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REVIEW ARTICLE

Utility of gingival crevicular fluid components for periodontal diagnosis

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Abstract

Periodontal diseases are highly prevalent chronic diseases, and severe periodontitis creates functional and esthetic problems and decreases self-esteem for a large percentage of the older population worldwide. In many cases of periodontitis, there is no distinct tell-tale pain that motivates a patient to seek treatment, rather the signs become clinically detectable late, and typically when the disease has progressed to a problematic level for the life of the dentition. Early periodontal screening and diagnostics tools will provide early recognition of periodontal diseases and facilitate timely management of the disease to reduce tooth loss. To this goal, gingival crevicular fluid is easily sampled, can be repeatedly and non-invasively collected, and can be tested for potential biomarkers. Moreover, the site specificity of periodontal diseases enhances the usefulness of gingival crevicular fluid sampled from specific sites as a biofluid for diagnosis and longitudinal monitoring of periodontal diseases. The present review aimed to provide up-to-date information on potential diagnostic biomarkers with utility that can be assayed from gingival crevicular fluid samples, focusing on what is new and useful and providing only general historic background textually and in a tabulated format.

KEYWORDS
biomarkers, diagnosis, gingival crevicular fluid, periodontitis

1 | INTRODUCTION

Periodontitis is a significant health burden that is considered as the sixth most common chronic disease worldwide[1] with an 11% prevalence of severe periodontitis which presents considerable functional, esthetic issues.[2] Bacterial and host factors together initiate and propagate periodontitis[3] with a specific combination of these factors resulting in microbial dysbiosis which triggers periodontal inflammation via release of numerous cytokines and enzymes. Microbial dysbiosis may result in activated host cells that cause irreversible destruction of hard and soft tissues. All of these dysbiotic elements are represented in the gingival crevicular fluid (GCF), and thus, these microbial and biochemical factors are relevant as biomarkers of periodontitis.[2–5] There are also various susceptibility factors related to genes, systemic health state, and environmental factors, which again can be assayed within the gingival crevice or derived from the patient's history.[5] For example, tobacco product usage and the presence of diabetes are the major factors that increase the risk of developing periodontitis and are thus specifically named and included in the current classification of periodontitis.[6]

Periodontal diseases are initiated by bacterial challenge; however, progression of the disease is defined by a complex set of

immune-and inflammatory processes such that the path from health to periodontal destruction is closely dependent on the host response. Despite significant efforts and advances in research into the etiology and pathogenesis of periodontal disease at the molecular level, periodontal diagnosis is still largely dependent on the clinical assessment of probing depth (PD), clinical attachment level (CAL), and gingival inflammation. Although detailed clinical and radiographical examination offers reliable data on the periodontal status of the patient at the time of presentation, it provides limited information on the future risk of progression. Within the context of the new classification system, clinical diagnosis of periodontitis also consists of assessment of progression rate and the risk of progression at patient level even in the lack of direct evidence.

2 | CLINICAL DIAGNOSIS OF PERIODONTAL DISEASES

Diagnosis of a disease is usually made by the clinician based on the patient's anamnestic history, symptoms and clinical signs, and symptoms that are detectable at the time of examination. In periodontitis, the complex and multifactorial nature of the disease is a challenging factor for accurate clinical diagnosis particularly for determination of the current state of disease activity. Basic clinical periodontal parameters are thoroughly described and discussed in the review by Heitz-Mayfield[7] in this volume and therefore are only briefly outlined here. The basic clinical periodontal parameters can be grouped as follows: signs of inflammation, signs of oral hygiene level, and signs of periodontal tissue destruction. These clinical signs and symptoms predominantly provide information on the history of the patient's periodontal disease but have limited value in determining the current disease activity or prognosis. Moreover, the reproducibility and accuracy of these clinical periodontal parameters are questionable. The measurement outcomes may vary depending on the clinician-related, site-related, and periodontal probe-related factors. All these shortcomings of clinical diagnostic methods have motivated researchers to look for alternative or complementary diagnostic tools basing on microbiological and/or biochemical data. Reliable, non-invasive, molecular-based methods are likely to reveal biomarkers for periodontal diagnosis. Early detection of severe periodontitis by the help of biomarkers and timely treatment could prevent premature tooth loss, thereby increasing individuals' physical, psychological, and esthetic confidence.

3 | BIOMARKERS FOR PERIODONTAL DIAGNOSIS

The National Institute of Health (NIH) defines a biomarker as a quantifiable biological parameter that is measured and evaluated as an indicator of normal biological, pathogenic, or pharmacological responses to therapeutic interventions.[8] The main challenges for a candidate biomarker in periodontics are to identify current disease activity, to differentiate active sites from inactive ones, to predict further disease progression, and to monitor the response to periodontal treatment.[9] Furthermore, an ideal biomarker should be reliable, safe, and easy to measure, cost efficient for follow-up, modifiable with treatment, consistent across gender and ethnic groups, and should provide information about diagnosis, staging, and prognosis of periodontitis.[9] Moreover, benefits in clinical practice are expected from a true biomarker, rather than just providing a confirmation of clinical findings. A true biomarker would serve as the diagnostic and prognostic tool and indicate the accurate diagnosis or even the treatment plan in the absence of a periodontist. This may sound rather unrealistic and utopic, but such circumstances already exist in various fields of medicine. If such a biomarker for periodontal diseases is revealed, then the need for detailed clinical periodontal examinations using a periodontal probe, which may absorb 20 to 40 min of precious clinical time, may have a secondary rather than a primary place for periodontal diagnosis. This may eventually decrease the subjectivity of diagnostic tools and tremendously impact the time spent in clinics for both dental care providers and patients.

The current classification of Periodontal Diseases and Periodontal Conditions acknowledges the impact of systemic health on periodontal health and encourages clinicians to handle a particular case with a holistic approach. The use of biomarkers in periodontal diagnosis was also proposed to be included in the current classification affecting grading of periodontitis.[10] However, with the strength of the available evidence at the time of the World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions, it was not possible to implement biomarkers into the classification schema. Periodontal disease severity, progression rate, and response to periodontal treatment show extensive variations between individual patients or even between individual teeth/sites. The current grading system allows clinicians to assess periodontitis progression rate on the basis of the direct or indirect evidence modified with the presence of risk factors such as smoking and diabetes.

The site-specific nature of periodontitis demands site-specific assessment of risk of progression. Therefore, during the last decades, a substantial number of molecules have been evaluated with the expectation of defining those that could be considered as "biomarkers" of periodontal diseases for the assessment of future risk of progression even before the clinical onset of the disease.

Biomarkers are also beneficial in understanding disease mechanisms and monitoring the host immune response before, during, and after the treatment.[11] When the complex nature of periodontitis is considered, it is unlikely that one single clinical or laboratory parameter can address all issues.[12,13] In spite of the persistent limitations related to the characteristics of periodontal diseases, there is substantial progress in the field. Extensive reviews were published on this subject,[9,14] and the current narrative review addresses the subsequent rather than historic literature on the potential of GCF components in periodontal diagnostics, which is both abbreviated for contextual sense and provided in tabular form.

4 | GCF AS A SOURCE OF BIOMARKERS FOR PERIODONTAL DIAGNOSIS

The source of the biological sample for potential biomarker search is another important issue. GCF, saliva, and serum samples are currently used as the biological fluids for laboratory analyses including biochemical and microbiological investigations. Among the various biofluids, GCF has a special place not only as it is capable of providing site-specific information about the periodontal lesion but it also reflects components within serum, for example glycosylated hemoglobin that might diagnose subclinical diabetes[15] or cotinine that would indicate smoking status,[16] both of which are major susceptibility factors for periodontitis. Moreover, GCF sampling is easy and non-invasive (Figure 1). GCF can be both a physiological and pathological fluid originating from the blood vessels of gingival connective tissue, subjacent to the epithelium of dentogingival space, and in some cases also from the periodontal lesion itself. Since, its composition is directly affected by the interaction between host cells and bacteria in the subgingival biofilm, GCF is composed of leucocytes, proteins, mediators of inflammation, host-derived enzymes, tissue breakdown products, antibodies, and ionic molecules. GCF samples have a wide spectrum of ingredients that can be grouped as host-derived enzymes, bacteria-derived enzymes, metabolic end products, electrolytes, and cellular elements.[17] Its content is very complex as it has components from serum, host resident cells, and subgingival biofilm. At least 90 different components in GCF samples have been evaluated as potential biomarkers of periodontal disease detection and prognosis.[14] From a traditional point of view, possible biomarkers can be evaluated under three major categories corresponding with the three phases of periodontitis which are inflammation, connective tissue degradation, and bone resorption.[18] Point-of-care diagnostics based on GCF biomarkers could provide an instant indication of the disease and susceptibility status allowing monitorization of periodontal health.

