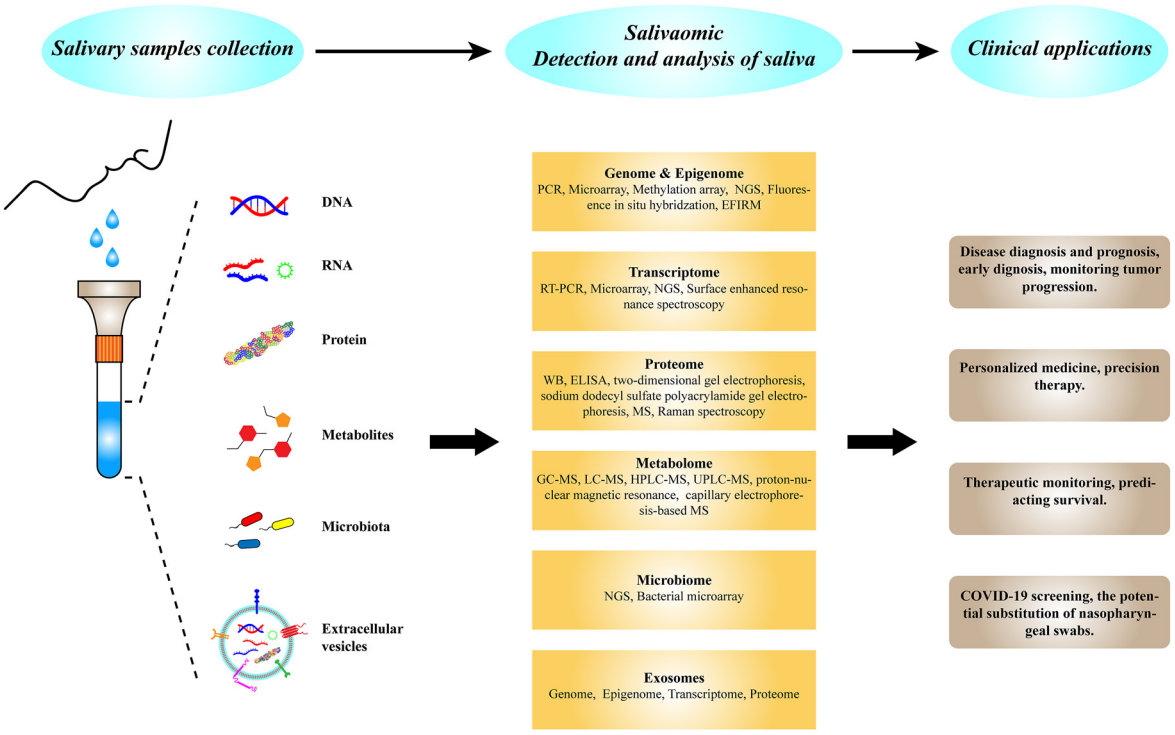

Figure 2: Process and application of salivary diagnosis.

cross-contamination is relatively low. All these are the advantage of saliva as a diagnostic fluid. However, compared with blood, the content of biomolecules in saliva is lower, and the microbial environment in saliva is more complex, which will have an unpredictable impact on the detection results. Nevertheless, the construction and development of a highly sensitive detection platform will gradually solve these problems, and saliva testing may also become a new opportunity for disease screening, detection and surveillance [131].

Salivaomics

Saliva contains various biological components, including DNA, RNA, proteins, microorganisms and metabolites, which are potential biomarkers. Biomarkers can reflect the physiological and pathological state of the body, which is an important basis of personalized medicine. Therefore, a comprehensive analysis and identification of various components in human saliva will greatly help us to develop biomarkers related to human health and disease status, early identification of diseases, assessment of disease prognosis and risk, and monitoring the effect of treatment [110]. Salivaomics is a broad collection of groups used to explore different types of components in saliva and their significance in health and disease states, which include genomics, epigenomics, transcriptomics, proteomics, metabolomics and microbiomics [132]. The research objects of genomics and epigenomics are the biochemical characteristics of DNA, genes and their methylation modification. Transcriptomics investigate RNA in cells and organs, including mRNA and non-coding RNA. Proteomics is the methods to detect the protein profile of the target. Metabolomics focuses on the overall metabolic profile of the samples. Microbiomics study the diversity of microbial flora and its relationship with the body during the development of diseases. Salivaomics will provide us with a complete and profound saliva profile, so we can better discover and develop more accurate biomarkers and contribute to precision medicine.

The genome and epigenome of saliva

The saliva genome contains human and microbial DNA, about 70% of which comes from humans, while the rest from microbiota in the mouth [133]. DNA in the saliva is of good quality and can be preserved for a long time with a low degradation rate. Abraham et al. [134] evaluated the quality of DNA in blood and saliva by measuring absorbance ratios at 260 and 280 nm (A260/A280). The results show that the average value in the saliva is 1.56, and blood is 1.71, indicating the DNA quality of saliva is comparable to the blood. In quantity, the total amount of DNA in the saliva is about

12 μg/mL, ranging from 0.1–26 μg/mL, nearly half of the total DNA in blood (6–73 μg/mL, with an average of about 26 μg/mL). The quantity of DNA in saliva can meet the requirements of chip genotyping but also be used for sequencing arrays and polymerase chain reaction analysis. Epigenetics means that when the DNA sequence of the gene does not change, the gene function changes heritable, and finally leads to the phenotypic change. DNA methylation is the covalent binding of a methyl group on the cytosine base in the genomic CpG island under the action of DNA methyltransferase. It is a common epigenetic process and the most well-studied epigenetic modification [135]. DNA methylation can cause changes in chromatin structure, DNA conformation, DNA stability and the interaction between DNA and protein, thus controlling gene expression, which is necessary to maintain cell growth and metabolism [136, 137]. With the passage of time, or environmental exposure, aberrant methylation of genes may appear, leading to the occurrence and development of the disease, especially in the tumor oncogenesis [138].

Recently, the detection and analysis techniques of saliva genome and epigenome are various and more accurate, such as DNA sequencing, methylation array, and quantitative PCR genotyping, showing excellent effects in detecting many diseases. Carvalho et al. [139] used the quantitative methylation-specific PCR to measure the tumor suppressor gene promoter in the saliva of patients with head and neck squamous cell carcinoma (HNSCC) before treatment. It showed that about 54% of patients occur methylation of more than one selected gene in saliva DNA. In addition, patients with hypermethylation in saliva DNA presented lower local disease control and overall survival. Schussel et al. [140] analyzed the methylation status of 9 genes related to HNSCC in saliva of 191 patients by quantitative methylation-specific PCR, compared with the risk assessment made by expert clinicians. It showed that the methylation of endothelin receptor type B (ENDRB) and deleted in colorectal cancer (DCC) in saliva was associated with premalignant or malignant oral cancer lessions. The sensitivity, specificity and area under the curve (AUC) of the ENDRB and DCC combined diagnosis were 46%, 72% and 0.60, respectively, while the clinical risk classification (CRC) evaluated by expert clinicians were 56%, 66%, and 0.61. Furthermore, the optimal AUC (0.67) occurred in the combination of ENDRB, DCC and CRC. The results suggested that the methylation levels of ENDRB and DCC in the saliva is comparable to the CRC of expert clinician, which is of considerable guiding significance. Obviously, the genome and epigenome of saliva can provide many vital information for diagnosis of disease. However, it cannot afford information about the up-regulation or down-regulation of gene expression, nor can it reflect the expression or deletion of specific genes and gene mutations.

The transcriptome of saliva

The mRNAs and non-coding RNAs (such as microRNAs, piRNAs and circular RNAs) produced by cells consist of the transcriptome of saliva and flow into the oral cavity from various sources, such as exfoliated oral epithelial cells, gingival crevicular fluid and salivary glands [129]. Abnormal expression of specific mRNAs or non-coding RNAs can be detected in many diseases, indicating that transcriptome analysis has a certain value in disease diagnosis.

