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CLINICAL RESEARCH:

Analysis of Exosomal Biomarkers in Oral Fluids of Periodontal Patients

Análisis de biomarcadores exosomales en fluidos orales de pacientes periodontales

Mohammed Rathan Sharfudeen[1] https://orcid.org/0009-0003-4607-7935
Shivakumar Baskaran[2] https://orcid.org/0009-0001-4743-3378
Swarna Alamelu[3] https://orcid.org/0009-0001-8526-6945
Karthick Baskar[4] https://orcid.org/0009-0002-4097-8807

[1]Post Graduate, Department of Periodontics, Ragas Dental College and Hospital, Chennai- 600119, Tamil Nadu, India.
[2]Professor and Head, Department of Periodontics, Ragas Dental College and Hospital, Chennai- 600119, Tamil Nadu, India.
[3]Professor, Department of Periodontics, Ragas Dental College and Hospital, Chennai- 600119, Tamil Nadu, India
[4]Senior Lecturer, Department of Periodontics, Ragas Dental College and Hospital, Chennai- 600119, Tamil Nadu, India

Correspondence to: Shivakumar Baskaran - shivakumar.dental@gmail.com

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ABSTRACT: Exosomes, small extracellular vesicles enriched with markers such as CD63, play essential roles in cell communication and inflammatory regulation. In periodontal disease, salivary and gingival crevicular fluid (GCF) exosomes may act as carriers of inflammatory mediators like interleukin-1 beta (IL-1β), a key factor in periodontal tissue destruction. Evaluating these exosomal biomarkers may support non-invasive assessment of periodontal disease activity. A total of 20 subjects were enrolled and categorized into two groups: 10 periodontally healthy individuals and 10 with chronic periodontitis. Saliva and GCF samples were collected and processed for extracellular vesicle (EV) isolation using ultracentrifugation. The size distribution of exosomes was assessed using Dynamic Light Scattering (DLS). Western blotting was performed to detect the exosomal marker CD63, and IL-1β expression was quantified via RT-PCR. Correlation analyses between exosome sizes in saliva and GCF were conducted. Salivary exosome size was significantly larger in the periodontitis group (419.21±181.95 nm) than in the healthy controls (285.24±76.95 nm; $p<0.001$). GCF exosome size showed no significant difference between the groups. Salivary IL-1β expression, measured by $2^{(-\Delta\Delta Ct)}$, was significantly higher in the periodontitis group (5.22±2.42) compared to healthy controls (1.11±0.53; $p=0.017$). A strong positive correlation ($r=0.967$, $p<0.001$) was found between salivary and GCF exosome sizes in the periodontitis group. Western blot confirmed CD63-positive exosomes in all samples, with stronger bands observed in the disease group. Exosomalprofiling of saliva, particularly IL-1β expression and CD63 detection, presents a promising, non-invasive approach for diagnosing and monitoring periodontitis. These findings highlight the potential of salivary exosomes as surrogate indicators of periodontal inflammation and emphasize the need for further research to improve diagnostic accuracy. Results should be interpreted cautiously due to the pilot nature and small sample size.

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KEYWORDS: Periodontitis; Exosomes; Biomarkers; Interleukin-1beta; Saliva; Gingival crevicular fluid.

RESUMEN: Los exosomas, pequeñas vesículas extracelulares enriquecidas con marcadores como CD63, desempeñan funciones esenciales en la comunicación celular y la regulación de la inflamación. En la enfermedad periodontal, los exosomas presentes en saliva y en el fluido crevicular gingival (GCF) pueden actuar como transportadores de mediadores inflamatorios, como la interleucina-1 beta (IL-1β). Se reclutaron 20 participantes y se clasificaron en dos grupos: 10 individuos periodontalmente sanos y 10 con periodontitis crónica. Se recolectaron muestras de saliva y GCF, las cuales se procesaron para el aislamiento de vesículas extracelulares (EV) mediante ultracentrifugación. La distribución del tamaño de los exosomas se evaluó mediante dispersión dinámica de luz (DLS). Se realizó Western blot para detectar el marcador exosomal CD63, y la expresión de IL-1β se cuantificó mediante RT-PCR. El tamaño de los exosomas salivales fue significativamente mayor en el grupo con periodontitis (419.21±181.95 nm) en comparación con los controles sanos (285.24±76.95 nm; $p<0.001$). El tamaño de los exosomas en GCF no mostró diferencias significativas entre los grupos. La expresión salival de IL-1β, medida mediante $2^{(-\Delta\Delta Ct)}$, fue significativamente mayor en el grupo con periodontitis (5.22±2.42) en comparación con los controles (1.11±0.53; $p = 0.017$). Se observó una fuerte correlación positiva ($r=0.967$, $p<0.001$) entre los tamaños de los exosomas salivales y de GCF en el grupo con periodontitis. El Western blot confirmó exosomas positivos para CD63 en todas las muestras, con bandas de mayor intensidad en el grupo con enfermedad. Estos hallazgos resaltan el potencial de los exosomas salivales como indicadores sustitutos de inflamación periodontal y subrayan la necesidad de realizar investigaciones adicionales para mejorar la precisión diagnóstica. Los resultados deben interpretarse con cautela debido al carácter piloto tamaño reducido de la muestra.

