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Impact of periodontitis on gingival crevicular fluid miRNAs profiles associated with cardiovascular disease risk

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Abstract

Background and Objective: Recent emerging evidence has shown that microRNA (miRNAs) is involved in several epigenetic processes linked with periodontitis, increased oxidative stress and cardiovascular disease (CVD). The present study aimed to assess the impact of periodontitis on gingival crevicular fluid (GCF) miRNAs expression associated with CVD risk and to evaluate possible confounders that influenced this association.

Materials and Methods: For the present study, healthy controls ($n = 28$) and subjects with CVD ($n = 28$), periodontitis ($n = 30$) and periodontitis + CVD ($n = 29$) were enrolled. All subjects underwent regular periodontal examinations and blood sampling. In addition, GCF sampling was performed, and miRNAs 7a-5p, 21-3p, 21-5p, 100-5p, 125-5p, 200b-3p, and 200b-5p expression was analyzed using a real-time quantitative polymerase chain reaction (RT-PCR).

Results: The results showed that periodontitis and periodontitis + CVD subjects presented significantly different GCF miRNAs expression compared to healthy controls and CVD subjects. More specifically, compared to healthy controls and CVD, subjects with periodontitis and periodontitis + CVD showed higher GCF miRNA 7a-5p, miRNA 21-3p, miRNA 21-5p, miRNA 200b-3p, and miRNA 200b-5p ($p < .05$) and lower miRNA 100-5p, miRNA 125-5p levels ($p < .05$). Furthermore, the multivariate regression analysis evidenced that periodontitis (miRNA 21-3p, 100-5p) and periodontal inflamed surface area (PISA) (miRNA 7a-5p, 21-3p, 21-5p, 100-5p, 125-5p, 200b-3p) were significant predictors of GCF miRNAs concentration ($p < .05$).

Conclusion: The results of the study highlighted that the periodontitis and periodontitis + CVD group showed higher GCF miRNAs expression than healthy controls and CVD subjects. Furthermore, periodontitis and its extent (PISA) were revealed as significant predictors of GCF miRNAs associated with CVD risk.

KEYWORDS

cardiovascular disease, c-reactive protein, gingival crevicular fluid, microRNA, oxidative stress, periodontitis

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1 | INTRODUCTION

Periodontitis is a multifactorial inflammatory disease with bacterial etiology affecting the periodontium and leading, if not properly treated, to progressive periodontal tissue attachment and alveolar bone loss.[1] In this regard, it was widely shown that periodontitis represents one of the most widespread diseases, as it is estimated to affect nearly half of the world's population,[2] closely correlated with various systemic conditions such as obesity,[3] metabolic syndrome, rheumatic diseases,[4] risk of premature birth,[5] and cardiovascular diseases (CVDs).[6,7]

In 2019 a workshop held by both the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) clearly concluded that periodontitis itself represents a true risk factor for CVDs[8] through a mechanism that involves, through an unbalanced oxidative stress pathway, the release of several systemic pro-inflammatory mediators, the dysregulation of reactive oxygen species (ROS) and reduction of nitric oxide (NO), which finally contributes to the genesis and the progression of endothelial dysfunction and atherosclerosis.[9]

MicroRNAs (miRNAs) are short noncoding single-stranded RNA molecules (17–24 nucleotides),[10] which control the post-transcriptional expression of certain genes implicated in ROS generation and antioxidant defense of vascular mechanisms, finally influencing tissue homeostasis.[11] In this regard, miRNAs dysregulation has been proven to be involved during the early stages of CVD through the post-transcriptional repression of protein expression pathway that, through the binding to the target mRNA 3′ untranslated region of the cardiac cell, negatively influences cardiomyocyte growth, cells contractility, and the overall cardiac tissue maintenance.[12] Similarly, some preclinical studies have proposed that miRNA-21[13] and miRNA-200,[14] through regulating target genes cardiac and endothelial cells, influence NO and ROS production, with a specific role in the regulation of endothelial functions, with a related role in some therapeutic target strategies of endothelial dysfunctions.[12]

Furthermore, some pivotal evidence has emphasized the modulating effects of miRNAs in periodontal tissue homeostasis and osteogenic differentiation of periodontal ligament stem cells.[11] More specifically, miRNA-7,[15] miRNA-100, and miRNA-125[16] have been related to the release of inflammatory cytokines and metalloproteases in gingival fibroblasts during the early stages of periodontitis, suggesting the critical modulatory role that miRNAs could play during the early stages and subsequent progression of the disease.[17] Moreover, a preliminary study by Rovas et al.,[18] who analyzed miRNA profiles in subjects affected by periodontitis and CVD, found higher miRNA-3198 and miRNA-4299 levels compared with healthy controls. In agreement, Bagavad Gita et al.[19] evidenced upregulated miRNA-146a concentrations in subgingival plaque of subjects with periodontitis, suggesting a possible role of miRNAs in the early immune response seen during periodontitis and CVD.

