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Review

Untargeted Salivary Metabolomics and Proteomics: Paving the Way for Early Detection of Periodontitis

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Abstract: Periodontitis is a chronic inflammatory disease that affects the supporting structures of the teeth and is a major cause of tooth loss worldwide. Early detection is critical to prevent disease progression and avoid irreversible tissue damage. Saliva, a non-invasive, easily accessible biological fluid, has emerged as a promising diagnostic tool for the early detection of various diseases, including periodontitis. This narrative review explores the potential of untargeted salivary metabolomics and proteomics in identifying biomarkers for the early diagnosis of periodontitis. Unlike traditional targeted approaches, untargeted analyses allow for the comprehensive exploration of a wide range of metabolites and proteins, without predefined hypotheses. This approach provides a deeper understanding of the disease's biochemical landscape and can reveal novel biomarkers associated with the inflammatory processes of periodontitis. Besides making an early diagnosis, detecting specific biomarkers of periodontitis may enable the clinician to make an extremely personalized treatment plan. The review highlights key findings in the field, discusses the challenges and limitations of these techniques, and presents future perspectives on how salivary metabolomics and proteomics could revolutionize early diagnostic strategies in periodontal management.

Keywords: technologies; metabolomics; proteomics; saliva biomarkers; periodontitis; early detection

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1. Introduction

Periodontitis is a chronic multifactorial inflammatory disease with bacterial etiology affecting tooth-supporting structures (periodontal ligament, gums, cementum, and alveolar bone), that if left untreated, may lead to tooth loss [1]. It is highly spread worldwide, especially in its severe form, which represents the sixth most prevalent condition globally, affecting 10% of adult individuals [2,3]. Poor oral hygiene and dysbiosis of oral microbiota, in genetically predisposed individuals, may lead to aberrant inflammation mediated by the host's immune system, which damages the periodontal tissues, leading to periodontitis [4]. During recent decades, increasing evidence has demonstrated that periodontitis is not just a local inflammatory condition, but it may also have systemic implications [5]. In fact, it is related to inflammatory systemic diseases, such as diabetes and cardiovascular diseases [6]. Periodontitis is evaluated by measuring clinical periodontal indices, among

which are clinical attachment loss (CAL), probing depth (PD), and gingival recession (REC). In particular, a patient is affected by periodontitis when interdental CAL is present in more than one non-adjacent tooth and periodontal charting shows the presence of PPD of 4 mm or more [1]. If left untreated, periodontitis leads to teeth loss, compromising the normal occlusion and the masticatory function. For this reason, the progression of the disease should be arrested as soon as possible, and early diagnosis is the main tool to control it. Traditional diagnostic procedures have some limitations: they require skilled dentists and assistants to assess and document periodontal clinical parameters properly; there may be differences in the clinical evaluation among operators; the probing process may cause patient discomfort; and the procedure is both time consuming and expensive [7]. Beyond the traditional diagnostic methods, research has been focusing on other methods, involving saliva and gingival crevicular fluid (GCF), in order to find out the effectiveness and the possibility of early diagnosis, overcoming the limitations of the traditional diagnostic method. In particular, saliva is a biofluid that contains a wide variety of biomarkers, which reflect both physiological and pathological conditions [8]. Its collection is non-invasive and particularly suitable for tests requiring repeated sampling (Figure 1). Moreover, advancements in test accuracy, sensitivity, and reliability have enhanced salivary diagnostic capabilities and have facilitated the identification of novel biomarkers, especially those involved in oral disease processes, including caries and periodontitis [9].

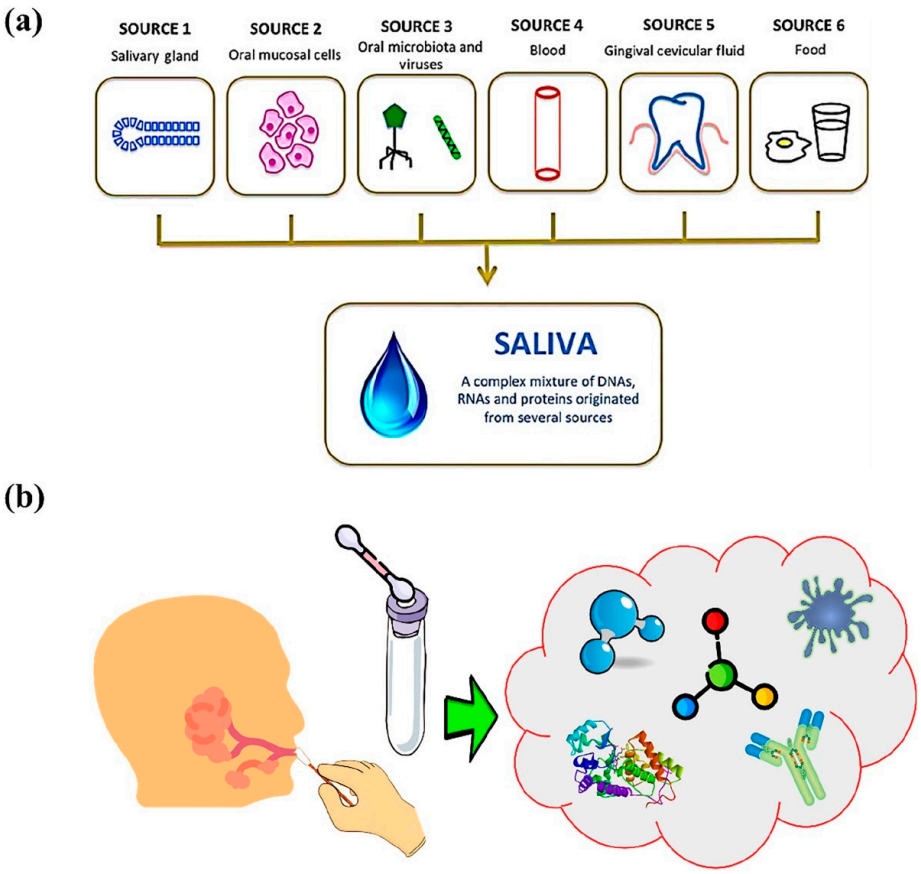

Figure 1. Saliva composition. (**a**) Saliva is composed of lots of components derived from different sources. (**b**) Salivary samples that contain water, electrolytes, proteins, mucus, and antibodies. From [10], under the terms of the Creative Commons CC BY license.

Metabolomics and proteomics are emerging as viable methods for assessing the salivary metabolites and proteins that are involved in biochemical processes, and their analysis helps elucidate the pathways underlying different oral and systemic diseases. Salivary pro-

tein and metabolite profiles can be regarded as valid tools for diagnosing and monitoring periodontitis [11,12]. In fact, the omics approach has already been used in some studies for periodontitis patients, giving promising results [13,14]. By metabolomic and proteomic analysis, it is possible to systematically screen metabolite profiles and protein expression by both quantitative and qualitative means [12,15]. There are two approaches to omics analysis: targeted and untargeted. The first one, that is the conventional analysis method, focuses on selected subtypes of metabolites; instead, untargeted analysis enables the comprehensive investigation of a broad spectrum of metabolites and proteins without prior assumptions. This approach offers a deeper insight into the biochemical processes of the disease and may uncover novel biomarkers linked to its inflammatory mechanisms. The means to get untargeted metabolomics and proteomics analysis are various: for metabolomics there are mass spectrometry (MS), liquid chromatography (HPLC-MS) or two-dimensional gas chromatography (2DGC-MS), nuclear magnetic resonance (NMR) spectroscopy, Fourier-transform infrared (FTIR), photoacoustic spectroscopy (PAS) [12,16,17]; for proteomics, there are liquid chromatography and tandem mass spectrometry (LC-MS/MS) [18].

Metabolomics and proteomics have been recently introduced, and their use represents an interesting research field in expansion. In this regard, the aim of this narrative review is to examine the potential of untargeted salivary metabolomics and proteomics in identifying biomarkers for the early detection of periodontitis. The review summarizes key findings, addresses the challenges and limitations of these techniques, and explores future directions for integrating salivary metabolomics and proteomics into early diagnostic strategies for periodontitis.

