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Artículo de Revisión

Salivary Collection for Periodontitis Biomarkers: A Review of Pre-analytical Variables and Recommendations for Standardization

Recolección de saliva para biomarcadores de periodontitis: una revisión de variables preanalíticas y recomendaciones para la estandarización

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ABSTRACT

Background: Periodontitis is a prevalent inflammatory disease affecting tooth-supporting structures. While clinical examination remains the diagnostic gold standard, saliva is an appealing medium for detecting biomarkers that may aid in screening and monitoring. However, differences in collection, handling, and storage can markedly influence biomarker stability and detection.

Objective: To summarize current evidence on how salivary collection and pre-analytical factors affect biomarker integrity in periodontitis.

Methods: A narrative review was conducted of English-language studies published between January 2020 and July 2025, identified through PubMed, Scopus, and Web of Science. Studies were included if they measured salivary biomarkers relevant to periodontitis and reported collection or handling protocols. Data on collection methods, sample source, timing, participant preparation, and storage were synthesized qualitatively.

Results: Unstimulated whole saliva collected by passive drooling or spitting was the most common method due to its simplicity and ability to preserve biomarker integrity. Stimulated saliva altered protein profiles but offered more stable circadian patterns. Cotton-based absorbent devices reduced analyte recovery, while gland-specific sampling was rarely used. Biomarker levels varied with sampling time and participant preparation. Proper handling—immediate centrifugation, aliquoting, and ultra-low temperature storage—was critical, as warmer storage conditions and repeated freeze–thaw cycles accelerated biomarker degradation.

Conclusion: Methodological heterogeneity in salivary biomarker studies limits reproducibility. Standardizing unstimulated collection protocols, controlling pre-collection variables, and adopting consistent handling and storage practices are essential to advance the clinical utility of salivary biomarkers in periodontitis.

RESUMEN

Introducción: La periodontitis es una enfermedad inflamatoria que afecta las estructuras de soporte dental. Si bien el examen clínico es la regla de oro para su diagnóstico, la saliva es un medio de interés para detectar biomarcadores que pueden ayudar en su detección y monitoreo.

Objetivo: Resumir la evidencia actual sobre cómo la recolección de saliva y los factores preanalíticos afectan la integridad de los biomarcadores en la periodontitis.

Métodos: Revisión narrativa de artículos en inglés publicados entre enero de 2020 y julio de 2025, en PubMed, Scopus y la Web of Science. Estos fueron incluidos si medían biomarcadores salivales relevantes para la periodontitis y reportaban protocolos de recolección o manejo. Los datos sobre métodos de recolección, el origen de la muestra, su temporalidad, la preparación del participante y su almacenamiento fueron cualitativamente sintetizados.

Resultados: La saliva entera no estimulada fue el método más común debido a su simplicidad y capacidad para preservar los biomarcadores. La saliva estimulada alteró los perfiles proteicos, pero ofreció patrones circadianos más estables. Los dispositivos absorbentes a base de algodón redujeron la recogida de analitos, mientras que el muestreo específico de glándulas se utilizó poco. Los niveles de biomarcadores variaron con el momento de toma de la muestra y la preparación del participante.

Conclusiones: La estandarización de protocolos de recolección no estimulada, el control de variables previas a la recolección y la adopción de prácticas consistentes de manejo y almacenamiento son esenciales para avanzar en la utilidad clínica de biomarcadores salivales en la periodontitis.

INTRODUCTION

As one of the most frequent oral health problems globally, periodontitis affects the supporting tissues of teeth (gingiva, periodontal ligaments, bone, and cementum) and has serious consequences for oral and systemic health.[1] Diagnosis, staging and grading are currently based on a comprehensive clinical periodontal examination that includes measurements of probing pocket depth, bleeding on probing, clinical attachment level and radiographic assessment following the guidelines of the 2017 World Workshop.[2] These clinical measurements remain the gold standard for diagnosing periodontal abnormalities, although early detection can be challenging. Accurate clinical evaluation—supported by radiographs when needed—allows practitioners to identify disease stage and grade and plan appropriate therapy.[3]