Diagnostic potential of GCF-derived molecules has been investigated extensively during the last five decades.[19–21] Numerous studies highlighted the inflammatory mediators in GCF samples such as interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor-α (TNF-α), host-derived enzymes such as matrix metalloproteinase (MMP)-8, oxidative stress markers, and tissue breakdown products. However, solely the presence of these molecules in GCF does not necessarily imply their diagnostic capability.[19,22] Recent systematic reviews/meta-analyses focused on the diagnostic accuracy of GCF-derived molecules indicated a potential for various combinations of biomarkers.[22–24] Arias-Bujanda et al.[22] evaluated the accuracy of single biomarkers in GCF samples and among the biomarkers evaluated MMP-8 was reported to have a good sensitivity and excellent specificity in the diagnosis of periodontitis. Considering the multifactorial pathogenic mechanisms involved in periodontitis, combination of different molecules for the diagnostic accuracy of biomarkers in different oral fluids was also assessed in another systematic review.[23] Although the available data did not enable performing a meta-analysis of GCF parameters, dual combination of IL-1α, IL-1β, or IL-17 with interferon gamma (IFN-γ) or IL-10 was suggested to have an accuracy level higher than 90%.[23] Augmentation of clinical periodontal data with appropriate biomarkers is demanded particularly in patients presenting rapid destruction of periodontium at rather early ages. Alamri et al.[24] evaluated serum, saliva, and GCF biomarkers associated with Stage III, Grade C, and molar–incisor pattern in children and young adults; however, due to the heterogeneity of the available data, a meta-analysis was inconclusive. In another systematic review, Baima et al.[21] examined GCF-derived metabolites for their diagnostic potential and reported that oxidative stress molecules were highly associated with clinical findings of

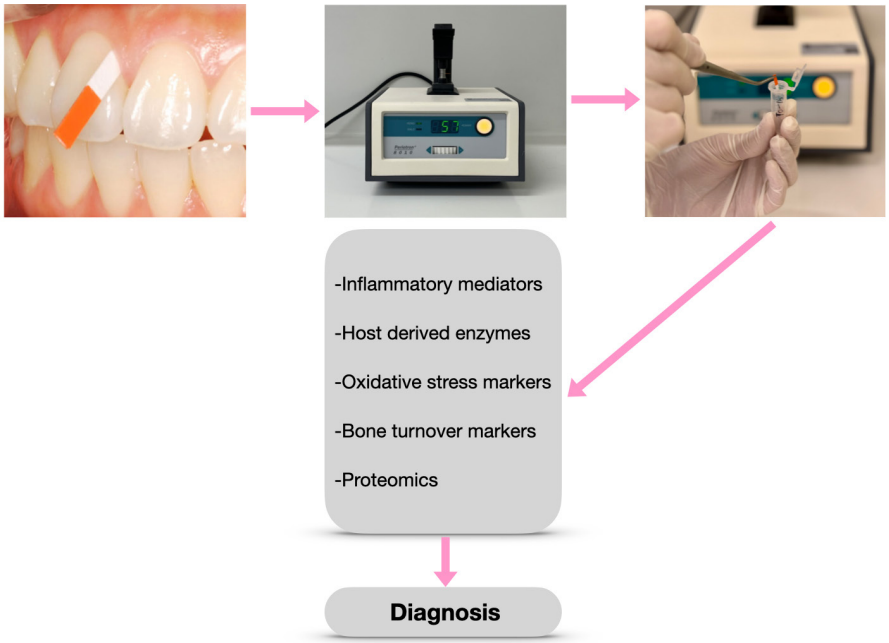

FIGURE 1 Major steps in the analysis of gingival crevicular fluid (GCF) samples; sample collection using filter papers, volume determination using a calibrated instrument, storage in appropriate conditions, and quantification of the ingredients with appropriate laboratory techniques.

periodontitis. The recent systematic reviews and their findings are outlined in Table 1. In the present review, the potential biomarkers of GCF are categorized into four main groups as inflammatory mediators, host-derived enzymes, oxidative stress molecules, and bone turnover markers (Table 2).

4.1 | Inflammatory mediators in GCF

Host immune responses triggered by bacteria in the biofilm attract neutrophils to the inflammation site, and cytokines are released to the crevicular environment by the cells of the gingival tissues through the junctional epithelium.[25] Cytokines are low molecular weight, soluble proteins that mediate the immune-inflammatory processes. Interleukin-1β (IL-1β), IL-2, IL-6, IL-8, IL-17, and tumor necrosis factor-α (TNF-α) are reported to play prominent roles in the pathogenesis of periodontal disease. GCF levels of IL-1β were found to be elevated in sites with progression and also were shown to decrease following non-surgical periodontal therapy.[25] In a molecular epidemiologic study conducted on 6768 individuals, clinical, microbiological, inflammatory, and host response data were used to categorize the clinical periodontal status.[26] The authors reported that among the five disease categories the study constituted, patients with deeper PDs and severe bleeding had the highest GCF levels of IL-1β, prostaglandin E_2 (PGE_2), and IL-6. Interestingly, patients with deep pockets exhibited severe bleeding and high levels of GCF IL-1β, but the microbial counts did not increase in parallel with the disease severity.

In their longitudinal cohort study, Kinney et al.[27] evaluated an array of GCF biomarkers including IL-1β, matrix metalloproteinase (MMP)-8, MMP-9, and osteoprotegerin (OPG) for their possible ability to predict periodontal disease progression. A total number of 100 individuals were included and divided into four groups according to their clinical periodontal diagnosis as healthy, gingivitis, mild chronic periodontitis, and moderate-to-severe chronic periodontitis. None of the individuals recruited in the study received any periodontal therapy at the initial phase of the study where they were followed for 6 months starting from the baseline. During this disease monitoring phase, patients were seen every second month for clinical periodontal evaluation and sampling of GCF and saliva. Radiographs were taken at baseline and at the end of this phase to evaluate disease progression. At 6 months, all individuals received periodontal therapy consisting of either oral hygiene instruction (OHI) and prophylaxis or scaling/root planning (Sc/RP) and OHI in accordance with their clinical periodontal diagnosis. In the second phase of the study, disease recovery was monitored. Any site revealing at least 2 mm clinical attachment loss compared with baseline was considered as site with progression. When individual analysis of GCF biomarkers at baseline was performed, significantly higher concentrations of GCF biomarkers were found in progressing patients compared with those patients with stable status and the statistical significance was higher for IL-1β. In a meta-analysis, evaluating predictive value of GCF cytokines and chemokines in chronic periodontitis patients, it was reported that IL-1β, IL-6, interferon (IFN)-γ, monocyte chemoattractant protein-1 (MCP-1), and chemokine ligand 2 (CCL2) exhibited higher levels in chronic periodontitis patients compared with healthy controls.[28] It was suggested that these biomarkers could distinguish chronic periodontitis and periodontally healthy sites. Not surprisingly, there were significant decreases in GCF levels of IL-1β and IL-17, while anti-inflammatory cytokine IL-4 was increased following the non-surgical periodontal therapy.[19] However, the authors claimed that there was no direct evidence of a predictive value for any of the investigated cytokines or chemokines other than IL-1β in the data derived from the aforementioned study by Kinney et al.[27,28]

In a study conducted to develop cytokine-based predictive models to estimate the probability of chronic periodontitis, Tomás et al.[29] performed quantitative analysis of 16 inflammatory mediators in GCF samples by using multiplexed bead immuno-assays. The pro-inflammatory cytokines including granulocyte-macrophage colony stimulating factor (GMCSF), IL-1α, IL-1β, IL-6, TNF-α, IL12p40, IL-17A, and IL-17F were all found to be significantly elevated in chronic periodontitis patients. When smoking-adjusted models were used, it was shown that IL-1α, IL-1β, and IL-17A in GCF were accurate biomarkers in distinguishing chronic periodontitis patients from periodontally healthy individuals. Two-cytokine models of IL-1α + IFNγ and IL-1β + IL-10 were shown to reveal better predictive ability than single cytokine models.[29] To a large extent, this is hardly surprising, inflammation and cytokine presence correlate well, and a question arises as to the utility of such biomarker-based diagnostics when simple assessments of redness, bleeding, or swelling would indicate the same thing. This point is relevant to all of these biomarker analyses, and the real utility for such biomarkers would be in disease or prognostic prediction.