Microarray analysis and high-throughput sequence analysis are the main technologies for studying the transcriptome. Li et al. [141] first discovered the human transcriptome of saliva through the microarray technology. They reported the salivary mRNA biomarkers detected in the malignant tumors and systemic disease, indicating high-throughput technology is promising in analysing salivary transcriptome. Gomes et al. [142] reported that the mRNA expression of interferon-γ (IFN-γ) was correlated with the severity of periodontitis in saliva samples of patients with chronic periodontitis and type 2 diabetes. Hu et al. [22] found that 3 mRNA biomarkers in saliva samples of patients with primary Sjogren syndrome were significantly higher than those of patients with systemic lupus erythematosus and healthy subjects. Some studies have found a simple and direct way to stabilize saliva mRNAs at room temperature without further processing [143, 144]. However, mRNA is still not a good candidate for salivary transcriptome analysis due to its highly fragmented under decomposition of RNA enzyme, and is easy to mix with bacterial RNA. Therefore, most salivary transcriptome studies are focused on the analysis of non-coding RNAs, which are the new regulatory factor with a variety of biological functions, and play an important role in cell development, differentiation, tumorigenesis and development. Compared with mRNA, the non-coding RNA (especially microRNA) in saliva is abundant, stable and shorter sequence, so it is not easy to be degraded by RNA enzyme and is more suitable for hybridization analysis with oligonucleotides on biosensors [145]. The microRNAs participate in various biological processes, regulating cell differentiation, proliferation and survival, especially in cancers [146–148]. Park et al. [149] detected microRNAs in saliva of patients with oral squamous cell carcinoma and healthy controls by reverse transcription-pre-amplification-quantitative PCR. The results showed that the contents of miR-125a and miR-200a in saliva of patients with oral squamous cell carcinoma were significantly lower

than those of healthy controls. Xie et al. [66] found the potential biomarkers of resectable pancreatic cancer by detecting the microRNA in saliva. The combination of miR-3679-5p and miR-940 presents well discriminatory ability to detect resectable pancreatic cancer with acceptable specificity and sensitivity. With the development of high-throughput RNA sequencing analysis technology, it is possible to analyze the whole RNA sequence in saliva, and explore more potential biomarkers in the disease. Wong et al. [73] investigated the saliva extracellular RNA biomarkers in evaluating the gastric cancer. Saliva samples from 63 patients with gastric cancer and 31 patients without gastric cancer were analyzed by transcriptome sequencing. The results showed that there were 30 mRNAs and 15 microRNAs expression patterns associated with gastric cancer. Among them, the biomarker group composed of 3 mRNAs (SPINK7, PPL and SEMA4B) and 2 microRNAs (miR140-5p and miR301a) had reliable performance, which was significantly down-regulated in gastric cancer, and the AUC reached 0.81.

The proteome of saliva

The salivary proteome mainly studies the protein profile in the oral cavity. These proteins participate in different biological functions in the oral cavity and are the most important components in saliva. Saliva contains more than 2000 proteins produced by salivary glands and microorganisms in mucous membrane and oral cavity [150]. The proteins secreted by acinar cells of salivary glands account for more than 85% of salivary proteins, while the proteins secreted by glandular cells, such as growth factors and immunoglobulins, have important biological functions [151]. The proteolytic enzymes in oral cavity can decompose salivary proteins into small molecular proteins or peptides, which account for 40–50% of salivary proteins [152, 153]. The molecular weights of various proteins in saliva vary greatly. The existence of high molecular weight proteins such as amylase and mucin will interfere with the detection and identification of relatively low abundance proteins, which may be potential biomarkers. Therefore, the ability of salivary proteome technology to detect and identify low abundance proteins is very important. The commonly used proteomic techniques include western blotting (WB), enzyme-linked immunosorbent assay (ELISA), two-dimensional gel electrophoresis (2D-GE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), quantitative mass spectrometry (qMS), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and Raman spectroscopy (RS). Mass spectrometry (MS) is a special technique for the identification of compounds, providing structural information with high specificity and accuracy. MS has an excellent ability to measure peptides and small molecular proteins, and has become the core technology of proteomics, which can greatly improve the research progress of salivary proteome, broadening our understanding of saliva proteins. Moreover, combined with advance separation techniques, such as high-performance liquid chromatography (HPLC), small proteins and peptides in saliva can be analyzed qualitatively and quantitatively. Pappa et al. [154] identified more than 2000 proteins in the whole saliva by HPLC-MS.

At present, four main salivary proteins have been identified on the research of human salivary proteome, which are proline-rich proteins (PRPs), cystatins, statherins and histatins [155]. These proteins play a vital role in maintaining the integrity of teeth by regulating the balance of enamel demineralization and remineralization [156]. It has been found that about 27% of plasma proteins are present in saliva, which reflects the contribution of vascular leakage or fluid from interstitial compartment to saliva components [154, 157]. The main components of human plasma protein are immunoglobulin and albumin, which represent 60–80% of the total weight [158]. The 22 most abundant proteins in plasma account for 99% of the total protein content in plasma, while the 20 most abundant proteins account for only 40% of the saliva protein content. For the abundant and wide dynamic range proteins in the plasma fluid, it is more difficult to identify the lower abundance proteins which are important in detecting disease. Comparatively, the proportion of high abundance proteins in saliva is moderate, which is more beneficial for detecting low abundance proteins. Furthermore, some of the most abundant proteins in plasma have moderate to high relative abundance in saliva, while only a small number of high abundance salivary proteins have an equally abundance in plasma [159]. These suggest that salivary proteins have certain potential as biomarkers. Xiao et al. [74] quantitatively identified more than 500 proteins by detecting the saliva protein components of gastric cancer patients and healthy controls, of which 48 were differentially expressed, 41 were down-regulated, and 7 were up-regulated in patients with gastric cancer. Cystatin B (CSTB), triosephosphate isomerase (TPI1) and deleted in malignant brain tumors 1 protein (DMBT1) can significantly distinguish gastric cancer patients from healthy controls. The sensitivity of the combination of these three protein markers for the detection of gastric cancer is 85%, the specificity is 80%, and the AUC is 0.93.

The metabolome of saliva

As the final product of gene expression, metabolites can reflect the actual biological status of the body. Metabolomics is the quantitative analysis of all metabolites in the

body, such as nucleic acids, amino acids, peptides, lipids, carbohydrates and vitamins, so as to find the relative relationship between metabolites and physiological and pathological changes. It is helpful for us to monitor the state of the body and make correct treatment decisions [160, 161]. Especially in cancer, changes in metabolites can occur before the symptoms of the disease so that abnormalities in the body can be detected as early as possible [162]. The metabolites in saliva can reflect the health and disease state in the oral cavity, but also the state outside the oral cavity. Most of the metabolites in the blood can transport to saliva due to the close relation between salivary glands and capillaries, and the small molecular weight of metabolites. Additionally, the changes of metabolites in saliva are generally consistent with the blood so that they can reflect the effect of disease, nutrition, drugs and environment on the body status [130]. Nonetheless, the fact is that the concentration of metabolites in saliva is much lower than that in blood, which requires the measure technique with a high level of sensitivity. The emergence of MS makes up for the deficiency of the detection of low molecular weight compounds, and it is a common research method in proteomics and metabolomics. The metabolomic technologies are usually based on various separation techniques combined with MS, such as liquid chromatography mass spectrometry (LC-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS), ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), gas chromatography mass spectrometry (GC-MS), plasma mass spectrometry, proton-nuclear magnetic resonance (H NMR) spectroscopy, and capillary electrophoresis-based mass spectrometry (CE-MS) [71].

Most salivary metabolomic studies focus on whole-mouth saliva (WMS). The composition of WMS is complex, including saliva secreted by three major glands and hundreds of small glands in the mouth, but a large number of microorganisms and host cells (such as neutrophils). These microorganisms and host cells may have continuous metabolic activities in saliva, which challenges the metabolomic analysis of WMS [156, 163]. Saliva collected directly from the gland can eliminate the interference of microorganisms to the sample (commonly collected in the parotid gland). The secretions collected by this method do not flow into the oral cavity, without modification from oral microorganisms, which can simply reflect the metabolic activity of the host [164]. Research found that oral microorganisms importantly contribute to the WMS metabolomes, and some WMS metabolites (such as lactate, citrate and urea) are derived from the blood circulation, indicating that WMS may play a special role in helping to diagnose bacteria dysbiosis [165]. Saliva metabolites are a collection of metabolic activities of a complex system. Combining hosts and microorganisms to study salivary metabolomics is a potential direction which is helpful for us to further understand the role of salivary metabolites in health and disease. Sugimoto et al. [71] used capillary electrophoresis time-of-flight mass spectrometry to study metabolites in saliva samples from healthy controls and patients with periodontitis, oral, breast and pancreatic cancer, finding 57 metabolites that predict these diseases. Among them, three metabolites (taurine, piperidine and a peak at 120.0801 m/z) are specific markers of oral cancer with an AUC of 0.865, and eight specific markers of pancreatic cancer (leucine, isoleucine, valine, tryptophan, glutamic acid, phenylalanine, glutamine and aspartic acid) with an AUC of 0.993. Song et al. [60] collected saliva samples from healthy people, patients with precancerous lesions and oral squamous cell carcinoma (OSCC). The abnormal metabolites and metabolic pathways were found by conductive polymer spray ionization mass spectrometry (CPSI-MS) analysis. Then the reliability of metabolic markers in diagnosis was verified by desorption electrospray ionization MS imaging (DESI-MSI) at the tissue level of the primary tumor. Finally, combined with machine learning (ML), it can distinguish OSCC, precancerous lesions and healthy people in real time, and the accuracy is as high as 86.7%. This method is a direction with huge potential for developing salivary metabolomics in point-of-care technology (POCT).