Saliva has emerged as a highly attractive biological matrix for periodontitis biomarker research.[4] Unlike blood collection that requires invasive procedures and specialized healthcare settings, saliva can be collected non-invasively, repeatedly, and in various clinical and research environments.[5,6] Saliva contains about 20–30% of the proteins found in blood, as well as more than 2,000 different proteins that offer a wealth of potential biomarkers. This composition reflects both systemic health status and local oral conditions.[7] However, salivary composition varies with age, sex, diet and medication use, and the absence of standardized collection and processing protocols undermines reproducibility and comparability across studies.[8]

Novel analytical technologies have detected and quantified salivary biomarkers associated with periodontal disease, such as interleukin-1β (IL-1β), matrix metalloproteinases (MMPs), chemokines, and growth factors.[9,10] Studies have shown that in patients with periodontitis, levels of biomarkers such as MMP-8 and IL-1β are higher than those in healthy individuals.[11,12] Despite these associations, current evidence emphasizes that salivary biomarkers are adjunctive tools and that further research and validation are needed before they can be used as standalone diagnostic tests.[13,14]

The critical barrier to the widespread clinical adoption of salivary biomarkers lies in the significant variability observed across different collection protocols, storage conditions, and analytical approaches employed by research laboratories worldwide.[15] Unstimulated versus stimulated saliva, morning versus evening collection, fasting status and handling procedures all influence biomarker stability and detectability. Addressing these pre-analytical variables through standardized protocols is essential if salivary biomarkers are to fulfill their promise for periodontitis screening and monitoring.[8,16]

This narrative review aims to synthesize current evidence on salivary collection and processing methods in periodontitis research, focusing on how unstimulated versus stimulated saliva, whole saliva versus gland-specific secretions, and key pre-analytical variables (such as timing, fasting status, and patient-related

factors) influence biomarker measurements. It will also summarize of the impact of storage conditions, preservation media, and freeze–thaw cycles on salivary biomarker stability.

METHODS

This article is a narrative review. The decision to adopt a narrative approach was made because the literature on salivary collection methods for periodontal biomarkers is highly varied in design, aims, and reporting, which precludes formal meta-analysis.[17] The review focuses on summarizing existing evidence and identifying methodological patterns and gaps.

The guiding question was: How do collection, pre-collection, and processing protocols in salivary biomarker studies of periodontitis influence biomarker stability, detectability and clinical applicability? Following recommendations for narrative reviews,[17] the scope was defined to include studies that (1) involved human participants with periodontitis or healthy controls; (2) measured salivary biomarkers relevant to periodontal inflammation (e.g., IL-1β, MMP-8, S100 proteins, cytokines, chemokines); and (3) explicitly described at least one aspect of saliva collection, handling or storage.

The search was limited to articles published in English between 2020 and July 2025 to focus on current practices.

Although narrative reviews do not require exhaustive searches, transparency remains essential. Accordingly, PubMed/MEDLINE, Scopus, and Web of Science were searched for studies published from January 2020 through July 2025. The search utilized broad keywords and Medical Subject Headings (MeSH) related to saliva, periodontitis, and biomarkers (e.g., "saliva," "salivary biomarkers," "periodontitis," "periodontal disease," "collection method"). The search strings were adapted for each database and refined iteratively as new relevant terms emerged. Additionally, the reference lists of key papers were screened to identify seminal or illustrative studies, a process recommended for narrative reviews.[17]

RESULTS

After removing duplicates, titles and abstracts were screened for relevance to periodontitis and salivary biomarkers. Studies were included if they reported experimental or observational data on saliva collection or pre-analytical variables (e.g., stimulation, sample source, timing, storage). Case reports, commentaries and letters without original data were excluded. Because narrative reviews are iterative, the inclusion criteria were refined as the review progressed; for example, if several papers used identical protocols without additional methodological insights, representative examples rather than all duplicates were selected.

For each included study, the authors noted the publication year, country, study design, participant periodontal status, saliva type (unstimulated vs. stimulated), collection method (passive drooling, spitting, Salivette®, chewing gum, etc.), sample source (whole saliva vs. gland-specific), timing and pre-collection conditions (time of day, fasting, abstention from oral hygiene), storage conditions (temperature, preservation media, freeze–thaw cycles), and the biomarkers measured. They then organized the extracted information into four thematic categories: (1) collection method; (2) sample source; (3) timing and pre-analytical variables; and (4) storage and handling. Within each theme the authors summarized trends, contrasted practices, and highlighted methodological variability.

