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Molecular signatures of extracellular vesicles in oral fluids of periodontitis patients

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Abstract

Objective: To characterize extracellular vesicles (EVs) in gingival crevicular fluid (GCF) and saliva samples from healthy/gingivitis and periodontitis patients and correlate them with clinical inflammatory periodontal parameters.

Material and Method: An exploratory study, including 86 subjects, was conducted. Clinical and periodontal data were recorded, and oral fluid samples were obtained. EVs were precipitated by ExoQuick-TC™ and characterized by nanoparticle tracking (NanoSight™), Western blot (WB), transmission electron microscopy (TEM), and ELISA analysis.

Results: TEM showed nanoparticles morphologically compatible with EVs, and WB analysis revealed bands of specific EV markers (CD9, TSG101, and Alix) in both oral fluids of periodontitis and healthy/gingivitis subjects. The total concentration of EVs in GCF was increased in periodontitis patients compared to healthy/gingivitis subjects ($p = .017$). However, we did not observe differences in the EV concentration of saliva samples ($p = .190$). The size of GCF-EVs was 144.2 nm in periodontitis and 160.35 nm in healthy/gingivitis patients ($p = .038$). The CD63 exosome marker was increased in GCF of periodontitis patients ($p = .00001$). The total concentration of EVs in GCF was correlated with bleeding on probing (rho = 0.63, $p = .002$), periodontal probing depth (rho = 0.56, $p = .009$), and clinical attachment level (rho = 0.48, $p = .030$).

Conclusion: Periodontitis patients have an increased concentration of EVs in GCF, and their role in periodontitis should be clarified.

KEYWORDS

exosomes, extracellular vesicles, micro-vesicles, periodontitis

1 | INTRODUCTION

Extracellular vesicles (EVs) are the smallest nanoparticles involved in cell communication and molecule transport between different cell types (Théry et al., 2009). Initially, they were considered as cellular metabolism waste. However, EVs are now considered biologically active elements, which reflect the state of cells, and serve as messenger vehicles that allow intercellular communication, and

contain cell-specific proteins, mRNA, and microRNAs, among other molecules that can contribute to intercellular interaction at distance from the originated cell (Théry et al., 2009; Valadi et al., 2007; Lee et al., 2012; Yuana et al., 2013). There are different types of EVs, differentiating themselves according to their biogenesis process and release mechanism, biophysical properties, size, and surface markers (Théry et al., 2009, György et al., 2011; Buzas et al., 2014). Even though there is still no consensus regarding the best way to classify EVs, the last position statement of the International Society for Extracellular Vesicles suggested to define EV as a generic term to refer particles naturally released from the cell that is delimited by a lipid bilayer and cannot replicate, that is, do not contain a functional nucleus. EV subtypes can be derived from endosome origin (exosomes) and plasma membrane-derived origin "ectosomes" (micro-vesicles). Physical characteristics such as size, "small EVs" (sEVs), with ranges defined < 200nm or > 200nm for medium or large EVs (Théry et al., 2018). EVs also contain components that take part in membrane transport and fusion processes (i.e., Alix and TSG101), lipid-related proteins, phospholipases, heat shock proteins (HSP70, HSP90), integrins, and tetraspanins (CD63, CD9, CD81) (Yuana et al., 2013; Boriachek et al., 2017: Théry et al., 2018).

Surprisingly, EVs can modify and activate target cells in a paracrine or endocrine pathways, mediating communication and immune responses between remote cells, and also, they have been recognized and involved in the pathogenesis of several chronic inflammatories, and autoimmune and infectious diseases (Buzas et al., 2014; Cecil, O'Brien-Simpson, & Lenzo, 2017; Yuana et al., 2013). Therefore, EVs have been emerging as potential diagnostic and therapeutic vehicles in several diseases and can be found in large amounts in different corporal body fluids including plasma, urine, saliva, and breastmilk among others (Théry et al., 2009; György et al., 2011; Zlotogorski-Hurvitz et al., 2015 a, b). For these reasons, EVs have been suggested as a liquid biopsy that could contribute to the diagnosis and prognosis of different inflammatory diseases. Recent studies showed that the total concentration of EVs is increased in patients with diseases such as atherosclerosis, diabetes mellitus, and metabolic syndrome, and other autoimmune diseases (Agouni et al., 2008; Revenfeld et al., 2014; Jeyaram & Jay, 2017).

