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Uncovering the molecular networks in periodontitis

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Abstract

Periodontitis is a complex immune-inflammatory disease that results from a preestablished infection in gingiva, mainly due to Gram-negative bacteria that colonize deeper in gingival sulcus and latter periodontal pocket. Host inflammatory and immune responses have both protective and destructive roles. Although cytokines, prostaglandins, and proteases struggle against microbial burden, these molecules promote connective tissue loss and alveolar bone resorption, leading to several histopathological changes, namely destruction of periodontal ligament, deepening of periodontal pocket, and bone loss, which can converge to attain tooth loss. Despite the efforts of genomics, transcriptomics, proteomics/peptidomics, and metabolomics, there is no available biomarker for periodontitis diagnosis, prognosis, and treatment evaluation, which could assist on the established clinical evaluation. Nevertheless, some genes, transcripts, proteins and metabolites have already shown a different expression in healthy subjects and in patients. Though, so far, ‘omics approaches only disclosed the host inflammatory response as a consequence of microbial invasion in periodontitis and the diagnosis in periodontitis still relies on clinical parameters, thus a molecular tool for assessing periodontitis lacks in current dental medicine paradigm. Saliva and gingival crevicular fluid have been attracting researchers due to their diagnostic potential, ease, and noninvasive nature of collection. Each one of these fluids has some advantages and disadvantages that are discussed in this review.

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Keywords

Diagnosis; Gingival crevicular fluid; ‘Omics; Periodontitis; Saliva

1 Introduction

Periodontitis is a multifactorial infectious and immuno-inflammatory disease that, together with gingivitis, belongs to a more broad group of pathologies termed “periodontal diseases” [1]. It results from a complex interaction between colonizing microorganisms and host immune-inflammatory response, being characterized by irreversible histopathological changes, such as destruction of the periodontal ligament, bone destruction, and deepening of periodontal pockets, which can converge to tooth loss (Fig. 1) [2, 3]. Its complexity arises from the interplay between microbial pathogens and the host’s inflammatory and immune response as well as environmental and genetic factors [1-3]. However, not all inflammatory conditions of the gingival *sulcus* seem to progress to periodontitis [3]. Indeed, some potential risk factors have been established, which can be subcategorized in local plaque accumulation/oral hygiene, tobacco use, malocclusion, dental restorative procedures, iatrogenic factors, and systemic factors, such as age, race, gender, socioeconomic environment, genetic influences, and other systemic conditions, such as psychosomatic, nutritional, endocrine, metabolic and immunodeficient-related disorders [2-4].

Since 1958, the knowledge of periodontitis pathogenesis has been growing, as depicted in Fig. 2. Nonetheless, periodontitis diagnosis is still performed with clinical tests and tools of low sensitivity and specificity. In addition, most of these clinical methods are fraught with a certain degree of subjectivity since they are examiner-dependent. Clinical evaluation relies on the assessment of oral hygiene, gingival status, clinical attachment loss (CAL), probing depth (PD), bleeding on probing (BOP), alveolar bone status, and other more involved procedures, such as periodontal microbiology testing, blood analysis for systemic health profiling, as well as histological studies [1,5]. Hence, the profession of periodontics lacks a reliable and objective arsenal to correctly perform diagnosis and prognosis of periodontitis-afflicted patients, which would allow an earlier diagnosis, and which could minimize interventions, such as periodontal surgery, in order to reduce periodontitis-related complications, such as tooth loss and subsequent rehabilitative therapeutics [1].

Following the ‘omics boom, researchers have made efforts to unravel molecular markers for periodontitis, resulting in the identification of several genes, transcripts, proteins, and metabolites related to periodontitis. While of interest, none of these parameters showed, so far, a highly selective and specific relationship to the disease [6]. In this pursuit, saliva and gingival crevicular fluid (GCF) have been the two main biofluids used to screen molecular profiles of periodontal disease, since they can reflect both the local oral microenvironment and the systemic environment related to health status [7, 8]. In this review, a brief account on the pathogenesis of periodontitis will be provided, highlighting saliva and GCF as diagnostic fluids, taking into account the contributions of several ‘omics perspectives focusing on the diagnosis of periodontitis. In an earlier review, Grant [6] provided an analysis of the major ‘omics contributions to the clinical field of periodontics. The current

review aims at a further and in-depth analysis of the ‘omics approaches and, in addition, examine the relevance of the microbiome findings with respect to periodontitis. Also discussed are data on the overall contribution of genomics, transcriptomics, and proteomics/peptidomics, using the bioinformatics tool ClueGO [9] to gain insights into the complex molecular interactions and to address future directions in periodontitis diagnosis and research.

2 Material and methods

In order to analyze the “omics” contribution to the molecular diagnosis of periodontitis, database searching was first carried out on Pubmed, Google Scholar, and Web of Knowledge using “periodontitis,” “omics,” “genomics,” “transcriptomics,” “proteomics,” “peptidomics,” “metabolomics,” “microbioma” as keywords to retrieve recent publications of omics studies. Those publications were limited to the ones in which samples collected were entirely from human subjects and included saliva, GCF, blood, polymorphonuclear leukocyte, epithelial cells, gingival tissues samples, and bacterial biofilm.

This was followed by a new research on Pubmed using “periodontitis” as the first keyword and “genomics,” “transcriptomics,” “proteomics,” “peptidomics,” “metabolomics”/“metabonomics,” or “microbioma” as second keywords. Publication dates were limited to 2011 until present, retrieving close to 200 publications. Exclusion criteria included reviews and original articles regarding periodontitis treatment and antibiotic therapy, oral diseases, rather than periodontitis itself, such as endodontic infections, acute apical abcesses, dental root canal infections, dental periradicular lesions, dental implants, necrotizing periodontal diseases, or related to animal studies. The major goal was to analyze novel data concerning ‘omics contributions to periodontitis since Grants’ review [6] was submitted in 2011.

3 Periodontal diseases

3.1 Gingivitis and periodontitis

Since 1960, the model of periodontitis’ pathogenesis has been adapted to new findings, as reviewed by Kornman [10]. It was first assumed that periodontitis was a bacteria-induced disease, with bacterial dental plaque being responsible for gingivitis onset and, later on, to its development into periodontitis [10]. Currently, it is accepted that despite the pivotal role played by microorganisms, a complex set of factors seems to balance the initiation and the progression of the initial lesion to periodontitis. Those include genetic and environmental factors, summarized in Table 1, and may explain the range of host responses and clinical presentations of periodontitis [2,3,10].