The microbiome of saliva

The oral cavity is the second largest microbial reservoir in the human body after the intestinal tract. The diversity of the microbiome is also second only to the intestinal tract, with more than 700 bacterial species [166]. As we all know, oral microflora is closely related to oral health and diseases. Studies have shown the relationship between oral bacteria and dental caries, periodontitis, dry socket, pulpitis and odontogenic infection [167, 168]. In addition, oral microbes are potentially associated with systemic diseases, such as diabetes, heart disease and pancreatic cancer [72, 169, 170]. Most microbial species detected in different parts of the mouth overlap, but there are usually some specific species in some parts. Saliva microbiome is basically composed of a mixture of microorganisms from all parts of the mouth, which can include microorganisms at almost all sites. It is a relatively ideal specimen for microbial detection. The common research methods of salivary microbiome are bacterial microarrays and next-generation sequencing technology (NGS), of which the NGS is the most important research method. Wei et al. [171] detected the samples of pancreatic cancer patients and healthy people by 16S rRNA sequencing,

and compared the difference of microbiome between them. The results showed that the carriage of *Streptococcus* and *Leptotrichina* had a higher risk of developing pancreatic cancer (PDAC), while those carrying *Veillonella* and *Neisseria* had a relatively lower risk of developing PDAC. These two microorganisms may be protective bacteria to prevent PDAC. Among PDAC patients, those with abdominal distension had a higher amount of *Porphyromonas* (p=0.039), *Fusobacterium* (p=0.024), and *Alloprevotella* (p=0.041), while patients with jaundice had a higher level of *Prevotella* (p=0.008), patients reporting diarrhea had a lower amount of *Neisseria* and *Campylobacter* (p=0.024 and p=0.034), and patients with vomit had a lower amount of *Alloprevotella* (p=0.036). These suggest that salivary microbiome was likely to distinguish patients with PDCA and healthy individuals. Furthermore, combining symptoms and microbiological assessment may be helpful for early diagnosis of PDCA. Jiang et al. [172] performed 16S rRNA sequencing and whole-genome shotgun metagenomic sequencing on saliva samples from 13 salivary adenoid cystic carcinoma (SACC) patients and 10 healthy controls to analyze microbial diversity, composition and function in both groups. The α diversity was not significantly different between SACC patients and healthy controls, while the β diversity showed a separation trend. And *Streptococcus* and *Rothia* were more abundant in SACC patients, while *Prevotella* and *Alloprevotella* were more abundant in the healthy controls. These results showed the potential of saliva to distinguish SACC patients from healthy controls.

The exosomes in saliva

Exosomes are a kind of nanoscale lipid bilayer vesicles (about 30–120 nm) secreted by nearly all cell types, mainly derived from multivesicular bodies (MVBs) formed by invagination of lysosomal particles. In forming MVBs, some small molecules in the cytoplasm, including mRNAs, microRNAs and proteins, are intake by budding inward to form small internal vesicles. Part of MVBs is fused by lysosomes and decomposes the contents. When the MVBs fuse with the cell membrane, the internal vesicles, as exosomes, explosively release out of the cell and act on the adjacent cells. Moreover, it can also spread to the distant tissue through the vascular system and promote the communication between cells, affecting the biological behavior of cells. Exosomes recognize target cells through surface-specific ligands and usually enter the target cells in two ways. One of them is that the target cells absorb it into the cytoplasm by endocytosis. The other is by fusing with the membrane of the target cell and releasing the contents directly into the cytoplasm [173]. Exosomes can be found in various biological body fluids, such as blood, saliva, urine, cerebrospinal fluid and breast milk [174–176]. A large number of high-throughput exosomes studies have shown that exosomes contain a variety of small molecular substances, including mRNA, non-coding RNA, mitochondrial DNA, proteins, lipids and metabolites, and their size and cargo are heterogeneous [177–179]. Exosomes exist widely in a variety of extracellular fluids, and transport cargo with diversity, which endows them with multiple and complex biological functions. Studies have shown that exosomes play an important role in cell development and differentiation, tumor growth and invasion. Sphingosine kinase 2 can be transferred into target hepatocytes to form sphingosine-1-phosphate in hepatocyte-derived exosomes, which leads to cell proliferation and liver regeneration [180]. The exosomes derived from breast cancer cells contain Dicer, AGO2 and TRBP. After being transported to the target cells, the cargo can process the precursor microRNA into mature miRNA, silence some tumor suppressor genes and induce non-carcinogenic epithelial cells to form tumors [181]. Additionally, the heterogeneity of exosome size and cargo can reflect the state and type of parental cells, which makes it a potential biomarker for disease diagnosis and prognosis. Some proteins in exosomes, such as CD9, tumor rejection antigen 1 (gp96) and caveolin 1 (CAV1), are valuable in the diagnosis of HNSCC, and can be used as potential biomarkers for diagnosis and prognosis [182]. The combined detection of exosome miR-21 and lncRNA HOTAIR as biomarkers for the diagnosis of laryngeal squamous cell carcinoma achieved a sensitivity and specificity of 94.2 and 73.5%, respectively [183]. For the study of exosomes, different methods can be adopted according to the research object, usually combined with proteomics, transcriptome or metabolomics to study the biomarkers. In the development of systemic cancers such as lung and pancreatic cancer, some specific biomarkers can be detected in saliva samples [67, 184]. This may all be due to the role of exosomes, which can carry substances from the source cells to drift through the vascular system, thus passing these goods into saliva and being detected.

The exosomes in saliva can be used as a new diagnostic platform for disease research. Saliva is easy to collect and non-invasive. Combined with the various and important biological functions of exosomes, it will become a new potential biomarker of oral and other systemic diseases and tumors. Machida et al. [185] found that miR-1246 and miR-4644 are highly expressed in plasma exosomes of patients with pancreatic cancer, and can also be identified in salivary exosomes, which can be used as potential biomarkers of pancreatobiliary tract cancer. Zhang et al. [186] identified cancer-enrich small tsRNA signature by RNA sequencing of salivary exosomes obtained from 3 patients

with esophageal squamous cell carcinoma (ESCC) and 3 healthy controls, and further verified in the discovery cohort (66 individuals). The results showed that tsRNA (tRNA-GlyGCC-5) and previously unrecorded small RNAs were specifically enriched in salivary exosomes of ESCC patients, ESCC tissues and ESCC cells. The bi-signature composed of these small RNAs can discriminate ESCC patients from healthy controls with high sensitivity (90.50%) and specificity (94.20%). Furthermore, a bi-signature Risk Score for Prognosis (RSP) was set up to evaluate the risk. The patients with high RSP present shorter overall survival (OS) and progression-free survival (PFS) than those with low RSP. In addition, adjuvant therapy improved OS and PFS only in patients with high RSP. These results are consistent in the training and validation cohort. It suggested that tsRNA-based signature has diagnostic and prognostic potential, but also can be used as pre-operative biomarkers to screen patients more sensitive to adjuvant therapy. This study broadens the prospect of salivary exosomes research and has considerable guiding significance for the future study of salivary exosomes. The study of salivary exosomes has a broad prospect, but it is still in the early stage, and a lot of research is still needed to lay the foundation for this hot direction.

Saliva collection and standardization procedure

Among the body fluid collection methods, saliva collection is the most simple and fast. Notably, the accurate participant identification, sufficient sample size and appropriate container type are indispensable for each collection methods. Additionally, maintaining consistency in the marking and processing of samples is critical, which can ensure the accession to the high-quality data [187]. Due to the influence of saliva flow rate, type of salivary gland, circadian rhythm, saliva stimulation type, age, sex, physiological status, diet and collection methods, the detection results of saliva samples will be some difference. Therefore, we need to further standardize the methods of saliva collection. It is also necessary to adopt corresponding methods for different saliva detection contents, which can reduce the error as much as possible.

The composition of saliva is complex, and will change with age. Compared with the young, the saliva flow rate and calcium concentration of the elderly decreased, while the ion concentration increased significantly [188]. Similarly, the salivary mucin levels between the young and the old present significant differences [189]. Moreover, the protein profiles between newborns and adults have been reported to differ [190]. In the aspect of gender, the size of salivary glands in males is generally larger than that in females, so it also has a certain effect on saliva secretion. The unstimulated saliva secretion of healthy men is higher than that of women, presenting gender-dependence [191]. Hence, subjects of different ages and genders should be classified, tested and counted to order to reduce errors. In addition, the flow rate of saliva of the same person is different at different time points, so it is best to collect saliva at the same time. In collecting large samples, it should also be considered to eliminate individuals with extremely large and small amounts of saliva to minimize errors.

Salivary glands include three major salivary glands and many small salivary glands. Although most of the components secreted by these salivary glands are the same, there are differences in concentration. The parotid gland mainly produces serous type of saliva, which contain high levels of α-amylase and proline-rich proteins. The sublingual gland is mainly mucous acini, which mainly secretes mucin and lysozyme. As a mixed gland, the submandibular gland secretes both α-amylase and mucin. Other minor salivary glands mainly secrete mucin and lipase. The saliva collected from each salivary gland is generally sterile, and has a similar composition to that of plasma. It is generally used to measure the flow of each gland and to detect for salivary gland diseases. Whole mouth saliva (WMS) is a mixture of salivary gland secretions, gingival crevicular fluid, nasal and bronchial secretions, food residues, bacteria and their metabolites [192]. It can be divided into stimulated whole saliva (SWA) and unstimulated whole saliva (USWA). The production, composition and main sources of these two types of saliva are different. The WMS is rich in ingredients, which is suitable for the assessment of systemic diseases. And the interference factors of USWA collection are small, so it is a commonly used sample in the study. In collecting saliva samples, it is necessary to consider the gland sources and the state of stimulation. We should ensure that the samples collected are consistent, and different types of saliva should be selected according to the content of the study.