In a recent systematic review, Blanco-Pintos et al.[23] evaluated the accuracy of biomarker combinations for the diagnosis of periodontitis in GCF and saliva. Given the fact that periodontal disease has a very complex nature, any effort to determine biomarker combinations rather than one single biomarker for diagnostic purposes is the preferential approach. Twenty-one biomarker combinations in GCF were analyzed for their diagnostic accuracy; however, the authors claimed that the heterogeneity of studies' methods hindered the use of meta-analysis. However, combinations of pro-inflammatory cytokines IL-1α, IL-1β, or IL-17A with anti-inflammatory cytokines IFN-γ or IL-10 showed at least >90% sensitivity and specificity.[23]

MCP-1 is an important chemoattractant for monocytes involved in periodontal tissue destruction as it plays a significant role in osteoclastic differentiation.[30] There are studies demonstrating increased GCF levels of MCP-1 with progression of periodontal disease.[31–34] Conversely, in a study exploring inflammatory mediators in GCF and systemic lipopolysaccharide (LPS) levels in localized aggressive periodontitis patients, MCP-1 levels in GCF samples were found to be higher in the healthy sites and there was a negative correlation with LPS.[30] It should be kept in mind that the immune-inflammatory response acting in the pathogenesis of periodontitis is highly complex and each single molecule in the process acts as a piece of a larger puzzle. MCP-1 expression is suppressed by GMCSF.[35] Therefore, the

TABLE 1 Main characteristics and findings of the recent systematic reviews on the potential of gingival crevicular fluid (GCF) biomarkers for periodontal diagnosis.

First Author	Database	Year range	Number of GCF articles included	Aim	Main findings
Stadler et al. (2016)	Medline Embase	Up to December 2015	57	Comparison of cytokines/Chemokines in CP	Significantly higher IL-1β, IL-6, IFN-γ, and MCP-1/CCL2 levels in CP compared with periodontally healthy subjects. No conclusion regarding the risk of disease progression
Arias-Bujanda et al. (2019)	PubMed (Medline), Embase, Cochrane Central Register of Controlled Trials and Trial Protocols, Scopus, Lilacs WoS	Up to October 25, 2018	9	Analysis of single molecular biomarkers in GCF for the periodontitis diagnosis	MMP-8 showed good sensitivity (77%) and excellent specificity (92%) and was followed by elastase (74.6% sensitivity and 81.1% specificity median) and cathepsin (72.8% sensitivity and 67.3% specificity)
Baima et al. (2021)	PubMed Embase Scopus Cochrane Library	Up to October 2020	15	Examination of periodontitis-specific metabolites in GCF	Among metabolites analyzed oxidative stress-related molecules such as MDA and 8-hydroxy deoxyguanosine were associated to periodontitis
Alamri et al. (2023)	Embase PubMed WoS Scopus Virtual Health Library	Up to February 08, 2023	2	Evaluation of molecular biomarkers in GCF, saliva, blood, and serum for the diagnosis of Stage III grade C periodontitis with molar–incisor pattern or previous equivalent definitions	Meta-analyses were conducted for GM-CSF, IFN-γ, IL1-β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, MCP-1, MIP-1α, and TNFα. No conclusions were drawn due high heterogeneity
Blanco-Pintos et al. (2011)	PubMed Embase Cochrane Scopus Lilacs WoS	Up to August 2023	10	Determination of the diagnostic accuracy of molecular biomarkers combinations in GCF and saliva for periodontitis	The dual combinations of IL-1β, IL-6, and MMP-8 in saliva had an excellent diagnostic accuracy to detect periodontitis. Although it was not possible to perform a meta-analysis of GCF biomarkers, dual combinations of IL-1α, IL-1β or IL-17A with IFN-γ or IL-10 were shown to have sensitivity and specificity of >90%

Abbreviations: CCL, chemokine ligand; CP, chronic periodontitis; GCF, Gingival crevicular fluid; GMCSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinases; SOD, superoxide dismutase; TNF, tumor necrosis factor; TOS, total oxidant status.

TABLE 2 Main characteristics and findings of the original studies investigating the potential of various biomarkers in gingival crevicular fluid (GCF) samples for periodontal diagnosis.

First author	Study design	Study groups	Follow-up period	Clinical parameters	Sample source	Technique	Biomarkers	Results
Kinney et al. (2014)	Longitudinal cohort	18 Healthy 32 Gingivitis 28 Mild CP 22 Moderate–Severe Periodontitis	12 months	PD CAL BOP	GCF Whole Saliva Serum Microbial Plaque Biofilm	Quantibody Human Cytokine Array	IL-1β, MMP-8, MMP-9, OPG CRP	Higher specificity of GCF biomarkers for the identification of periodontal disease progression with higher statistical significance for IL-1β
Tomas et al. (2017)	Cross-sectional	75 periodontally healthy controls 75 chronic periodontitis		PD CAL BOP BPL	GCF	Multiplexed bead immuno-assays	GMCSF, IFNγ, IL-1β, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, IL-17F, TNF-α	Higher GCF levels of pro-inflammatory cytokines GMCSF, IL-1α, IL-1β, IL-6, TNF-α, IL12p40, IL-17A, IL-17F, and anti-inflammatory cytokines IFNγ, IL-2, IL-3, IL-4 in CP Better predictive ability of IL-1β, IL-1α, IL-17A in smoking-adjusted models
Balci et al. (2021)	Cross-sectional	20 Stage III/Grade B periodontitis 20 healthy controls		PPD CAL PI BOP REC	GCF Serum	ELISA	CX3CL1 CX3CR1 IL-1β	Significantly higher GCF levels of CX3CL1, CX3CR1, and IL-1β in periodontitis Positive correlations between clinical parameters and biomarkers
Huang et al. (2023)	Cross-sectional	29 Stage III Periodontitis 11 Stage IV Periodontitis		PD CAL PI BOP PISA PESA	GCF Gingival tissue	ELISA Immunohistochemical Western blot	C3b C4b	C3b and C4b have shown to differentiate diseased sites from healthy sites, particularly when used in combination
Shaddox et al. (2011)	Cross-sectional	34 LAP 10 healthy siblings 9 healthy unrelated control individuals		PD CAL Plaque Radiography	GCF Plasma	Fluorescence detection kits	IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, EGF, Exotoxin, Fractalkine, GCSF, GMCSF, IFN-γ, IP10, MCP-1, MIP-1α, MIP-1β, sCD40L, TGF-α, TNF-α, VEGF	Higher GCF levels of TNFα, INFγ, IL-1β, IL-2, IL-6, IL-10, IL-12p40, GMCSF, and MIP1α in LAP Higher GCF MCP-1, IL-4, IL-8 levels in healthy sites Higher plasma levels of endotoxins in LAP Correlation of endotoxin levels with Clinical inflammation and cytokine levels
Gupta & Chaturvedi (2013)	Intervention	30 severe CP 15 healthy controls	6 weeks	PD CAL GI PI	GCF Serum Saliva	ELISA	MCP-1	GCF and saliva MCP-1 levels may be reliable indicator of disease severity
Leppilahti et al. (2014)	Cross-sectional	8 moderate-to-severe periodontitis patients (19 sites) 6 Gingivitis patients (19 sites) 9 Healthy individuals (20 sites)		PD CAL	GCF	ELISA IFMA	Azurocidin, CXCL5, MPO, TIMP-1, MMP-8, MMP-13, MMP-14	MPO had highest site-specific diagnostic accuracy which is followed by MMP-8 (IFMA), MMP-13, MMP-14, MMP-8 (ELISA), and azurocidin

TABLE 2 (Continued)