Based on these findings, the aim of this study was to assess differences in miRNAs associated with CVD risk in the gingival crevicular fluid (GCF) of subjects with periodontitis, CVD and periodontitis + CVD. Furthermore, the secondary objective was to identify, among the other confounders, the impact of periodontitis and CVD as possible significant predictors of GCF miRNA levels in the enrolled population.

2 | MATERIALS AND METHODS

2.1 | Study design

Between December 2018 and February 2022, 348 consecutive subjects were first assessed for eligibility at the Dental School of the University of Catania, Catania, Italy.

The study was performed in accordance with the guidelines for strengthening the communication of observational studies (STROBE)[20] (Table S1) and followed the declaration of Helsinki on medical research guidelines reviewed in 2016. The study protocol was registered on ClinicalTrials.gov (NCT05383794), and ethical approval was obtained from the local Institutional Review Board (215/21/PO). Before the enrolment stage, each participant signed an informed consent specifying the protocol's risk and characteristics.

2.2 | Study sample

During the first visit, all subjects underwent an anamnestic examination, including recording the clinical history, pharmacological therapy and previous clinical records. Periodontitis was diagnosed in accordance with the classification of periodontal diseases[1] with the following inclusion criteria: (1) presence of ≥16 teeth; (2) ≥40% of periodontal sites with a probing depth (PD) ≥ 4 mm and a clinical attachment level (CAL) ≥ 2 mm; (3) bleeding on probing (BOP) in ≥40%; (4) alveolar bone loss (ABL) ≥ 2 mm in ≥2 sites, verified through Rinn X-rays. Subjects were categorized as stage II, grade C, generalized periodontitis.

CVD was diagnosed with ≥50% stenosis of at least one coronary artery observed through a percutaneous coronary intervention/coronary angiography. CVD was diagnosed by the same calibrated operator who analyzed the medical records, and CVD subjects underwent an electrocardiogram to detect the possible presence of atrial fibrillation or other pathologies.[21] Subjects of the periodontitis + CVD had to meet the combined inclusion criteria for periodontitis and CVD, while individuals were considered healthy controls if not affected by any systemic disease and did not take drugs. Furthermore, healthy and CVD subjects had no periodontal sites with PD and CAL ≥3 mm, BOP <10% and ABL of ≥2 mm, verified on periapical Rinn X-rays.

The following exclusion criteria were adopted for all four groups: (1) contraceptives in the last 6 months before the study; (2) antibiotics, immunosuppressants, anti-inflammatories or any other drugs, which could induce gingival hyperplasia in the last 6 months before the study; (3) history of alcohol abuse; (4) drug allergies/intolerances; (5) lactation or pregnancy; (6) diabetes or rheumatic diseases; (7) periodontal therapy in the last 6 months preceding the study; (8)

COVID positivity status. The selected participants were divided into four groups: healthy controls (group 1), CVD (group 2), periodontitis (group 3), and CVD + periodontitis (group 4).

2.3 | Sample selection

After an initial screening, 233 participants were excluded because they did not meet the inclusion criteria (n = 166), declined to participate (n = 39), or were absent at the first appointment (n = 28) (Figure 1). Therefore, for the study, 115 subjects were finally enrolled, allocated, and categorized into four groups as: healthy controls (n = 28), CVD (n = 28), periodontitis (n = 30), and CVD + periodontitis (n = 29) (Figure 1).

2.4 | Outcomes

During the first visit, each selected subject was registered for the demographic characteristics: age, sex, BMI (body mass index), smoking habit, comorbidities/medications and level of education (primary school, college, university). Furthermore, the subjects were classified into three socioeconomic status (SES) levels (low, middle and high) as a combined measure of education, income and occupation.[21] Fasting glucose levels ≥125 mg/dl or related medical history information were considered indicative of diabetes. Regarding smoking habits, all the selected subjects were distinguished as: smokers, past smokers (at least ≥ 5 years) and non-smokers. From February 2020 during the first visit, all patients performed a COVID test in order to be enrolled. In the case of positivity, patients were excluded from the study.

Two independent calibrated examiners carried out the periodontal indices recording. During the intraoral examination, periodontal status was obtained in all included subjects by a standardized periodontal probe, at six sites per tooth in all present teeth, excluding wisdom teeth.* More specifically, PD, CAL, BOP, plaque index (PI),[22] Full-Mouth Plaque Score (FMPS), Full-Mouth Bleeding Score (FMBS), and ABL were recorded. CAL was assessed by calculating PD and the gingival recession (REC) levels using the cementoenamel junction (CEJ) as a reference and Periodontal Inflamed Surface Area (PISA) was calculated as previously reported.[23] ABL was measured on the distal and mesial bone levels at the root surfaces of each tooth using periapical Rinn X-rays.[24]

Inter-and intra-examiner reliability was assessed using PD and CAL as reference values, using the intraclass correlation coefficient (ICC) analysis. There was good agreement among examiners for both PD (ICC = 0.822) and CAL (ICC = 0.819). The intra-examiner reliability for both examiners was performed on only eight random subjects per group (32 total subjects). For the first examiner, reliability showed a good level of agreement for both PD (ICC = 0.819) and CAL (ICC = 0.823); for the second examiner (control), reliability was also good for both PD (ICC = 0.819) and CAL (ICC = 0.822).