2. Materials and Methods

The articles included in this narrative review were identified using the major search engines: Pubmed and Google Scholar. The keywords used in all search engines were as follows: “salivary diagnosis in periodontal disease”, “metabolomics in periodontitis”, and “proteomics in periodontitis”. Articles were included in the present review after the exclusion of duplicate papers and articles that did not fit the topic and inclusion criteria. At least two independent researchers reviewed titles and abstracts for inclusion. Full articles were requested for all articles that passed the initial screening. Each full article was evaluated by two researchers for final inclusion/exclusion. In case of disagreement, a third researcher was consulted, and the decision was made by consensus. Initial screening was based on the following criteria: RCTs, cohort studies, case-control and case-series studies, meta-analysis, and systematic review. Only studies in the English language were included in this review.

3. Periodontitis: Pathogenesis and Early Diagnosis

Periodontitis is a chronic inflammatory disease affecting the tooth-supporting structures, including the periodontal ligament, cementum, gums, and alveolar bone. Pathogenesis of periodontitis involves a complex interplay between microbial factors and the host immune response (Figure 2).

At first, there is microbial colonization, periodontitis pathogens, including *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, organized in biofilm, colonize tooth surfaces [19]. In particular, the key periodontal pathogen is *Porphyromonas gingivalis*, which exhibits a very large number of virulence factors that, in the presence of nutrients, are fundamental for colonization and persistence, immune system evasion, impairment of inflammatory cell function, and cause immune subversion and consequent inflammation. *Porphyromonas gingivalis* cannot induce tissue destruction or bone loss by itself, but manages to do it by manipulating other commensals and modulating the host response, sub-

sequently disrupting host homeostasis and inducing dysbiosis [20]. In fact, in response to the microbial invasion, the host has an inflammatory reaction that involves the recruitment of neutrophils, macrophages, and T-cells, and the release of pro-inflammatory cytokines (e.g., IL-1β, TNF-α, IL-6) [21]. Chronic inflammation leads to the release of matrix metalloproteinases (MMPs) and other enzymes that degrade connective tissue and bone. The persistence of chronic inflammation leads to severe damage to tooth-supporting structures, including CAL and bone resorption, and if it is left untreated, it may lead to tooth loss [22].

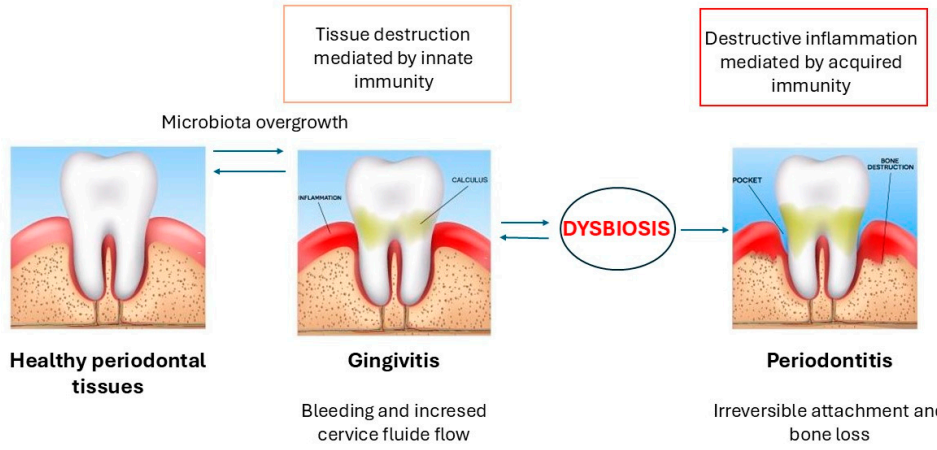

Figure 2. Schematic representation of pathogenesis of periodontitis. From healthy periodontal tissues, microbiota overgrown leads to a reversible condition called gingivitis, which consists of inflammation of gums mediated by innate immunity with bleeding and increased cervical fluid flow. In presence of dysbiosis gingivitis can get worse, leading to periodontitis, in which is a condition in which there is destructive inflammation mediated by acquired immunity with irreversible attachment and bone loss.

3.1. Early Detection and Salivary Diagnosis of Periodontitis

Early detection of periodontitis would allow prevention of irreversible tissue damage, consequently improving treatment outcomes. In fact, early detection of periodontitis makes early treatment possible, preventing CAL and bone loss, which are irreversible once destroyed [23]; in addition, periodontal therapies, including scaling, root planing, and antimicrobial therapy, are more effective when conducted at early stages [24].

Moreover, it is known that periodontitis is linked with systemic health, in particular with cardiovascular disease, diabetes, and adverse pregnancy outcomes. Early detection of periodontitis may help reduce systemic inflammatory condition and reducing risks of the correlated systemic implications [25]. Finally, intercepting periodontitis at early stages implicates early management, which reduces the need for complex and costly treatment, such as surgery or prosthetic rehabilitation [26].

A promising means of early detection of periodontitis is the analysis of salivary samples. Saliva has the following advantages: it contains biomarkers, its collection is easy and non-invasive, and it provides real-time information. Saliva contains a wide array of potential biomarkers, including cytokines (e.g., IL-1β, IL-6), enzymes (e.g., MMP-8), immunoglobulins, and microbial DNA/RNA, reflecting the health status of the patient, including periodontium condition [27]; its collection is simple, painless, and does not require specialized doctors, making it ideal for routine screening and epidemiological studies [28]; salivary diagnostics can provide real-time information on disease status, progression, and response to therapy [29]. All the characteristics of salivary diagnosis of periodontitis are very advantageous, making it a good potential periodontitis diagnostic tool.

3.2. Saliva Compared with Gingival Crevicular Fluid

The advantages of salivary analysis are its non-invasive collection and being suitable for tests requiring repeated sampling, but it has some limitations too [8]. In particular, when compared to GCF analysis, saliva analysis can be less accurate in representing periodontal condition. According to the existing analyses of GCF, its composition has similarities and differences with saliva composition [30]. This is because saliva collects compounds from many other sources than GCF and it reflects both the individual systemic condition [31] and the whole mouth infectious-inflammatory condition [32], while GCF reflects a specific site of the mouth, that is to say gingival sulcus or periodontal pocket. For this reason, GCF characteristics are more influenced by the quality of the subgingival ecological niche [33]. On the other hand, the collection of GCF may be more difficult and uncomfortable than salivary collection. GCF sampling is made with strips inserted in two different sites of the gingival sulcus for each tooth. Collection times for GCF samples can vary from 30 s, 60 s to 3 min. Such variation depends on the fluid-flow dynamics of the host. Healthy patients have a slower GCF rate, while unhealthy patients (i.e., periodontal disease) have faster rates. Moreover, the clinician should be careful about the possible contamination of blood, especially for periodontitis patients. GCF may also be contaminated by saliva; for this reason, the area should be isolated with cotton rolls [34].

4. Salivary Samples: The Importance of the Correct Collection and Preparation

The pre-analytical processing of biological samples is a crucial aspect of metabolomic and proteomic research; in fact, it directly impacts the accuracy and reliability of metabolite and protein measurements, ultimately influencing the overall quality of the data. This stage involves various procedures, including sample collection, quenching, extraction, and storage.

Sample preparation protocols are essential for providing an accurate representation of the metabolic and protein profile of the biological system that is studied [35]. In human metabolomics and proteomics research related to oral diseases, participants are typically advised to refrain from eating, drinking, chewing gum, using oral hygiene products, taking medication, or smoking before sample collection to minimize potential factors that could affect the results [36]. Consequently, incorrect sampling timing and inadequate sample collection or preparation can significantly compromise the accuracy and reliability of the results [37] (Figure 3).