The standard periodontal diagnosis is based on accumulated periodontal destruction instead of current or future disease. Because of this, the clinically periodontal diagnosis should be complemented with the use of oral biomarkers related to the early inflammation and periodontal tissue destruction. In this sense, oral fluids, such as saliva and gingival crevicular fluid (GCF), show a huge potential for diagnosis and monitoring of periodontal status (Giannobile et al., 2009; Giannobile, McDevitt, Niedbala, & Malamud, 2011; Kinney et al., 2014; Nomura et al., 2012; Barros et al., 2016), and they have probably been poorly explored as a molecular clinical tool for periodontal monitoring. Due to their accessibility, both oral fluids represent an attractive source for EVs for diagnostic, monitoring, and prognostic purposes (Giannobile et al., 2009, 2011; Barros et al., 2016; Bostanci et al., 2017). Moreover, later studies suggest that EVs from saliva, isolated by non-invasive methods, are promising biomarkers in different diseases, including periodontal diseases (Ogawa et al., 2011; Zheng et al., 2014; Huang, Hu, Zhao, & Zhang, 2019; Tobón-Arroyave, Celis-Mejía, Córdoba-Hidalgo, & Isaza-Guzmán, 2019; Michael et al,. 2010). Also, new findings suggest that EVs derived from osteoclasts could be involved as regulators of osteoclastogenesis by the expression of receptor activator of nuclear factor κB (RANK) and imbalance of Th17 cells (Zhao et al., 2019; Zheng et al., 2019).

Considering that the field of personalized medicine is actively entering in clinical research and dental practice and that research in novel biological biomarkers could help to discover new insights into the molecular diagnosis of periodontitis, we hypothesize that the EVs in oral fluids (saliva and GCF) are related to periodontitis severity and clinical inflammatory periodontal parameters. Also, we sought to characterize small EV (exosomes) and medium/large EV (MVs) profiles in saliva and GCF of healthy/gingivitis subjects and periodontitis patients.

2 | MATERIAL AND METHODS

2.1 | Study design

An exploratory cross-sectional study was conducted in the Health Care Centre of the Universidad de Los Andes, Santiago, Chile. Enrollment, clinical, physical, and periodontal data were recorded of 86 subjects. Of them, 41 patients diagnosed with periodontitis (stages II, III, and IV) and 45 subjects with gingival health or gingivitis were included. All subjects were systemically healthy (both genders), aged between 30 and 60 years old. Complete full-mouth periodontal examinations were performed by a periodontist, including bleeding on probing (BOP), periodontal probing depth (PPD), clinical attachment loss (CAL), plaque index (PI), gingival index (GI), and periodontal inflamed surface area (PISA). Exclusion parameters for the study were as follows: chronic inflammatory diseases (diabetes, cardiovascular, chronic inflammatory or autoimmune diseases, infectious diseases), less than 18 teeth, history of previous periodontal treatment in the previous 3 months, and systemic or topical antimicrobial/anti-inflammatory therapy for the previous 3 months. After a periodontal examination, GCF and saliva samples were taken. All clinical data for the study were recorded. The control group was selected from the same dentistry health center and was matched for age, body mass index, gender, and tobacco use. All patients were instructed in oral hygiene education and then were derived for periodontal therapy. This study was reviewed and approved by the Universidad de Los Andes Scientific Ethics Committee. All patients participating in the study consent to participate by signing the appropriate informed consent form. The variables studied were as follows: total small and medium/large EV concentration, EV size distribution, periodontal clinical measures, and clinical diagnoses.

2.2 | Diagnostic criteria

Periodontitis was defined as stated by the 2017 World Workshop, as interdental CAL is detectable at ≥ 2 non-adjacent teeth, or buccal or oral CAL ≥ 3mm with pocketing > 3mm is detectable at ≥ 2 teeth. Gingivitis was defined as subjects, who did not exhibit PPD greater or equal to 3mm, without CAL and positive BOP in ≥ 10%. Gingival health was defined as < 10% bleeding on probing sites, with PPD ≤ 3mm (Caton et al., 2018; Chapple et al., 2018).