PALABRAS CLAVE: Periodontitis; Exosomas; Biomarcadores; Interleucina-1 beta; Saliva; Fluido crevicular gingival.

INTRODUCTION

Periodontal diseases comprise a range of inflammatory conditions that primarily result from bacterial presence and their metabolic byproducts. The immune system's reaction to these microbial agents involves the release of various local mediators from inflammatory cells (1). Current understanding suggests that periodontitis should not be viewed solely as a typical bacterial infection, but rather as an immune-driven inflammatory disorder initiated by the host's response to a complex microbial biofilm (2). This immune-inflammatory activity includes the secretion of proinflammatory cytokines, prostanoids, and matrix-degrading enzymes, which collectively contribute to the breakdown of the connective tissues supporting the teeth.

Exosomes are vesicles that are secreted from most cell types into the extracellular environment and range in size in nanometers (3). They are produced in the endosomal compartment, where multivesicular bodies merge with the plasma membrane and excrete them (4). They are present in all body fluids, such as blood, saliva, urine, and here in the gingival crevicular fluid (GCF) (5).

Exosomes are critical cell-to-cell communicating molecules that pass proteins, lipids, messenger RNAs (mRNAs), and microRNAs (miRNAs) between

cells. Exosomes have important functions in many biological processes and disease pathologies such as immune modulation, angiogenesis, and tissue repair (6). The cellular material of exosomes is very similar to the cell type and health status of the secreting cells. They are also selectively enriched with typical proteins such as tetraspanins (CD9, CD63, CD81), heat shock proteins (HSP70, HSP90), and membrane-trafficking proteins such as Alix and TSG101 (7).

CD63 is a member of the tetraspanin family of transmembrane proteins and is widely used as a canonical marker for exosomes. It is primarily localized in late endosomes and lysosomes but becomes enriched on the surface of exosomes during their formation. CD63 participates in various cellular processes, including vesicle trafficking, membrane fusion, and immune regulation (8).

Logozzi *et al*. (9) demonstrated that CD63 expression is directly proportional to the concentration of exosomes in biological fluids. CD63, along with CD9 and CD81, is typically used in combination with antibodies or microbeads to isolate and quantify exosomes using methods such as ultracentrifugation, size-exclusion chromatography, or immunoaffinity capture. Because of its unique and specific, CD63 used as a good indicator of exosomal content in diagnostic procedures.

In inflammatory diseases, exosomes regulate immune function by modulating the behavior of immune cells. They can transport pro-inflammatory or anti-inflammatory agents, thus either intensifying or suppressing the inflammatory response (10). Inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are known to stimulate the formation of osteoclasts by upregulating the expression of RANKL and triggering downregulating pathways like NF-κB and MAPK (11). These molecular events collectively contribute to the degradation of alveolar bone, resulting in periodontal disease.

Interleukin-1 beta (IL-1β), an important pro-inflammatory cytokine plays a role in inflammatory and immune processes of the innate immune system. It facilitates the release and activation of multiple inflammatory molecules. IL-1β increases the expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and triggers the production of the chemokine CXCL8 (IL-8), both of which assist in chemotaxis. In addition, IL-1β collaborates with other cytokines and prostaglandin E2 (PGE2) promoting the breakdown of bone (12).