DISCUSSION

Collection methods: unstimulated vs. stimulated

Most periodontitis biomarker studies collect unstimulated whole saliva via passive drooling or spitting. A recent meta-analysis of matrix metalloproteinase-8 (MMP-8) reported that 68 % of included studies used unstimulated saliva.[11] Passive drooling is considered the "gold standard" for unstimulated collection because it preserves the natural composition of saliva.[7] In this method, participants allow saliva to pool in the mouth and expectorate into a tube over 5-15 minutes, yielding 2-3mL without artificially stimulating salivary glands.

Several studies have compared unstimulated and stimulated saliva. Stimulation achieved by chewing paraffin or applying citric acid increases flow rate but alters protein composition. A pilot study measuring 71 cytokines, chemokines and growth factors over a 12-h period found that concentrations of some mediators (e.g., IFN-γ, IL-7) decreased after stimulation, while others (e.g., IL-8, CCL5) increased.[7] Stimulated saliva exhibited more stable diurnal patterns, whereas unstimulated saliva showed significant circadian variability.[7] Proteomic analyses of pregnant women likewise reported that unstimulated samples contained more proteins involved in immune response and inflammation than stimulated samples, suggesting that stimulation may dilute or remove

biomolecules of interest.[18]

Sampling devices can also affect biomarker detection. Cotton-based swabs such as the Salivette® are convenient but problematic for certain analytes: when salivary amyloid-β peptides were collected with a Salivette®, Aβ42 and Aβ40 were not detected because the peptides bound to the cotton.[19] In contrast, the same biomarkers were detectable in unstimulated saliva collected by passive drooling.[19] These examples illustrate that seemingly small methodological choices, such as stimulating saliva or using absorbent devices, can markedly influence biomarker concentrations and even lead to false-negative results.

Whole Saliva vs. Glandular Secretions

Periodontal biomarker research overwhelmingly relies on whole saliva, a mixture of secretions from all major and minor salivary glands along with gingival crevicular fluid.[20] Whole saliva is easy to collect and represents the entire oral environment. Proteomic studies have cataloged 1,939 proteins in whole saliva, of which 740 overlap with glandular saliva proteomes and 597 overlap with plasma.[21] This rich composition makes whole saliva attractive for detecting systemic and local biomarkers.

Some investigators advocate gland-specific sampling to increase anatomical specificity (e.g., isolating parotid or submandibular secretions). However, glandular collection requires specialized devices, careful positioning, and trained personnel, and yields smaller volumes.[22] None of the recent periodontitis studies identified in our search directly compared glandular and whole saliva for biomarker detection, reflecting a gap in the literature. Current evidence therefore supports whole saliva as the pragmatic choice for periodontal biomarker research, while acknowledging that gland-specific studies may provide additional insights into tissue-specific pathology.[22,23]

Timing & Physiological Factors

The time of saliva collection is an important variable that may affect the concentrations of analytes and the reproducibility of the study; however, it is not well standardized in periodontal studies.[7] Circadian rhythm modulation of salivary content has been identified as a significant source of variation in biomarker research, and it has been shown recently that the time of sampling has a major influence on the detection and interpretation of inflammatory markers in saliva.[24,25]

Over the course of the day (7:30 AM to 7:30 PM), an analysis of 71 inflammatory markers sampled at 3-hour intervals observed that these biomarkers fall into three clusters, each with its own circadian rhythm.[7] This result illustrates that variation in the timing of sample collection can have substantial effects on biomarker levels but may also be of sufficient magnitude to impact study conclusions if not well controlled. Morning collection has become the most

common time of day for recovering human salivary biomarkers, with the period between 7:30 and 11:00 AM yielding the most stable and repeatable measures.[24]

Fasting status is another essential physiological factor that greatly affects saliva composition and the concentration of biomarkers.[26] Presently, it is justified to advise fasting periods of 2-3 hours prior to saliva sampling to reduce the impact of food intake on biomarker concentrations.[26] Physical activity and oral health behaviors are further physiological parameters that should be standardized in saliva collection, with guidelines advocating refraining from oral hygiene for at least 30 minutes before collection and avoiding strenuous exercises for 1 hour before sampling.[27]