2.3 | Sample size calculation

This is a secondary pilot study from a principal cohort study, with an objective related to extracellular vesicles as an oral biomarker of type 2 diabetes mellitus (T2DM). Given that this is an exploratory study, we were not able to perform a previous sample calculation and the sample size was established arbitrarily based on the number of the available samples of subjects without T2DM. From them, we calculated the power of our sample, based on alpha = 0.05, which was 0.75.

2.4 | Gingival crevicular fluid and saliva: collection and elution

GCF samples were collected using Periopaper™ strips (Oraflow, Smithtown, NY, USA). All samples were collected from the mesiobuccal site of the sulcus/pocket. In periodontitis patients, 4 periodontal pockets (1 x quadrant) (PPD > 3mm and CAL ≥ 3mm) were selected. In the healthy/gingivitis group, 4 shallow periodontal sulcus sites (1 x quadrant) (PPD < 3mm, without CAL and positive BOP until 10%) were selected. Briefly, the supragingival plaque was removed using curettes without contacting the marginal gingiva, and the gingival sulcus/pocket was then dried gently with an air syringe. The strips were inserted 2-3mm into the sulci/pocket for 30 s. Strips contaminated by saliva and blood were discarded. Samples were then stored in 1.5-mL tubes at −80°C until elution. For elution of GCF, 4 strips were placed into a 1.5-ml tube containing 160 µl of phosphate-buffered saline (PBS) (Corning, Mediatech Inc, NY, USA) and protease inhibitor cocktail (EDTA cOmplete™, mini, EDTA-free Protease Inhibitor Cocktail, Roche, USA). Tubes were vortexes and incubated on ice for 30 min, and then centrifuged at 12,000 x g for 5 min at 4°C. The eluate was collected and placed on ice. The elution procedure was repeated, and both eluates were pooled and stored at −80°C until analysis (Leppilahti et al., 2014).

For saliva samples, subjects were asked to refrain from eating 2 h and drinking 30 min before saliva collection. Before saliva collection started, subjects rinse their mouth with clear water for 30 s, then, a 50-ml Falcon tube was used to collect for 1 min the passive drooled saliva from the patients. Immediately, after saliva collection, proteinase inhibitor cocktail (Sigma-Aldrich, v/v 1:20) was added. Further, the samples were centrifuged for 10 min at 2000 g and the supernatant was rescued and used for the EV isolation.

2.5 | EV isolation from GCF samples

EVs from GCF were isolated by precipitation with the commercial reagent ExoQuick™ (System Biosciences Inc., Mountain View, CA, USA) (EQ system) according to the manufacturer's recommendations. In brief, 300 µl of GCF eluate was mixed with 150 µl of the EQ system and incubated overnight at 4°C. The day after, the EQ-GCF complex was centrifuged at 1,500 g for 30 min at room temperature to obtain the EV precipitate that was subsequently suspended in 200 µl of PBS.

2.6 | EV isolation from saliva samples

EVs from saliva samples were isolated by precipitation with the reagent ExoQuick-CG™ (System Bioscience™ EXOCG50A-1, CA, USA) (EQ system), according to the manufacturer's instructions. Briefly, 5 ml of saliva supernatant was mixed with 1.6 ml of EQ system reagent and incubated overnight at 4°C. Later, the mix was centrifuged at 1,500 g x for 30 min at room temperature to obtain the EV pellet, and suspended in 100µl of PBS 1x filtered.

2.7 | Nanotracking particle analysis

The size distribution and concentration of EVs were analyzed by nanotracking particle analysis (NTA) using the NanoSight NS300 instrument (Malvern, Worcestershire, UK). Non-diluted EVs isolated from GCF and saliva samples were evaluated, and their size distribution (mode) and their concentration were determined. The samples were automatically introduced into the sample chamber and were recorded using a camera level of 10 (slide shutter of 10; slider gain of 73). NTA postacquisition settings were optimized and kept constant between samples. Each video was analyzed to give EV size distribution and concentration measurement.