Despite significant advances in understanding periodontal disease, early detection and accurate assessment of active disease remain challenging. Conventional diagnostic methods such as probing, attachment level measurements, and radiographs primarily reflect past tissue destruction and provide limited insight into ongoing inflammation or future disease progression. To address these limitations, recent research has focused on identifying non-invasive biomarkers for early detection and monitoring (13).

Exosomes are small, stable extracellular vesicles present in saliva and gingival crevicular fluid (GCF) have emerged as promising diagnostic tools due to their ability to carry molecular cargo reflective of cellular health or disease (14). This study aimed to evaluate the diagnostic potential of exosomal biomarkers, particularly CD63 and interleukin-1 beta (IL-1β), in saliva and GCF from individuals with chronic periodontitis and those with healthy gingiva. By assessing exosome size and inflammatory marker expression, the study sought to identify reliable, non-invasive indicators of periodontal disease activity.

MATERIALS AND METHODS

Study population enrolled in the study were individually seeking dental treatment who reported to the Department of Periodontics, Ragas Dental College and Hospital in Chennai. Ethical clearance was obtained from the Institutional Review Board (ref no: EC/NEW/INST/2023/4006) of Ragas Dental College and Hospital, Chennai, prior to initiating the study. Following a detailed clinical examination, subjects were categorized into two groups based on the 2017 Classification of Periodontal Diseases and Conditions: one group with clinically healthy gingiva and the other with chronic periodontitis. A total of 20 participants were included, comprising 10 with clinically healthy gingiva and 10 diagnosed with chronic periodontitis.

GROUP A (PERIODONTAL HEALTH GROUP)

Group A consisted of 10 subjects who presented with clinically non-inflamed, healthy gingiva, characterized by bleeding on probing in less than 10% of sites, probing depths of ≤ 3 mm, and absence of clinical attachment loss.

GROUP B (PERIODONTAL DISEASE GROUP)

Group B consisted of 10 subjects who had probing depth ≥6 mm, clinical attachment loss ≥3 mm with bleeding on probing in >20% of sites examined.

SELECTION CRITERIA

Inclusion Criteria:

- Individuals diagnosed with periodontitis based on the current classification of periodontal diseases.
- Systemically healthy patients.
- Age- and gender-matched participants.

Exclusion Criteria:

- Individuals with a history of periodontal treatment or antibiotic usage within the past six months.
- Patients with any systemic conditions known to influence periodontal health.
- Pregnancy or Lactating woman.
- Smokers.

GINGIVAL CREVICULAR FLUID COLLECTION

GCF samples were collected using microcapillary pipettes with lines from 1- 5μl. Supragingival plaque was removed using curettes without contacting the gingival margin, and the gingival sulcus/pocket was then dried gently with an air syringe. Then micropipette was placed at the entrance of sulci/pocket for 30 seconds. Micropipette contaminated by saliva and blood were discarded. For elution of GCF, the collected pipettes were placed into a 1.5-ml tube containing 200 μl of phosphate- buffered saline (PBS). Samples were then stored at −20ºC until further analysis.

SALIVA COLLECTION

For saliva samples, subjects were asked to refrain from eating & drinking 1 hour before saliva collection. Before saliva collection started, subjects asked to rinse their mouth with clear water for 30s, then, a 50-ml specimen container tube was used to collect for 1 min the passive drooled saliva from the patients and stored at -20ºC until further analysis.

EXOSOME ISOLATION (ULTRACENTRIFUGATION AND ULTRAFILTRATION)

Samples were processed by sequential centrifugation at 300 × g (5 min), 1200 × g (10 min), and

10,000 × g (30 min) at 4°C to remove debris. Supernatants were concentrated using a 100 kDa molecular weight cut-off ultrafiltration unit (Amicon Ultra, Millipore, USA) and subjected to ultracentrifugation at 150,000 × g for 3 h. The pellet was washed with DPBS, recentrifuged, and resuspended in 100 μl DPBS for further analysis (15).

EXOSOME CHARACTERIZATION

Dynamic Light Scattering (DLS): Exosome size distribution and polydispersity were analyzed using a Zetasizer Nano ZS (Malvern Instruments, UK) under standard nanoparticle analysis settings (14).

Western Blot (CD63 Detection): Exosomal protein CD63 was detected using a rabbit monoclonal anti-CD63 antibody (ABclonal, Cat. No. A19023, Woburn, MA, USA) following the manufacturer's protocol (8).