As such, the first hypothesis for periodontitis pathogenesis was replaced by a nonlinear model (1997; Fig. 3), where the interplay between pathogens, host inflammatory and immune response, connective and bone tissue metabolism, and the systemic environment were considered [10]. Additional risk factors, whether local or systemic, genetic, environmental, or acquired, were not excluded, and credited to modulate the clinical expression of the disease.

Depending on the clinical manifestation of periodontitis, seven classes of periodontal diseases were defined at the 1999 World Workshop for the Classification of Periodontal Diseases and Conditions [11]: chronic periodontitis (CP), localized aggressive periodontitis (LAP), generalized aggressive periodontitis (GAP), periodontitis as a manifestation of systemic disease associated with hematologic or genetic disorders, Necrotizing Ulcerative Periodontitis, abscesses of the periodontium, and combined periodontic-endodontic lesions. Although some of these conditions result from local manifestation of the disease, others result from systemic conditions. From those periodontitis subtypes, only CP and aggressive periodontitis (AP) will be discussed due to their epidemiologic relevance. CP is commonly found in the adult population, and its rate of progression is slow to moderate. Bacterial biofilms are consistent with the degree of tissue destruction depicted, even though there is some variability regarding the distribution of the lesions, which can be localized (LCP) or generalized chronic periodontitis (GCP). AP has a distinctly higher rate of progression and shows a remarkable familiar association. This particular form of periodontitis is less usual and can be further divided into LAP and GAP. The localized form is usually found in the adolescent population, while the latter generally affects people under 30 years of age. In LAP, the microbial biofilms are not consistent with the degree of tissue destruction, the defects are mainly localized in the region of the permanent first molars and incisors, whereas in GAP tissue defects are more largely distributed and microbial plaque accumulation is sometimes consistent with the severity of the periodontal tissue destruction [5].

3.2 Pathogenesis of periodontitis

In spite of their distinct clinical presentation, CP and AP share some common features in terms of their pathogenesis, such as the activation of a pathogen-mediated inflammatory host response (Fig. 4). Several Gram-negative anaerobic and microaerophilic bacterial species have already been associated with periodontitis, noticeably, bacteria belonging to “red complex,” comprising *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*. These species represent a typical group of pathogens active in periodontitis, which are able to adhere to host surfaces and coaggregate with other microorganisms, yielding biofilms [2, 3, 12]. That ability depends essentially on adhesins that recognize and interact with host elements, such as extracellular matrix components, or proteins expressed on host cell surfaces [12]. When the barrier to bacterial colonization and invasion is overcome, these pathogens induce a local inflammatory response through antigen stimulation and release of toxic products [13]. The defense response includes activation of both innate and acquired immunity with infiltration of the gingival tissues bordering the sulcular space with neutrophils and expression of antibodies by B cells. In an attempt to overcome the microbial burden, epithelial cells, periodontal ligament fibroblasts, leukocytes, osteoblasts, and dendritic cells release cytokines and chemokines, including interleukin 1 (IL-1), IL-6, chemokine (C-X-C motif) ligand 8, tumor necrosis factor α (TNF-α), and others as well as proteases, including matrix metalloproteinases (MMPs), prostaglandins, and other inflammatory mediators [3, 10, 13]. Despite the initial protection, these inflammatory molecules and proteases lead to the breakdown of the major tooth supporting structures affecting connective tissue and bone [13]. Furthermore, bacterial products can directly destruct supporting tissues and lead to further infiltration. For instance, *P. gingivalis* produces gingipains, extracellular cysteine proteinase–adhesin complexes that are able to

adhere and digest fibronectin, collagen type V, and laminin [12, 14] as well as immune-related molecules, such as β-defensin 3 [15], IgG1, and IgG3 [16], and host protease inhibitors such as secretory leukocyte protease inhibitor [17] and even osteoprotegerin, which prevents osteoclastogenesis [18]. Thus, through bacterial and host inflammatory/immune factors, the periodontal tissue appears swollen, infected, and inflamed, leading to severe histological changes, namely, apical migration of the junctional epithelium, deepening of the periodontal pockets, destruction of connective tissue and bone, and, eventually, tooth loss [3].

4 Saliva and GCF as diagnostic fluids

So far, periodontitis diagnosis has been accomplished predominantly with the periodontal probe measuring specific clinical parameters. These include the CAL. This measurement defines the distance from the cementoenamel junction—a fixed anatomical location that does not change throughout life—to the base of the probable base of the periodontal pocket. Other parameters include PD (a measure of the distance from the gingival margin to the base of the probable base of the periodontal pocket), BOP, and radiographically determined alveolar bone level [1, 5]. Hence, a more sensitive and specific molecular diagnostic tool is still missing, which could facilitate an earlier diagnosis, even before clinical manifestation of periodontitis, in generating a better and more accurate prognosis, and providing guidance in the decision for periodontal therapeutic interventions. In addition, the detection of biomarkers for active disease could be of tremendous benefit if they could be used for the assessing current disease activity and determining the risk of developing the disease.

Researchers have become increasingly interested in saliva and GCF as diagnostic fluids, since these biological fluids have shown the potential as diagnostic predictors of several conditions, ranging from systemic conditions, such as Sjögren syndrome, systemic sclerosis, diabetes mellitus, cerebrovascular/cardiovascular diseases, and acquired immunodeficiencies [7, 19, 20], to local manifestations, such as gingivitis, periodontitis, and caries [19], and even neoplasic disorders, such as salivary gland tumor, head and neck carcinoma, tumors of oral cavity, larynx carcinoma, and breast cancer [7,19].

Saliva is a complex body fluid composed by the secretions from major salivary glands comprising the parotid, sublingual, and submandibular glands and from about 600 minor salivary glands that are dispersed throughout the oral mucosa [7]. With regard to the molecular composition, saliva contains water (accounting for 99%), proteins, peptides, small organic molecules, electrolytes, nucleic acids, and hormones [7, 16]. Over 3000 proteins have been identified in saliva [21] with proline-rich proteins, mucins, cystatins, amylases, histatins, and statherin, representing the major families present in saliva [22, 23]. GCF exists as a serum transudate, changing into an inflammatory exudate as the inflammatory events progress. This fluid can be collected from the gingival *sulcus* or periodontal pocket. Like saliva, GCF is a complex fluid composed of molecules from different sources. The predominant components originate from serum or interstitial fluid, but other constituents reflect connective tissue and bone-derived molecules, inflammatory mediators, antibodies, and breakdown products from the periodontium [8,20].