First author	Study design	Study groups	Follow-up period	Clinical parameters	Sample source	Technique	Biomarkers	Results
Baeza et al. (2016)	Cross-sectional	11 CP (31 GCF samples) 38 AP (44 GCF samples) 13 healthy individuals (31 GCF samples)		PD CAL BOP Radiographic evaluation	GCF	ELISA IFMA Zymography	MMP-8 MMP-2, MMP-9, MPO, IL-1, IL-6, TNF-α, Dkk1, periostin, TRAP-5, osteonectin OPG	Significantly higher GCF levels of MMP-8 and MMP-9 in CP and AP
Sorsa et al. (2020)		15 Stage I 81 Stage II 23 Stage III Periodontitis 14 Grade A 91 Grade B 14 Grade C 31 Healthy		PD CAL BOP VPI	Mouthrinse	aMMP-8 PoC/ chairside mouthrinse test	aMMP-8	aMMP-8 levels may be utilized diagnosis of periodontitis and to identify the active periods and to monitor disease progression
Gupta et al. (2023)	Cross-sectional	15 Stage I 81 Stage II 23 Stage III Periodontitis 31 Healthy		PD CAL BOP	Mouthrinse Capillary blood	aMMP-8 PoC/ chairside mouthrinse test ELISA Western blot	aMMP-8 Total/latent MMP-8 proMMP-8 activation by *T.d.* dentilisin HbA1c	Increased aMMP-8 level is related to severity and progression rate of periodontitis particularly in the presence of prediabetes
Deng et al. (2021)	Cross-sectional	408 participants		PPD CAL FMBS FI Mobility Number of teeth lost due to periodontitis	Mouthrinse	aMMP-8 PoC/ chairside mouthrinse test	aMMP-8	aMMP-8 is able to detect periodontitis severity with better specificity than sensitivity
Deng et al. (2022)	Cross-sectional	95 adult subjects		PPD CAL FMBS FI Mobility Number of teeth lost due to periodontitis	Whole Saliva Oral rinse (twice)	aMMP-8 PoC/ chairside mouthrinse test	aMMP-8	Oral rinse without prerinse may be valid approach for the discrimination of periodontal disease and health However, whole saliva and oral rinse with prerinsing with water had lower diagnostic accuracy
Öztürk et al. (2021)	Cross-sectional	18 Stage III 19 Stage IV periodontitis 21 gingivitis 22 clinically healthy periodontium		PD CAL PBI PI	GCF Saliva	aMMP-8 PoC/ chairside test IFMA		GCF and saliva levels of a-MMP-8 levels were increased with disease severity with the highest levels in Stage IV periodontitis, absence or low level of aMMP-8 was associated with healthy conditions

(Continues)

TABLE 2 (Continued)

First author	Study design	Study groups	Follow-up period	Clinical parameters	Sample source	Technique	Biomarkers	Results
Yakob et al. (2013)	Cross-sectional	56 periodontitis 45 periodontally healthy		PD CAL GI PI CI	GCF	IFMA qPCR	MMP-8 MMP-9 *A. a.* *P. g.* *P. i.* *T. f.* *T. d.*	Significantly higher *T. d.* levels in periodontitis group Increased GCF levels of MMP-8 are associated with the presence of *T. f.* and *T. d.*
Wei et al (2010)	RCT	48 CP 16 healthy controls	16 weeks	PD CAL GI PI GBI	Serum Whole saliva GCF	HPLC for MDA Total oxidant status assay Enzyme activity assay for SOD	MDA TOS SOD	At baseline, only GCF levels of MDA, and Serum, saliva, GCF TOS, and SOD levels were higher in CP Non-surgical periodontal therapy restored GCF levels of MDA, and serum, saliva, GCF levels of TOS and SOD
Srinath et al. (2010)	Cross-sectional	15 moderate-to-severe CP 15 gingivitis 15 healthy		GI Russel Periodontal Index	Saliva GCF	ELISA	Melatonin	Salivary and GCF melatonin concentrations were lowest in the CP group
Almughurabi et al. (2013)	Cross-sectional	20 CP 20AP 10 gingival inflammation 10 healthy		PD CAL GI Tooth mobility	Saliva GCF	ELISA	Melatonin	Saliva and GCF melatonin levels were higher in the healthy group compared with AP, and CP groups AP group had the lowest melatonin levels
Ghallab et al. (2016)	Cross-sectional	25 CP 25 GAP 15 healthy		PD CAL GI PI	GCF	ELISA	Melatonin SOD MDA	Significantly higher GCF levels of MDA in GAP Significantly higher SOD and melatonin GCF levels in healthy group, GCF levels of SOD and melatonin were significantly lower in GAP compared with CP
Hassan et al. (2015)	RCT	20 CP 10 periodontally healthy	Baseline and 3 and 6 months after OFD	PD CAL GI PI	Saliva GCF Gingival tissue	ELISA Immunohistochemistry	OPG	Saliva and GCF levels of OPG were higher in the healthy control group Statistically significant increase in salivary and GCF levels of OPG following OFD
Segerra-Vidal et al. (2017)	RCT	40 periodontitis (SRP only group ($n = 20$), SRP + PDT group ($n = 20$)) 20 healthy controls	One week prior to intervention, 5, 13, and 25 weeks following non-surgical periodontal therapy	PD CAL BOP PI REC	GCF	RT-PCR ELISA	*A. a.*, *T. f.*, *P. g.*, *T. d.*, *P. i.*, and *C. r.* IL-1β, TNF-α, IL-6 RANKL OPG	Baseline GCF levels of IL-1β, TNF-α, IL-6 RANKL, RANKL/OPG is higher in CP compared with healthy controls while baseline GCF level of OPG is lower in CP. At Week 13, significant decrease in RANKL in the SRP + PDT group

TABLE 2 (Continued)

First author	Study design	Study groups	Follow-up period	Clinical parameters	Sample source	Technique	Biomarkers	Results
Bostanci et al. (2011)	Intervention	14 CP 13 GAP	2, 3, and 4 months following SRP	PPD CAL GI PI	GCF	ELISA	OPG RANKL	Increase in GCF OPG levels at 4 months after SRP, no significant changes in RANKL/OPG
Becerik et al. (2011)	Cross-sectional	20 CP 20 GAP 20 gingivitis 20 healthy		PPD CAL PBI PI	GCF	ELISA	Osteocalcin NTx Calprotectin	Lower osteocalcin and NTx levels in CP and GAP groups Higher calprotectin levels in CP and GAP groups
Afacan et al. (2020)	RCT	60 CP (20 Q-SRP, 20 FMD, 20 FMUD)	1, 3, and 6 months after periodontal treatment	PPD CAL PBI PI	GCF Plaque	ELISA RT-PCR	Osteocalcin NTx Calprotectin *A. a.*, *P. g.*, *P. i.*, *T. f.*, *F. n.*, TBC	Increase in calprotectin levels after therapy in all study groups Osteocalcin levels remained unchanged
Choi et al. (2011)	Cross-sectional	161 healthy samples 229 chronic gingivitis samples 296 moderate periodontitis samples 183 severe periodontitis samples		PD CAL BOP GI	GCF Gingival tissue	LC–MS/MS analysis Western blot ELISA	GCF proteome	Total number of 305 proteins were identified, 45 of those proteins were differentially expressed in moderate periodontitis Also, azurocidin is identified in periodontitis patients and suggested to be a possible biomarker
Nalmpantis et al. (2020)	Cross-sectional	53 CP 48 periodontally healthy		PD CAL REC BOP PL	GCF	ELISA	Azurocidin	3.6 times higher GCF azurocidin levels in CP
Baliban et al. (2012)	Cross-sectional	12 CP 12 periodontally healthy		PD REC BOP	GCF	HPLC MS/MS	GCF proteins	432 human, 30 bacterial proteins identified. High levels of angiotensinogen, clusterin, and thymidine phosphorylase were found in healthy sites Neutrophil defensin-1, carbonic anhydrase-1, and elongation factor-1 gamma were associated with CP
Ngo et al. (2010)	Cross-sectional	N/A		N/A	GCF	HPLC nano-LC-ESI-MS/MS MALDI-TOF/TOF MS	GCF proteins	Total of 66 proteins were identified from diseased sites 43 of those were novel proteins including actin and actin-binding proteins, such as gelsolin, profilin, cofilin, and histones
Bostanci et al. (2010)	Cross-sectional	5 GAP 5 healthy		PPD REC BOP FMBS FMPS	GCF	Label-free LC/MS ELISA	GCF exudate	Host defense associated proteins Cystatin-B and defensins were found only in healthy sites Newly identified protein L-plastin was found only in diseased sites

(Continues)

TABLE 2 (Continued)