2.5 | Biological samples collection

During the first visit, before the intraoral examination, a single operator took Crevicular Gingival Fluid (GCF) and blood samples from each subject between 8:00–10:00 a.m. All subjects were instructed not to drink, eat, or perform any oral hygiene maneuvers within the 12 h prior to sampling collection. For GCF sampling, the supragingival plaque and saliva around the teeth were gently removed using a cotton pellet, and the teeth were air-dried and isolated with cotton rolls. Two sterile paper strips† were gently inserted into the periodontal pocket until mild resistance for 30 s to collect GCF samples from 2 non-contiguous interproximal deepest periodontal sites per each subject as previously described.[25] The strips contaminated with blood were discarded. Immediately after collection, blood samples were centrifuged (1000 rpm for two minutes) at 4°C and stored at −80°C. A nephelometric assay kit was used to measure hs-CRP (high-sensitive C-reactive protein, mg/L). Glucose, triglycerides, total cholesterol, and low/high-density lipoproteins (LDL/HDL) cholesterol levels were analyzed through routine techniques.

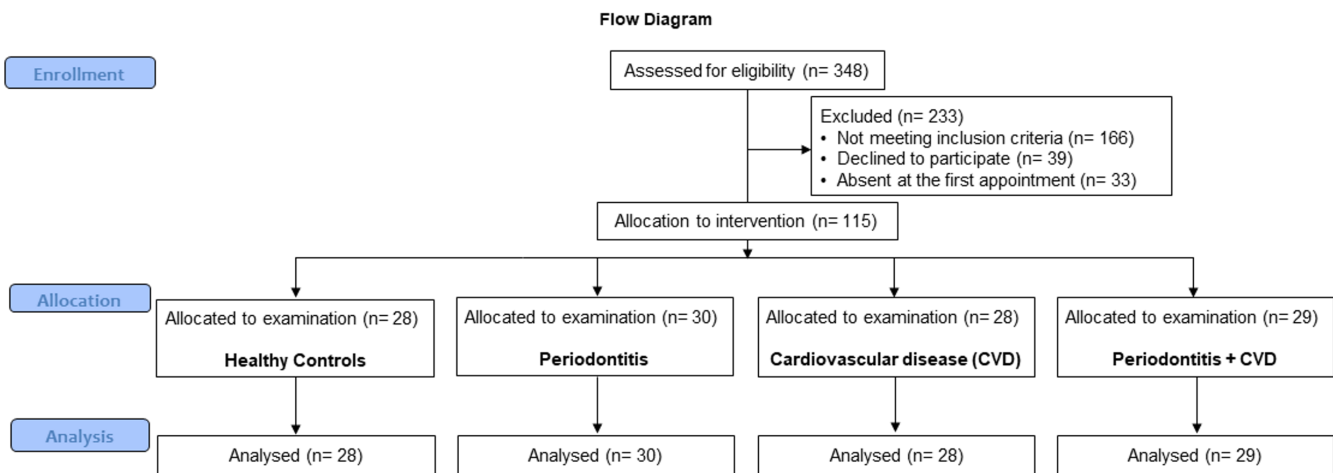

FIGURE 1 Flowchart of the study

2.6 | MiRNA extraction and analysis: Extraction of miRNA from GCF

The GCF strips were stored at −70°C and inserted into sterile microtube vials until analyzed. Any strips with visible blood contamination were discarded. Small RNA was isolated from GCF using a kit‡ according to the manufacturer's protocol and stored at −80°C for further study. Considering the volume of GCF fully absorbed by a single strip corresponding to 1.2 µl, the sample volume was estimated according to the absorption levels of collected strips. miRNAs were extracted from all samples, and nucleic acid was measured in order to load the same quantity for all reactions. Furthermore, miRNAs were normalized to a reference miRNA U66 as previously described.[26] The concentration of RNA was determined by a NanoDrop ND-1000 spectrophotometer§ for quantification and purity analysis. The GCF amount of each strip was also measured before storage by an electronic GCF measuring device (Periotron 8000, Oraflow, NY, USA) and reported in picograms (pg)/microliter.