Both saliva and GCF have advantages and disadvantages as diagnostic fluids, which are summarized in Table 2. Saliva collection is highly advantageous due to its noninvasive and painless nature, ease of collection that eliminates the need for technically trained professionals, and its minimal biological risks. Additional advantages of saliva collection relate to its higher safety when compared to other body fluids, lower cost of technical procedures and materials, applicability for large-scale populations, and higher collaboration from children, elderly people, handicapped, or anxious patients. In addition, saliva has a lower salt concentration when compared to urine, and the total protein concentration does not mirror the concentration of the most abundant proteins in blood and blood-derived products, such as serum albumin and globulins [7,24]. Nevertheless, the use of saliva as a diagnostic tool has some disadvantages, including the variation of molecular composition due to circadian rhythm, age, gender, dietary habits, method of collection, and the possible use of a stimulation technique. Moreover, special attention to sample collection and storage is needed, since saliva contains microorganisms, proteases, and proteins that can be enzymatically or physically destroyed [7,19,24]. In spite of these disadvantages, saliva remains as a resourceful diagnostic biofluid, as proteome coverage is not significantly affected by different collection methods and stimulation techniques [25].

While saliva is produced in large volumes, ranging from 500 to 1500 mL per day in healthy adults and is easier to collect [7], GCF is produced in only microliter volumes that makes its collection technically more challenging. Furthermore, sampling times are longer, especially in healthy subjects, to achieve appreciable quantities of material. GCF collection is also prone to contamination during the commonly used paper strip collection method. This contamination includes components of saliva, blood, or bacterial plaque. This is not the case with saliva, since all nonexocrine contributions to saliva are part of its composition. Finally, GCF sampling can induce some discomfort, particularly if using “deep” intracrevicular method with paper strips [8, 20, 26]. Regardless of these disadvantages, GCF keeps attracting researchers because this fluid is more “periodontal specific” since it derives from local site involved in the actual periodontal disease manifestation. It is therefore considered to represent a mirror of the periodontal health state of an individual [8]. GCF can also be collected with a minimally invasive methods and in this case GCF is easier to handle than saliva due to its reduced viscosity when compared to whole saliva samples [8,24].

5 “Omics” approaches to periodontitis

Efforts have been made in several ‘omics disciplines in an attempt to achieve molecular tools for the diagnosis of periodontitis. Genomics, transcriptomics, proteomics/peptidomics, and metabolomics studies have a common goal that is the identification and characterization of a molecular signature of periodontitis, either on the level of DNA, RNA, proteins, or metabolites that hopefully could predict the development of periodontal disease, its prognosis, and assess the follow-up of periodontal treatment. Supporting Information Table 1 illustrates the published literature on the human periodontitis ‘omics studies.

As shown, some insight into potential diagnostic targets, such as single nucleotide polymorphisms (SNPs), genes, transcripts, proteins and metabolites, that could contribute to earlier diagnosis, prognosis, and follow-up of periodontitis have already been reported..

Nevertheless, none of the addressed markers has been validated as a clinical diagnostic tool of periodontitis. It is therefore not surprising that the classical clinical determinants, CAL, PD, BOP, and alveolar bone status, remain the gold standards of the clinical diagnostic armamentarium [1,5].

5.1 Genomics and transcriptomics approaches to periodontitis

Genomic and transcriptomic approaches aim to identify potential genes and SNPs associated with an increased risk to develop periodontitis, as well as to detect differences in gene expression in periodontitis-afflicted individuals. Genomic and transcriptomic studies have been focusing on one or a few candidate genes/transcripts [27-38]. There are also studies where genome- or transcriptome-wide approaches have been conducted, omitting an a priori positive discrimination of certain gene(s) [39-47]. Regardless of the approach, all studies require the extraction of DNA or RNA from blood, epithelial cells collected from an oral swab, gingival tissue, among other sources. While DNA can be directly amplified through PCR, purified RNA is first reverse-transcribed yielding cDNA. cDNA is then amplified in a quantitative real-time PCR device and is detected through fluorescence-based assays [48]. Depending upon the goal, the PCR method can be modified to increase sensitivity using sequence-specific primers to detect specific alleles in real time or to amplify several DNA templates with multiple primers (multiplex PCR) [49]. With regard to SNP or gene detection, different methods, including PCR-RFLP, TaqMan® Allelic Discrimination, Tetra-Primer Amplification Refractory Mutation System PCR, DNA arrays, among others, have been used in periodontitis biomarker studies (Supporting Information Table 1) [49,50].

Since periodontitis is characterized by an inflammatory burden, it is not surprising that several molecular markers related to inflammation are found differently expressed in periodontitis in omics studies. Indeed, classical proinflammatory cytokines, such as IL-1α, IL-1β, and TNF-α; key mediators of periodontal inflammation; and inducers of bone resorption by osteoclasts [13,40] have been linked to periodontitis. Moreover, RNA sequences of other transcripts of inflammatory mediators, such as IL-6, chemokine (C-X-C motif) ligand 8, regulated on activation normal t-cell expressed and secreted and macrophage chemotactic protein 1, were found to be expressed to higher degree in periodontitis-afflicted gingiva compared to healthy sites [40].

Also, several genomic studies showed an association of specific gene polymorphisms to different clinical presentations of periodontitis. For instance, the combination of IL-1A (−889, allele 2) with IL-1B (+3953, allele 2) genotypes are correlated with severe periodontitis [37]. Moreover, IL-1B (+3953TT) [35] and tumor necrosis factor α gene (−1031CC) genotypes are associated with CP, and tumor necrosis factor α gene (−308AA) with AP [34]. However, these genomic studies display some limitations, such as the use of highly homogeneous populations, which make conclusions valid only for the specific population studied. The limitations of these data relate to the focus on only a few genes, excluding the important possibility of gene–gene and gene–environment interactions. This aspect is important in an etiologically complex disease such as periodontitis. An additional confounder is the low number of evaluated subjects used in contrast to genome-wide association studies (GWAS) allowing the discrimination of intrapopulation genetic

variability. Nevertheless, in large-scale studies [43-46], where these limitations are at least in part overcome, results are not very promising. For instance, in a report by Suzuki et al. [43], five promising genomic markers for periodontitis were detected. These are gonadotropin-releasing hormone 1 gene, phosphatidylinositol 3-kinase regulatory 1 gene, dipeptidylpeptidase 4 gene, fibrinogen-like 2 gene, and calcitonin receptor gene. These results, however, lack large-scale validation, due to the relative low number of participants (n = 41) and due to the homogeneity of the population consisting exclusively of Japanese subjects. DNA arrays, such as Affymetrix® Genome-Wide Human SNP Array 6.0 used by Teumer et al. [46], in a GWAS, allowed for the screening of millions of SNPs in a single assay, in a fast, accurate, and low-cost manner [50]. Despite the high number of evaluated subjects, GWAS yielded only one marker representing the glycosyltransferase 6 domain containing 1, GTL6D1 gene [45] and no genetic markers for periodontitis [46] after adjustment of the data for gender, age, diabetes, and smoking.