2.8 | Transmission electron microscopy

The EVs isolated from GCF and saliva samples were assessed by transmission electron microscopy (TEM) in the TEM facility of the Faculty of Biological Sciences (Pontificia Universidad Católica de Chile, Santiago, Chile), Briefly, 5 µl of EV suspension was diluted 10 times in PBS and deposited on Formvar-carbon-coated electron microscopy grids and left to adsorb for 20 min. The grids were then stained with 5% uranyl acetate for 5 min and washed with distilled water. After drying for 5 min at 60°C, the grids were examined in the Phillips CM100 TEM at 80 kV.

TABLE 1 Description of periodontal clinical parameters of subjects included in the study

Variables	Gingival crevicular fluid samples			Saliva samples		
	Healthy/gingivitis subjects (*n* = 18)	Periodontitis patients (*n* = 16)	*p*-value	Healthy/gingivitis subjects (*n* = 27)	Periodontitis patients (*n* = 25)	*p*-value
BOP (%)	16.83 (2.08–20)	71.90 (30.86–100)	**< 0.00001**	33.33 (2.08–20)	73 (30.86–100)	**0.0002**
PI (%)	25.5 (5.35–59.52)	82.11 (44–100)	**< 0.00001**	45 (5.35–64)	78 (36–100)	**< 0.00001**
Mean of PPD (mm)	2.3 (1–2.5)	2.9 (2.5–4.7)	**< 0.00001**	2.3 (1.8–2.6)	3 (2.7–4.7)	**< 0.00001**
Mean of CAL (mm)	2.1 (1.2–2.8)	3.2 (1.8–5.9)	**< 0.00001**	1.9 (1.2 –2.8)	3 (1.7–5.9)	**< 0.00001**
PISA (mm^2)	189.99 (14.88–1355)	1,117.77 (437.57–2604.3)	**< 0.00001**	390.31 (14.88–1604.1)	1,085.25 (437.57–2286.13)	**< 0.00001**

Note: BOP: bleeding on probing; PI: plaque index; PPD: periodontal probing depth; CAL: clinical attachment level; PISA: periodontal inflamed surface area; mm: millimeter. % Percentage. ***(Bold)** significance p-value (< 0.05, *Mann–Whitney test*). Results are expressed in median values (P50) with minimum and maximum values.

2.9 | Western Blot

EV markers were identified by Western blot (WB) in GCF and saliva samples. Briefly, protein concentration was measured using BCA assay. Twenty micrograms of EV proteins was separated by 10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane for 1 hr at 100 V. The membranes were blocked with 5% skimmed milk and incubated overnight at 4°C with primary antibody anti-CD9 (1:1,000), anti-TSG101 (1:1,000), and anti-Alix (1:1,000). After overnight incubation, the membranes were washed and incubated with the appropriate secondary antibody for 2 hr, and the immunoreactivity was detected using horseradish peroxidase linked to anti-rabbit or anti-mouse and visualized using ECL detection system.

2.10 | Quantification of CD63 in EVs from GCF samples by ELISA

EVs were isolated from GCF by EQ, and total protein concentration was measured using Qubit Protein Assay Kit (Thermo Fisher Scientific). The presence of the tetraspanin exosome membrane marker, CD63, was assessed by ELISA, using the EXOEL-CD63A-1 kit (System Biosciences, Palo Alto, CA, USA). The protocol applied was according to the manufacturer's instructions.

2.11 | Statistical analysis

The normality of the data distribution was tested using the Shapiro–Wilk test. The concentration of total EVs was not normally distributed; therefore, the non-parametric test was used. Mann–Whitney U test was used for comparisons of continuous variables. Correlation coefficients were tested using Spearman's test. Dichotomy data were compared using Fisher's exact test. Data were analyzed using Stata v14 software (StataCorp, College Station, TX USA) and GraphPad software. A p-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Demographic and periodontal characteristics of the study population

A total of 86 subjects were recruited in the present pilot study, 53% of them corresponded to female and 47% were males. The mean age was 31.6 years (*SD* 10.73), mean weight of 67.7 kg (*SD* 11.23), the mean height of 1.65mt (*SD* 0.53), and body mass index (BMI) of 24.4 (*SD* 4.28). 34% of the subjects reported being smokers. Forty-five subjects were diagnosed as healthy or gingivitis clinical status, and 41 patients were diagnosed with periodontitis. Following the sample matching criteria, there were no significant differences in the variables of age, gender, BMI, and smoking habit between study groups. From the recruited population, we took saliva and GCF samples from 52 and 34 subjects, respectively. The periodontal clinical parameters of both groups are summarized in Table 1.