The common methods of collecting WMS include the draining method, the spitting method, the suction method, and the swab method [193]. And the collection of saliva from a single gland needs some special methods and equipment [194]. As mentioned above, WMS is more suitable for evaluating systemic diseases for containing more analyzable substances. Therefore, the methods and equipment for collecting WMS are more commonly used. Currently, there is still no generally accepted WMS sample collection technique, which will also result in poor reliability and reproducibility of the results, hindering the process of saliva

research [195]. The UWS collected by swab method or suctioning will increase the variability of data, because the wiping action will cause certain stimulation [193]. The samples collected by spitting may contain more microbe than the draining method, which can be used to identify microorganisms, but can also affect the storage of saliva and the follow-up analysis of some compounds [196]. At present, passive drainage of UWS from the lower lip and spitting directly into the tube is considered the most promising and recommended method to minimize the error in the saliva collection process. This method has the following advantages: 1. collecting a large number of samples in a short time [197]; 2. containing a variety of substances in the samples, which can be used to detect a variety of biomarkers, and is suitable for most of the studies of omics; 3. can be preserved for a long time without affecting the detection. In addition, it is suggested that only one type of collection method and device should be used in a given study, and some guidance should be given to the sample provider. At present, many companies have produced special equipment for saliva collection, and even special versions for children, infants or for the detection of some specific compounds. These commercial salivary collectors include: (1) Salivette (Sarstedt AG & Co, Germany); (2) Oragene saliva kit (DNA Genotek, Canada); (3) Aware Messenger Device (Calypte Biomedical Corp, USA); (4) BBL Culture swab (BD, USA); (5) Intercept Oral Specimen Collection Device (OraSure Technologies Inc, USA); (6) UpLink saliva collector (OraSure Technologies Inc, USA); (7) Saliva Collection device (DNA Genotek, Canada) [198]. Although these devices are varied, they all have a similar protocol: subjects are told to avoid brushing for at least 30 min before collecting saliva, and not to eat food, drinking (except water) or chew gum, then gargle and spit into the collection tube. Saliva samples can universally be preserved at room temperature for 30–90 min. Low temperature can reduce the degradation rate of protein in saliva [196, 199]. In terms of storage, saliva samples should be frozen at −20 °C or below immediately after collection. Without this condition, the sample can also be stored at 4 °C to prevent bacterial growth and degradation of saliva components [196]. Generally, the sample can be stored at −80 °C for several years without degradation [187]. It is a routine process to add inhibitors before storage. However, for the RNA analysis, QIAzol method has been reported that could separate high yield RNA without additional RNA stabilizers. By using this method, saliva samples can be successfully stored at −80 °C for more than 2 years without adding RNase inhibitors [200].

Regardless of saliva collection methods, pre-analysis and analytical variables need to be standardized, such as collection and storage methods, circadian variation, sample recovery, sample contamination prevention and analytical procedures. The salivary flow rates, age, gingival health and physiological status of subjects can also affect the concentration of biomarkers. This significant range of variables raises concerns about the accuracy and repeatability of diagnosis using salivary biomarkers [195, 201]. How to overcome these challenges will be one of the keys to develop salivaomics research, which requires us to carry out a large number of samples to determine.

Clinical application of salivary biomarkers

Disease detection

Oral disease and cancer

Caries

Microorganisms are an important cause of dental caries. The colonization, proliferation and metabolism of cariogenic bacteria, especially *Streptococcus mutans* and *Lactobacillus*, have been widely used to identify individuals prone to caries. Samaranayake et al. [202] collected the salivary sample of the high and low caries activity population by paraffin wax stimulation method, and compared them after incubation. It suggested that the densities of *Streptococcus mutans* and *Lactobacillus* in people with high caries activity were much higher than those with low caries, and the difference was about an order of magnitude. With the application of high-throughput sequencing technology in microbiome, microorganisms in saliva are expected to become potential markers. Yang et al. [203] analyzed the salivary samples of 19 caries-active and 26 healthy control by 16S rRNA sequencing. The results showed that the *Prevotella* genus increased significantly in the caries active group, distinguishing the caries microbiota from the healthy ones. The results are also cross-verified by whole-genome-based deep-sequencing data. More importantly, it suggested that a single microbe change is not enough to identify caries, but it needs to be verified by the changing whole microbiome structure. Moreover, the changes of electrolytes in saliva can also be used as predictors of caries, and can be combined with microbiome to construct a diagnostic model [204].

Periodontal diseases

Periodontal diseases (PD) are serious infection of gingival tissue caused by many factors, which can lead to the destruction of periodontal ligament and alveolar bone. The microbes play a vital role in the occurrence and development

of periodontal disease. The pathogenic bacteria can produce a variety of toxic factors to reduce the resistance of host, and directly destroy the periodontal tissue. In addition, they also stimulate the host's immune response, and induce the release of a variety of inflammatory factors to further aggravate the destruction of periodontal tissue. Therefore, the pathogenic bacteria and inflammatory cytokines will be significant factors in predicting periodontal disease. *Porphyromonas gingivalis* has been proved to be closely associated with periodontitis. Currently, an ELISA kit has been developed for the special detection of *P. gingivalis* in saliva. Compared with qPCR, its detection speed is faster, sensitivity and specificity are as high as 92 and 96%, respectively. Hence, it is expected to become an easy and rapid chair-side diagnostic tool for the detection of *P. gingivalis*, and is of great potential in rapid screening of periodontitis [205]. Nonetheless, most studies have focused on the detection of inflammatory cytokines in periodontal disease as diagnostic markers. Kim et al. [9] used ELISA kit to detect matrix metalloproteinase-9 (MMP-9) and S100A8 (a molecular subgroup of acid calcium binding protein S100) in saliva of 99 patients with periodontitis and 50 healthy controls. In addition, patients with periodontitis were processed with non-surgical treatment, and saliva MMP-9 and S100A8 were detected again 3 months later. The results showed that MMP-9 and S100A8 were associated with periodontitis. Using saliva MMP-9 and S100A8 algorithm (combining age, sex, smoking, drinking and other factors), the screening ability of periodontitis can reach 0.86. Comparing saliva samples of patients with periodontitis before and after treatment, it was found that S100A8 and MMP9 decreased by 83.7% and 23.5%, respectively, suggesting that MMP-9 and S100A8 have the potential to be used as models for diagnosis and prognosis of periodontitis. Junior et al. [206] evaluated the level of myeloid-related proteins in saliva in relation to PD, and their potential screening ability. The results showed that the colony-stimulating factor-1 (CSF-1), S100A8/A9, S100A12, interleukin-1β (IL-1β), matrix metalloproteinase-8 (MMP-8) and hepatocyte growth factor (HGF) in saliva of patients with periodontitis and gingivitis increased significantly, while IL-34 of patients with periodontitis was significantly lower than that of healthy people and patients with gingivitis. After 3 months of treatment, IL-34 increased significantly, whereas IL-1β and MMP-8 decreased 1 month after treatment. In addition, patients with periodontitis clustered in high and low levels of S100A8/A9, and the patients with high levels had deeper pockets, more bleeding, and higher S100A12. It suggested that the salivary level of myeloid-related proteins changes in periodontitis and is partially regulated by periodontal treatment. The S100A8/A9 level in saliva may be helpful in identifying different groups of periodontitis patients. Sukriti et al. [207] used the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool to assess the methodological quality of articles searching for periodontal disease and related saliva diagnostic markers, and seven studies were selected in the evaluation system. The results showed that macrophage inflammatory protein-1α (MIP-1α), IL-1β, IL-6 and MMP-8 were identified as acceptable biomarkers for the diagnosis of PD. Generally, the combination of MMP-8 and IL-6 showed the best diagnostic performance. Furthermore, the combination of IL-1β, IL-6, MMP-8 and MIP-1α presented promising results for distinguishing gingivitis from periodontitis, as well as periodontitis from gingival health. In addition to bacteria and inflammatory cytokines, microRNA and exosomes are also reported to be of certain value in the diagnosis of periodontitis [6, 7]. Overall, we need to expand the sample size and further implement structured research to verify these biomarkers.