2.7 | RNA extraction and reverse-transcriptase PCR (RT-PCR)

Total RNA was isolated from small swabs using TRIzol reagent¶ and cDNA was synthesized using the miRCURY LNA RT Kit (cat. no. 339340) to produce a suitable template for PCR. The PCR reactions were run on a QuantStudio™ 3.** The quantification of miRNA was carried out using the miRCURY LNA SYBR® Green PCR Kit.†† For the present study, miRNA 7a-5p, miRNA 21-3p, miRNA 21-5p, miRNA 100-5p, miRNA 125-5p, miRNA 200b-3p, miRNA 200b-5p levels were evaluated. Triplicate values for real-time PCR were handled by calculating the mean of triplicates.

Briefly, 10 ng of template RNA was reverse-transcribed (in 10 µl) using a stem-loop primer. For the PCR reaction, 3 µl cDNA template (diluted 1:60) of RT-product was used. Both RT- and PCR reactions were performed in triplicate in 3 separate experiments. The expression level was defined based on the threshold cycle, and relative expression levels were calculated as △△Ct after normalization with reference control. The U6 snRNA was used as the housekeeping gene. The primers for U6 snRNA are forward, CTTCGGCAGCACATATACT, and reverse, AAAATATGGAACGCTTCACG. The forward- and reverse-transcribed primers for different miRNAs are listed in Figure 2.

2.8 | Power sample size analysis

The power sample was determined using statistical software.‡‡ The sample size was calculated using miRNA-7 as a primary outcome variable and considering four groups, a power level of 80%, an effect size of 0.35, and a 2-sided level of 0.05.[27] Therefore, it was fixed a priori that at least 24 subjects per group were needed in order to achieve a power level of 80%. However, to avoid potential drop-outs, 115 subjects were finally enrolled in order to achieve a power level of 88%.

2.9 | Statistical analysis

For each of the four groups, numerical data was expressed as a median and interquartile range (IQR) and categorical variables as absolute frequencies and percentages.

Examined variables did not present normal distribution as verified by the Kolmogorov–Smirnov test; consequently, a non-parametric approach was used. The Kruskal-Wallis test was applied to compare the four groups regarding numerical variables, so two-by-two comparisons were performed using the Dunnet test. For these multiple comparisons, Bonferroni's correction was applied, for which the significance alpha level 0.050 was divided by the number of possible comparisons. So, the "adjusted" significance level for this analysis was equal to 0.050/6 = 0.008. The Chi-Square test was applied in order to compare the four groups regarding categorical variables such as sex, smoking, hypertension and CVD drugs.

Firstly, a logarithmic transformation of each miRNA was performed and, conditionally to the obtained normality condition after a logarithmic transformation, uni- and multivariable linear regression models were estimated to assess the dependence of each GCF miRNAs concentrations from possible variables such as age, sex, BMI, smoking, CRP, HbA1c, fasting glucose, CVD, number of teeth, FMBS, FMPS and PISA (as a measure PD and CAL). Smoking and CVD were inserted in the model as dichotomous variables (yes/no). Statistical analyses were performed using a statistical software§§ by a skilled statistician blinded to the study groups. A p-value lower than .05 was considered statistically significant.

3 | RESULTS

The clinical characteristics of the sample is represented in Table 1. All participants were aged 40 to 65 years old with a 1:1 female/male ratio to avoid any difference and were well matched for age (p = .939), sex (p = .755), and the number of smokers. The results of the Dunnet Test revealed that, in comparison with healthy controls, the CVD group showed higher hs-CRP levels ($p < .001$), as well as PD, CAL, FMBS, FMPS, PISA and a lower number of teeth ($p < .001$ for all comparisons) (Table 1 and Table S2). Compared to periodontitis, the periodontitis + CVD group presented slightly higher fasting glucose values (p = .021) (Table S2). However, in comparison with CVD, subjects in the periodontitis group showed higher CRP levels ($p < .001$), as well as PD, CAL, FMBS, FMPS, PISA and a lower number of teeth ($p < .001$ for all comparisons).

3.1 | Primary outcome

Values of miRNAs expression among groups are represented in Figure 3. The pairwise comparison highlighted that, compared to healthy controls, CVD subjects showed higher miRNA 7a-5p ($p < .002$), miRNA 21-3p ($p < .001$), miRNA 21-5p ($p < .002$), miRNA 200b-3p ($p < .001$), miRNA 200b-5p ($p < .001$) and lower miRNA 100-5p ($p < .001$), miRNA 125-5p ($p < .001$) expression. In comparison