3.2 | Nanotracking particle analysis (NTA) and transmission electron microscopy (TEM)

Isolation of EVs from saliva and GCF samples was performed by ExoQuick™ system (EQ system); this is a chemical-based agent designed to precipitate EVs in samples with a small volume (Figure A). NTA was performed with NanoSight™ to estimate the size distribution and total concentration of EVs in both oral fluids. The size distribution (mode) of GCF-derived EVs was 144.2nm in periodontitis patients and 160.35nm in healthy/gingivitis patients (*p-value = 0.038)* (Table 2). However, the EV size distribution in saliva samples was not different between both groups (*p-value = 0.379*) (Table 2). In addition to these clinical results, the TEM analysis confirmed the NTA data, showing the clear presence of EVs surrounded by bi-layered lipid membrane compatible with the EV morphology in GCF and saliva samples (Figure B).

The total concentration of GCF-derived EVs was significantly increased in periodontitis patients in comparison with healthy/gingivitis subjects (*p-value = 0.017*) (Table 2) (Figure D). The total

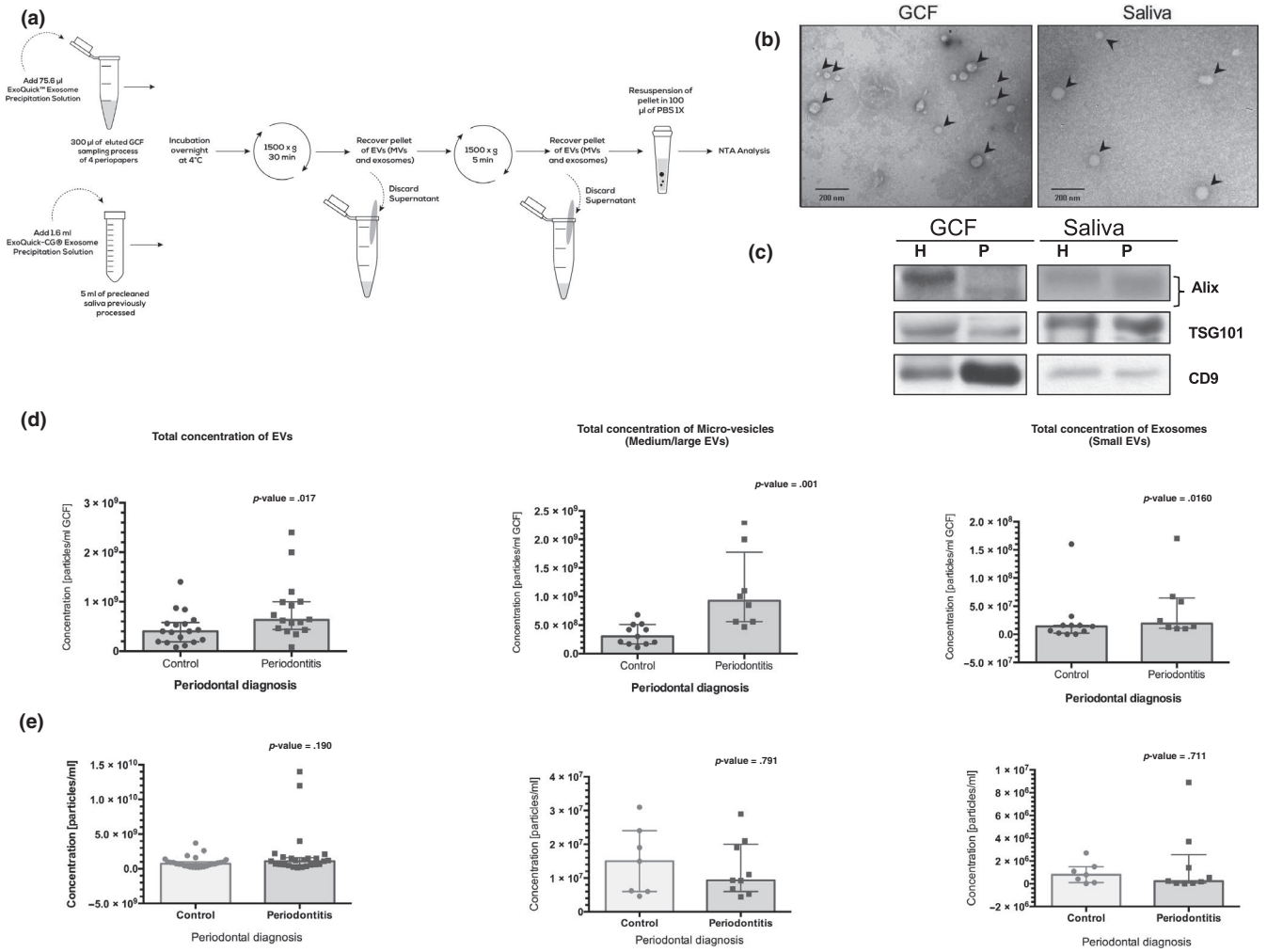

FIGURE 1 EV precipitation and isolation protocol with ExoQuick™ from GCF and saliva samples (a). Morphology of EVs in the pellet obtained with ExoQuick™ by TEM from GCF and saliva samples (b). EVs/exosome markers (Alix, TSG101, and CD9) detected by Western blot analysis in periodontitis (P) and healthy/gingivitis (H) from GCF and saliva samples (c). Total concentration of EVs, MVs, and exosomes in GCF and saliva samples according to periodontitis diagnosis (d and e)