Real-Time PCR (IL-1β Expression): DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. IL-1β expression was quantified using SYBR Green real-time PCR (Rotor-Gene Q, Qiagen), with β-actin as the reference gene. Expression levels were calculated using the $2^{(-\Delta\Delta Ct)}$ method (16,17).

STATISTICAL ANALYSIS

Data were analyzed using SPSS v21.0 (IBM, USA). Exosome size differences were assessed using independent sample t-tests. Correlations between GCF and salivary exosomes were analyzed using Pearson's correlation. IL-1β expression differences were compared using independent sample t-tests. A p-value <0.05 was considered statistically significant.

RESULTS

COMPARISON OF SALIVARY EXOSOME SIZE (DLS) BETWEEN CONTROL AND TEST GROUPS

Analysis revealed that the test group demonstrated a significantly larger average salivary exosome size (419.21±181.95 nm) compared to the control group (285.24±76.95 nm), with the statistically significant difference (P<0.001). These results showed that individuals with periodontitis have larger exosomes in saliva of healthy subjects (Table 1), (Figure 1).

Table 1. Comparison of Salivary and GCF exosomes isolation (Dynamic Light Scattering) between Control group and Test group among the samples.

Parameter	Measurement	Test group	Control group	P-value
Saliva	Mean ± SD	419.21± 181.95 nm	285.24 ± 76.95 nm	0.001*
GCF	Mean ± SD	359.81± 150.97 nm	396.64 ± 203.89 nm	0.652

*Significance level P<0.05.

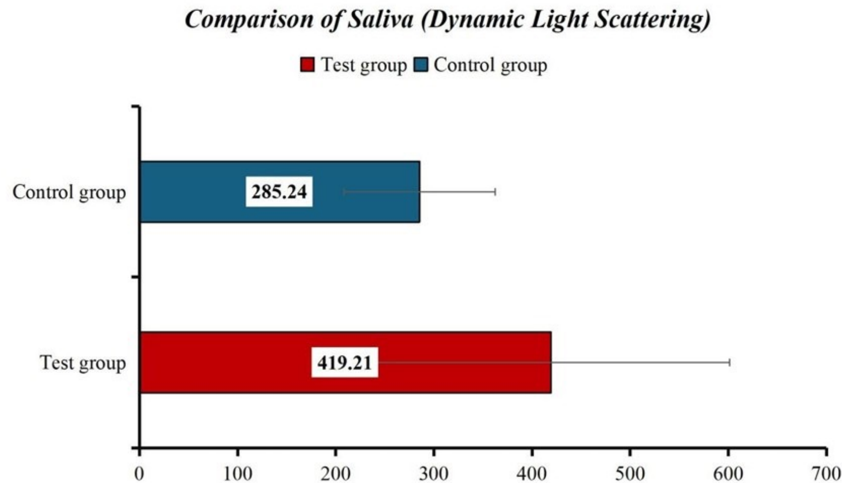

Figure 1. Comparison of Saliva (Dynamic Light Scattering) between Control group and Test group among the study participants.

COMPARISON OF GCF EXOSOME SIZE (DLS) BETWEEN CONTROL AND TEST GROUPS

In contrast, the test group showed a slightly smaller mean exosome size in GCF (359.81±150.97 nm) compared to the control group (396.64±203.89 nm); however, the difference was not statistically significant (P=0.652). These results indicate that exosome size in GCF is relatively similar between periodontitis patients and healthy controls (Table 1), (Figure 2).

CORRELATION BETWEEN SALIVARY AND GCF EXOSOME SIZES IN HEALTH AND DISEASE

Among healthy individuals, a moderate positive correlation was identified between exosome sizes in saliva and GCF (r=0.546, p=0.102), though not statistically significant. In contrast, patients with periodontitis showed a very strong and statistically significant positive correlation (r=0.967, P<0.001). This suggests a coordinated increase in exosome size across both fluids in the presence of periodontal disease (Table 2), (Figure 3).

Comparison of IL-1β Expression Between Control and Test Groups The relative expression of salivary interleukin-1β (IL-1β) was evaluated using quantitative real-time PCR, with β-actin as the endogenous control. Fold change values calculated by the $2^{(-\Delta\Delta Ct)}$ method were significantly higher in the periodontitis group (5.22±2.42) compared to the healthy group (1.11±0.53), indicating substantial upregulation of IL-1β in diseased subjects (p=0.017). These findings highlight the diagnostic potential of IL-1β expression in saliva as a non-invasive biomarker for periodontal disease (Table 3).