Storage and Handling

Salivary biomarker stability is highly sensitive to storage conditions. Most studies centrifuge saliva to remove cells and debris, and then freeze aliquots at -70°C or -80°C. A scoping review of saliva collection methods found that freezing at -70°C to -80°C was the most common practice and recommended immediate analysis where possible.[25] A separate review on oral microbiome samples concluded that storing specimens at -80°C for up to 1-2 years best preserves composition and that multiple freeze-thaw cycles should be avoided.[6]

An experimental study comparing storage at -20°C and -80°C showed that both temperatures maintain total antioxidant capacity and total oxidant status of saliva for up to 60 days, but degradation begins after 90 days.[28] These data support the use of ultra-low-temperature freezers for long-term storage and indicate that -20°C may be acceptable for short-term storage if samples are analyzed within two months. Only a minority of studies reported adding protease inhibitors or preservatives to saliva; whether such additives improve stability remains unclear.[27]

The effect of freeze-thaw cycles is an important but neglected component of salivary biomarker storage procedures.[19] Multiple freeze-thaw cycles can lead to protein denaturation, thus causing a reduction in target protein activity. Current guidelines state that no more than 2 freeze-thaw cycles should occur, with aliquoting at the time of initial processing to avoid repeated thawing.[9]

Impact on Biomarker Detection and Reproducibility

Across the literature, methodological heterogeneity in collection, timing and storage translates into variability in biomarker levels and hampers cross-study comparisons. For instance, the MMP-8 meta-analysis cited above found a high degree of heterogeneity among studies (standardized mean difference 2.71 [95% CI: 1.04-4.38]),[11] and this variability was largely attributed to differences in collection protocols rather than biological factors. Experimental evidence

shows that using absorbent swabs or storing samples at suboptimal temperatures can result in complete loss of specific biomarkers.[19]

Conversely, adopting consistent protocols—unstimulated whole saliva collection by passive drooling, morning sampling after fasting, immediate centrifugation and storage at -80°C—improves reproducibility and allows for more meaningful comparisons across studies. The narrative synthesis also highlights the absence of multicenter validation studies and the paucity of research on pediatric or special-population samples. Future investigations should prioritize standardized methodologies and report detailed metadata on pre-analytical variables to advance the clinical translation of salivary biomarkers.

This narrative review summarized recent evidence on pre-analytical factors affecting salivary biomarkers in periodontitis research. Four key domains—collection method, sample source, timing and pre-collection conditions, and storage/handling—were identified as major sources of variability.

Collection Method. The preference for unstimulated saliva, typically collected via passive drooling, is well founded: most MMP-8 studies use this method,[11] and comparative analyses show that unstimulated samples better reflect baseline salivary composition.[7] Stimulated saliva increases flow rate but alters protein expression; concentrations of some cytokines decrease while others increase after stimulation.[7] Moreover, stimulated saliva exhibits more stable circadian patterns than unstimulated saliva,[7] which may be advantageous for longitudinal sampling, though it risks diluting analytes. Device selection also matters: absorbent swabs such as Salivette® can bind amyloid-β peptides, leading to false-negative results.[19] These findings underscore the need for clear reporting and careful choice of collection technique.

Sample Source. Whole saliva remains the primary matrix for periodontal biomarker studies. It is easy to collect and contains a rich proteome—1,939 proteins, including hundreds shared with gland-specific secretions and plasma.[21] Although gland-specific samples could offer greater anatomical precision, they are rarely used due to technical challenges and limited volumes. No recent periodontitis studies directly compared glandular and whole saliva for biomarker detection, indicating an evidence gap that warrants future research.

Timing and Pre-Collection Variables. Salivary biomarkers exhibit circadian patterns: unstimulated saliva shows significant diurnal fluctuations, whereas stimulated saliva remains relatively stable.[7] Morning collection (7:30—11:00 AM) is generally favored, especially for cortisol and related hormones.[25] Most studies require participants to fast for at least two hours and avoid smoking, toothbrushing and mouthwash immediately before sampling. The influence of physical activity is less well documented, but it is common practice to instruct participants to refrain from strenuous exercise for at least one hour before collection. These pre-collection controls are essential to minimize variability.