Another critical factor to consider is metabolic flux, which is the movement of metabolites through a reaction system over time. To ensure that samples remain metabolically inactive after collection, it is essential to prevent biochemical reactions that can lead to rapid metabolite turnover within seconds to minutes [38]. Sample quenching plays an important role in arresting or slowing down these metabolic processes, preserving the metabolic profile during sample handling. This step is typically performed at the moment of collection and before extraction [39]. To minimize potential alterations after sample collection, samples are typically flash-frozen in liquid nitrogen and stored at −80 °C immediately. If −80 °C freezers are unavailable at the clinical site, an alternative approach involves freezing samples at −25 °C or collecting them in sterile containers with a preservative like sodium azide, followed by immediate freezing in liquid nitrogen [40–42]. Before analysis, saliva samples should be thawed in ice and then centrifuged at low temperatures to remove cell debris. There is a two-step centrifugation protocol. An initial centrifugation at 2600–3000× g for 10–15 min at 4 °C is performed to remove large debris, epithelial cells, and food particles. This is followed by a higher-speed centrifugation at 10,000–15,000× g for 10–15 min to eliminate residual cellular material and microorganisms. For studies focusing on extracellular vesicles or aiming for maximum sample purity, an optional ultracentrifugation step at ≥100,000× g for

60–90 min may be applied [43]. After centrifugation, metabolite extraction is conducted using solvents that provide broad coverage of both polar and non-polar compounds. Commonly used solvents include methanol: water (80:20 or 70:30, v/v) for effective protein precipitation and polar metabolite recovery, or methanol:chloroform: water (2:2:1.8, $v/v/v$) for biphasic extraction of both hydrophilic and lipophilic molecules. Acetonitrile: water mixtures (1:1 or 3:1) are also widely used due to their efficiency in protein removal and compatibility with both MS and NMR platforms [44].

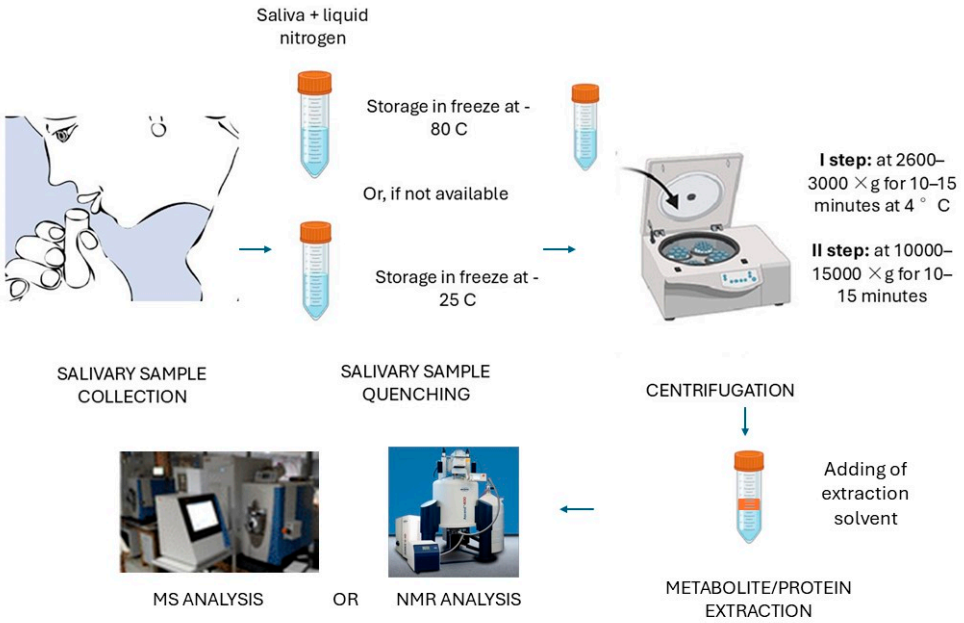

Figure 3. Collection and preparation of salivary samples. Sample collection and preparation are crucial steps in salivary analysis. If some step is incorrectly performed, the whole analysis may be damaged. The patient is invited to spit into a test tube an amount of 1.5–2 mL of saliva. The collected sample undergoes a quenching process, that is, the inhibition of all the biochemical reactions by freezing the sample at −80 °C or −25 °C, to preserve the real composition of such sample. When it is ready to be analyzed, the sample is centrifuged to remove all cell debris. Centrifugation is followed by metabolite extraction. At the end, the analysis may be conducted by using MS or NMR.

In an untargeted approach, the extraction process aims to recover the majority of metabolites and proteins from the biological sample while ensuring reproducibility and accuracy, particularly when analyzing a large number of samples. In contrast, a targeted approach tailors the extraction process to the specific metabolites or proteins of interest. Additionally, the extraction method should be simple and efficient to prevent alterations in metabolite or protein composition due to prolonged preparation times [41,45–48].

5. Salivary Metabolomics: Techniques and Applications

Salivary metabolomics differs among individuals; for this reason, the most appropriate way to analyze them is through longitudinal studies, instead of cross-sectional studies. The composition of salivary metabolomics reflects the physiological or pathological condition of the individual and depends on the characteristics of the individual's oral microbiome [49]. Moreover, variation in the microbiome influencing the characteristics of salivary metabolomics may reflect a local or systemic disease [50,51]. Untargeted metabolomics gives the possibility to examine the whole spectrum of metabolites contained in the saliva. In this way, we can find metabolites that would have been discarded previously, but that can be involved in some disease's biochemical process. Due to the vast diversity of metabolites in the metabolome, each with distinct chemical and physical

properties, relying on a single analytical method for their detection is impractical. Therefore, adopting an approach that integrates multiple analytical techniques is essential to ensure comprehensive metabolite coverage [52]. The analysis of salivary metabolomics is driven by different methods, including MS in conjunction with either HPLC-MS or 2DGC-MS, and NMR spectroscopy. FTIR spectroscopy analyzes molecular composition using infrared light. Different molecular bonds absorb specific wavelengths; thus, by examining the resulting spectra, it is possible to determine compounds and their concentrations. PAS is a variation of FTIR, and it is particularly effective for gas analysis. However, both techniques share the same limitation: they are highly sensitive to water, requiring sample drying before measurement to ensure accuracy [12,16,17] (Table 1) (Figure 3).

Table 1. Characteristics of metabolomics sequencing techniques.

Metabolomics Sequencing Techniques	Methodologies	Strengths	Limitations	Potential Role in Early Diagnosis of Periodontitis	References
NMR Spectroscopy	Detects nuclear magnetic resonance signals (commonly ^{1}H, ^{13}C); non-destructive and quantitative.	- High reproducibility - Non-destructive - Absolute quantification	- Low sensitivity - Signal overlap - Costly instruments	Reliable for saliva metabolite fingerprinting and longitudinal studies.	[42,46,53,54]
GC-MS	Separates volatile/derivatized compounds; detects by MS.	- High sensitivity - Good for small molecules - Established protocol	- Requires derivatization - Limited to volatile analytes	Useful for SCFAs and volatile microbial metabolites.	[55–57]
LC-MS	Separates polar/non-polar metabolites in liquid phase; detects ions by MS.	- Broad coverage - High sensitivity - Versatile	- Ion suppression - Requires complex QC	Gold standard for untargeted saliva metabolomics.	[58,59]
FTIR Spectroscopy	Measures infrared absorption of molecular bonds; generates spectral fingerprints.	- Rapid, label-free - Minimal preparation	- Lower specificity - Mostly qualitative - Sensitive to water	Distinguishes healthy from diseased saliva via biochemical fingerprinting.	[16,60,61]
PAS (Photoacoustic Spectroscopy)	Measures acoustic waves generated after absorption of modulated light; used to assess biochemical changes.	- Non-invasive - High sensitivity to molecular vibrations - Can be miniaturized	- Lower metabolite specificity - Limited commercial systems - Sensitive to water	Emerging tool for real-time screening of periodontal changes in saliva.	[12,62,63]

In 2015, Dame et al. [64] gave a panoramic view of salivary metabolomics by analyzing salivary metabolomics using NMR. In their research, they identified a total of 76 different metabolites, some of which were previously reported as detected but not quantified. The researchers detected the following salivary compounds: short-chain organic acids (acetic, acetoacetic, butyric, citric, formic, glycolic, lactic, propionic, and pyruvic acids); amino acids (l-alanine, d-glutamic acid, l-valine, l-methionine, l-threonine, l-leucine, l-tyrosine, and l-phenylalanine); alcohols (e.g., ethanol), amines (e.g., ethanolamine, dimethylamine, and methylamine); sugars (e.g., d-glucose); and pharmaceutical adjuvants (e.g., propylene glycol). It was reported that the most representative compounds were short-chain organic acids, with acetic acid being the most abundant. The scientists of the study also reported higher rates of concentrations of acetic acid (6.8 ± 4.3 mM) and succinic acid (0.125 ± 0.181 mM) than previously reported in the literature [65] (Table 2) (Figure 4).