miR-ID	Sequence	Forward primer	Reverse primer
hsa-let-7a-5p	UGGGAUGAGGUAGUAGGUUGUAUAGUUUUAGGGUCACACCCACCACUGGGAGAUAACUAUACAAUCUACUGUCUUUCCUA	UGGGAGAGAGUAGGUUGUAUAGUUC	AUCCUUUCUCAUCUAACAUAUCAAA
hsa-miR-21-3p	UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUCUGACA	GUCGGAGCUUAUCGACUGUGUUGCUGUGA	CAGUCUCGGGUAGCUGACACAACGGUACC
hsa-miR-21-5p	UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUCUGACA	GUCGGAGCUUAUCGACUGUGUUGCUGUGA	CAGUCUCGGGUAGCUGACACAACGGUACC
hsa-miR-100-5p	CCUGUUGCCACAAACCCGUAGAUCCGAACUUGUGGUAUUAGUCCGCACAAGCUUGUAUCUAUAGGUAUGUGUCUGUUAGG	CCUGGCACAACCUAGAUCGACUUGUGGU	GGAUCGUGUUGGAUCUAGUUGAACACCA
hsa-miR-125b-5p	UGCGCUCCUCUCAGUCCCUGAGACCCUAACUUGUGAUGUUUACCGUUUAAAUCCACGGGUUAGGCUCUUGGGAGCUGCGAGUCGUGCU	GCGCCUCCAGCCAGACCUAACUUGUGGUUUAC	CGUGGAGGUCGGUCUGGAUUGGGCACUAAAUU
hsa-miR-200b-3p	CCAGCUCGGGCAGCCGUGGCCAUCUUACUGGGCAGCAUUGGAUGGAGUCAGGUCUCUAAUACUGCCUGGUAAUGAUGACGGCGGAGCCCUGCACG	GCGGGCCCGUGCAUCUUACGGCAGAUUGGAGGU	CGCCCGGGCGCGUAGAAUGCCGUCUAAUCUCUA
hsa-miR-200b-5p	CCAGCUCGGGCAGCCGUGGCCAUCUUACUGGGCAGCAUUGGAUGGAGUCAGGUCUCUAAUACUGCCUGGUAAUGAUGACGGCGGAGCCCUGCACG	GCGGGCCCGUGCAUCUUACGGCAGAUUGGAGGU	CGCCCGGGCGCGUAGAAUGCCGUCUAAUCUCUA

FIGURE 2 Forward- and reverse-transcribed primers for different analyzed miRNAs

TABLE 1 Patients clinical characteristics

Characteristics	Controls (*n* = 28)	CVD (*n* = 28)	Periodontitis (*n* = 30)	Perio + CVD (*n* = 29)	*p*-value*
Age	52.5 (49.0–55.8)	52.5 (51.0–56.0)	53.0 (50.5–56.0)	52.0 (51.0–55.5)	.939
Sex (male/female)	14/14	14/14	15/15	15/14	.755
Education n (%)					
Primary school *n* (%)	15 (53.6)	16 (57.1)	14 (46.7)	14 (48.3)	.235
College *n* (%)	8 (28.7)	9 (32.2)	10 (33.3)	10 (34.5)	.332
University *n* (%)	5 (17.7)	3 (10.7)	6 (20)	5 (17.2)	.547
Smokers n (%)					
Never smokers *n* (%)	26 (92.8)	25 (89.3)	26 (86.6)	24 (82.8)	.235
Past smokers *n* (%)	1 (3.6)	2 (7.1)	2 (6.7)	3 (10.3)	.331
Current smokers *n* (%)	1 (3.6)	1 (3.6)	2 (6.7)	2 (6.9)	.109
hs-CRP (mg/L)	3.50 (3.10–3.75)	6.03 (5.93–6.58)	6.40 (6.53–7.30)	6.90 (6.60–7.55)	<.001
BMI	24.85 (22.08–27.13)	25.00 (22.08–26.90)	25.75 (22.23–27.55)	26.70 (22.35–28.25)	.453
N° tooth	26.00 (25.00–27.00)	24.00 (23.00–26.90)	21.50 (20.00–23.25)	21.00 (19.50–23.00)	<.001
HbA1c	5.20 (4.95–5.60)	5.50 (5.20–5.60)	5.58 (5.38–5.6)	5.56 (5.30–5.6)	.136
Fast glucose (mg/dl)	111.00 (106.00–122.00)	115.00 (105.00–123.00)	117.50 (120.50–124.00)	119.00 (95.00–123.50)	.188
PD (mm)	2.12 (1.56–2.30)	2.31 (2.19–2.65)	4.41 (4.23–4.79)	4.36 (4.07–4.92)	<.001
CAL (mm)	2.09 (1.88–2.24)	2.21 (2.02–2.43)	3.93 (3.71–4.26)	4.12 (3.78–4.74)	<.001
FMBS (%)	12.60 (11.83–13.58)	15.75 (13.30–16.40)	45.00 (42.30–48.70)	45.80 (44.20–46.60)	<.001
PISA (mm^2)	423.60 (415.60–446.60)	657.00 (601.50–698.30)	1750.05 (1526.30–1848.60)	1856.90 (1255.75–2219.60)	<.001
FMPS (%)	14.55 (13.20–15.50)	23.60 (22.10–25.40)	50.10 (47.90–54.20)	52.30 (48.90–53.60)	<.001

Note: Values are indicated as: 50° percentile (25°–75° percentiles) or number and percentage.