Primary Sjögren's syndrome

Primary Sjögren's syndrome (pSS) is a chronic systemic autoimmune disease that damages the exocrine glands (mainly the salivary and lacrimal glands), characterized by dry mouth and keratoconjunctivitis [208]. The decrease of saliva flow rate and the change in saliva composition are important manifestations of the progression of pSS. These symptoms can lead to serious complications such as dental caries, periodontal disease, and *Candida albicans* infection. The B cell hyperactivity expressed as hypergammaglobulinemia, the existence of several serum autoantibodies and the activation of type I interferon (IFN-1) pathway are the immunopathogenic mechanisms of pSS, which lead to the excessive production of immunoglobulin [209, 210]. In addition, the secretory function of salivary glands is impaired, which makes the salivary protein profile of pSS patients change. Hu et al. [211] identified saliva autoantibodies of pSS using immune-response protoarrays. Of the 24 candidate autoantibody biomarkers, anti-histone, anti-transglutaminase, anti-SSA and anti-SSB were validated in a cohort of 534 pSS patients, 534 SLE patients and 534 HC. All four autoantibodies were overexpressed in pSS patients. The AUC of anti-histone, anti-transglutaminase, anti-SSA and anti-SSB to pSS (relative to HC) were 0.95, 0.87, 0.93 and 0.94, respectively. Lee et al. [24] found abnormally high expression of soluble sialic acid-binding immunoglobulin like lectin (siglec-5) in serum and saliva of patients with pSS. Then investigated the correlation between the level of siglec-5 in saliva of pSS patients and clinical parameter. The results showed that the level of siglec-5 was negatively correlated with saliva flow rate, but positively correlated with dry mouth scale (XI) score and ocular staining score (OSS). The ROC

analysis showed that when the cutoff value was 400 pg/mL and the area under the siglec-5 curve was 0.774, the sensitivity and specificity were 0.69 and 0.70, respectively. In the verification cohort, the sensitivity and specificity of siglec-5 in distinguishing pSS patients from non-SS patients were 64.4 and 77.8%, respectively. It is possible to be used as a new saliva biomarker of pSS.

Oral lichen planus

Oral lichen planus (OLP) is a chronic inflammatory disease that often affects the tongue, gums and buccal mucosa. As a potential malignant disease, OLP has a certain carcinogenic rate, so early diagnosis and intervention treatment are particularly important [212]. Abnormal activation of the immune system is an important mechanism for the occurrence and development of OLP. Therefore, various signal molecules in the immune system pathway may be potential biomarkers for diagnosing OLP. Liu et al. [28] found that IL-4 was significantly increased, while IFN- γ decreased in salivary sample of OLP patients. IL-4 and IFN- γ are the principal cytokines of Th1 and Th2 cells, respectively. This result is consistent with the phenomenon that Th2 cells are predominant in saliva of patients with OLP. These also reflect the potential of IL-4 as a biomarker to detect the severity of OLP. Additionally, it has also been reported that other inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF- α in saliva can be used as biomarkers for the diagnosis and prognosis of OLP [213, 214]. On the other hand, oxidative stress and impaired antioxidant defense are also possible causes of OLP. Many studies have shown that the levels of oxidative stress molecules such as lipid peroxide malondialdehyde, 8-Hydroxy-deoxy (8-OHdG) and nitric oxide (NO) in saliva of patients with OLP are significantly increased [215, 216]. Although no studies have shown the relationship between the concentration of these substances in saliva and the severity of OLP, they have a potential role in monitoring the progress of the disease. Recently, Mehdipour et al. [26] found that miR-21 was significantly increased in salivary samples of OLP and OSCC patients, while miR-125a levels were significantly decreased. These results may become the research direction of the salivary transcriptome in OLP.

Oral leukoplakia

Oral leukoplakia (OLK) is a white plaque on oral mucosa that cannot be removed by scratching. It is the most common precancerous lesion in oral cavity and has a high carcinogenic rate. Traditionally, resection and biopsy are the main diagnostic methods, which are more traumatic. Therefore, many diagnostic studies pay more and more attention to less invasive methods, such as the analysis of inflammatory cytokines in saliva. Previous studies have shown that the levels of IL-6 and TNF-α in saliva of patients with OLK are significantly higher than those of healthy people, which can be used as potential biomarkers of OLK [33, 217]. However, many reports show no significant difference between TNF- α and ILs in saliva of healthy individuals and patients with OLK, even decreased [218–220]. In addition to cytokines, other salivary proteomic studies have reported possible markers. Sivadasan et al. [32] used liquid chromatography and tandem mass spectrometry (LC-MS/MS) to analyze protein components in saliva samples of healthy controls, patients with leukoplakia, OSCC patients with negative and positive lymph node metastasis. The results showed that the expression of S100A7, S100P, CD44 and COL5A1 increased in leukoplakia and tumor patients. Subsequently, through the detection of these proteins in the verification queue by ELISA, it was found that CD44, S100A7 and S100P could distinguish dysplastic leukoplakia from normal people with high sensitivity (CD44: 91.67%; S100A7: 81.82%; S100P: 81.82%), but relatively low specificity (S100A7, S100P: 72.73%; CD44: 54.55%). The result suggested that the saliva levels of these proteins can be used as potential markers for predicting patients with OLK, but further sample expansion and clinical verification are necessary. Precancerous lesions involve changes in some special genes and proteins, but also changes in the concentration of endogenous metabolites. Wei et al. [221] found that threonine, isoleucine, phenylalanine, n-tetradecanoic acid, homocysteine and 4-methoxyphenylacetic acid in saliva of patients with OLK were significantly higher than those of healthy people. Sridharan et al. [222] also reported the differential expression of 157compounds in patients with OLK compared with healthy people. All these provide a direction for applying salivary metabolomics in OLK, which is worthy of our in-depth study. There is no clear clinical data to support the effective diagnostic markers of OLK in saliva. Further research is needed to find and construct a biomolecule model of saliva diagnosis which can be used for rapid clinical detection.

Oral cancer

Oral cancer is a malignant tumor occurring in the oral cavity, and OSCC is the most common, accounting for about 95%. Most of the OSCC cases in the world are concentrated in Asia, and the prognosis is very poor. Despite the improvement in surgical and chemotherapy techniques, the 5-year overall survival rate of patients was only about 64.4% [223]. Hence, the early diagnosis and prevention of OSCC are particularly important, which is conducive to treatment and prognosis. Especially in densely populated Asia, simple and rapid techniques for extensive scale screening is necessary. OSCC can occur in many factors, including smoking, drinking and microbial infection. The DNA mutation, abnormal

transcription, abnormal protein expression, metabolic disorder and microbial composition will change, and this abnormal information can be spread throughout the oral cavity. Long-term direct contact between saliva and oral tissue can better reflect the information on oral diseases, which is also the reason why early saliva screening and diagnosis have become a research hotspot in OSCC. At present, there are many high-quality saliva diagnostic markers in OSCC, including genome, transcriptome, proteome, metabolic group and microbiome.

Gene mutation is one of the markers of carcinogenic progression, and the mutation of cancer-specific gene can accurately distinguish all kinds of tumors. The mutated DNA can flow into various body fluids, including saliva, which is the basis of the saliva genome. The lesions of OSCC patients are immersed in saliva for a long time, and the released factors or exfoliated cells also become components of saliva. Therefore, salivary genomics has a natural advantage in the early screening and diagnosis of OSCC. Preston et al. [224] found that HOXA9 and NID2 in saliva samples can distinguish between OSCC patients and healthy controls, with a certain sensitivity (75 and 87%, respectively) and specificity (53 and 21%, respectively). The AUC is 0.75 and 0.73, respectively, which can be used for early detection and follow-up patients with OSCC. Nagata et al. [225] analyzed the methylation status of genes in salivary samples from patients with OSCC and healthy people. It was found that the DNA methylation level of 8 genes was significantly increased in the OSCC group. The methylation status of combination of ECAD, TMEFF2, RAR β and MGMT showed high sensitivity (100%) and specificity (87.5%) in detecting OSCC. Shanmugam et al. [226] designed a custom next-generation sequencing panel with a unique molecular identifier that covers the coding regions of seven frequently mutated genes (CASP8, PIK3CA, FAT1, CDKN2A, NOTCH1, HRAS and TP53) in OSCC. It was used to detect saliva samples from patients with OSCC, and the results showed a detection rate of 95.87%. This shows that the targeted next generation sequencing technique is feasible and has a good prospect in the early screening and diagnosis of OSCC. Notably, in these studies, salivary samples were collected by salt water to reduce the degradation of free DNA in saliva, which can increase the content of DNA in the samples as much as possible.