Storage and Handling. Biomarker integrity is highly sensitive to post-collection handling. The majority of studies centrifuge saliva and store aliquots at -70°C or -80°C, which preserves many analytes for months.[25] Storing samples at -20°C maintains antioxidant capacity for up to 60 days, but degradation begins thereafter.[28] Reviews recommend immediate freezing at -80°C and limiting storage to one to two years to minimize microbiome and protein changes.[6] Repeated freeze-thaw cycles degrade proteins and should be avoided.[6] Few studies report adding preservatives or protease inhibitors, leaving uncertainty about their efficacy.

Collectively, these findings highlight substantial methodological heterogeneity across periodontal biomarker studies. Differences in collection technique, sample source, timing and storage can produce widely divergent results, complicating comparisons and meta-analyses. In turn, this heterogeneity may contribute to the broad confidence intervals observed in MMP-8 meta-analyses.[11] Without standardized protocols, it will be difficult to translate salivary biomarkers into clinically useful diagnostic tools. Our synthesis supports the view that salivary biomarkers are promising adjuncts to clinical periodontal assessment but remain insufficient as standalone diagnostics. Rigorous protocol harmonization and multicenter validation studies are needed to realize their clinical potential.

Recommendations for Future Research and Practice

Based on the evidence summarized, several recommendations can improve the reliability and comparability of salivary biomarker studies in periodontitis:

Use unstimulated whole saliva collected by passive drooling as the primary sampling method to preserve biomarker concentrations and avoid device-related losses.[7,19] Reserves stimulated sampling for specific research questions, and report the stimulation protocol in detail.

Avoid cotton-based swabs (e.g., Salivette®) for analytes prone to adsorption, such as amyloid-β peptides.[19] When swabs are used, validate recovery for each biomarker of interest.

Standardize pre-collection conditions by collecting samples in the morning after a fasting period of at least two hours, instructing participants to abstain from smoking, oral hygiene and vigorous exercise shortly before sampling.[25] Document these conditions in detail.

Process samples promptly, centrifuging to remove cells and aliquoting the supernatant to minimize freeze-thaw cycles. Store saliva at -80°C or colder for long-term preservation and at -20°C only for short-term storage (<60 days).[6,28]

Collect and report metadata on collection time, fasting duration, medication use, dental status and storage history to facilitate cross-study comparisons.

Investigate understudied areas, including gland-specific saliva, pediatric populations and the effects of physical activity and diet on biomarker levels. Multi-center studies using harmonized protocols will be critical to validate biomarkers across diverse populations.

Implementing these recommendations will improve reproducibility, enable meaningful meta-analyses and accelerate the clinical translation of salivary biomarkers.

Limitations of this Narrative Review

As a narrative synthesis, this review does not aim to exhaustively capture all relevant literature. The search was limited to English-language articles published from 2020 through July 2025; earlier studies or non-English publications were not systematically explored. The authors prioritized studies that described collection and handling protocols, which may have excluded biomarker studies that did not report pre-analytical details. They did not perform a formal risk-of-bias assessment; instead, the included studies were discussed qualitatively. These limitations mean that these conclusions should be interpreted as reflective of current trends rather than definitive estimates of effect.

CONCLUSION

Saliva remains a practical, noninvasive matrix for adjunctive assessment of periodontal inflammation, but its reliability is highly protocol-dependent. This review indicates that unstimulated whole saliva collected by passive drooling or gentle spitting, in the morning between approximately 7:30 and 11:00 a.m., after at least 2 hours of fasting and abstaining from toothbrushing, mouthrinses, smoking, and vigorous exercise, provides the most consistent conditions for periodontal biomarker analysis. Cotton—based devices and other absorbent swabs should be avoided for protein—and peptide-based biomarkers prone to adsorption, and stimulated saliva should only be used when justified by the research question and reported in detail. Samples should be centrifuged promptly, aliquoted to prevent repeated freeze-thaw cycles, and stored at -80°C (with -20°C reserved for short-term storage up to 60 days).

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