Other evidence of inflammation in periodontitis were derived from microarrays studies, a higher throughput technology for transcriptome analysis, which were employed to assess differences in gene expression between healthy and periodontitis patients [39, 41, 42]. The high-throughput nature of the transcriptomic and genomic techniques requires an equally high-throughput bioinformatic tools to analyze long lists of genes and validate its statistical significance using annotation databases, such as gene ontology [51]. It was found, for example, that the leukocyte transendothelial migration pathway was upregulated in periodontitis, which can be explained by the dependency on leukocyte migration through endothelial cells being part of the first line of defense against bacterial invasion [39]. Moreover, genes related to apoptosis, antimicrobial humoral response, antigen presentation, regulation of metabolism, signal transduction and angiogenesis were found to be differently expressed in periodontitis and healthy subjects [41]. With the aid of microarray technologies, it could be shown that cell communication pathways were downregulated in periodontitis-affected tissues, specifically connexin, desmogrein 1, desmocollin 1, and nestin. This finding could result from attachment loss of communicating structures, either in cell-to-cell communications at the soft tissue level, or in cell-to-tooth signaling as a consequence of the inflammatory status of the periodontium [39]. Despite enabling the detection of distinctive patterns of biological pathways in health and periodontitis, the gene expression data obtained were gathered from heterogeneous cell populations that comprised epithelial cells, connective tissue fibroblasts, and several infiltrating cell types. While these data are important, additional approaches using proteomic/peptidomic tools are required for further confirmation and validation of the reported data [41].

5.2 Proteomics and peptidomics approaches to periodontitis

Evaluation of the complete protein and peptide profile in health and periodontitis has been the aim of proteomics/peptidomics approaches. Concerning the methodological strategy, both bottom-up approaches, where proteins are digested to peptides, and top-down approaches, where proteins are kept intact, have been employed [52]. The majority of the studies depicted in Supporting Information Table 1 follow a bottom-up approach [53-61], while just two studies [62, 63] follow a top-down approach. In both methods sample collection and processing occur prior to proteomic analysis. Whole saliva, stimulated or

unstimulated, and GCF are widely used as starting materials. To remove cell debris, food remnants, and to prevent protein digestion, whole saliva is centrifuged at low temperatures with a cocktail of protease inhibitors or kept on ice, and the recovered supernatant is immediately frozen at −80°C [53,55,56,59,61-64]. GCF is recovered from paper strips with different organic or aqueous elution methods [54,57,58,60]. In bottom-up proteomics, peptides are fractionated with multidimensional resolving techniques before MS analysis in order to decrease the complexity of the mixture [24,65]. Electrophoresis, either 1DE or 2DE, is one of the most widely used techniques to separate the proteins in the sample. In 2DE, proteins are separated according to their p*I* in the first dimension, and in the second dimension according to their molecular weight, using denaturing conditions, in the presence of SDS-PAGE. In the bottom-up approach, resolved proteins present in bands (SDS-PAGE, 1DE) or in spots (2D-PAGE) are digested with trypsin for identification by MS [65, 66].There are, however, also gel-free-based approaches to decrease the complexity of protein mixtures, such as chromatographic separation methods. RP chromatography and ion-exchange chromatography (IEC) represent the most common chromatographic procedures used in peptide fractionation. RP chromatography separates proteins based on their hydrophobicity, and IEC protein separation is related to the differences in net charge of each protein or peptide at the prevailing pH. IEC resins can be classified into strong cation or anion exchangers or into weak cation or anion exchangers. The salient difference of these resins relates to the charge of the functional groups covalently bound to the matrix and the degree of retaining this charge at the employed pH [65]. In the majority of bottom-up proteomic studies summarized in Supporting Information Table 1, a combination of both electrophoretic and chromatographic separation methods is used for achieving multidimensional separation of proteins. In top-down proteomic/peptidomic studies, as carried out by Taiyeb-Ali and his colleagues [63] and Zhang and his colleagues [62], a combination of electrophoretic and chromatographic separation techniques was employed for protein fractionation without any protein digestion. After these steps, the resolved proteins/peptides are analyzed by MS. First, proteins are ionized by ESI or by MALDI. Once ionized, proteins are resolved in mass analyzers accordingly to *m/z* stability, using instrumentation parameters such as quadrupoles, TOF, or *m/z* resonance frequency (ion trap, orbitrap and ion cyclotron resonance). Some experiments use hybrid technologies with more than one analyzer to further resolve proteins [52]. Finally, detected proteins are identified using algorithms like SEQUEST or MASCOT [66]. In proteomic studies, the technique of zymography also plays an important role for the analysis of proteases. In this electrophoretic technique, a substrate for a specific protease is copolymerized within the polyacrylamide gel matrix to assess enzymatic activity within specific zones of the electrophoretogram. After the run, SDS is washed out and the gel is stained to distinguish between digested and nondigested zones [67].

Some studies have been aimed at the characterization of the GCF proteome, identifying between 199 [57] and 327 [54] proteins. These included proteins related to early inflammation, immune response, protease activity, modulators of cytokines, response to stimulus, biological regulation, metabolic processes, and extracellular matrix constituents [54,57]. There are studies that have provided some insight into the proteomic GCF patterns in healthy and in periodontitis-related conditions. For example, Nagata et al. [60] identified

64 proteins specific to periodontally healthy sites and 63 proteins specific to periodontally affected sites. These proteins were related to blood, cytoskeleton, immunity, inflammation, lipids, and some enzymes [60]. Another study used label-free quantitative proteomics (LC/MS^E) of the GCF exudatome to compare healthy with periodontally diseased sites. The results showed that the sites from the latter group displayed higher amounts of microbial proteins and ʟ-plastin, and lower amounts of annexin-1, neutrophil defensins, cystatin B, and IgG [58]. Similarly to these GCF data, plastin-2, profilin-1, neutrophil collagenase, α-2-macroglobulin have been found to be differently expressed in health and periodontitis with the aid of salivary proteomics. Other salivary proteome studies demonstrated complement C3, lactotransferrin, MMP-9, serum albumin, Ig γ2 and α2 chain C region, vitamin D binding protein, α-amylase, zinc-α2 glycoprotein, S100A8, –A9, –A6, Ig heavy chain V-III region, and hemoglobin to be overexpressed [53, 55, 56, 59] and lactotransferrin, elongation factor 2, 14–3–3 sigma, short palate lung and nasal epithelium carcinoma-associated protein 2 precursor, carbonic anhydrase 6 and cystatin SN precursor to be underexpressed in periodontitis [55,59].