concentration of medium/large EVs (micro-vesicles) was threefold higher in periodontitis patients compared to the healthy/gingivitis group (*p-value = 0.001*) (Figure D). Nevertheless, the concentration of small EVs (exosomes) was not different between periodontitis patients and healthy/gingivitis group (*p-value = 0.160*) (Figure D3). In the NTA of saliva samples, no significant differences were observed in relation to the total concentration of EVs, micro-vesicles, or exosomes (*p-value = 0.190*; *p-value = 0.791*; and *p-value = 0.711*, respectively) (Figure E).

Furthermore, independently of the periodontal diagnosis, the total concentration of GCF-derived EVs was positively correlated with periodontal clinical parameters as: BOP (rho = 0.63, *p-value = 0.002*), PPD (rho = 0.56, *p-value = 0.009*), CAL (rho = 0.48, *p-value = 0.030*), and PISA (mm^2) (rho = 0,64, *p-value = 0.002*). The Spearman correlation analysis by group showed that in periodontitis patients, BOP and CAL were correlated with the total concentration of GCF-derived EVs (rho = 0.55, *p-value = 0.027*; rho = 0.54, *p-value = 0.028*, respectively). In the saliva samples, we do not observe significant correlations between the periodontal clinical variables and the concentration of the different populations of EVs.

3.3 | Western blotting and ELISA analysis of EV markers

Evaluation of the positive markers by Western blotting shows the identification of EVs/exosomal markers including the tetraspanin CD9 and the cytosolic markers TSG101 and Alix in both oral fluids (Figure C). The intensity of the band was higher for the cytosolic marker TSG101 in saliva samples and the tetraspanin CD9 in GCF samples of periodontitis patients (P) compared to healthy/gingivitis (H) subjects. Next, to corroborate the differences observed in the total concentrations of EVs in GCF, we quantify the tetraspanin CD63 by ELISA. The total concentration of tetraspanin CD63 was 1.37 x 10^9 (1.10 x 10^9 – 1.70 x 10^9) in gingival health/gingivitis subjects compared to 4.77 x 10^9 (3.85 x 10^9 – 6.29 x 10^9) (*p-value = 0.00001*) in periodontitis patients.