Table 2. The characteristics of biomarkers found in metabolomic untargeted analysis.

Biomarkers	Molecular Categories	Biological Pathways	Functions and Significance	References
Acetic, acetoacetic, butyric, citric, formic, isovalerate, formate, glycolic, lactic, propionic, and pyruvic acids.	SCFAs	They result from the fermentation of sugars and proteins from anaerobic bacteria (*Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella intermedia*), and some of them, like formate, may result from purine breakdown under oxidative stress.	Inducing apoptosis of gingival epithelial cells, impairing fibroblast function, suppressing immune cells, promoting dysbiosis and neutrophil recruitment, and decreasing pH.	[64,66–71]
L-alanine, d-glutamic acid, l-valine, l-methionine, l-threonine, l-leucine, isoleucine, l-tyrosine, and l-phenylalanine.	Amino acids	They are energy sources for anaerobic bacteria (*Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella intermedia*, and they result from the inflammation and tissue damage process.	Markers of tissue degradation, elevated in inflammation, reflecting the host's immune response involved in energy metabolism, and bacteria.	[64,72,73]
Taurine, ethanolamine, dimethylamine, and methylamine.	Amines	They are released by the host immune system.	Antioxidant effect, in particular, Taurine. Maintaining cellular homeostasis under inflammatory conditions.	[64,74–76]
D-glucose	Sugar	They are an energetic source for periodontal bacteria.	Augmented energy demand of bacteria.	[64,77]
Hypoxanthine	Purine nucleobase	It is a byproduct of purine degradation mediated by bacteria.	Its presence indicates periodontal pathogens' activity.	[78]

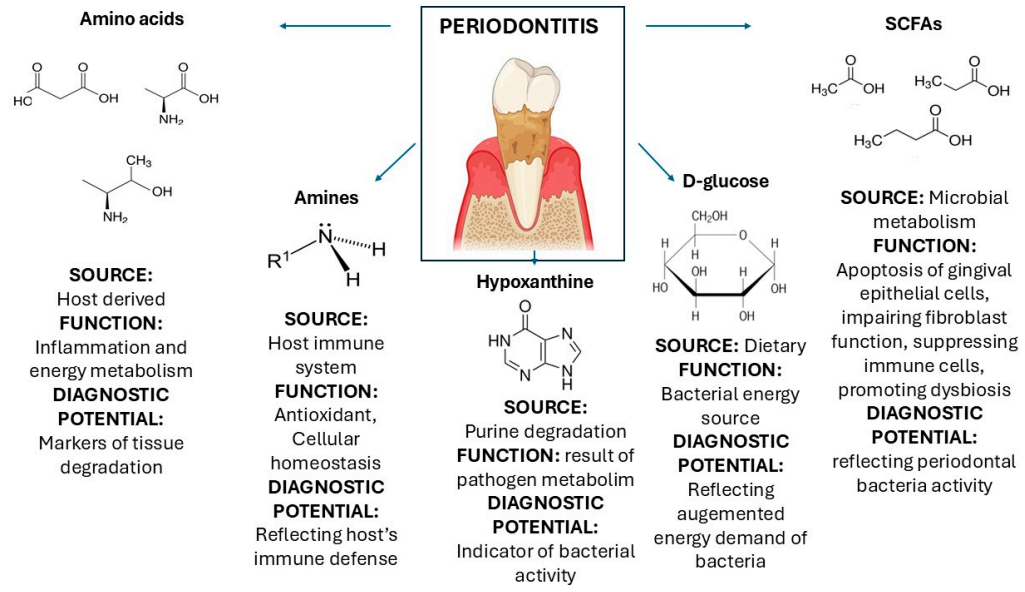

Figure 4. The characteristics of periodontal metabolites. Metabolites involved in periodontitis differ in sources, function, and periodontitis diagnostic potential. The figure summarizes such characteristics of the principal groups of periodontitis metabolites.

The affirmation of Dame et al. [64] was that such differences may be due to differences in ethnicity, oral microflora, the time of sample collection, diet, and other associated factors. Concerning the research about variation in metabolic profile during periodontitis, some preliminary studies have investigated it, giving good potential results [42,79,80] (Table 3).

Table 3. Main findings of the studies about metabolomics in periodontitis.

References	Study Design	Sample Size	Method of Analysis	Variation in Metabolomic Profile
Aimetti et al. [79]	Cross-sectional study	32 cases, 22 controls	Nuclear Magnetic Resonance	Higher concentrations of acetate, γ-aminobutyrate, *n*-butyrate, succinate, trimethylamine, propionate, phenylalanine, and valine, and decreased concentrations of pyruvate and *N*-acetyl in GCP patients compared with controls.
Rzeznic et al. [42]	Cross-sectional study	26 cases, 25 controls	Nuclear Magnetic Resonance	Higher concentrations of short-chain fatty acids and lower concentrations of lactate, γ-amino-butyrate, methanol, and threonine in periodontitis.
Romano et al. [80]	Cross-sectional study	33 cases with GCP, 28 cases with GAgP; 39 controls.	Nuclear Magnetic Resonance	Higher concentrations of pyruvate, *N*-acetyl groups, and lactate, and higher levels of proline, phenylalanine, and tyrosine in GCP and GAgP patients compared with controls.
Kim et al. [74]	Cross-sectional study	129 cases, 92 controls.	Nuclear Magnetic Resonance	Higher concentrations of taurine, isovalerate, butyrate, and glucose in periodontitis.
García-Villaescusa et al. [39]	Case-control study	91 cases, 39 controls.	Nuclear Magnetic Resonance	Higher concentrations of caproate, isocaproate, butyrate, isovalerate, isopropanol, methanol, 4-aminobutyrate, choline, sucrose, sucrose-glucose-lysine, lactate-proline, lactate, and proline in periodontitis.

GCP: Generalized Chronic Periodontitis. AgP: Generalized Aggressive Periodontitis.