The inflammatory picture of periodontitis can clearly be shown with a network of genes, transcripts, and proteins, constructed according to molecular function with the bioinformatic tool ClueGO [9] depicted in Cytoscape [68] (Fig. 5). This network was built based on the molecular findings of the studies described in Supporting Information Table 1, with genomics, transcriptomics, or proteomics/peptidomics methodology. The scheme aims to translate the reported data from saliva or GCF obtained with the various omics approaches into a perspective view of their potential use for specific marker application in the field of periodontology. As can be seen in Fig. 5, several genes and proteins are up-regulated in periodontitis, remarkably those that belong to cytokine receptor binding, cytokine activity, lipopolysaccharide (LPS) receptor activity, haptoglobin binding, copper ion binding, and phenylpyruvate tautomerase activity. Only genes with cysteine-type endopeptidase inhibitory activity were downregulated, which include cystatin SN and cystatin B. Low levels of protease inhibitors are consistent with high proteolytic activity, which can explain matrix degradation and connective tissue and bone loss in periodontitis. On the other hand, the upregulation of cytokine receptor binding and activity genes as well as genes of LPS receptor activity and phenylpyruvate tautomerase activity could be a consequence of the infectious and inflammatory process underlying the pathogenesis of periodontitis. Moreover, upregulation of haptoglobin binding genes could be indicative of increased level of ruptured erythrocytes and the liberation of free hemoglobin in inflammation sites.

There is only a single peptidomic study [62] showing none of the identified peptides to be clustered with the other markers. The promise and potential of peptidomics for diagnostics considering the high proteolytic activity associated with inflammatory lesions justify further investigation in the peptidomics field. A few genes/proteins have already been validated with more than one ‘omics methodology as indicated in the graphic representation with overlapping circles in Fig. 5. For instance, upregulation of S100A8 and S100A9 in periodontitis was corroborated by transcriptomics and proteomics studies. Overexpression of IL-1α, IL-1β, toll-like receptor 9, and TNF-α is another example of validated data by two ‘omics approaches (genomics and transcriptomics). MMP-9 upregulation in periodontal disease was shown by genomics and proteomics. Nevertheless, a similar trend has also been

observed for other diseases such as Sjögren syndrome, diabetes mellitus, acquired immunodeficiency syndrome (AIDS) [20,24,34,69,70], or neoplasic conditions such as oral squamous cell carcinoma [71], which highlight a degree of nonspecificity of these targets.

5.3 Microbioma findings in periodontitis

To date, the majority of microbiome studies have relied on genomics approaches, such as 16S rRNA genes pyrosequencing, DNA–DNA hybridization, and microarrays [72-74]. They attempt to find possible associations among bacterial species identified from subgingival and supragingival biofilm samples with different periodontitis conditions [75-80], clinical parameters regarding periodontal health [81, 82], with systemic conditions, such as diabetes mellitus [83-86], and rheumatoid arthritis [87], Down syndrome [88], or with other factors, such as smoking, race, or presence of caries [89]. Overall, the results show that a community of microorganisms rather than a single identity should be associated to periodontitis pathogenesis. For instance, Griffen et al. [80] used a genomic methodology to identify CP-specific bacteria showing that Spirochaetes, Synergistetes, Bacteroidetes, Clostridia, Negativicutes, and *Erysipelotrichia* were disease-associated, while *Proteobacteria* and the class *Bacilli* were health-associated. Another research group was able to link Aggregatibacter actinomycetemcomitans, Filifactor alocis, Tannerella sp., *Solobacterium moorei*, *Parvimonas micra*, and *Capnocytophaga* sp. to LAP [79].

Proteomics and metabolomics represent an important complement/alternative to genomics and transcriptomics studies on microbiome, since it is possible to address bacterial phenotypic profiles in gingival *sulci* rather than their genotype. These approaches avoid problems related to cultivation-based methods, such as the cultivation of fastidious bacteria, which are difficult or impossible to culture and characterize in available culture media [90]. So far, proteomics and metabolomics studies regarding microbiome of periodontitis had distinct purposes. For instance, a proteomic study aimed to unravel potential bacterial biomarkers in GCF [91]. Another studies focused on the characterization of red complex biofilms proteome [92] and on the identification of immunoreactive antigens from *A. actinomycetemcomitans* infected subjects [93].

It is noticeable, though, the lack of global-approached proteomics/peptidomics and metabolomics studies regarding microbiome, particularly with the same goals as the ones included in Supporting Information Table 1, that is to say, unraveling a molecular signature of periodontitis. For instance, more ‘omics studies are needed to identify bacterial adhesins, their targets, and virulence factors as well as human proteases, which can be potential predictors of disease progression and a discriminatory tool between different clinical presentations of periodontitis. For example, a glycomic study was already performed, which revealed the importance of fucose in host glycan moieties to *P. gingivalis*’ fimbriae binding [94]. Moreover, *P. gingivalis* ATCC 33277 fimbriae have been shown to adhere to acidic proline-rich proteins, proline-rich glycoproteins, and statherins, proteins present in parotid-derived salivary secretion [95]. Another study found that LPS of the same bacteria can bind to α-amylase, cystatins, prolactin-inducible protein, lysozyme C, Ig components, serum albumin, lipocalin-1, and submaxillary gland androgen-regulated protein 3B, using *P. gingivalis* LPS-immobilized beads [96]. Indeed, the proteins referred to above are

glycoproteins and present themselves differentially expressed in CP [53, 59]. AP has been associated with increased levels of several salivary glycoproteins as well. These include serum albumin, Ig γ2 chain C region, Ig α2 chain C region, vitamin D binding protein, α-amylase, and zinc-α2 glycoprotein [55]. Hence, glycoproteomics may be also a helpful tool to identify distinctive glycoproteomic profiles among the different clinical stages of periodontitis.