First author	Study design	Study groups	Follow-up period	Clinical parameters	Sample source	Technique	Biomarkers	Results
Ozturk et al. (2015)	Cross-sectional	21 CP 20 GAP 20 non-periodontitis		PD CAL BOP PI	GCF Saliva Serum Gingival tissue	Immunohistochemistry RT-PCR ELISA	L-plastin	GCF levels of L-plastin and gene expression of L-plastin in gingival tissue were higher in CP and GAP compared with healthy controls No differences in saliva and serum L-plastin levels between 3 study groups
Silva-Boghossian et al. (2013)	Cross-sectional	5 CP 5healthy		PD CAL BOP Plaque (dichotomous)	GCF	LC-ESI-MS/MS ELISA	GCF proteins	APOE was found exclusively in periodontitis patients and in greater abundance in periodontitis sites undergoing destruction
Torres et al. (2023)	Longitudinal	5 periodontitis patients (10 sample sites: 5 progression sites, 5 non-progression sites)	12 weeks (in order to monitor progression)	PD CAL Radiographic alveolar bone loss	GCF	PRLC-MS/MS Western blot	GCF proteins	1504 and 1500 proteins were identified in NP and P sites 48 and 52 of those proteins were identified exclusively in P and NP sites, respectively Most relevant protein interactions in P sites were IL-1 response, infection and stress response, and acetylation process
Baliban et al. (2013)	Cross-sectional	51 periodontally diseased 45 periodontally healthy		PD REC BOP	GCF	HPLC MS/MS	GCF proteins	MILP model was tested in its accuracy to identify optimal biomarker combination to distinguish healthy or diseased sites and predictive accuracy of the model was 95%. The protein CA1 was identified as potential biomarker

Abbreviations: *A.a.*, *Aggregatibacter actinomycetemcomitans*; AP, apical periodontitis; APOE, apolipoprotein E; BOP, bleeding on probing; *C.r.*, *Campylobacter rectus*; CAL, clinical attachment level; CCL, chemokine ligand; CI, calculus index; CP, chronic periodontitis; CRP, C-reactive protein; CXCL5, CXC chemokine ligand 5; Dkk1, pre-resorptive factors such as dickkopf related protein 1; ELISA, enzyme-linked immunosorbent assay; FI, furcation involvement; FMBS, full mouth bleeding score; GAP, generalized aggressive periodontitis; GCF, gingival crevicular fluid; GI, gingival index; GMCSF, granulocyte-macrophage colony stimulating factor; HPLC, liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS); IFMA, immunofluorescence assay; IFN, interferon; IL, interleukin; LAP, localized aggressive periodontitis; MALDI-TOF/TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; MILP, mixed-integer linear optimization; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinases; NTx, N-telopeptide of type I collagen; OPG, osteoprotegerin; *P.g.*, *Porphyromonas gingivalis*; *P.i.*, *Prevotella intermedia*; PBI, papilla bleeding index; PD, Probing depth; PESA, periodontal epithelial surface area; PI, plaque index; PISA, periodontal inflamed surface area; qPCR, q polymerase chain reaction; RANKL, receptor activator of nuclear κB ligand; REC, recession; ROS, reactive oxygen species; RT-PCR, real-time polymerase chain reaction; SOD, superoxide dismutase; *T.d.*, *Treponema denticola*; *T.f.*, *Tannerella forsythia*; TBC, total bacterial count; TIMP, tissue inhibitor of matrixmetalloproteinases; TNF, tumor necrosis factor; TOS, total oxidant status; TRAP-5, tartrate-resistant acid phosphatase-5.

authors claimed that the increased GCF levels of GMCSF in the diseased sites found in the study might be responsible for lower levels of MCP-1.[30]

IL-8 is another chemokine that regulates neutrophil activity by inducing neutrophil adhesion to endothelial cells and also transendothelial migration.[36] IL-8 is related to subclinical inflammation at the initiation of periodontal lesions, and although it has been reported to increase in periodontitis patients,[25,37,38] the available findings in the literature are controversial. In their systematic review, Stadler et al.[28] reported that following non-surgical periodontal therapy, there were no significant changes in GCF levels of several cytokines and chemokines including that of IL-8. The possible explanation for the lack of significant differences may be the limited number of studies with rather small sample sizes. The controversial findings from studies with large sample sizes for effect size estimation make the clinical utility of IL-8 questionable.[28]

Fractalkine (CX3CL1) is also a member of the chemokine family which is known to promote cell survival during inflammation and homeostasis, and it is expressed from endothelial cells, smooth muscle cells, and monocytes.[39] There are two different forms of CX3CL1 both of which bind to the receptor CXCR1. The selective receptor CX3CR1 is shown to be related to vascular pathologies by mediating the adhesive and migratory functions of fractalkine on leukocyte and coronary artery small muscle cell proliferation.[40–44] Therefore, interaction of CX3CL1 may contribute to the progression of inflammatory diseases and is shown to increase in the presence of systemic diseases such as angina pectoris, diabetes mellitus, and rheumatoid arthritis (RA).[45–47] In a cross-sectional study, Balci et al.[48] evaluated the GCF and serum levels of CX3CL1, its receptor CX3CR1 and IL-1β in stage III, grade B periodontitis patients in comparison with periodontally healthy controls. The total amounts and concentrations of CX3CL1, CX3CR1, and IL-1β were significantly higher in GCF samples obtained from periodontitis patients. Also, there was positive correlations between the clinical periodontal parameters and GCF levels of fractalkine, its receptor, and IL-1β. As there are no other published studies with a similar scope, it is too early to implicate their role in the pathogenesis of periodontitis.[48] Yet, the findings of this study may inspire further studies with larger study populations that would aim to clarify the role of CX3CL1 and CXCR1 in periodontal disease progression and the underlying mechanism.

The complement cascade plays a role in the regulation of immunoinflammatory pathways, and it can be activated through three distinct pathways: classical, lectin, and alternative pathway.[49] Periodontal diseases, either gingivitis or periodontitis primarily, activate the complement system via the alternative pathway.[25] The complement components C3b and C4b facilitate phagocytosis by opsonizing the surface of recognized pathogenic substances,[49] and both C3b and C4b have been shown to have positive correlations with periodontal disease severity.[50] In an experimental periodontitis study conducted on rats, single and multiple injections of C3b/C4b inhibitors have shown therapeutic and preventive effects on inflammatory bone loss.[50] In a very recent clinical study[51] by the same group, gingival tissue and GCF levels of C3b and C4b were investigated for their correlation with disease severity and their potential role as a complementary diagnostic biomarker. Both of the complement components were shown to be positively correlated with the clinical periodontal parameters either alone or in combination. When further analysis was performed on their diagnostic accuracy, both C3b and C4b were shown to differentiate diseased sites from non-diseased sites with a specificity of 97.4% and 97.5% and a sensitivity of 31.6% and 51.4%, respectively. The utility of the combination of C3b and C4b was found to have better diagnostic ability (94.3% specificity, 62.9% sensitivity) rather than single use. In conclusion, it is suggested that further understanding of the C3b–C4b network would help in the development of new screening, diagnostic and therapeutic strategies.[51]

4.2 | Host-derived enzymes in GCF

Matrix metalloproteinases (MMPs) are proteolytic enzymes that are involved not only in physiological processes such as angiogenesis, embryogenesis, cellular differentiation, wound healing, and immune responses but also in pathological processes such as destructive periodontal diseases.[52] More than 23 members of the MMP family have been defined so far which are divided into six groups according to the substrate specificity and molecular structure: (1) collagenases, (2) gelatinases, (3) matrilysins, (4) stromelysins, (5) membrane-type MMPs, and (6) other MMPs.[52] Although MMPs were initially thought to be expressed only by neutrophils, it has been shown that they are released by various other cell types such as macrophages, endothelial cells, epithelial cells, and fibroblasts.[25] Following the bacterial trigger, activated cells release MMPs and other inflammatory molecules.[53] IL-1, IL-6, and TNF-α are the major cytokines stimulating MMP expression with specific relations between certain cytokines and MMPs. It has been shown that IL-1β and TNF-α stimulate the release of MMP-3, MMP-8, and MMP-9 from gingival fibroblasts and MMP-13 from osteoblasts.[25]