Among the cited studies, the most recurrent metabolites that were found after metabolomic untargeted analysis are as follows: short-chain organic acids and amino acids, both detected in six studies [40,42,64,74,79,80]. Kim et al. [74] identified five periodontal biomarkers in salivary samples. They conducted a study comparing periodontitis patients to healthy controls and subdivided the periodontitis patients according to the severity of the disease (stage II and stage III periodontitis). According to their study, age does not produce any change in salivary metabolomics, and there is no correlation between the clinical severity of pathology and the metabolites; instead, some of them are indicators of disease. The researchers made both discovery and validation for biomarkers, identifying five metabolites: ethanol, taurine, isovalerate, butyrate, and glucose. Such metabolites have a significant correlation with periodontal clinical parameters, being candidates for an accurate diagnosis of the disease. In particular, taurine, isovalerate, butyrate, and glucose augmented significantly in periodontitis patients. An increase in butyrate and isovalerate-major short-chain fatty acids (SCFAs) in periodontitis [30,40], and, in opposition to the study of Kim et al. [74], demonstrated the existence of the. association of the increase in butyrate with the progress of the periodontitis stage [77,81]. In fact, SCFAs

are the end-products of bacterial metabolism; they are involved in periodontitis [70] and are associated with deep probing depth, loss of attachment, and bleeding on probing [42]. Moreover, butyrate and isovalerate in high concentrations were found in sites positive to *Porphyromonas gingivalis*, which is one of the most important periodontal bacteria [71]. Periodontal bacteria products induce tissue damage, and for this reason, the host tries to defend itself by metabolite relapse with antioxidant effects, in particular Taurine. Such a metabolite accumulates in damaged tissues to preserve them by its antioxidant effect, maintaining cellular homeostasis under inflammatory conditions [75,76]. The increase in taurine in periodontitis shows its role in tissue protection and repair [82] and its diagnostic importance [74]. The increase in glucose levels demonstrates that the energy demand of pathogenic bacteria was increased and a more favorable energy environment for oral bacteria was generated during periodontitis [74]. Ethanol concentrations decrease significantly in periodontitis patients in comparison with healthy controls [74,82]. This volatile organic compound is present in saliva and is produced by bacterial alcohol dehydrogenase. It can be converted into acetaldehyde, a known carcinogen. The bacterial conversion of ethanol to acetaldehyde in saliva is associated with poor oral health and may play a role in oral cancer development. The fact that a more significant reduction in ethanol concentration was observed in periodontitis patients indicates that ethanol oxidation is highly activated in the presence of periodontitis [74]. The aforementioned metabolites are the results of preliminary research; further investigations are needed. The discoveries should find the most accurate methods of identification of periodontitis-related metabolites to integrate them into daily clinical practice. In addition, metabolites identified as related to periodontitis can help in tailoring periodontitis treatment and evaluating the therapeutic response, too. For example, Citterio et al. [73] demonstrated that concentrations of some metabolites detected in patients with generalized periodontitis changed after non-surgical therapy (NST). In particular, they observed a significant decrease in the levels of valine and isoleucine ($p < 0.005$). These amino acids are known to reflect the host's immune response to the oral microbiota and have previously been linked to active periodontal disease [83]. Hypoxanthine levels also decreased after NST, suggesting that the treatment effectively mitigates the cellular metabolic variations caused by bacterial activity. Hypoxanthine is a byproduct of purine degradation mediated by bacteria, particularly under oxidative stress conditions, which is common in sites affected by periodontitis [84]. Moreover, an increase in formate levels was observed, which may indicate a decrease in anaerobic bacterial populations within the oral cavity. Lastly, the reduction in phenylalanine concentration reflects a reduction in host tissue damage following successful periodontal therapy [78,85]. These discoveries of Citterio et al. were limited, due to the small sample size, but suggests that metabolomic profiling can detect elevated inflammatory markers or oxidative stress metabolites, which may guide decisions regarding the most suitable therapy [86] and may predict how well a patient responds to periodontal therapy, aiding in treatment planning [87].

Moreover, post-treatment shifts in metabolomic profiles may indicate therapeutic success or persistent inflammation earlier than clinical signs. Monitoring the therapeutical effects through saliva-based metabolomic testing is very advantageous because it is non-invasive and can be repeated easily; the molecular-level feedback, may help clinicians in adjusting periodontal treatment basing on biological evidence [44].

6. Salivary Proteomics: Techniques and Applications

Untargeted salivary proteomics is emerging as a promising approach to identifying proteins involved in pathological processes, including periodontitis. In fact, as for untargeted metabolomics, untargeted proteomics consists of the complete analysis of salivary compounds without preselection, with the difference that it analyzes only salivary

proteins. Shotgun proteomics is an untargeted analysis approach to examine the whole proteins contained in the sample analyzed. This method relies on bottom-up proteomics, where proteins are enzymatically digested—usually with trypsin—into peptides before being identified and quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Unlike targeted proteomics, shotgun proteomics allows for the broad detection of thousands of proteins in a single experiment, making it particularly useful for the discovery of new biomarkers involved in specific diseases [18,88,89]. Most salivary proteins are secreted by salivary glands, their role is to provide viscoelasticity and to allow the coating of oral surfaces made by saliva. Proteins from blood enter saliva in relatively small amounts as part of a transudate, crossing tight junctions between epithelial cells, which present a diffusion barrier. Mucin glycoproteins (MUC5B and MUC7), statherin, proline-rich proteins, carbonic anhydrase 6, histatins, secretory component, secretory IgA, IgG, albumin, lysozyme, lactoferrin, matrix metalloproteinase-8, interleukin 8, nerve growth factor, leptin, LL37, and alpha-defensin are the principal proteins and peptides detected in saliva that may serve as biomarkers [90]. Besides the role of protecting oral surfaces and providing lubrication [91], salivary proteins are involved in the following processes: antimicrobial defense, in particular, lysozyme, lactoferrin, and defensins are active against bacteria, viruses and fungi, contributing to the immune defense system; anti-inflammatory process, in fact, histatins and cystatins suppress the production of pro-inflammatory cytokines and inhibit proteases involved in tissue degradation, reducing inflammation and promoting tissue repair and wound healing; digestion; pH regulation; mineralization; and tooth protection [92,93]. Considering their involvement in the aforementioned processes, variations in their concentrations may reflect para-physiological or pathological conditions. Changes in the salivary levels of components linked to oral diseases have been detected and have been studied in the last decades. For instance, antimicrobial peptides, including LL37 (cathelicidin), alpha-defensins, and beta-defensins, which originate from neutrophils and epithelial cells, can act as indicators of the innate immune response [94]. Moreover, matrix metalloproteinase-8, an enzyme that breaks down collagen and that is released by neutrophils and fibroblasts, has been found in higher concentrations in the saliva of patients with periodontitis. Elevated levels of matrix metalloproteinase-8 and other proteins in the saliva of these patients could serve as indicators for monitoring disease progression and evaluating responses to treatment [95] (Table 4) (Figure 5).

Table 4. The characteristics of biomarkers found in proteomic untargeted analysis.

Biomarker	Molecular Category	Biological Pathway	Function and Significance	Reference
Matrix metalloproteinase-8	Enzyme	It is released by neutrophils and fibroblasts	Breaking down collagen	[95]
Complement C3	Protein part of the complement system	It is triggered by immune complexes	Enhancing recognition and clearance of periodontal pathogens by phagocytes, recruiting neutrophils and other immune cells to the gingival tissues, leading to chronic inflammation	[96,97]
Profilin-1	Actin-binding protein	Rho GTPase signaling → Profilin-1 → Actin polymerization → Cell movement and adhesion.	Regulating actin polymerization and cytoskeletal growth	[98–100]

Table 4. *Cont.*

Biomarker	Molecular Category	Biological Pathway	Function and Significance	Reference
S100A8	Pro-inflammatory mediator	S100A8/A9 → TLR4/RAGE → NF-κB → Inflammation	Calcium ion binding Cytokine activity (in inflammatory states)	[18,101–103]
Fibrinogen	Glycoprotein	Fibrinogen → Thrombin cleavage → Fibrin → Clot formation & tissue scaffolding	Blood coagulation factor, acute-phase reactant, ligand for integrins (e.g., Mac-1, αIIbβ3), interacts with toll-like receptors (e.g., TLR4).	[96,104,105]
Cystatin-SN	Cysteine protease inhibitor	Cystatin-SN → Inhibition of cathepsins/proteases → Protection of connective tissue & regulation of inflammation	Cysteine-type endopeptidase inhibitor activity, protease binding	[106–108]