5.4 Metabolomics approaches to periodontitis

Among the omics studies, metabolomics approaches are of a more recent vintage, but could represent a very productive avenue regarding potential contributions with regard to the diagnosis of periodontitis. Metabolomics ultimate concern is to obtain a complete screen of all metabolites of a given biological sample and interpret how the metabolic profile changes with a given pathophysiological state. The key techniques used in this field are NMR, GC-MS LC-MS [97]. The results of an NMR-based study of periodontitis in which the metabolic profiles of GCP patients were compared to those of healthy subjects are provided in Supporting Information Table 1. NMR spectra from processed saliva samples were obtained by applying Fourier transform. An unsupervised principal component analysis was performed to determine the variance of the NMR profiles [98]. NMR patterns of whole saliva showed a distinct metabolic profile in GCP patients, which revealed increased levels of acetate, γ-aminobutyrate, *n*-butyrate, succinate, trimethylamine, propionate, phenylalanine, and valine, and decreased levels of pyruvate and *N*-acetyl groups, which could be explained by host tissue degradation and metabolic and fermentative activity of the pathogens [98].

5.5 “Omics” challenges for the next decade

The challenge for the field of omics in the next decade will be to unravel new molecular patterns of biomarkers useful for the field of periodontics. Particularly promising to achieve this goal are approaches in peptidomics and metabolomics. While few studies have been conducted in these fields, they appear promising tools to achieve the desired molecular information for the development of a diagnostic tool applicable for the diagnosis, monitoring, and prospective evaluation of this widespread oral disease. Peptide fingerprinting in addition to enzymatic studies, such as zymography, can be the solution to address which proteases and peptides are differentially expressed in the various clinical presentations of periodontitis, and may detect specific types or families of microorganisms being particularly virulent and destructive for the periodontium. Glycoproteomics will also be an important tool to detect molecular targets for pathogen adhesins, and metabolomics approaches may help to define characteristic patterns associated uniquely with CP, AP, and the healthy state of the periodontium.

6 Concluding remarks

In summary, periodontitis is a complex inflammatory disease that leads to the destruction of the periodontium and ultimately to loss of the dentition. It is clearly known that genetic, environmental, and microbiological factors seem to determine periodontitis predisposition, onset, and progression. In the molecular realm, the various omics studies conducted so far

have pointed to several genes, SNPs, transcripts, and proteins/peptides, revealing a significant association with the different clinical presentations of periodontitis. The few studies comprising larger numbers of subjects have not confirmed these associations. Some proteomics studies of saliva and GCF have uncovered distinct protein profiles in health and periodontitis, but closer inspection of these protein markers reveals a lack of specificity. Proteolytic activity underlying periodontitis is at the outset of being explored showing collagenolytic and gelatinolytic activities being more intense in periodontitis patients. Metabolomics and peptidomics studies have begun to disclose the pattern of metabolites and the “fragmentome” signature of periodontitis. So far, ‘omics studies have described the host inflammatory response as a consequence of pathogen invasion. In the future, a comprehensive study of bacterial adhesins and virulence factors, their molecular targets, and host proteases through proteomic and peptidomic approaches could be valuable for finding molecular predictors of disease progression and to discriminate between the various manifestations of periodontitis.

From the molecular studies carried out so far, it is increasingly evident that adequate specificity and sensitivity for a diagnostic tool is required. The complexity of the disease makes it clear that this can only be achieved by a multiplex approach in which more than one biomarker is measured simultaneously. It also requires study cohorts of adequate size to maximize the chances to reach the goal of validity. The ultimate diagnostic tool will be acceptable by the clinicians only if the application of such a tool provides data superior or at least adding so far elusive and critically important information to what can be achieved by currently employed clinical assessment procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP	aggressive periodontitis
BOP	bleeding on probing
CAL	clinical attachment loss
GAP	generalized aggressive periodontitis
GCF	gingival crevicular fluid
GCP	generalized chronic periodontitis
GWAS	genome-wide association study

IEC	ion exchange chromatography
IFN-α1	interferon α 1
IL	interleukin
LAP	localized aggressive periodontitis
LCP	localized chronic periodontitis
LPS	lipopolysaccharide
MIP-1α	macrophage inflammatory protein 1α
MMP	matrix metalloproteinase
NOS3	endothelial nitric oxide synthase
PD	probing depth
SNP	single nucleotide polymorphism
TNF-α	tumor necrosis factor α

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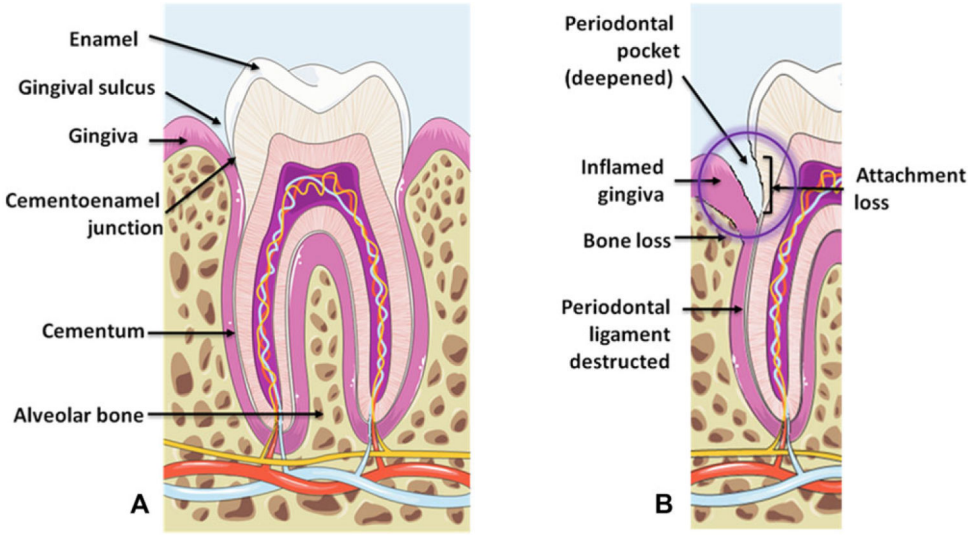

Figure 1.

Overview of the major histopathological changes found in periodontitis. (A) A periodontally healthy site with the most important histological structures depicted. (B) A periodontally affected site showing an inflamed gingiva due to microbial colonization, a deepened periodontal pocket, attachment loss and loss of periodontium structures (bone and periodontal ligament). The purple circle illustrates a critical area of inflammation and disease progression. Images were adapted from Servier Medical Art (http://www.servier.com).