The microRNA is stable in biological fluids and differentially expressed in all kinds of cancers. It is a commonly used fluid biopsy marker in transcriptome. Previous studies have shown that the levels of miR-125a and miR-200a in saliva of patients with OSCC are significantly decreased and can be used to detect oral cancer [149]. It was also found that the miR-31 in saliva was more abundant than in plasma, suggesting miR-31 in saliva is more sensitive in detecting OSCC. Additionally, the miR-31 decreased significantly after focal resection. These results indicated that salivary miR-31 could be used as a biomarker for early detection and post-operative follow-up of OSCC [227]. Recently, Romani et al. [34] collected saliva from patients with untreated primary OSCC and healthy controls, screening the differentially expressed miRNA through microarray analysis, using RT-qPCR for signature validation. In the training set, the saliva of patients with OSCC present high levels of expression of miR-106b-5p, miR-423-5p and miR-193b-3p. The accuracy of miR-106b-5p, miR-423-5p and miR-193b-3p as diagnostic biomarkers was evaluated by logical model prediction. The results showed that the combination of the three had excellent sensitivity (0.974) and specificity (0.942), and AUC was 0.98. The results in the validation set also confirmed this result, indicating that the combined application of miR-106b-5p, miR-423-5p and miR-193b-3p can be used as biomarkers for the detection and diagnosis of OSCC. Furthermore, it was also found that the high expression of miR-423-5p was an independent predictor of poor disease-free survival (DFS) when positive lymph nodes (the number of positive lymph nodes was the only important clinical prognostic factor) were included in multivariate survival analysis. Finally, it was also found that miR-423-5p decreased significantly after tumor resection, which may be a specific prognostic biomarker of OSCC progression and metastasis. In addition to microRNA, other RNAs can be used as diagnostic markers. Li et al. [36] found and identified 7 transcripts (DUSP1, H3F3A, IL1B, IL8, OAZ1, S100P and SAT) up-regulated in saliva samples of OSCC patients, which could distinguish OSCC patients from healthy controls. Tang et al. [228] found that all OSCC saliva samples contained lncRNA MALAT-1, indicating that lncRNA in saliva could also be used as a potential marker for OSCC diagnosis.

Abnormal protein expression often occurs in the process of tumor occurrence and development. These proteins usually enter the bloodstream and can be detected in the early stage. Saliva contains more than 2000 kinds of proteins, similar in composition to those in plasma, most of which are blood sources. Tumor antigen CA15-3 and tumor protein markers CA-125, c-erbB2 and p53 in saliva present increased expression in oral cancer and other malignant tumors, which can be used as biomarkers [229]. Yu et al. [51] detected and analyzed saliva from healthy people, oral potentially-malignant disorders (OMPD) patients and OSCC patients by LC-MS combined with multiple reaction monitoring (MRM). The MMP1, KNG1, ANXA2 and HSPA5 were selected from the differentially expressed proteins to form the diagnostic model. The model shows high sensitivity (87.5%) and specificity (80.5%) in distinguishing OSCC from non-OSCC patients. Moreover, a risk scoring scheme is constructed based

on this model. When the risk scores >0.4, 84% of OSCC patients and about 42% of high-risk OPMD can be detected. In addition, of the 88 high-risk OPMD patients followed up, 18 developed to OSCC within 5 years, and 14 of them had risk scores >0.4. Chu et al. [56] screened 6 proteins (APOA1, APOA4, CFH, FGA, SERPINA1 and SERPIND1) by targeted and non-targeted quantitative proteomic approaches, which can effectively distinguish OSCC from healthy controls (AUC ≤0.8). Among them, the levels of CFH, FGA and SERPINA1 in saliva of patients with advanced primary OSCC are much higher than those of patients with early primary OSCC. In addition, some studies have found that proteases are related to metastasis and translocation of cancer. Inhibition of these proteases can reduce the invasion and metastasis of cancer cells [230–232]. Feng et al. [43] found that the salivary protease spectrum of OSCC patients is significantly different from that of health and patients with other oral diseases, such as jaw bone ossification fibroma (JBO) and mild chronic periodontitis (CPD). The saliva of patients with OSCC present more protease types, and the levels of MMP-1, MMP-2, MMP-10, MMP-12, A disintegrin and metalloprotease (ADAM) 9, A disintegrin and metalloprotease with thrombospondin type 13 motifs (ADAMST13) and cathepsin V and kallikrein 5 increased significantly. The combination of cathepsin V, kallikrein 5 and ADAM9 has good sensitivity (85.0%), specificity (93.3%), and the AUC was 0.963.

Metabolites can reflect the real state of the body. Changes in cell metabolism have been identified as a new marker of cancer [233]. In the process of tumorigenesis and development, it is usually accompanied by changes in metabolic pathways, resulting in some abnormal metabolites, forming a unique metabolic profile of tumors. Saliva contains a large number of metabolites, especially the metabolites from the primary site of oral cancer, which will be mixed directly into saliva. At present, more than 100 metabolites have been reported to change with the malignant progression of OSCC, such as lactate, choline, glutamate, histidine, sialic acid and trimethylamine N-oxide [221, 222, 234]. Song et al. [60] used conductive polymer spray ionization mass spectrometry (CPSI-MS) to detect the salivary metabolic profiling of healthy people, patients with premalignant lesions and patients with OSCC. The results showed that the metabolic profiles of the three groups could be distinguished from each other and had considerable stability (the similarity of the metabolic maps of saliva collected at different times was as high as 86%). Further analysis showed that 58 metabolites changed significantly between healthy group and premalignant lesions group, while 116 metabolites increased or decreased significantly between premalignant lesions group and OSCC group. These results are also similar to those in the validation set. In addition, the Lasso regression was introduced to construct a machine learning (ML) model for OSCC prediction. The model showed an accuracy of up to 86.7% when it was applied to validation set. These results indicate that the metabolomic has a broad prospect in the development of saliva diagnostic markers for OSCC. In particular, CPSI-MS has the following excellent: rapid and direct metabolic analysis of biological liquids; low cost; cleanliness; and wide coverage of metabolites, which will make the point-of-care test (POCT) of OSCC possible, and will be the hot direction of large-scale early screening in the future.

Oral microorganisms play an important role in the health and disease of the host. In recent years, some studies have shown that the change of oral microflora will destroy the balance between microorganisms and human body, coupled with the influence of risk factors, which will lead to the occurrence of OSCC [235, 236]. Oral microorganisms, epithelial barrier, immune system and chronic inflammation constitute the four important factors leading to oral cancer [237]. Therefore, oral microorganisms are also promising potential biomarkers for early diagnosis of OSCC. Single microorganism, such as *P. gingivalis*, *Fusobacterium nucleatum* and *Staphylococcus aureus* have all been reported to play an important role in the occurrence and development of OSCC [238–240]. However, a single microbe is not enough for the diagnosis of OSCC. It is necessary to identify the core microbiome that causes OSCC for developing a clinically applicable and highly accurate diagnostic model. Zhou et al. [65] sequenced saliva samples from 47 OSCC patients and 46 healthy subjects based on 16SrRNA sequencing, and established a random forest model. The results show that the accuracy of the model is 95.70%, and the sensitivity is 100%. Additionally, they found that even in healthy controls, saliva samples from different regions showed different microbial compositions. Finally, they also put forward the strategy of developing the prediction model: on the basis of establishing small samples, and gradually adding new samples to update the model, so as to improve the accuracy. This method is also applicable to healthy individual groups. The results suggest that salivary microbiome combined with machine learning is a new direction for early detection of OSCC and can be used in extensive scale population screening in the future.

Systemic malignant tumor

Pancreatic cancer

The researches of saliva diagnostic markers of pancreatic cancer are still in preliminary stage. There have been some reports on transcriptome in pancreatic cancer. Zhang et al. [67] analyzed the transcriptome of saliva samples from pancreatic cancer patients, pancreatitis patients and healthy controls. They found that the combination of 4 mRNA (KRAS,

MBD3L2, ACRV1 and DPM1) could be used as markers to distinguish pancreatic cancer patients from non-pancreatic cancer patients (sensitivity of 90.0%, specificity of 95.0%, and AUC of 0.971). In addition to mRNA, more than 10 kinds of saliva microRNA have been reported [66, 68, 185, 241]. Humeau et al. [68] found that hsa-miR-21, hsa-miR-23a, hsa-miR-23b, and miR-29c in saliva of patients with pancreatic cancer were significantly up-regulated compared with the control group with 100% specificity, but relatively low sensitivity. The concentration of endogenous metabolites in saliva of patients with pancreatic cancer often changes, which can be used as a target for detection. Asai et al. [70] found that the combination of alanine, N1-acetylspermidine, 2-oxobutyrate and 2-hydroxybutyrate had ability to distinguish pancreatic cancer patients from non-pancreatic cancer patients, with an AUC of 0.887. Sugimoto et al. [71] found that eight metabolites, leucine, isoleucine, valine, tryptophan, glutamic acid, phenylalanine, glutamine and aspartic acid, could distinguish healthy controls from pancreatic cancer patients, with an AUC of 0.993. Additionally, Farrell et al. [72] found significant differences in salivary microbiota between pancreatic cancer patients and healthy controls based on human oral microbe identification microarray (HOMIM) and verified by qPCR in an independent cohort. The results showed that the combination of *Neisseria elongata* and *Streptococcus mitis* had a sensitivity of 96.4% and a specificity of 82.1% in distinguishing pancreatic cancer patients from healthy subjects, with an AUC of 0.90. Torres et al. [242] reported similar results in saliva samples from pancreatic cancer and healthy people by 16SrRNA sequencing. These reports confirmed that salivary microbiota could be used as an important informative source for exploring biomarkers of systemic malignant tumors.