TABLE 2 Description of nanoparticle tracking analysis of EVs isolated from GCF and saliva samples in healthy/gingivitis subjects and periodontitis patients

	GCF samples			Saliva samples		
Variables	**Healthy/gingivitis**	**Periodontitis**	***p*-value**	**Healthy/gingivitis**	**Periodontitis**	***p*-value**
Total concentration of EVs	4.01×10^8 (1.92×10^8– 5.56×10^8)	6.32×10^8 (4.47×10^8 – 1.00×10^9)	**0.017**	7.33×10^8 (3.88×10^8- 1.02×10^9)	1.14×10^9 (5.86×10^8- 1.54×10^9)	0.190
Total concentration of medium/large EVs	2.99×10^8 (1.72×10^8– 5.12×10^8)	9.26×10^8 (5.59×10^8 – 1.58×10^9)	**0.001**	1.45×10^7 (6,043,408 – 2.39×10^7)	9,299,960 (6,826,609 – 1.91×10^7)	0.791
Total concentration of small EVs	1.38×10^7 (2,143,113 – 1.63×10^7)	1.87×10^7 (1.17×10^7- 6.25×10^7)	0.160	799,409.7 (106,246 – 1,463,468)	230,831.6 (65,022.7 – 1,394,442)	0.711
Median size distribution of EVs (nm)	234.75 (201 – 255.1)	193.55 (181.9 – 218.55)	0.063	245.4 (218 – 277.9)	232.4 (217.8 – 246.1)	0.238
Mode of size distribution of EVs (nm)	160.35 (133.5 – 185.6)	144.2 (131.7 – 151.15)	**0.038**	173.6 (150.3 – 195.4)	167.3 (150.3 –175.6)	0.379

Note: GCF: gingival crevicular fluid; EVs: extracellular vesicles; MVs: micro-vesicles; nm: nanometer. ***(Bold)** significance p-value (< 0.05, *Mann–Whitney test*). Results are expressed in median (P50) with (P25-P75).

4 | DISCUSSION

EVs are heterogeneous populations of membrane-derived vesicles that play different and relevant functions in diverse physiologic and pathological cellular processes (Buzas et al., 2014; Thery et al., 2018). At present, there is a growing interest in using EVs found in body fluids as predictive and diagnostic biomarkers (Lee et al., 2012; Yuana et al., 2013; Cecil et al., 2017). These EVs are released from a diversity of cell types, including epithelial, endothelial, and immune, neurons, and platelets, and it seems to reflect the disease state of the releasing cells (Thery, Ostrowski, & Segura, 2009; Turchinovich, Weiz, & Langheinz, 2011). EVs are also responsible for delivering signals and transferring proteins and nucleic acids from a donor to a target cell locally, or at distance, and therefore, they could affect the function and phenotype of the target cells (Valadi et al., 2007; Reiner, Witwer, & Balkom, 2017).

In this exploratory study, we demonstrate the feasibility to isolate salivary and GCF-EVs by ExoQuick™ system, and also, we verify their presence by NTA, TEM, WB, and ELISA techniques to characterize the EVs (small and medium/large EVs). EVs isolated from saliva and GCF samples showed a round-shaped structure surrounded by a bi-layered membrane that corroborates the typical morphology of EVs characterized in other biologic fluids (Thery et al., 2009; Sharma et al., 2010; Zlotogorski-Hurvitz et al., 2015 a, b). Also, the EV size distribution from both oral fluids revealed the presence of EVs with the correct range of size as definition recognized (*< 200nm*) compatible with small EVs (Thery et al., 2018). Furthermore, our results suggest a smaller EV size in GCF samples of patients with periodontitis compared with non-periodontitis subjects.

The data obtained in the present study support the potential diagnostic utility of EV total concentration in GCF in identifying periodontitis patients. Indeed, the results showed that total EVs and medium/large EVs (MVs) in GCF were significantly higher in periodontitis patients. Specifically, periodontitis patients have more than threefold of medium/large EVs in comparison with gingival health/gingivitis subjects. Furthermore, the total concentration of these EVs was significantly correlated with periodontal clinical measures such as BOP, PI, PPD, CAL, and PISA.