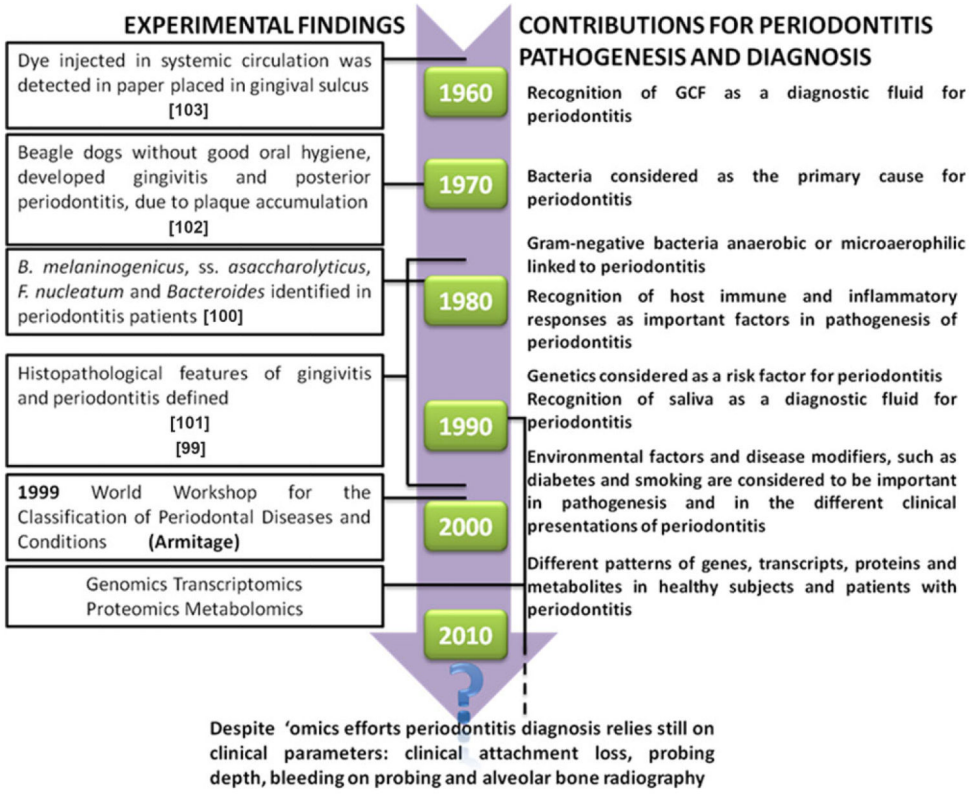

Figure 2.

Evolution on the view of periodontitis pathogenesis and diagnosis since 1958. The time frame was built based on published reports [5, 10, 11, 20, 99-103].

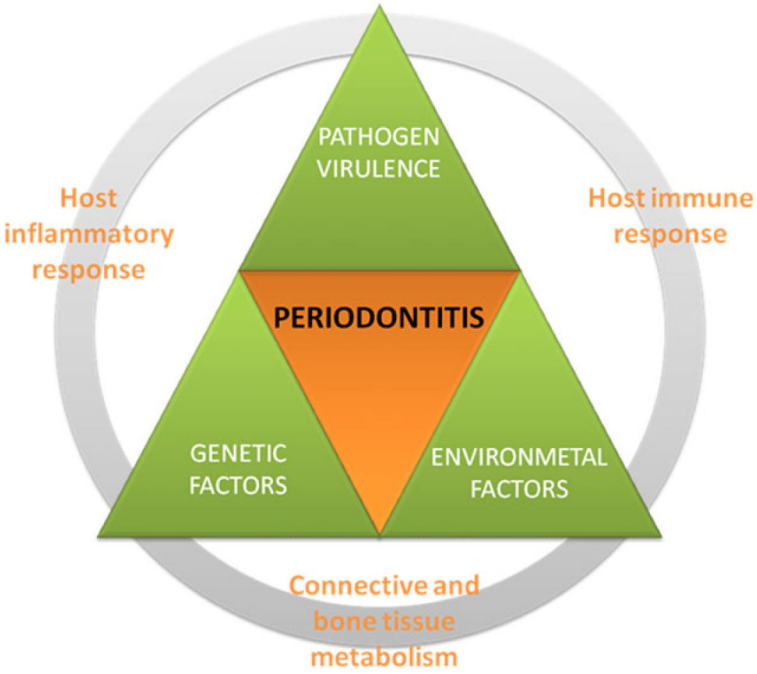

Figure 3.

Current view of periodontitis pathogenesis—a nonlinear model (1997) [10]. In the present model, microbial, genetic, and environmental factors play important roles in periodontitis pathogenesis. Besides, it recognizes the importance of host inflammatory and immune response and connective and bone tissue metabolism to the progression of periodontitis [2,3,10].

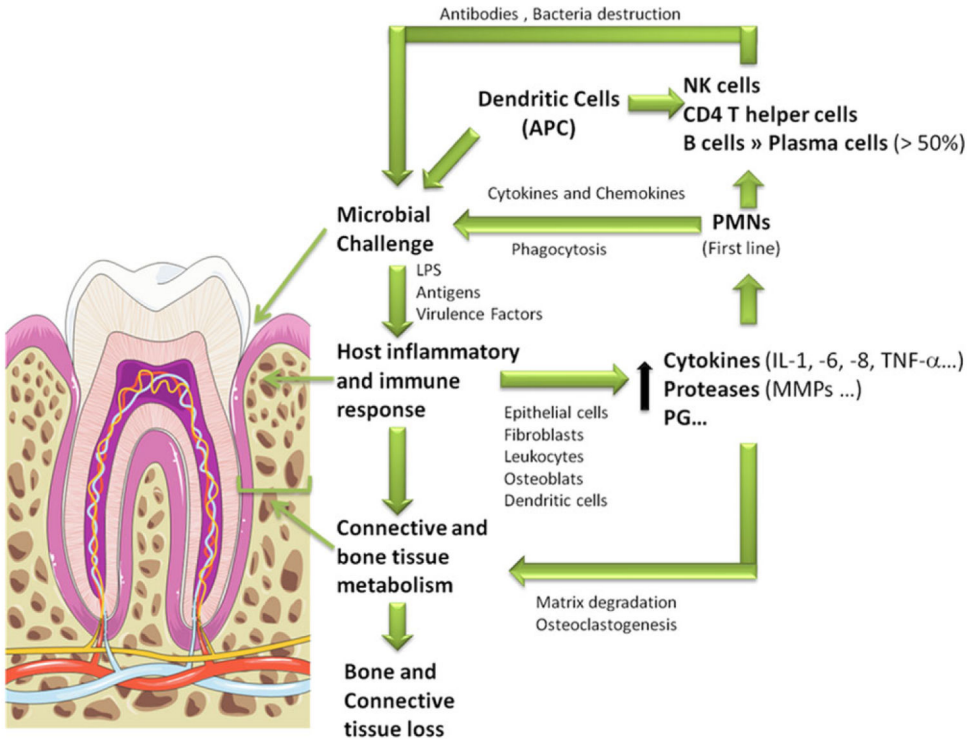

Figure 4.