Gastric cancer

Compared with the traditional liquid biopsy (such as blood, gastric juice), saliva biopsy of gastric cancer still lacks the consensus of analytical methods and clinical verification, which is also a problem faced by most systemic malignant tumors. During the development of gastric cancer, salivary glands are stimulated by nerve growth factors released from distant tumors, which can lead to significant changes in salivary RNA profiles. These differentially expressed RNA can be used to detect gastric cancer [243]. Li et al. [73] identified 30 candidate mRNAs and 15 candidate microRNAs expression patterns associated with gastric cancer. Through another independent cohort verification, it was found that the biomarker combination of 3 mRNAs (SPINK7, PPL and SEMA4B) and 2 microRNAs (miR-140-5p and miR-301a) could be used to distinguish gastric cancer patients from non-gastric cancer patients, with a sensitivity of 75% and specificity of 83%. This study proved for the first time that saliva extracellular RNA biomarkers have potential utility in early detection and risk assessment of gastric cancer. Xiao et al. [74] identified and quantified more than 500 proteins from saliva. Among them, 48 were differentially expressed between patients with gastric cancer and the control group. And the combination of Cystatin B, triosephosphate isomerase and malignant brain tumors 1 protein can be used to distinguish between gastric cancer patients and normal controls. The sensitivity and specificity were 85 and 80%, respectively, and the AUC was 0.93. Huang et al. [244] compared salivary microflora profiles of patients with gastric cancer, superficial gastritis and atrophic gastritis based on 16SrRNA sequencing. Through the construction of random forest model, it can accurately distinguish gastric cancer patients from non-gastric cancer patients, with an AUC of 0.91.

Breast cancer

At present, the evidence for saliva biomarkers of breast cancer is limited, and there are many interference factors. The chronic gingival inflammation has been proved to be the main confounding factor, reducing the clinical effectiveness of saliva biomarkers of breast cancer [76]. Through the detection and analysis of transcriptome and proteome of saliva from patients with breast cancer and normal controls, 8 mRNAs and 1 protein were found as biomarkers. The sensitivity and specificity of the combination in distinguishing breast cancer patients from normal controls were 83 and 97%, respectively, and the AUC was 0.92 [67]. However, the results of most proteome studies are not ideal, such as CA15-3. The CA15-3 is a serum proteomic biomarker approved by FDA to monitor breast cancer metastasis, but its use in saliva is still immature [245, 246]. Recently, metabolomics is becoming more and more important in the study of salivary biomarkers of breast cancer. Zhong et al. [83] proposed for the first time a method based on UPLC-MS and multivariate data analysis for salivary metabolomics analysis of breast cancer, and 18 potential metabolites were identified. Among them, three upregulated metabolites, LysoPC (18:1), LysoPC (22:6) and MG (0:0/14:0/0:0), had higher curvilinear AUC values of 0.920, 0.920 and 0.929, respectively, presenting potential to be used as biomarkers. Assad et al. [81] performed non-targeted metabonomic analysis of saliva samples from breast cancer patients and healthy controls, and 31 compounds up-regulated in the breast cancer group were identified. Among them, the lipid PG14:2 has a good sensitivity (65.22%) and specificity (77.14%) in distinguishing breast cancer patients from healthy controls, and the AUC is 0.73, which has the potential to be used as a saliva biomarker of breast cancer.

Lung cancer

Compared with other systemic malignant tumors, there are relatively many salivary studies on lung cancer, and many biomarkers have been found. Nonetheless, how to make the most effective use of these biomarkers in clinic needs further research.

Zhang et al. [85] identified and verified 7 highly discriminatory salivary biomarkers in saliva transcriptomes of patients with lung cancer. The logistic regression model conbined with CCNI, EGFR, FGF19, FRS2 and GREB1 could distinguish lung cancer patients from normal controls, with a sensitivity of 93.75%, a specificity of 82.81%, and an AUC of 0.925. Epidermal growth factor receptor (EGFR) is a membrane receptor frequently expressed in non-small cell lung cancer (NSCLC), which is also one of the most frequently studied genetic markers in the diagnosis of lung cancer. It is really vital to identify the presence and type of EGFR mutation in NSCLC, which is important for the treatment, for the common mutations, exon 19 deletion and exon point mutation 21-L858R of EGFR are sensitive to the treatment of tyrosine kinase inhibitors [247, 248]. Wei et al. [84] developed electric field-induced release and measurement (EFIRM), an electrochemical based technology using specific mutation-detecting probes, which can be used for cell-free DNA analysis of samples. Then they established EFIRM for detecting the EGFR mutation, and applied it in saliva samples of 40 NSCLC patients. The ROC analysis suggested that EFIRM can detect the exon 19 deletion (AUC=0.94) and the L858R mutation (AUC=0.96). These results indicate that the EFIRM for detecting EGFR is rapid and simple, meeting many clinical requirements for successful and efficient detection, and may become a clinical method in the future, either alone or in conjunction with supplementary analysis of biopsies.

The proteomic researches of lung cancer are mostly used for blood analysis, while saliva samples are relatively seldom used. Xiao et al. [86] identified 16 proteins for salivary biomarkers in patients with lung cancer. The combination of haptoglobin, zinc-a-2-glycoprotein and calprotectin presented a sensitivity of 88.5% and a specificity of 92.3%, promising saliva biomarkers. In addition, the proteins in salivary exosome have some potential biomarkers that need to be further verification [249].

As well as other systemic malignant tumors, the study of salivary metabolomics in lung cancer is still in its infancy. Li et al. [184] used Surface-enhanced Raman spectroscopy (SERS) to identify biomarkers in saliva of patients with lung cancer. They found 9 differential peaks between patients with lung cancer and healthy controls, most of which were nucleic acid bases and amino acids. Through the analysis of SERS data by PCA-LDA algorithm, it is found that lung cancer patients can be well distinguished from healthy controls, with a sensitivity and specificity of 94 and 81%, respectively. Jiang et al. [250] established an ultralow noise tip-enhanced laser desorption/ionization (TELDI) platform, combined with MS, to study the metabolic profile of saliva samples from patients with early lung cancer and healthy volunteers. After multivariate analysis, 23 metabolites were selected. Combined with artificial neural networks, the prediction model was constructed and verified by another independent queue. The results show that the model can distinguish between patients with early lung cancer and normal controls, with a sensitivity and specificity of 97.2 and 92%, respectively. And the AUC is 0.986. This study provided a new platform for the study of salivary metabolomics in lung cancer, and can also be used for the analysis of other diseases.

There are also relatively few studies on salivary microbiome of lung cancer. Yan et al. [89] proved the relationship between salivary microflora and lung cancer for the first time. Saliva from patients with lung cancer and normal controls were studied by 16S rRNA sequencing. It was found that the abundance of *Veillonella* and *Capnocytophaga* in saliva of patients with lung cancer increased, and their enrichment characteristics could distinguish lung cancer patients from normal controls. The sensitivity and specificity of the combination of *Veillonella* and *Capnocytophaga* to distinguish lung squamous cell carcinoma from normal controls were 84.6 and 86.7%, respectively, and the sensitivity and specificity of distinguishing lung adenocarcinoma from normal controls were 78.6 and 80.0%, respectively. It suggested that *Veillonella* and *Capnocytophaga* may serve as potential biomarkers for the detection or classification of lung cancer.

Systemic diseases

Cardiovascular disease

The salivaomics study of cardiovascular disease is mainly focused on proteome. C-reactive protein (CRP), salivary creatinine kinase myocardial band (CK-MB), myoglobin (MYO), troponin-1 (Tn1), myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9) are the most studied salivary biomarkers of cardiovascular diseases. Among them, CRP, CK-MB, MYO and Tn1 are the most promising biomarkers, and the results of others are controversial. Miller et al. [251] studied the role of serum and saliva as diagnostic fluids for identifying biomarkers of acute myocardial infarction (AMI). Through logical regression analysis, it was found that CRP was the best biomarker for predicting AMI. Floriano et al. [252] showed that the combination of salivary biomarkers containing CRP and electrocardiogram (ECG) could effectively distinguish AMI from healthy controls (AUC=0.96),

which was much higher than that of ECG alone. These reports indicate that salivary CRP has considerable potential in the diagnosing AMI. Other biomarkers such as CK-MB, MYO and Tn1 present consistent increases in blood and saliva in patients with AMI, significantly higher than in healthy controls [93, 252, 253]. However, the current research evidence is insufficient to support them as saliva biomarkers of AMI, which needs to further expand the sample for research.