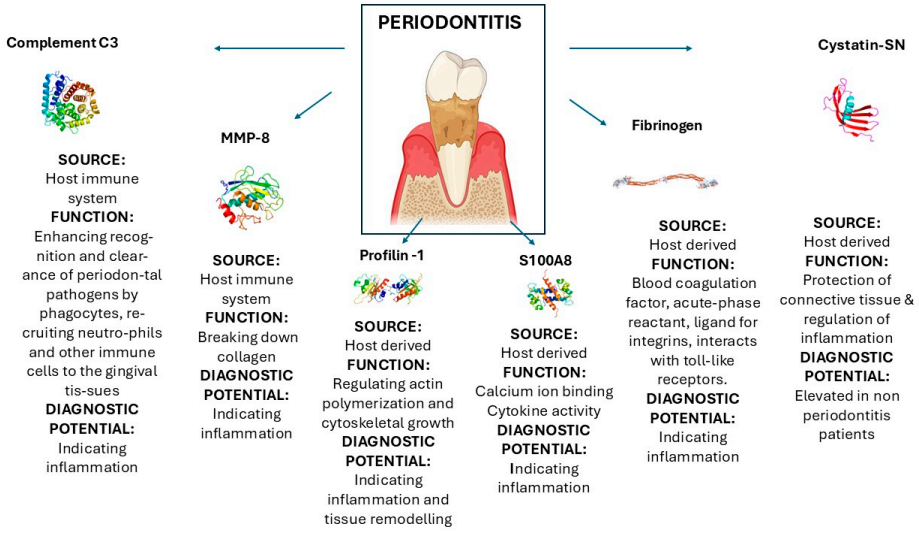

Figure 5. The characteristics of periodontal proteins. Proteins involved in periodontitis differ in sources, function, and periodontitis diagnostic potential. The figure summarizes such characteristics of the principal groups of periodontitis proteins.

Some recent studies applied mass-spectrometry-based untargeted proteomics to salivary samples of individuals affected by periodontitis [18,87,96,103,106,107,109–115] (Table 5). Among the cited studies, the most recurrent proteins which were found as promising biomarkers after proteomic untargeted analysis are S100A8, found in three studies [18,100,103] and Cystatin SN, found in two studies [106,107].

Table 5. Main findings of the studies about proteomics in periodontitis. CP: Chronic Periodontitis; AgP: Aggressive Periodontitis; AP: Advanced Periodontitis; MMP: Mild to Moderate Periodontitis; UP: Untreated patients; TP: Treated patients.

References	Study Design	Sample Size	Method of Analysis	Variation in the Proteomic Profile
Shin et al. [18]	Cross-sectional study	36 cases, 36 controls	Mass spectrometry-based untargeted proteomics	S100A8 and S100A9 higher in periodontitis patients.
Belstrøm et al. [87]	Cross-sectional study	10 cases, 10 controls	Mass spectrometry-based untargeted proteomics	Proteins associated with innate immune response were higher in Periodontitis patients

Table 5. *Cont.*

References	Study Design	Sample Size	Method of Analysis	Variation in the Proteomic Profile
Bostanci et al. [107]	Cross-sectional study	17 CP, 17 AgP; 16 controls	Mass spectrometry-based untargeted proteomics	Lactoferrin, lacritin, sCD14, Mucin 5B, and Mucin 7 down-regulated in AP and SLC4A1 was upregulated. Cystatin SN higher in controls
Mertens et al. [109]	Cross-sectional study	10 CP, 11 AgP; 12 controls.	Mass spectrometry-based untargeted proteomics.	Hemopexin, plasminogen, and α-fibrinogen were higher in periodontitis patients.
Tang et al. [110]	Cross-sectional study	16 cases, 17 controls.	Mass spectrometry-based untargeted proteomics.	Two peptide peaks had a lower level of intensity in the CP group, while the rest of the differentially expressed peptides had a higher level of intensity.
Antezack et al. [111]	Cross-sectional study	67 cases, 74 controls	Mass spectrometry-based untargeted proteomics	No identification of specific proteins
Hartenbach et al. [112]	Cross-sectional study	30 cases, 10 controls.	Mass spectrometry-based untargeted proteomics.	Salivary acidic proline-rich phosphoprotein, submaxillary gland androgen-regulated protein, histatin-1, fatty acid binding protein, thioredoxin, and cystatin-SA higher in periodontitis patients.
Grant et al. [103]	Cross-sectional study	10 MMP, 10 AP; 10 controls.	Mass spectrometry-based untargeted proteomics.	MMP9, S100A8, A1AGP, and pyruvate kinase higher in periodontitis patients.
Casarin et al. [113]	Cross-sectional study	12 cases, 13 controls	Mass spectrometry-based untargeted proteomics	GHG1, CSTB, KRT9, SMR3B, IGHG4, and SERPINA1 were higher in periodontitis patients.
Romano et al. [106]	Cross-sectional study	15 UP, 15 TP; 15 controls.	Mass spectrometry-based untargeted proteomics.	Cystatin SN higher in healthy patients and in patients under periodontal active treatment.
Gonçalves et al. [114]	Cross-sectional study	10 cases, 10 controls	Mass spectrometry-based untargeted proteomics	Periodontitis patients had higher levels of albumin, hemoglobin, and immunoglobulin, and they had a lower abundance of cystatin compared to the control group.
Salazar et al. [96]	Cross-sectional study	20 cases, 20 controls.	Mass spectrometry-based untargeted proteomics	alpha-2-macroglobulin, ceruloplasmin, complement C3, alpha-2-HS-glycoprotein, fibrinogen alpha chain higher in periodontitis patients-
Chaiyarit et al. [115]	Cross-sectional study	30 cases, 30 controls.	Mass spectrometry-based untargeted proteomics	No identification of specific proteins

These analyses identified several proteins consistently overexpressed in periodontitis patients, including complement C3, profilin-1, S100A8, and fibrinogen. Conversely, proteins, such as cystatin-SN and leukocyte elastase inhibitors, were found at elevated concentrations in healthy individuals. These findings suggest that specific salivary proteins could serve as potential biomarkers for distinguishing periodontal health from periodontitis [11]. The progress and efficacy of the untargeted proteomic methods in periodontal diagnosis have been studied to assess their clinical utility. For instance, Blanco-Pintos et al. [100] studied the diagnostic accuracy of salivary protein biomarkers for periodontitis, considering the impact of age and smoking. They collected salivary samples from healthy and periodontitis patients and analyzed them by sequential window acquisition of all theoretical mass spectra (SWATH-MS), and proteins were identified by employing the UniProt database. They discovered that eight single salivary proteins

had a bias-corrected accuracy (bc-ACC) of 78.8–86.8% (bc-sensitivity/bc-specificity of 62.5–86.9%/60.9–98.1%) to diagnose periodontitis. Predictive capacity increased more by adjusting for age (bc-ACC: 94.1–98.2%; bc-sensitivity/bc-specificity: 90.2–98.6%/93.6–97.2%) than smoking (bc-ACC: 83.9–90.4%; bc-sensitivity/bc-specificity: 73.6–89.9%/76.2–96.4%). The proteins involved were as follows: keratin, type II cytoskeletal 1, protein S100-A8, β-2-microglobulin, neutrophil defensin 1, lysozyme C, ubiquitin-60S ribosomal protein L40, isoform 2 of tropomyosin α-3 chain, and resistin. Two dual combinations showed bc-sensitivity/bc-specificity of >90%: β-2-microglobulin with profilin-1, and lysozyme C with zymogen granule protein 16 homologue B. Thus, the authors concluded that salivary biomarkers have good diagnostic relevance, and that age is better than smoking in influencing the accuracy of the single biomarkers. Moreover, they also added that clinical diagnosis remains the gold standard, but untargeted analysis may be helpful in early diagnosis, considering the lack of clinical signs at early stages and the elevated accuracy of the untargeted method. Despite these promising developments, challenges persist, including data complexity, variability in study designs, and the need for standardized protocols [11]. Further research is crucial for the successful translation of proteomic findings into clinical practice, potentially leading to improved diagnostic and therapeutic strategies for periodontitis.