Cellular and molecular hallmarks of periodontitis—a summary scheme. In the presence of a microbial challenge, epithelial cells, fibroblasts, dendritic cells, leukocytes, and osteoclasts release inflammatory cytokines and chemokines that attract other leukocytes. MMPs are also released to allow leukocytes infiltration in periodontium. PMNs and dendritic cells activate both innate and acquired immune systems, which contributes to infection control through phagocytosis and destruction of bacteria [3, 10, 13]. The extensive release of cytokines, prostaglandins (PG), MMPs, and other proteases result in bone and connective tissue loss [13]. The tooth image was retrieved from Servier Medical Art (http://www.servier.com).

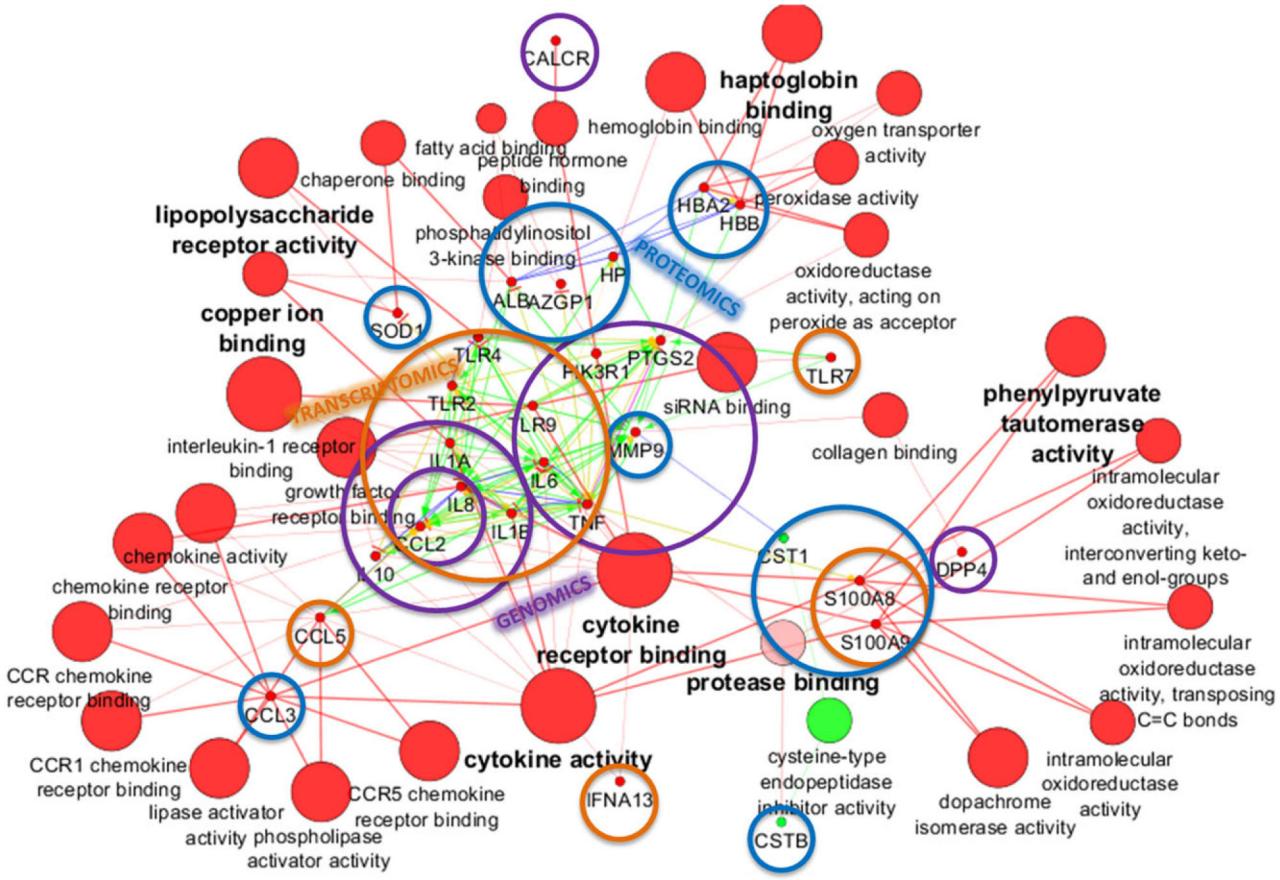

Figure 5.

Network of genes, transcripts, and proteins differentially expressed in periodontitis built with the ClueGO tool. Red nodes represent upregulated molecular markers in periodontitis, while green nodes represent downregulated molecular markers. Enrichment analysis was performed with CluePedia: activation, green arrows; binding, blue arrows; expression, yellow arrows; and PTMs, pink arrows. Contributions of genomics, transcriptomics, and proteomics are shown with transparent purple, orange, and blue circles, respectively.

Table 1

Possible risk factors for periodontitis

Nonmodifiable	Modifiable
Age	Oral hygiene
Gender	Smoking
Ethnicity	Diabetes control
Socioeconomic status	Obesity control
Genetic predisposition	Microbial flora
Some systemic diseases (Down's syndrome, neutropenia, Papillon–Lefèvre syndrome, Chédiak–Higashi syndrome, AIDS, osteoporosis)	Low dietary intake of calcium and vitamin D
Host immune factors	Local risk factors (restoration overhangs or deficiencies)

Table adapted from [1,3,4, 104].

Table 2

Advantages and disadvantages of saliva and GCF as diagnostic fluids

Fluid / Parameter	Saliva	GCF
Collection difficulty	Easy	Easy–medium
Yielded volume	High (>1.0 mL)	Very low (around 1 μL)
Invasiveness	Noninvasive	Can be minimally invasive
Speed of collection	Fast	Slow
Sensitivity to contamination	Insensitive	Sensitive
Specificity to periodontitis	Less specific than GCF	Specific

Table adapted from [7,8, 19, 20, 24, 26].