EXOSOMES IDENTIFICATION USING WESTERN BLOT (CD63)

Both healthy and periodontitis salivary samples (Lanes 1-4 and 6-10) shows visible bands around 100 kDa, confirming the presence of exosomes via CD63 detection (Figure 4).

The CD63 protein band at ~100 kDa supports successful exosome isolation. Stronger bands in patient samples may reflect increased exosomal release due to periodontal inflammation. Thus, the Western blot confirms that exosomes are present in both healthy and periodontitis salivary samples.

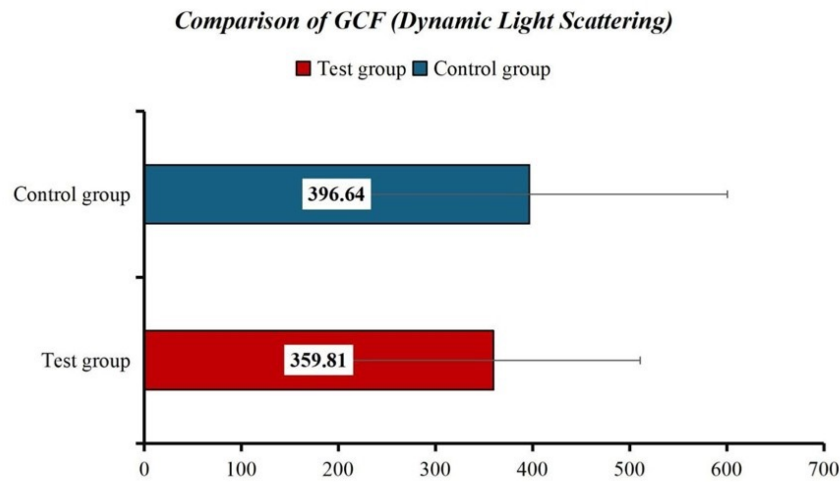

Figure 2. Comparison of GCF (Dynamic Light Scattering) between Control group and Test group among the study participants.

Table 2. Correlation between exosomes of GCF and saliva in healthy and periodontitis groups.

Variable	Healthy group GCF and salivary exosomes	Periodontitis group GCF and salivary exosomes
r	0.546	0.967
P-value	0.102	0.001*

*Significance level P<0.05.

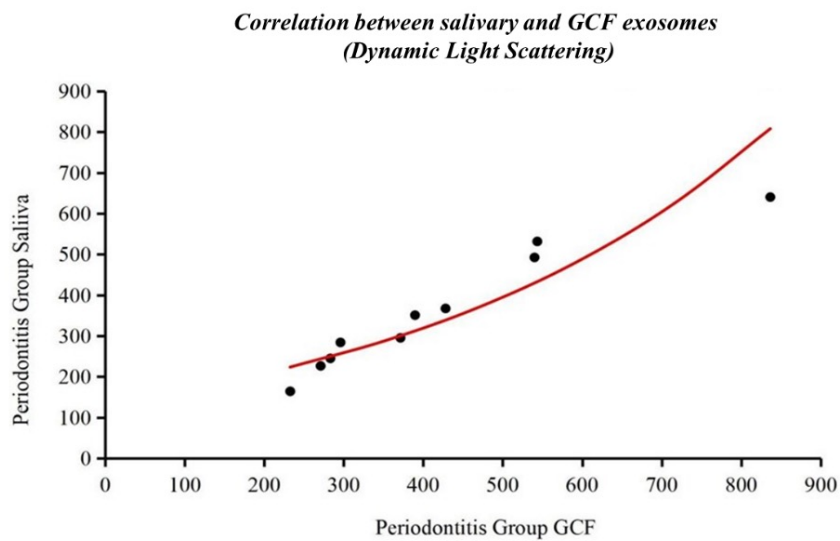

Figure 3. Correlation between salivary and GCF exosomes (Dynamic Light Scattering) in periodontitis groups.

Table 3. Comparison of IL-1 beta expression between Control group and Test group among the samples.

Parameter	Measurement	Control group	Test group	P-value
2^(-ΔΔCt) IL-1β	Mean ± SD	1.11 ± 0.53	5.22 ± 2.42	0.017*

*Significance level P<0.05.