The neutrophil response is crucial for the homeostasis of periodontal tissues; however, neutrophil hyper-responsivity is also related to periodontal destruction.[54] CXC chemokine ligand 5 (CXCL5) plays a role in the chemotaxis, activation, and degranulation of neutrophils which in turn results in the release of azurocidin, myeloperoxidase (MPO), and MMP-8.[55] In a cross-sectional study, GCF levels of azurocidin, CXCL5, MPO, tissue inhibitor of matrix metalloproteinases (TIMP)-1, MMP-8, MMP-13, and MMP-14 were evaluated as candidate biomarkers for their site-specific diagnostic accuracy to discriminate periodontitis sites from inflamed and healthy sites.[55] Fifty-eight GCF samples obtained from sites representing healthy, gingivitis and periodontitis conditions were analyzed by ELISA or activity tests for all of the studied biomarkers other than MMP-8. GCF levels of MMP-8 were analyzed by ELISA and by immunofluorometric assay (IFMA). Among the biomarkers evaluated, MPO had the highest diagnostic accuracy which is followed by MMP-8 analyzed

by IFMA, MMP-13, MMP-14, MMP-8 analyzed by ELISA, and azurocidin. The authors also claimed that MPO and MMP-8 by IFMA had diagnostic utility for the discrimination of periodontitis sites from those of gingivitis sites with high sensitivity, specificity, and positive and negative predictive values. The more accurate results obtained by IFMA in comparison with ELISA in the detection of GCF MMP-8 levels support the use of quantitative methods.[55] A further study evaluated the diagnostic accuracy of a range of GCF biomarkers for apical and chronic periodontitis, together with MMP-8 levels analyzed by ELISA and IFMA.[56] Both methods showed high diagnostic accuracy for MMP-8 to discriminate chronic periodontitis and apical periodontitis from healthy sites, with no significant differences between the two methods. The other biomarkers evaluated were MMP-2, MMP-9, MPO, IL-1, IL-6, TNF-α, and pre-resorptive factors such as dickkopf related protein 1 (Dkk1), periostin, tartrate-resistant acid phosphatase-5 (TRAP-5), and the non-collagen matrix protein osteonectin. Chronic periodontitis and apical periodontitis sites had significantly higher GCF levels of MMP-8 and MMP-9 compared with healthy sites. Moreover, ProMMP-2, ProMMP-9, and TRAP-5 had the highest diagnostic accuracy for chronic periodontitis.[56]

The activity of MMPs is regulated by their endogenous inhibitors and tissue inhibitor of matrix metalloproteinases (TIMP). In humans, four types of TIMPs (TIMP 1–4) have been identified and TIMP-1 is more effective on interstitial collagenases.[9] The remodeling of the extracellular matrix is implemented by the coaction of MMPs and TIMPs. A recent systematic review and meta-analysis reported that periodontal diseases are not associated with a statistically significant change in GCF TIMP-1 concentration.[57] Various types of MMPs have been demonstrated in gingival tissue samples and MMP-8, MMP-9, and MMP-13 are those most widely studied in GCF samples, with MMP-8 the most prominently reported.[58] It has been reported that in chronic periodontitis patients 80% of the total collagenase content in GCF samples consisted of MMP-8.[59–61] MMPs are known to be released in latent form, and they are then activated in the tissue. The active form of MMP-8 (aMMP-8) is known to degrade the collagen in the gingival and periodontal ligament tissues. Particularly activity of MMP-8 is elevated in patients suffering from periodontal diseases as it causes destruction and digestion of type I collagen, which is dominant in periodontal connective tissue.[62]

In a recent study,[63] the utility of incorporating aMMP-8 levels into the 2017 classification system for the diagnosis of periodontitis is investigated. In the new classification system, future risk of periodontitis progression is evaluated by grading.[10] Sorsa et al.[63] suggested the inclusion of aMMP-8 levels into the 2017 classification system for the diagnosis of periodontitis in order to identify the active periods of periodontitis and to monitor the disease progression. Cleavage of latent MMP-8 by periodontal pathogens and their virulence factors like Td-dentilisin leads to release of active MMP-8 molecules and also smaller MMP-8 fragments which are detectable by aMMP-8 point-of-care test kits that are readily available on the market.[64–66] These kits are lateral flow immunoassay-based kits utilizing detection of aMMP-8 in oral biofluids such as GCF, peri-implant crevicular fluid, and mouthrinse.[67] The sensitivity of the aMMP-8 point-of-care test (POCT) is 75%–85%, and the specificity is 80%–90%.[61] It was concluded that the use of aMMP-8 mouth rinse PoC/chairside test could be implemented to identify the progression rate of the periodontal disease as aMMP-8 level < 20 ng/mL is associated with no/slow rate of progression, aMMP-8 level ≥ 20 ng/mL with moderate rate of progression, and aMMP-8 level > 30 ng/mL with rapid rate of progression.[68] In another cross-sectional study, aMMP-8 PoC/chair-side test was evaluated for its screening and prevention potential at different stages of periodontitis, gingivitis, and health.[69] It was found that a-MMP-8 levels in both GCF and saliva were increased with disease severity and the highest levels were detected in stage IV periodontitis, while the absence or low level of aMMP-8 was associated with healthy conditions. The diagnostic accuracy of the test in GCF was 87% (AUC = 0.87, 95% CI 0.799–0.958), and the number of positive sites were 94.7%, for stage IV periodontitis, 94.4% stage III periodontitis, 81% for gingivitis, and 18.02% for health. Furthermore, the sensitivity and the specificity of the test were 83.9% and 79.2%, respectively. Chair-side tests as an adjunctive tool in the diagnosis of different stages of periodontal diseases may be helpful for the clinician.[69] This test is already available to clinicians commercially and has shown promising potential for detecting or excluding periodontitis.[65,66]

In a systematic review[22] evaluating the accuracy of molecular biomarkers for diagnostic purposes in periodontitis patients, GCF MMP-8 level was found to have good sensitivity and excellent specificity (76.7% and 92.0%, respectively). When the overall prevalence of periodontitis is considered as 45%, it was estimated that 88.8% of the tests with positive MMP-8 would indicate a true positive and 82.8% of the tests with MMP-8 negative would indicate a true negative.[53] GCF MMP-8 and MMP-9 levels correlated with clinical signs of disease activity in patients with chronic periodontitis.[63,70] In a cross-sectional study, increased GCF levels of MMP-8 were associated with the presence of *Treponema denticola* and *Tannerella forsythia* in periodontitis patients.[71]

However, in the recent study by Deng et al.[6] logistic regression analysis revealed only minor additive benefit from doing aMMP-8 point-of-care test and self-reported gingival bleeding on brushing, which also added time and cost for quite a minimal added value. The combination of aMMP-8 POCT and bleeding on brushing performed best for discrimination of periodontal health from disease and detection of gingivitis but not, importantly, for discriminating periodontitis from gingivitis, according to this study. The authors hypothesized that "gingival bleeding on brushing" analysis captures superficial inflammation that is gingivitis and aMMP-8 POCT captures periodontal breakdown that is periodontitis.[6]

4.3 | Oxidative stress markers in GCF

There is a large body of evidence demonstrating the role of oxidative stress (OS) in the pathological processes in periodontal disease progression.[72] OS occurs as a result of the imbalance between reactive

oxygen species (ROS) and antioxidants caused by excess production of ROS and relative deficiency of antioxidants. The balance between oxidants and antioxidants are maintained in healthy individuals; however, in the presence of a pathological condition this balance is shifted toward oxidative stress. Since ROS have a very short life, they are not easily detectable and the role of OS in periodontal disease pathogenesis may be evaluated by indirect methods such as investigation of ROS-related destruction products, enzymatic activity, and non-enzymatic antioxidants.[72] ROS are produced by various inflammatory cells, and furthermore, by resident cells such as fibroblasts, vascular endothelial cells, and osteoclasts.[73] ROS induce lipid peroxidation (LPO), which may cause OS and disruption of cell integrity. Malondialdehyde (MDA) is the most widely studied product of polyunsaturated fatty acid peroxidation, and higher levels of MDA may indicate OS status.[72,73]

In a meta-analysis performed by Chen et al.,[72] it was reported that saliva and GCF levels of MDA were significantly increased in GCF samples of periodontitis patients. The authors claimed that an explanation for the increased GCF MDA level could be superoxide anion production, as a result of bacterial challenge or as a by-product of neutrophil interaction. Superoxide dismutase (SOD), on the other hand, is an important antioxidant found in mammalian tissues.[73] There are conflicting data in the literature regarding the SOD levels in GCF samples.[73–75] In a study,[74] SOD activity in GCF and gingival tissues of chronic periodontitis patients and periodontally healthy controls was analyzed. Gingival SOD activity was found to be significantly higher in chronic periodontitis patients compared with healthy controls, whereas there were no differences in GCF SOD activity between the study groups.[74] Similarly, in another study GCF SOD levels were higher in chronic periodontitis group compared with control group.[73] The authors also reported decreased GCF SOD levels following non-surgical periodontal therapy. In contrast, Canakci et al.[75] reported significantly lower SOD activity in GCF, saliva, and serum of pre-eclamptic women with periodontal disease. In their meta-analysis, Chen et al.[72] found no significant differences between chronic periodontitis and healthy controls in terms of GCF levels of SOD.