In periodontal tissues, GCF-EVs could originate from the epithelial cells of the junction and/or sulcus epithelium, immune-inflammatory cells, bacteria's, circulating EVs, or a combination of all of them. The increased GCF-EV concentrations observed in periodontitis patients compared to healthy patients represent a very interesting finding. Given the immune-inflammatory nature of periodontitis and considering that GCF reflects the local and systemic inflammatory condition (Giannobile et al., 2009; Nomura et al., 2012; Kinney et al., 2014; Barros et al., 2016; Bostanci et al., 2017), these increases in the concentration of GCF-EVs probably are related to the periodontal inflammation. In this sense, it is interesting to mention that miRNAs are found inside EVs, and diverse miRNAs are implicated into osteoclastogenesis process and thereby in periodontitis (Huynh, VonMoss, & Smith, 2016; Zhao et al., 2019, Zheng et al., 2019). Furthermore, cytokines such as IL-6, IL-1B, IL-17, and TNF-α are released and transported by EVs (Huynh et al., 2016, Zhao et al., 2019; Zheng et al., 2019). Besides, increased levels of EVs in GCF could reflect a perturbation in cell homeostasis and should be researched about the content and function of these EVs during the periodontal breakdown tissues.

The role of EVs and exosomes in periodontal disease is still new and requires to be elucidated. Recent reports have suggested an increased secretion of exosomes when periodontal ligament fibroblasts are stimulated with LPS, participating in the inflammatory

signal transduction into osteoblast by inhibition of their osteogenic activity (Zhao et al., 2019). Meanwhile, two recent researchers report different findings. On the one side, it seems to be overexpression of salivary exosomal proteins in severe periodontitis patients (Huang et al., 2019). But on the other hand, the second report suggests a reduced salivary concentration of tetraspanin CD9/CD81 exosomes in periodontitis patients compared to healthy controls (Tobon-Arroyabe et al., 2019). In this study, we did not detect significant differences in the salivary concentration of EVs, but there were significant increases in total concentration of EVs and micro-vesicles in the GCF.

If the identification of specific profiles of EVs in GCF of patients with periodontitis can be achieved, it would provide a chance for early identification risk of periodontal tissue loss and would allow early clinical management to improve periodontal outcomes. Since GCF-EVs include locally and systemically derived markers of the immune-inflammatory response, they could offer the basis for a site-specific biomarker assessment for chronic periodontitis or systemic diseases in oral fluids (Nomura et al., 2012; Ogawa et al., 2011; Giannobile et al., 2011). Also, due to the non-invasive nature of collecting them, characterization and quantification of the amount of EVs in GCF may be especially helpful in the follow-up of progressive periodontal pockets, and a promising reliable prognostic tool to help us predict at-risk patients, minimizing the loss of periodontal tissues and teeth for these reasons. More likely, the monitoring of the levels of EVs in the GCF will allow us to establish thresholds for the amount of EVs associated with health or pathological conditions in the periodontal sulcus. However, the research of EVs in oral fluids only begins and their potential interest concerning their pathophysiology, their development, and compromise in oral or systemic pathologies is under development, denoting an immense field in which knowledge must be established because EVs are ubiquitously present in all body fluids and are deemed ideal as diagnostic biomarkers.

Finally, data from the present study must be interpreted with caution and needs to be further validated in subsequent prospective cohort studies regarding various periodontal conditions with larger sample size and also analyze their level of changes during and after periodontal treatment. Also, these results should be complemented by research in the biological function and content of EVs in periodontal inflamed tissues and gingival health, and probably, during the near future, they could greatly impact the development of valuable tools for clinical practice in periodontology.

5 | CONCLUSION

Whiting the limitations of the present pilot study, we can conclude that there are molecular signatures of extracellular vesicles in oral fluids. These EVs are increased in GCF samples of periodontitis patients and correlated with the clinical inflammatory periodontal parameters. Their role and utility as oral biomarkers of periodontitis remain to be established and validated in larger prospective cohort studies.

CONFLICT OF INTEREST

There are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTION

All authors have made a substantial contributions to the conception and design of the study. Conceptualization: Alejandra Chaparro, Marcela Hernández , Andrés pascual, Antonio Sanz and José Nart. Data curation: Laura Weber, Stephanie gallardo and Ornella Realini Formal analysis: Valeria Ramírez and Daniela Albers. Funding acquisition: Alejandra Chaparro. Investigaction: Alejandra Chaparro, Ornella Realini, Laura Weber. Project administration: Marcela Hernandez, Ornella Realini and Victor Beltran. Supervision: Victor beltran and Alejandra Chaparro Writing original draft: Alejandra Chaparro, Marcela Hernández, Antonio Sanz and Victor Beltran.

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