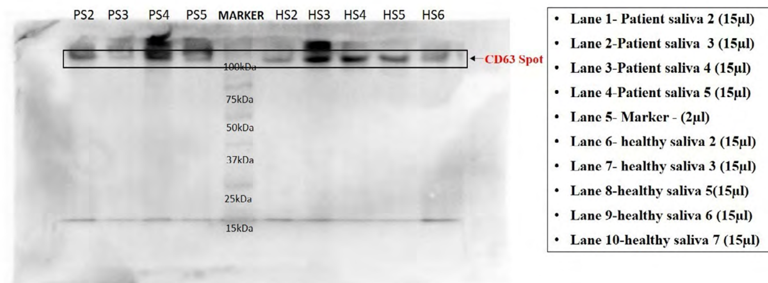

Figure 4. Exosomes identification using CD63 Western Blot analysis.

DISCUSSION

Periodontal disease is now well established as a chronic, immune inflammatory condition triggered by a dysbiotic microbial biofilm rather than a classical infection alone. While bacterial biofilms initiate the breakdown of periodontal tissues, it is the host's exaggerated inflammatory response marked by a surge in pro inflammatory cytokines that largely determines the extent of tissue destruction and disease progression (2,18). Among the cytokines implicated, interleukin 1 beta (IL 1β) plays a central role by promoting osteoclast differentiation, enhancing matrix metalloproteinase expression, and driving neutrophil infiltration (19).

Recent attention has turned to exosomes, nano sized extracellular vesicles released by nearly all cell types and can be found in a wide range of body fluids, including blood, urine, saliva, cerebrospinal fluid, and gingival crevicular fluid (GCF) (14). Their formation is a highly regulated process governed by the endosomal sorting complex required for transport (ESCRT), which involves crucial proteins like Alix, TSG101, and the tetraspanins CD9, CD63, and CD81.These surface markers are commonly used to identify and isolate exosomes from complex biological mixtures (5).

CD63 is a tetraspanin commonly enriched on the surface of exosomes and is widely used as a hallmark marker for exosomal identification. The observed band around 100 kDa likely represents glycosylated forms or aggregates of CD63, which are frequently reported in Western blot analyses of exosomes (20).

Because their molecular cargo cytokines, lipids, microRNAs and proteins mirrors the physiological or pathological state of their parent cells, exosomes are increasingly recognised as both biomarkers and active mediators of periodontal inflammation (4). In periodontitis, exosomes enriched with IL 1β and IL 6 have been detected in oral fluids, implicating them in disease propagation (21).

This study investigated the variation in exosome size within saliva and gingival crevicular fluid (GCF) between periodontally healthy individuals and those with periodontitis, utilizing dynamic light scattering (DLS) for analysis, along with the expression levels of interleukin-1 beta (IL-1β) in saliva. By also analysing the correlation between salivary and GCF exosome profiles, we assessed whether saliva could serve as a reliable surrogate for local periodontal changes.

Our findings revealed a significantly larger mean size of salivary exosomes in the periodontitis group (419.21 ± 181.95 nm) compared to the healthy control group (285.24 ± 76.95 nm, P<0.001).

Similarly, Han *et al*. (22) noted that the salivary EV concentration for the size range of 50-150 and 150-200 nm was slightly increased in the periodontitis patients compared to that of the healthy controls; however, the difference was not statistically significant.

In contrast studies by Chaparro *et al*. (24) reported Exosome's size distribution in saliva samples was not different between both groups.

This suggests that periodontal inflammation may alter the characteristics of salivary exosomes, possibly due to increased secretion from inflamed periodontal tissues or altered biogenesis under pathological conditions. Exosomes are play an important role in intercellular communication, and changes in their size or content can reflect the host response to inflammatory processes (21).

In this study, the mean size of exosomes in GCF of periodontitis group is smaller than control group but it did not differ significantly (P=0.652).

Similar studies by Chaparro et al (23) reported a smaller exosomes size in GCF samples of patients with periodontitis compared with non-periodontitis subjects.

This could imply that the inflammatory microenvironment influences GCF exosomes differently than saliva. Some studies suggest GCF exosomes may be more consistent in size, while their cargo (e.g., cytokines, miRNAs) varies with disease severity (4).