Melatonin is another antioxidant and anti-inflammatory molecule that has received significant interest in the field of periodontology.[19] There are studies reporting decreased GCF melatonin levels with increasing periodontal disease severity.[76,77] Ghallab et al.[78] found increased GCF melatonin levels in healthy controls compared with chronic periodontitis and generalized aggressive periodontitis groups. Melatonin has been suggested to inhibit bone resorption via its regulatory effects on PGE_2, IL-1, IL-6, and OPG/RANKL system.[79–82] Also, it has been shown that melatonin increases osteoblast differentiation and bone formation.[83] Due to its effects on bone metabolism along with anti-inflammatory and antioxidant properties, melatonin deserves further investigation.

4.4 | Bone turnover markers in GCF

Inflammation-induced alveolar bone loss is the consequence of the imbalance between osteoblastic and osteoclastic activity due to the disturbance caused by bacterial products and inflammatory cytokines.[25] Osteoclastic activity is mainly regulated by members of the TNF receptor superfamily, receptor activator of nuclear κB (RANK), its ligand (RANKL), and OPG.[25] RANKL is a cell membrane bound ligand that is expressed by a range of cells such as osteoblasts, stromal cells, chondrocytes, and fibroblasts and also by activated T or B cells.[84,85] When RANKL binds to RANK receptor on the surface of preosteoclasts, it stimulates differentiation into mature osteoclasts which in turn cause bone resorption.[85] OPG, on the other hand, is a soluble receptor that is expressed by osteoblasts, endothelial cells, fibroblasts, and periodontal ligament cells.[84,86] OPG is known to inhibit bone resorption by binding to RANK, and thereby suppressing osteoclastic differentiation and blocking osteoclastogenesis.[87–89] In physiological conditions, RANKL is produced by mesenchymal cells[85] for the adaptation of the periodontium to excessive occlusal forces or forces applied during orthodontic treatment.[90–92] Within the healthy conditions, OPG is expressed by fibroblasts and endothelial cells of periodontium and together with RANKL they regulate the physiological bone remodeling process.[93] When there is an inflammatory response, the presence of inflammatory mediators such as IL-1β, IL-6, IL-11, IL-17, and TNF-α increases the expression of RANKL,[9] while causing a decrease in the expression of OPG by osteoblasts.[94] Therefore, RANKL/OPG ratio is important in determining whether bone resorption will occur or not. Decreased levels of OPG and increased levels of RANKL have been reported in the presence of periodontal inflammation.[95,96] Previously, it was shown that RANKL and OPG gene expressions differ from each other in gingival tissues of patients diagnosed with various forms of periodontal diseases with stronger RANKL expression in generalized aggressive periodontitis patients compared with those with chronic periodontitis.[97] Accordingly, higher GCF levels of OPG were reported in different forms of periodontitis, while periodontally healthy and gingivitis groups exhibited reduced GCF levels of RANKL.[97] The findings of increased RANKL/OPG ratio in the periodontitis groups in comparison with the periodontally healthy and gingivitis groups are in line with the findings of other studies.[84,98,99] Periodontal treatment has been shown to decrease the RANKL/OPG ratio, mainly by causing a decrease in RANKL while there was no significant change in OPG.[100] This discrepancy might be explained by either normalization of the RANKL levels by the periodontal treatment or binding of OPG to the excess RANKL which results in undetectable levels of OPG.[100,101] There are conflicting results on the effects of periodontal therapy on the GCF OPG levels.[84,102,103] In a study evaluating the effects of open flap debridement on the GCF, saliva, and gingival tissue levels of OPG, in chronic periodontitis patients, it was found that OPG levels were elevated at 3- and 6-month reevaluations compared with the baseline.[84] Furthermore, Bostanci et al.[102] reported that OPG levels in GCF samples increased 4 months after completion of the non-surgical periodontal therapy. However, the difference was not statistically significant and RANKL/OPG ratio remained unchanged. Another study[103] reported no change in GCF levels of RANKL, while OPG levels were decreased 4 weeks after completion of the non-surgical periodontal therapy. RANKL/OPG ratio may be considered

as a valuable predictor of sites at risk of further disease progression, whereas it does not seem to be a good indicator of clinically successful periodontal treatment.

Another biomarker of bone homeostasis is carboxyterminal-telopeptide pyridinoline cross-links of type I collagen (ICTP), which is a 12–20 kDa fragment of type I collagen. ICTP is released into periodontal tissues when type I collagen of bone is digested by bacterial collagenase or trypsin.[104] GCF ICTP levels have been shown to correlate with clinical periodontal parameters and to decrease accordingly following periodontal therapy, suggesting that it may be utilized as a biomarker to distinguish diseased sites.[9,104–106]

Osteocalcin (OC) is a calcium-binding protein of bone, synthesized by osteoblasts, odontoblasts, and chondrocytes.[107] Increased OC levels in GCF samples have been found in periodontal diseases.[108] Becerik et al.[109] found lower GCF levels of OC in chronic and generalized aggressive periodontitis patients and concluded that fluctuating osteocalcin and N-telopeptide of type I collagen (NTx) levels might indicate abnormal bone turnover in periodontitis. There is conflicting data regarding the GCF levels of OC.[110–113] In a randomized controlled clinical study,[114] GCF levels of OC following different modalities of non-surgical periodontal therapy in chronic periodontitis were evaluated. No significant differences were found in GCF OC levels at 6 months follow-up versus control, regardless of the periodontal treatment modality. The authors concluded that OC levels may be related to bone formation during physiological turnover of tissues rather than being associated with periodontal disease status.[114]

5 | PROTEOMICS ANALYSIS OF GCF SAMPLES

Proteomics is a general term used to define the studies investigating proteins present in cells, tissues, biofluids, organisms, or proteins that are expressed by a genome.[115] Measurement and analysis of the immune-inflammatory components of GCF is a valuable approach to better understand the pathogenesis of periodontal diseases. The studies analyzing single proteins as potential biomarkers of periodontal disease led to the identification of key inflammatory molecules. However, as the pathogenesis of periodontal disease consists of chorus of multiple proteins rather than single molecules, traditional methods may provide limited data on the complex interactions between the vast number of molecules that are present in GCF.[115,116] Therefore, proteomics analysis of GCF using techniques such as polyacrylamide gel electrophoresis (PAGE), high-pressure liquid chromatography (HPLC), mass spectrometry (MS), matrix-assisted laser desorption ionization (MALDI), and surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS) may facilitate further clarification of the complex protein–protein interactions in healthy state and in different periodontal diseases.[117–119]

Choi et al.[120] investigated the potential protein biomarkers in GCF of periodontitis patients using LC–MS/MS and reported overexpression of proteins hemoglobin alpha 2 (HBA2) and hemoglobin delta (HBD) by more than 2-fold in periodontitis patients compared with healthy patients. Moreover, the authors suggested azurocidin as a promising biomarker for early diagnosis of periodontitis due to its higher GCF levels in gingivitis and moderate periodontitis, compared with severe periodontitis cases.[120] As previously addressed, Leppilahti et al.[55] investigated the diagnostic accuracy of a range of GCF biomarkers in chronic periodontitis, gingivitis, and healthy sites. Azurocidin levels of GCF were significantly higher in chronic periodontitis group with 90% diagnostic accuracy. The sensitivity and specificity were calculated as 87% and 84%, respectively.[55] In another study,[121] GCF azurocidin levels in chronic periodontitis patients and periodontally healthy subjects were analyzed by ELISA and chronic periodontitis patients exhibited 3.6 times higher levels compared with healthy individuals. Azurocidin protein is derived from neutrophils; therefore, it should be kept in mind that azurocidin levels in clinically healthy sites may be related with subclinical inflammation.[121]

Furthermore, a total number of 462 proteins were identified in another study[122] comparing the GCF proteome between periodontitis patients and healthy individuals. Of these investigated proteins, 232 were found both in the healthy and diseased states, whereas 123 proteins were found to be unique to periodontitis. Neutrophil defensin 1, carbonic anhydrase 1, and elongation factor-1 gamma were present in chronic periodontitis more frequently, and HBD was found to be overexpressed in the periodontitis patients.[122]

Ngo et al.[123] explored the proteome of GCF, and 66 proteins were identified from diseased sites with 43 novel proteins that were previously not known to be present in GCF. Among these proteins were actin and actin-binding proteins, such as gelsolin, profilin, cofilin, and histones.[123] Qualitative proteomic analysis of GCF is important to reveal the existence or non-existence of particular proteins but quantitative analysis is also crucial, since specific proteins may reveal significant variation in concentration depending on disease severity.[115]