Diabetes mellitus

In the past, many studies have investigated the salivary protein profile of patients with diabetes mellitus (DM), and identified a large number of differentially expressed proteins, such as S100 calcium-binding protein A7, alpha-2 macroglobulin, alpha-1 antitrypsin and complement component 3, which can change with the development of diabetes [94, 154, 254]. However, most of these studies have failed to find diagnostic markers or combinations. The 1,5-AG ($C_6H_{12}O_5$) can reflect the average glycemic level in the past one week, and is more sensitive to postprandial hyperglycemia and blood glucose fluctuations. During long-term hyperglycemia, a large amount of urine glucose was filtered out continuously, which inhibited the reabsorption of 1,5-AG in the proximal renal tubules, resulting in the loss of 1,5-AG. As a result, the level of 1,5-AG in diabetes patients was lower. Jian et al. [95] detected the level of 1,5-AG in saliva of DM patients and subjects without DM by liquid chromatography-mass spectrometry (LC-MS). It was found that the level of 1,5-AG in patients with DM was lower, and it was positively correlated with serum 1,5-AG, and negatively correlated with blood glucose and glycosylated hemoglobin. Further analysis showed that the best cutoff points of saliva 1,5-AG_0 and 1,5-AG_{120} for DM screening were 0.436 μg/mL (sensitivity: 63.58%, specificity: 60.61%, AUC: 0.657) and 0.438 μg/mL (sensitivity: 62.25%, specificity: 60.41%, AUC: 0.660), respectively. Compared with fasting blood glucose (FPG) alone, the combination of FPG and saliva 1,5-AG reduced the proportion of people who needed oral glucose tolerance test (OGTT) by 47.22%. It is suggested that saliva 1,5-AG is a convenient and non-invasive tool for screening DM.

Other diseases

Differential expression of 8 proteins (calgranulin A, calgranulin B, apolipoprotein A-1, 6-phosphogluconate dehydrogenase, peroxiredoxin 5, epidermal fatty acid-binding protein, 78 kDa glucose-regulated protein precursor (GRP78/BiP), and 14-3-3 proteins) can be detected in saliva of patients with rheumatoid arthritis, which may play a role in diagnosis [96]. The level of salivary lactoferrin in patients with Alzheimer's disease (AD) is significantly decreased, which can distinguish prodromal AD/AD from healthy controls, and may have a good diagnostic performance for the detection of AD [255]. Twenty-five proteins were identified as overexpressed in saliva of children with autistic spectrum disorders (ASD). Eight have been identified as potential biomarkers of ASD [256]. Salivary cortisol and α-amylase levels have long been reported as routine biomarkers reflecting psychological stress [257].

Personalized medicine

Classifying the patients according to the changed salivaomics profile, and then using statistical analysis to develop targeted therapy for different types of patients can greatly improve the therapeutic effect. EGFR in lung cancer is a good example. Some common mutations, exon 19 deletion and exon point mutation 21-L858R of EGFR are sensitive to tyrosine kinase inhibitors, such as Osimertinib, gefitinib and erlotinib [247, 248]. The mutation of EGFR in saliva can be directly detected by EFIRM, and the treatment plan can be made according to the results. HER2 overexpression occurs in 15–20% of breast cancer patients, which is an indication of trastuzumab therapy [258]. Detecting the level of HER2 in saliva may help to classify breast cancer patients, and identify subgroups of patients who will benefit from trastuzumab therapy. In addition, studies have shown that salivary IL-6 and IL-8 levels can distinguish patients who are more sensitive to gingival inflammation [259].

Therapeutic effect monitoring

Saliva biomarkers can be used in the diagnosis of diseases, but also can be used to detect the therapeutic efficiency of some diseases. Sexton et al. [260] found that the salivary levels of IL-1β, MMP-8, OPG and MIP-1α can reflect the severity of periodontitis and its response to treatment, which has potential use in monitoring the status of periodontal disease. The miR-31 and miR-423-5p in saliva decreased significantly after OSCC resection, which can be used as biomarkers of therapeutic effect and postoperative follow-up monitoring of OSCC [34, 227].

COVID-19 screening

Since the end of 2019, coronavirus disease2019 (COVID-19) has swept the world, bringing the most serious crisis to

global health. Over the past two years, COVID-19 continues to wreak havoc worldwide because of its high mutagenicity and strong infectious ability. How to quickly detect the virus, identify, isolate and control positive infected person as soon as possible is an important measure. Therefore, efficient, rapid and accurate screening is the cornerstone of successful public health response to COVID-19. At present, nasopharyngeal sampling for nucleic acid amplification testing (NATT) is a non-invasive standard test for the diagnosis of COVID-19. However, this method requires trained personnel and specially designed swabs. On the other hand, the process of collecting nasopharyngeal swabs is painful for the subjects, and some elderly people or children cannot easily cooperate with the collection. And there is also a risk of infection when taking samples from some patients who have been diagnosed. Furthermore, although RT-qPCR is the gold standard for the diagnosis of COVID-19, the time it takes to provide results (a few hours to 1 day) does not meet the requirements of public health and epidemic prevention to respond quickly to emergencies in the face of large-scale screening. Therefore, saliva samples instead of nasopharyngeal swab samples, looking for faster detection methods than RT-qPCR, and developing kits or tools convenient for people to detect by themselves will be the direction of diagnosis of COVID-19 in the future. A recent systematic review and meta-analysis comparing saliva samples with nasopharyngeal swab RT-qPCR for COVID-19 indicated that the sensitivity and specificity of saliva samples were 83.2 and 99.2%, respectively, while the NATT with a sensitivity of 84.8% and a specificity of 98.9%. The results showed that the diagnostic accuracy of saliva samples was similar to that of NATT, which supported the use of saliva samples as a substitute for nasopharyngeal swabs for extensive scale screening [261]. On the other side, finding a faster and more convenient detection method than RT-qPCR is also on the agenda. Huang et al. [262] developed reverse transcription loop-mediated isothermal amplification (RT-LAMP) to rapid screening of COVID-19 in saliva. It is confirmed that multiple RT-LAMP can directly detect COVID-19 particles as low as 1.5 copies/ul in saliva without isolating RNA. It has the same sensitivity and specificity as standard RT-qPCR. Wood et al. [263] used synchrotron-Fourier transform infrared (FTIR) and purified virus Raman spectra to study the characteristic spectral signals of COVID-19 biomarkers in saliva samples. Through the construction of the model to detect COVID-19 in saliva samples, they achieved 93% sensitivity and 82% specificity. The above researches shows that the using saliva samples, combined with new detection techniques for COVID-19 screening, will be the future research direction, which is of great significance for the global prevention of COVID-19.

The limitations and future prospects

With the in-depth study of salivomics, saliva plays an increasingly important role in fluid biopsy, considered a reliable biological diagnostic fluid. Simple collection, convenient storage, non-invasive and containing a large number of diagnostic biomolecules are the advantages of saliva. It makes the impression that saliva will replace blood as the main body of fluid biopsies. However, saliva diagnosis is still in the early stage of development, and further research is necessary to determine and consolidate its position. Generally, the current studies on saliva biomarkers also show a lot of limitations.

Firstly, saliva analysis does have its limitations. The volume of saliva in the body is much lower than that of blood, and the concentration of saliva biomarkers is very low, which poses a challenge to the analysis. And the overlap of specific saliva markers in some diseases makes it difficult to clearly associate the disease with salivary biomarkers. For example, the level of MMP has significant changes in chronic periodontal diseases, oral mucosal diseases, and even cardiovascular diseases. This makes it difficult to accurately determine whether changes in biomarkers are caused by one particular disease or another. If a specific link between these biomarker levels in serum and saliva can be established, clinicians will have another tool to improve patient diagnosis and care.

Secondly, a single biomarker is not enough to define the pathogenesis of the disease, and using a variety of biomarkers to build a diagnostic model seems to be the most likely method. Additionally, to identify specific salivary biomarkers for specific diseases, the biomarkers need to be studied in large cohorts and long-term follow-ups to verify their accuracy. The clinical-oriented research will fundamentally improve the level of existing supporting evidence. The best diagnostic model of saliva may come from the combination of biomarkers of each pathological condition, with clinical effectiveness and higher accuracy and specificity.

Thirdly, the composition of saliva may vary with the method and time of collection, thus affecting the concentration of salivary biomarkers. Standardized programmes should be developed to reduce heterogeneity between studies on a given disease. In addition, continuous measurements may be more valuable than single-point measurements, and are more capable of analyzing biomarkers in saliva.

Finally, it must be pointed out that although there have been tremendous studies related to saliva diagnosis, the development of related kits is still in the exploratory stage.

On the one hand, disease-related salivary biomarkers require more research to be identified. On the other hand, saliva testing requires some nanoscale devices with high sensitivity and specificity for accurate detection. In addition, current gold standard detection methods, such as PCR, gel electrophoresis, chromatography, microarray, are time-consuming and require trained professionals for analysis. These are important factors that limit the commercialization of salivary kits for disease diagnosis. Therefore, the development of fast, convenient and inexpensive detection methods will also be the focus of future research on saliva diagnosis, which will make saliva diagnosis go further.

Conclusions

As mentioned above, saliva biopsy will bring new opportunities for fluid biopsies. It is expected that with the accumulation of salivomics research data, a salivary biomarker group will be established for most diseases, through which diagnosis will become more and more common. Furthermore, it will also lead to the development of real-time testing and screening tools, providing faster and more effective treatment and patient management. As the researches progress, we believe that evidence will emerge to determine the relationship between various diseases and salivary biomarkers. Most of the evidence may come from large-scale longitudinal studies, and will be applied in the clinic, such as diagnostic testing, therapy management and monitoring. This field is still in the infant. However, salivary biomarker research is receiving rapid attention, and significant progress is expected in the future.

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