Interestingly, a very strong positive correlation was observed between salivary and GCF exosome sizes in the periodontitis group (r=0.967, P<0.001), indicating a synchronized response of exosomal release across these two fluids during active inflammation. This was not evident in the healthy group (r=0.546, P=0.102), suggesting that coordinated exosomal communication may be upregulated under inflammatory stress (24).

And in this study using western blot, the presence of distinct protein bands around ~100 kDa in both healthy (Lanes 1-4) and periodontitis (Lanes 6-10) salivary samples confirms successful isolation of exosomes, as indicated by the detection of the exosomal marker CD63. Notably, the bands in the periodontitis samples appear more intense compared to the healthy controls, which could reflect an increased release of exosomes in response to chronic inflammation associated with periodontal disease. Inflammatory conditions are known to enhance the biogenesis and secretion of exosomes, potentially as a mechanism for intercellular communication and immune modulation. This aligns with previous studies suggesting that salivary exosome concentration and cargo composition are altered in periodontal disease states (22). Therefore, the Western blot analysis not

only confirms the successful isolation of salivary exosomes but also hints at their potential role in the pathophysiology of periodontal inflammation.

Moreover, IL-1β expression was significantly elevated in the periodontitis group compared to controls, corroborating its well-established role in periodontal disease pathogenesis.

This outcome aligns with earlier research by Al-Musawi *et al*. (25) on salivary IL-1β level revealed a progressive increase from healthy to periodontitis groups and theoretically showed the potential to differentiate between healthy and unhealthy periodontium. The result is consistent with many studies. Also, Tan *et al*. (26) reported periodontitis showed a statistically significant difference with the healthy control group.

Liukkonen *et al*. (27) reported Patients with generalized periodontitis exhibited significantly elevated salivary IL-1β levels compared to those with localized periodontitis, while no notable difference was observed between individuals with localized periodontitis and healthy controls. And they confirmed the IL-1β is a useful biomarker of more advanced periodontitis.

Conversely, Teles *et al*. (28) who found no statistically significant change between a healthy periodontium and periodontal disease groups

Taken together, the salivary exosomes and elevated IL-1β levels in periodontitis patients underscore the diagnostic potential of these biomarkers. The strong correlation between saliva and GCF exosomes in diseased individuals also supports the notion that saliva may serve as a reliable, non-invasive medium for periodontal diagnostics. Additional research analyzing the molecular contents of these exosomes may offer a better understanding of their involvement in the progression of periodontal disease and the host's immune response. Future investigations should adhere more closely to the MISEV 2018 guidelines for extracellular vesicle characterization to enhance methodological rigor and reproducibility.

LIMITATIONS

The present study has certain limitations. The relatively small sample size may affect the statistical power and generalizability of the results. While exosomal CD63 was confirmed through Western blot analysis, a more comprehensive characterization using multiple exosomal markers or electron microscopy was not performed. Additionally, potential pre-analytical and assay-related variability could have influenced measurement outcomes. Future studies with larger cohorts and adherence to MISEV 2018 recommendations are warranted to validate and expand upon these preliminary findings.

CONCLUSION

This study highlights the potential of salivary and GCF-derived exosomes, particularly CD63 and IL-1β, as non-invasive biomarkers for chronic periodontitis. While the findings are promising, they should be interpreted within the context of the study's limitations and pilot-scale design. Periodontitis patients showed significantly larger salivary exosomes and elevated IL-1β levels, reflecting active inflammation. Although GCF exosome size did not differ significantly, a strong correlation with salivary exosomes suggests a coordinated inflammatory response. CD63 detection via Western blot confirmed successful exosome isolation, and elevated IL-1β levels affirmed its diagnostic relevance. These findings support salivary exosomal profiling as a valuable exploratory tool for early detection

and monitoring of periodontal disease, warranting further research with larger and more standardized cohorts.

AUTHOR CONTRIBUTION STATEMENT: Conceptualization and design: M.R.S. and S.A.; Literature review: M.R.S., S.B. and S.A.; Methodology and validation: M.R.S., K.B. and S.A.; Investigation and data collection: M.R.S., S.B. and S.A.; Formal analysis and data interpretation: M.R.S., K.B. and S.B.; Writing-original draft preparation: K.B.; Writing-review and editing: M.R.S., S.B. and K.B.; Supervision and project administration: M.R.S.

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