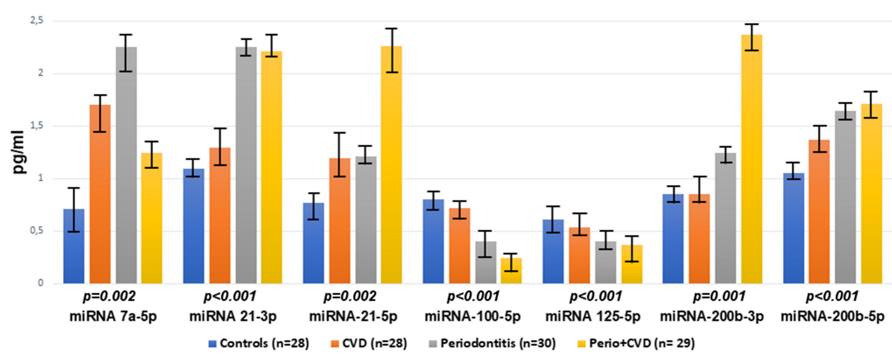

FIGURE 3 miRNAs expression among analyzed groups. Values are expressed in picograms per milliliter (pg/ml).

with healthy controls and CVD group, the periodontitis group showed higher miRNA 7a-5p ($p = .036$), miRNA 21-3p ($p = .044$), and lower miRNA 100-5p ($p = .029$) miRNA 125-5p ($p < .001$) expression. When compared to CVD, the periodontitis + CVD group presented significantly higher miRNA 21-3p ($p = .002$), and lower miRNA 100-5p ($p < .001$) expression (Figure 3).

3.2 | Secondary outcome

The uni- and multivariable regression models were estimated in order to identify significant predictors of each GCF miRNAs expression (Table 2). The multivariate model, in which only significant variables in univariate analysis were inserted, evidenced that HbA1c ($p < .001$), PISA ($p = .027$) and FMPS ($p = .021$) were significant predictors of miRNA 7a-5p; CVD ($p < .022$) and PISA ($p = .002$) were significant predictors of miRNA 21-3p, while FMPS ($p = .002$), fasting glucose ($p = .025$) and PISA ($p = .014$) were significant predictors of miRNA 21-5p (Table 2).

Furthermore, FMPS ($p = .004$) and PISA ($p < .001$) were significant predictors of miRNA 100-5p; CRP ($p < .001$), number of tooth ($p = .013$), FMBS ($p = .045$) and PISA ($p = .011$) were significant predictors of miRNA 125-5p; BMI ($p = .046$), HbA1c ($p = .015$) and PISA ($p = .010$) were significant predictors of miRNA 200b-3p; number of tooth ($p = .023$), and PISA ($p = .018$) were significant predictors of miRNA 200-5p (Table 2). The other analyzed variables were considered to be not significant.

4 | DISCUSSION

The aim of this study was to assess miRNA levels associated with CVD risk in GCF of healthy individuals and in subjects with periodontitis and CVD and to identify the impact of periodontitis and CVD on GCF miRNA expression.

The results evidenced that there were significant differences in the GCF miRNAs expression even if both healthy controls, periodontitis and CVD subjects, were well matched for age, sex, and number of smokers. In agreement, a pilot study by Saito et al.,[17] in which both periodontitis and healthy subjects were similarly matched for age and sex, found significant unbalanced expression of several GCF miRNAs profiling.[17] Regarding smoking, different from the present study results, some reports[17,18] showed that smoking habits might influence the expression of GCF miRNAs.[28] This may be because long-term smoking habits could negatively impact wound healing, and extracellular matrix protein homeostasis and accelerate cellular senescence of gingival fibroblasts through specific mechanisms involving some miRNAs, which code the type I and III collagen, interleukins and p53.[29]

Furthermore, the pairwise analysis evidenced that, compared to healthy controls, the CVD group showed higher miRNA 7a-5p, -21-3p, -21-5p, -200b-3p, and -200b-5p and lower miRNA 100-5p, and -125-5p expression. In this regard, it has been shown that endothelial dysfunction and atherosclerosis during CVD could impact circulating miRNAs expression.[30] More specifically, a previous study has reported an increase in some muscle-enriched miRNAs (-133 and -208a) of patients with coronary artery disease, particularly in miRNAs that are highly expressed by endothelial cells[30]; the increased miRNAs have been directly linked with an unbalanced mobilization of endothelial progenitor cells and a relative risk of endothelial dysfunction, indicating miRNAs -133 and -208a as potential early biomarkers of CVD risk.[30] Other evidence has shown that miRNA-21 is directly implicated in NO inhibition mechanisms, causing a pathway linked to unbalanced oxidative stress to negatively impact the defenses of endothelial progenitor cells, significantly increasing the risk of endothelial damage.[31]