7. Targeted and Untargeted Approaches in Biomarker Discovery

Early diagnosis of periodontitis through a rapid, accurate, and non-invasive method is highly desirable to minimize the individual and epidemiological impact of this widely prevalent disease. Targeted and untargeted salivary metabolomics and proteomics analyses are potential valid approaches that may enable clinicians to get to an early diagnosis of periodontitis. Untargeted analysis aims to detect and compare as many signals as possible related to unknown metabolites in a set of samples. Each metabolite and each protein is identified by referencing a metabolic and proteomic database. This approach can be divided into two categories: fingerprinting, which focuses on analyzing intracellular metabolites and proteins, and footprinting, which examines metabolites and proteins found in the surrounding environment or culture medium. Both methods involve rapid analysis and typically do not include precise quantification of the metabolites [116]. Targeted analysis focuses on selected groups of metabolites and proteins, aiming to detect and accurately measure their concentrations in biological samples [117,118]. This method is often employed for metabolomic and proteomic profiling when the aim is to examine a limited set of metabolites and proteins involved in specific biological pathways. As previously mentioned, untargeted approaches are better than targeted approaches because they involve a complete analysis of the whole composition of saliva, without a prior selection. This allows for the identification of salivary components as biomarkers that would have been discarded if a targeted analysis had been conducted (Table 6) [119].

Table 6. Differences between targeted and untargeted analysis. Target studies focus research on several known metabolites, while untargeted studies allow for a more comprehensive evaluation of metabolomic and proteomic profiles [119].

Targeted Analysis	Untargeted Analysis
Hypothesis-driven	Hypothesis-generating
Subset analysis	Global/Comprehensive analysis
Correlated to reference standards	Correlated to the database/libraries
Identification already know	Qualitative identification
Absolute quantification	Relative quantification

Using untargeted analysis methods on salivary samples to reveal novel biomarkers for early detection of periodontitis is advantageous. In fact, in the early stages of periodontitis, changes in known biomarkers may be subtle or absent. Untargeted approaches can uncover novel, low-abundance molecules or complex biomarker signatures that targeted approaches would miss [120,121]. In addition, due to the multifactorial nature of periodontitis, involving host immune responses, microbial dysbiosis, and inflammatory signaling, untargeted approaches can profile the full molecular spectrum, offering a comprehensive view of pathogenesis. Untargeted analyses are not limited to preselected markers; thus, they reduce the risk of overlooking important but unknown indicators of early disease [121]. Finally, even though untargeted approaches are very advantageous, there is still the need to combine them with targeted approaches. Compounds found by untargeted analysis needs to be confirmed and validated; thus, targeted analysis provides a precise quantification of specific biomarkers identified during the exploratory phase, conducted with an untargeted approach [122,123]. It offers sensitivity, specificity, and quantitative accuracy, which are essential to confirm and validate the results of untargeted analysis [124]. For example, in the study of Bostanci et al. [107], the research of biomarkers associated with periodontitis was conducted at first with a non-targeted shotgun proteomics combined with a label-free quantitative approach. Among the found proteins, only 7 were represented by more than one peptide; therefore, they were included in further comparative quantification. Further comparative analysis aimed to determine whether significant quantitative differences could be found between healthy controls and the diseased groups they found that only nine proteins were different between the healthy and gingivitis and groups. Therefore, untargeted analysis is a good tool to avoid missing the identification of some biomarkers, but it still needs to be followed by a targeted analysis in order to validate its results.

8. Analytical Technologies and Data Interpretation

Untargeted salivary metabolomics and proteomics rely on advanced analytical technologies to explore the comprehensive molecular composition of saliva without prior knowledge of specific targets. In metabolomics, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the primary tools. NMR is non-destructive, highly reproducible, and quantitative, but has lower sensitivity and signal overlap limitations [54]. MS, particularly when coupled with chromatography (UPLC-MS or GC-MS), offers high sensitivity and wide metabolite coverage, though it is destructive and susceptible to ion suppression [125]. Proteomics predominantly employs liquid chromatography-tandem mass spectrometry (LC-MS/MS), which allows sensitive and high-throughput protein identification. Techniques like label-free quantification (LFQ) and isobaric tagging (e.g., TMT or iTRAQ) support relative protein quantification, though they vary in cost and complexity [126,127]. Some variables in the results among the cited studies may be due to the variability of interlaboratory calibration, even though using NMR Spectroscopy guarantees more accuracy in the reproducibility of the data than using MS (Table 7) [125].

Table 7. Characteristics of NMR Spectroscopy and MS.

Feature	NMR Spectroscopy	Mass Spectrometry (MS)	References
Sensitivity	Low (µM range), although improving with hyperpolarization techniques.	Very high (nM–pM range), especially with HR-MS.	[125,128–131]
Quantification	Highly accurate, can quantify multiple analytes.	Quantification is relative unless internal standards are used	[125,128–131]

Table 7. *Cont.*

Feature	NMR Spectroscopy	Mass Spectrometry (MS)	References
Reproducibility	High, robust across time and between labs.	Moderate; targeted methods are reproducible, and untargeted methods need strict quality control.	[125,132,133]
Data Complexity	Moderate; relatively easier to interpret.	High; requires extensive data processing and normalization.	[125]
Throughput	Moderate (10–20 samples/day); limited by long acquisition times in 2D.	High (50–200+ samples/day), depending on the platform.	[125]
Method Development	Stable protocols; recent improvements in 2D NMR and hyperpolarization.	Rapidly evolving, flexible with multiple ionization and chromatographic modes.	[125]
Limitations	Low sensitivity–Long acquisition times for complex spectra.	- Ion suppression - Requires strict QC - Less robust than NMR	[125,128–131]

Data interpretation in both omics fields involves preprocessing (normalization, transformation, scaling), statistical analysis (univariate and multivariate approaches, such as PCA or PLS-DA), and machine learning for classification. Identification of molecules relies on databases like HMDB, METLIN, UniProt, and KEGG, with annotation tools such as XCMS or Mzmine [134]. Finally, pathway analysis using platforms like MetaboAnalyst or Reactome helps contextualize findings within biological systems, such as inflammation or immune response pathways relevant to periodontal disease [44,135].

9. Challenges and Limitations

Besides the advantages of untargeted analyses, some limitations should be faced and solved. First of all, saliva has the limitation of having low site specificity for periodontitis, as GCF does. In fact, periodontal inflammation is site-specific, and saliva may not reflect localized metabolic changes, making it limited in the evaluation of the severity of periodontitis [32,33]. However, other limitations concern the fact that all the present studies lack homogenization [136]. All the existing studies differ in the sample size, case definition, saliva collection methods, analytical platform, and protocols adopted, making it difficult to assess whether a metabolite or a protein variation has evidence to diagnose periodontitis [11]. The cross-sectional nature of some studies makes it difficult to assess the causal relationship between metabolic and protein profile changes with the disease condition and progress. Furthermore, the majority of studies focus on older adults, reducing the applicability of findings to younger populations [136]; thus, there is a need for further research to validate these biomarkers across various age groups. Taken together, these limitations accentuate the importance of introducing standardized methodologies, larger and more varied study populations, and longitudinal approaches to improve the reliability and relevance of metabolomic and proteomic biomarkers in periodontitis research [30,61]. Moreover, once all the results are taken, they should be categorized using a standardized method for metabolomics and proteomics alone and their combination too to interpret variations correctly, without bias. In particular, for untargeted metabolomics, the limita-