In an earlier study[124] comparing GCF protein profiles of patients with periodontitis patients and healthy individuals, a total of 154 proteins of human, viral, bacterial, and fungal origin were identified and quantified. The most abundant protein was reported to be human albumin, which was followed by immunoglobulins and various keratins. The protein L-plastin (LCP1) was identified as exclusively present in periodontitis patients.[124] Quantitative analysis revealed higher expression of GCF L-plastin levels in both chronic and aggressive periodontitis patients compared with periodontally healthy individuals.[125] Silva-Boghossian et al.[126] quantitatively analyzed the GCF proteome composition in periodontally healthy and periodontitis groups to identify exclusive proteins and more abundant proteins related to different clinical conditions. The authors found apolipoprotein E (APOE) exclusively in periodontitis patients and in greater abundance in periodontitis sites undergoing destruction.[126]

In a very recent study by Torres et al.,[127] proteomic analysis was performed in periodontitis patients with progressive and non-progressive sites in an effort to explore qualitative and quantitative

differences in GCF protein profiles. High-throughput proteomic approaches were used for global protein identification, and their relative abundances were determined by label-free analysis. A total of 1504 and 1500 proteins were identified in non-progressive and progressive sites, respectively. The number of proteins that were identified exclusively in progressive sites was 48, and 52 in non-progressive sites. Protein profiles representing catabolic processes, immune response, and in response to cellular stress were abundant in progressive sites, while non-progressive sites showed a protein profile that was associated with wound repair, healing, and regulation of cell death. The researchers also performed western blot analysis for the proteins CA1, CA2, MMP-8, and IL-6 in the GCF samples of both groups to confirm their proteomic findings: Significantly higher expression of these proteins was found in the progressive sites compared with the non-progressive sites.[127] The protein CA1 has been identified as a potential biomarker of periodontitis in other studies with similar methodology.[128,129] Proteomic technology for the identification and quantification of large number of proteins in GCF obtained from sites with different disease severity may help to further clarify the complex molecular interactions between various proteins acting in the pathogenesis of periodontitis and to detect disease progression with a higher reliability. Proteomics may thus identify novel biomarkers that can be more readily detected by lateral flow or other less cumbersome techniques than mass spectrometry assay.

6 | STRENGTHS AND LIMITATIONS OF GCF ANALYSIS FOR PERIODONTAL DIAGNOSIS

GCF is both a physiological and often pathological fluid defined as a serum transudate or an inflammatory exudate and is particularly useful as the samples can be easily obtained non-invasively, with minimal discomfort, and comprise of molecules that are both locally synthesized and systemically derived.[115] Yet, GCF-based tests are not a routine component of today's clinical dental practice. There are limiting factors even for the research such as the rather low volume of GCF that can be sampled, the difficulty in eliminating contamination of blood and saliva, the rather low quantities of various proteins that may remain below the detection limit for many assays, the high standards that are required for not only obtaining but also storing the GCF samples until the laboratory analysis, the technological and financial needs for laboratory analysis, etc.

Biological databases focusing on the microbiome, metabolome, or proteome are enlarging with the technological developments in the field. Measurement of any potential biomarker may be affected by dynamic changes in the context of the disease state. One of the most challenging issues in the search for a reliable biomarker is to understand which measurable changes are transient, physiological, and which persistent at all times. These molecular changes need to be related to clinical changes which are again microcosmic and difficult to temporally relate accurately. The feasibility of biomarkers for periodontal diagnostics is closely dependent on the progress in manufacturing reliable and rapid assays that are preferentially applicable at point-of-care and the subsequent thorough testing of their utility.

This literature search indicates that there is a paucity of standardized studies investigating possible biomarkers in GCF samples and not many robust conclusions can be drawn with the available reports. The published studies differ in clinical definition of periodontitis and/or healthy periodontium, the methodology of GCF sampling, storage conditions of samples, laboratory techniques used, statistical handling, and data interpretation. Such variations make it difficult to combine data from different studies and build up a consensus. Moreover, there are conflicts between data from different study groups in terms of increased, decreased, or indifferent levels of specific inflammatory markers in GCF samples. The findings of the biomarker studies focusing on GCF as the biofluid are quite encouraging; however, clinical implication of the findings has been much slower than expected. This can be explained by lack of a standardized process of development and validation of GCF biomarkers in large-scale and longitudinal studies.

Moreover, the natural course of progression of periodontitis with periods of exacerbations and remissions can be considered as a limiting factor for biomarker search.

7 | CONCLUSION

Looking at the current literature, it can be concluded that there is a vast number of studies using GCF as a biofluid for biomarker search and various analytes so far have been related to the presence and extent of periodontitis. Personalized periodontal diagnostics to-date include screening of saliva and GCF, and these tools can either use enzyme-based methods such as substrates for *Porphyromonas gingivalis* proteases[129] or antibody-based tools. The self-test assay of active MMP-8 has been tested in various populations by different research centers.[63,66,67] The available data from studies using this active MMP-8 test suggest that it can predict undiagnosed periodontitis, provides higher sensitivity than presence or absence of bleeding on probing, has a good specificity on disease severity, and possibly may supplement the current classification of periodontal and peri-implant diseases. However, in the recent study by Deng et al.[6] aMMP-8 POCT differentiated periodontitis from health and gingivitis but added minimal value to discrimination of periodontitis from gingivitis. Moreover, one major limitation of the available POCTs is the use of conventional clinical diagnostic parameters for validation of test outcomes. Another limitation is the requirement for the artificial dichotomization of the diagnostic question: health and gingivitis versus periodontitis, or health versus gingivitis and periodontitis.[6] Moreover, despite several reports suggesting decreased or increased levels of specific inflammatory and tissue degradation markers in GCF, meta-analysis is unlikely for most of them due to the variations in methodology. Another difficulty for

performing meta-analysis is the lack of the specificity and sensitivity reports in many of the papers. This fact was recently pointed out by Alamri et al.[24] in their systematic review, as they could perform meta-analysis only for total IgG levels in serum among many other biochemical parameters investigated in various biofluids.

Reliable biomarkers in monitoring periodontal diseases should have direct and indirect influence on clinical practice and if not they are far from being useful. During follow-ups after completion of successful periodontal therapy, lack of a change in the biomarker should confirm the healthy state and such information will help the clinician to positively encourage the patient to preserve and persevere with on-going behavioral and dietary habits. In cases where there are clearly early signs of deterioration in the healthy biomarker levels or increase in the disease related biomarkers, the clinician may be alerted to reinforce oral hygiene and behavioral and/or dietary changes and to increase the frequency of recalls. Late or advanced signs or findings may indicate a need for referral of the patient to a secondary health care for remedial action such as further treatment, retreatment, or adjunctive antimicrobial treatment. Reliable biomarkers for periodontal diagnostics need to be optimized by studies combining biochemical and clinical periodontal data. Moreover, validation of the candidate biomarkers in large populations is required. Ideally, reliable biomarkers should be combined with user-friendly software tools.

Never-the-less the promising biomarkers in GCF can be listed as PGE_2, aspartate aminotransferase (AST), IL-1b, IL-8, IL-10, neutrophil elastase (NE), osteocalcin (OC) and calprotectin, alkaline phosphatase (ALP), macroglobulins, MMP-8, and MMP-9.[14] Currently, MMP-8 is considered as one of the most robust biomarkers that can be used for the anticipation, diagnosis, prognosis of treatment, and classification of periodontal diseases. There is a basis to think that in the near future combined GCF analyte data can provide additional information to define the clinical case in a clearer manner and to make an individually tailored treatment plan.

8 | IMPLICATIONS FOR FUTURE RESEARCH

Despite the development of novel therapeutic interventions for periodontal diseases, the prevalence of severe periodontitis leading to tooth loss continues to be around 10%. Considering that populations are aging globally, there is an increasing demand for predictive, preventive, personalized, and participatory approach for the management of periodontal diseases.

Larger scale and follow-up studies with standardized methodology are warranted to better clarify the potential role of GCF biomarkers in periodontal diagnostics. Moreover, balancing the study populations according to gender, age, environmental factors such as usage of tobacco products, systemic diseases such as diabetes are required for obtaining reliable and clinically meaningful findings. Finally, artificial intelligence tools may be utilized in future to assist both the search for periodontal diagnostic biomarkers and also be incorporated in the diagnostic process such that we enhance the speed and utility of the diagnostic process generally.

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CONFLICT OF INTEREST STATEMENT

The authors state that they have no conflict of interest related to this manuscript.

DATA AVAILABILITY STATEMENT

No new data is associated with this review.

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