Furthermore, a preliminary report demonstrated that also the gingival inflammation during periodontitis can triggers the activation of miRNA-21, which, through a mechanism mediated by asymmetrical dimethylarginine (ADMA) and hs-CRP, determines the Erk Map kinase activation and a relative inhibition of the superoxide dismutase 2 enzyme, a key mediator for negative ROS release, contributing to the genesis and the progression of the CVD risk.[32] In this regard, periodontitis has been widely related to a negative pathway linked with a reduction of the NO bioavailability and angiogenic progenitor cell dysfunction, which may represent a further negative stimulus in the early risk for future CVD events.[33] In agreement, the results of the present study highlighted an increase of GCF miRNA-21 in both CVD and periodontitis patients, suggesting the possible role of miRNA-21 as an early CVD biomarker.[31]

Furthermore, the results of the present study showed that in comparison with the CVD group, subjects with periodontitis and periodontitis + CVD presented higher GCF expression of miRNA 7a-5p, -21-3p, and lower miRNA expression of -100-5p and -125-5p. In this regard, another study[34] reported significantly increased subgingival levels of the miRNA-146a in CVD + periodontitis patients compared to those affected by periodontitis alone, indicating how miRNA-146a is associated with an increased risk of coronary artery plaque accumulation, especially during CVD. Similarly, the present study evidenced upregulated in miRNA 7a-5p and reduced miRNA-100-5p and -125-5p levels; about that, both miRNAs have previously clearly been shown to be mediators of the CVD and endothelial damage risk.[35]

The multivariate linear regression analysis aimed at identifying possible predictors of GCF miRNAs expression highlighted that, among the analyzed confounders, the extent of periodontitis (PISA) represented one of the main significant predictors of several GCF miRNAs expression. In agreement with the present results, it has been previously shown that the local miRNAs upregulation may be directly correlated to the extent of periodontitis[36] and also that the severity of periodontitis could in turn influence the serum (miRNA-199a-5p) and local levels of several miRNAs in both gingival tissues (miRNA 199a-5p, -483-5p, -3198, -4299), GCF (miRNA-140-3p, -145-5p, 146a-5p, -3198, -4299) and saliva (miRNA-381-3p).[18,37]

One of the reason may be due that periodontitis contributes, through its chronic pathogenic biofilm burden, to a continuous negative stimulus on the local host response, with a further secretion of hs-CRP, NO and many other inflammatory mediators in a mechanism locally regulated by different miRNAs,[38] which could determine, in turn, a final negative stimulus further to develop CVD

TABLE 2 Uni- and multivariable linear regression analysis for GCF miRNAs expression in all enrolled patients

	MiRNA 7a-5p				MiRNA 21-3p			
	Univariate		Multivariate		Univariate		Multivariate	
Variables	B	*p*-value	B	*p*-value	B	*p*-value	B	*p*-value
Age	0.632	.633	–	–	0.348	.906	–	–
Sex	0.985	.436	–	–	0.127	.701	–	–
BMI	0.058	.087	–	–	0.244	.446	–	–
Smoking	0.839	.128	–	–	0.401	.110	–	–
hs-CRP	0.671	.113	–	–	0.308	.951	–	–
HbA1c	0.484	.002	0.417	<.001	0.434	.743	–	–
Fasting glucose	0.678	.219	–	–	0.371	.146	–	–
CVD	0.368	.196	–	–	0.298	.016	0.277	.022
No. of teeth	0.932	.419	–	–	0.202	.463		
FMBS	0.985	.712	–	–	0.317	.742		
FMPS	0.158	.012	0.137	.021	0.268	.163		
PISA	0.389	.021	0.270	.027	0.488	.003	0.457	.002
	MiRNA 21-5p				MiRNA 100-5p			
	Univariate		Multivariate		Univariate		Multivariate	
Variables	B	*p*-value	B	*p*-value	B	*p*-value	B	*p*-value
Age	0.492	.739	–	–	0.348	.906	–	–
Sex	0.485	.936	–	–	0.248	.701	–	–
BMI	0.058	.387	–	–	0.148	.307	–	–
Smoking	0.267	.187	–	–	0.257	.910	–	–
hs-CRP	0.191	.913	–	–	0.396	.806	–	–
HbA1c	0.295	.820	–	–	0.069	.778	–	–
Fasting glucose	−0.008	.021	−0.009	.025	0.174	.446	–	–
CVD	0.312	.715	–	–	0.134	.317	–	–
No. of teeth	0.438	.194	–	–	0.434	.105	–	–
FMBS	0.196	.123	–	–	0.179	.886	–	–
FMPS	0.028	.012	0.023	.002	−0.048	.002	−0.036	.004
PISA	0.289	.021	0.272	.014	−0.788	<.001	−0.707	<.001
	MiRNA 125-5p				MiRNA 200b-3p			
	Univariate		Multivariate		Univariate		Multivariate	
Variables	B	*p*-value	B	*p*-value	B	*p*-value	B	*p*-value
Age	0.532	.134	–	–	0.445	.196	–	–
Sex	0.589	.416	–	–	0.548	.170	–	–
BMI	0.268	.178	–	–	−0.088	.036	−0.057	.046
Smoking	0.745	.128	–	–	0.305	.632	–	–
hs-CRP	−0.361	.001	−0.273	.001	0.351	.743	–	–
HbA1c	0.484	.428	–	–	0.304	.013	0.293	.015
Fasting glucose	0.567	.251	–	–	0.486	.841	–	–
CVD	0.360	.936	–	–	0.261	.338	–	–
No. of teeth	0.072	.019	0.061	.013	0.302	.794	–	–
FMBS	−0.017	.037	−0.013	.045	0.222	.146	–	–
FMPS	0.457	.671	–	–	0.192	.168	–	–