tions are represented by metabolite identification and analytical variability. Translating analytical parameters into specific metabolite identities is a big problem, often caused by incomplete reference databases. For this reason, untargeted analysis should be followed by a targeted analysis, which helps in identifying metabolites and proteins found by the non-targeted approach. Meanwhile, differences in sample collection, storage, and preparation can impact metabolite stability and reproducibility [137]. On the other hand, proteomics has some limitations and challenges, too. For instance, a limitation is represented by comprehensive protein coverage: mass-spectrometry-based proteomics may not detect the entire protein spectrum, potentially missing proteins present in smaller quantities. Additionally, proteomics has to face data analysis challenges; in fact, analyzing proteomic samples requires advanced computational methods, and variability in methodologies can make cross-study comparisons difficult [138,139]. Facing these challenges is crucial for the effective application of untargeted metabolomics and proteomics in understanding and diagnosing periodontitis. Moreover, the implementation of salivary biomarker testing in clinical practice also presents considerable ethical and practical challenges. Ethically, patient privacy and data security must be ensured, as the collection and analysis of salivary biomarkers involve sensitive health information. Informed consent is another fundamental aspect, patients must fully understand the purpose, the risks, and the implications of testing to make informed decisions [140,141]. Additionally, issues of equity and accessibility must be considered to prevent healthcare disparities and to ensure that these diagnostic tools are available to diverse populations. Another challenge is represented by the integration of salivary biomarker testing into routine clinical workflows, requiring proper training for dental professionals and updates to electronic health record systems. Regulatory approval is another significant obstacle, as agencies such as the FDA or EMA must evaluate and approve these tests before they can be widely adopted. The classification of salivary biomarker tests also has an impact on their regulatory pathways and reimbursement policies [142]. Furthermore, the cost and economic feasibility of these diagnostics must be carefully assessed, as developing and maintaining reliable biomarker tests can be expensive. Evaluating their cost-effectiveness compared to traditional diagnostic methods is essential for sustainable implementation and for ensuring equal access to new diagnostic methods for people worldwide, without leaving aside the populations that cannot afford their costs [10,143–147]. Solving these ethical and practical challenges is important to successfully integrating salivary biomarker testing into clinical practice, ensuring both patient benefit and scientific reliability.

10. Future Perspectives

In the future, metabolomics and proteomics can be developed by integrating their efficacy with other diagnostic technologies, such as AI and machine learning. These last ones have gained a lot of popularity in recent years in clinical applications. Artificial intelligence (AI) is the utilization of computers or computer-controlled systems to reproduce human intelligence. It consists of various specialized areas, including machine learning, expert systems, natural language processing, planning, robotics, affective computing, and computer vision [148]. In particular, expert systems, machine learning, and computer vision are important for selecting and validating biomarkers in liquid biopsy samples [149–151]. Using these techniques, AI algorithms can extract knowledge from data provided by the user or identify patterns in multidimensional data derived from salivary biomarker analysis and patient information from electronic health records, all without explicit request [152–155]. AI models can subsequently predict new outcomes for independent patients or samples, based on the patterns learned from the biomarker dataset, like SalivaDB, Salivaomics Knowledge Base (SKB), which are open-source salivary biomarkers databases [149,156,157] (Figure 6).

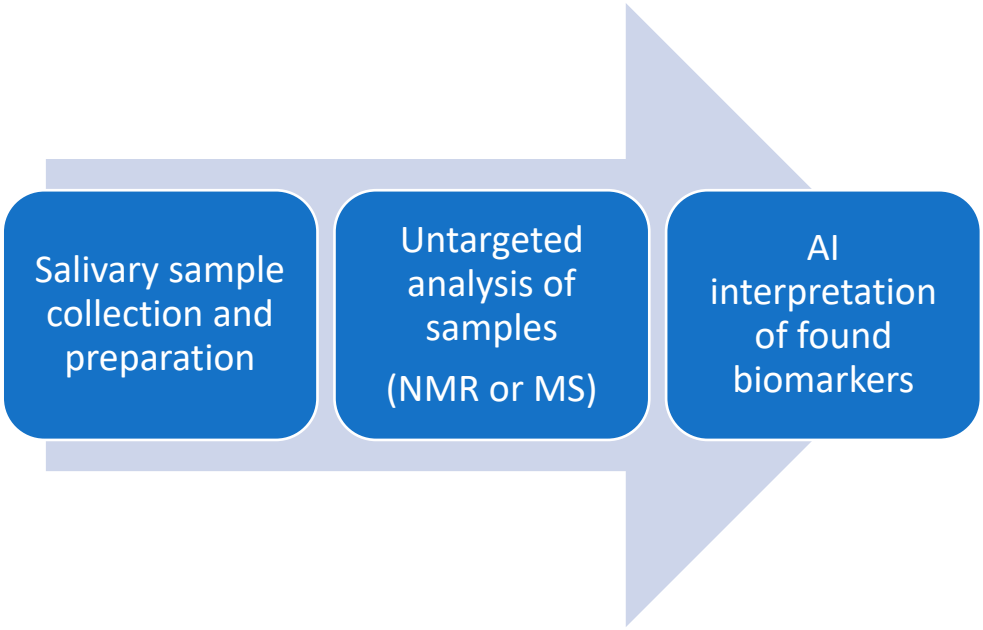

Figure 6. Workflow: from the collection of saliva to data interpretation. The first step is to collect and prepare salivary samples from the patients. Then, untargeted analysis is driven by Nuclear Mass Resonance (NMS) or Mass Spectrometry (MS). The last step is to interpret the data. If AI were employed, the results of the analysis could be easily interpreted, associating the found biomarkers with the related diagnosis, the most suitable treatment plan, and its prognosis.

Integrating salivary metabolomics and proteomics with advanced diagnostic technologies, such as artificial intelligence (AI) and machine learning, is a promising method for enhancing periodontal diagnostics. AI-assisted saliva liquid biopsy platforms have already been evaluated for diagnosing periodontitis, showing the potential of these technologies in identifying specific biomarkers related to periodontitis and improving diagnostic accuracy. It still should be evaluated what the clinical applicability of this method and its costs are. Moreover, there is still the question of whether AI can substitute the clinician in its diagnostic capability. However, some scientists demonstrate that the clinician cannot be substituted by the AI, with the clinician still having the most accurate diagnostic power [158]. The validation of metabolomics and proteomics diagnosis of periodontitis will be very useful for clinicians. In fact, the incorporation of salivary biomarker analysis into clinical practice paves the way for personalized periodontal care. Salivary biomarkers have shown high diagnostic accuracy in distinguishing periodontal health from periodontitis, facilitating early detection, monitoring disease progression, and tailoring treatment strategies to individual patient needs [159,160]. In particular, identifying specific metabolites and specific proteins related to periodontitis makes it possible to personalize the treatment plan and to define more precise prognostic categories [161]. The identification of periodontitis-related proteins and metabolites taken separately represents a promising source, but the combination of both would give a more complete panoramic view of periodontitis. In fact, the combination of metabolomics with proteomics will provide more information on the biological stratification of periodontitis and help construct the best discriminative diagnostic model within the framework of personalized and precision medicine [162,163]. Metabolomic and proteomic profiles may help in building prognostic models, predicting clinical outcomes of periodontitis, such as probing depth or alveolar bone loss; in order to achieve this, there is the need for further studies in which clinical outcomes are associated with the progression of metabolomic and proteomic profiles in 1, 3, and 5 years [33,164]. The limitation of this would be the standardization of the methods. To fully realize the

potential of salivary biomarker testing in periodontitis, collaborative research is essential to establish standardized protocols. The harmonization of saliva-sampling protocols and definitions would significantly enhance the comparability of studies and the reliability of identified biomarkers, facilitating their integration into clinical practice [165].

11. Conclusions

Early diagnosis of periodontitis is a major challenge. Intercepting this condition at early stages is fundamental to arrest the progression of the disease and irreversible tissue damage, avoiding consequent teeth loss. Untargeted salivary detection of metabolites and proteins as biomarkers for diagnosing periodontitis has the potential to improve early diagnosis of periodontitis. This new method allows the finding of potential early biomarkers related to periodontitis that would have been discarded in a targeted analysis, with the potential to improve traditional clinical diagnosis, when periodontitis does not show noticeable clinical signs. However, although the results from untargeted salivary diagnostics in periodontitis are promising, untargeted approaches represent only the first step in the identification of new biomarkers because subsequent validation and quantitative experiments still require targeted approaches. Moreover, most studies on salivary untargeted approaches are limited by high heterogeneity. For this reason, further research is needed to assess properly the salivary compounds associated with periodontitis and elaborate a standardized protocol potentially applicable in clinical practice.

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