(Continues)

TABLE 2 (Continued)

	MiRNA 125-5p				MiRNA 200b-3p			
	Univariate		Multivariate		Univariate		Multivariate	
Variables	B	*p*-value	B	*p*-value	B	*p*-value	B	*p*-value
PISA	−0.589	.009	−0.505	.011	0.489	.006	0.445	.010

	MiRNA 200-5p			
	Univariate		Multivariate	
Variables	B	*p*-value	B	*p*-value
Age	0.601	.383	–	–
Sex	0.544	.634	–	–
BMI	0.158	.617	–	–
Smoking	0.733	.551	–	–
hs–CRP	−0.501	.112	–	–
HbA1c	0.248	.452	–	–
Fasting glucose	0.367	.565	–	–
CVD	0.307	.544	–	–
No. of teeth	0.068	.018	0.051	.023
FMBS	−0.017	.087	–	–
FMPS	0.158	.718	–	–
PISA	−0.789	.016	−0.705	.018

Note: Significant values $p < .05$.

and related endothelial dysfunction in predisposed subjects.[19,39] The multivariate analysis also showed that fasting glucose and hbA1c were significant predictors of miRNA -7 and 21. In this regard, several reports have shown how differential miRNAs profiles have emerged as potential regulators of certain mechanisms such as increased advanced glycation end products (AGEs), receptor AGEs, and increased free radicals formation among prediabetic, diabetic individuals, and diabetic patients with vascular complications,[40,41] acting also as ideal biomarkers for diabetes associated complications.

The results of the present study have, however, some limitations that need to be reported, such as the study design, which does not permit a longitudinal evaluation of the effect of miRNAs as well as the limited number of enrolled subjects, an issue that should be kept in mind when interpreting the present results.

Many studies have been performed during the last few decades to find even more useful biomarkers for the subclinical diagnosis of CVD and endothelial disease risk. The results of the present study showed that, in comparison to healthy subjects, different GCF miRNAs were significantly increased in subjects with periodontitis (miRNA -7 and -21), with CVD (miRNA -7, -21, and -200) and with periodontitis + CVD (miRNA-21). Furthermore, periodontitis and its extent (PISA) have been demonstrated to predict almost all miRNAs strictly associated with subclinical increased CVD risk. Furthermore, the present results provide insights into how GCF miRNAs may serve as potential risk biomarkers and therapeutic targets for both generalized periodontitis and CVD. However, further studies are needed to understand better the impact of oral miRNAs levels during periodontitis and CVD.

AUTHOR CONTRIBUTIONS

Gaetano Isola and Giovanni Li Volti conceived the research, planned and performed the experimental procedures, and wrote the manuscript. Alessandro Polizzi, Simona Santonocito, and Manuel Vaccaro performed the procedure. Alfio Distefano, Giuseppina Raciti, and Giovanni Li Volti performed the analyses. Angela Alibrandi performed the statistical analysis concealment. This manuscript is dedicated to the lovely memory of Prof. Ray Clayton Williams, University of North Carolina at Chapel Hill who was a giant of Periodontology and a guide for all of co-authors.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest in the present study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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ENDNOTES

* UNC-15, Hu-Friedy, Chicago, IL, USA.

† Periopaper, Oraflow, NY, USA.

‡ miRNeasy® Mini Kit (Qiagen, #217004).

§ Thermo Scientific, Milan, Italy.

¶ Life Technologies, Gaithersburg, MD.

** Applied Biosystems Thermo Fisher scientific, Milan, Italy.

†† cat.no. 339346.

‡‡ G* Power version 3.1.9.4, Universitat Dusseldorf, Germany.

§§SPSS 22.0 for Windows package, SPS srl, Bologna, Italy.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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