Comparison of miRNA expression profiles in individuals with chronic or aggressive periodontitis

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INTRODUCTION

Periodontitis is an infectious disease characterized by the inflammation of periodontal tissues leading to attachment loss, bone resorption and eventually tooth loss (Kinane, Stathopoulou, & Papapanou, 2017). Thus, the progression of periodontitis may compromise functional aspects of the stomatognathic system including mastication, speech, aesthetics and, consequently, self-esteem (Borges et al., 2013).

There are two distinct forms of periodontitis. The formulation of a diagnosis is based on multiple clinical parameters, all of which may not be simultaneously required (American Academy of Periodontology, 2015). Chronic periodontitis (CP) is the most common form of the destructive periodontal disease in adults, although it may also occur in other age groups. It is associated with a variable microbial pattern; the amount of destruction is consistent with the presence of local factors and usually has a slight to moderate rate of
Aggressive periodontitis (AP) comprises a less frequent group of periodontitis with rapid attachment loss and bone destruction. It is associated with a more specific high virulence microbial pattern, and the amounts of microbial deposits are inconsistent with the severity of the disease. It also has a familial aggregation profile (American Academy of Periodontology, 2015; Armitage, 1999). Genetic predisposition plays a fundamental role in the development of AP and contributes to the early onset of tissue destruction (Albandar, 2014). Individuals with AP also display a heightened immune response (Shaddox et al., 2010). Phagocyte abnormalities and hyper-responsive macrophage phenotype have been reported (Armitage, 1999).

Overall, bacterial-induced inflammatory response is known to be involved in the pathogenesis of periodontitis. The severity of the disease depends, in some way, on the balance and interactions between the bacterial challenge and the host immunoinflammatory response, influenced by modifiable and non-modifiable environmental and host risk factors (Kinane et al., 2017; Kornman, 2008). The mechanisms of immunoinflammatory regulation in periodontitis are complex, and host modulation has been recently reviewed (Bartold & Van Dyke, 2017; Hajishengallis & Korostoff, 2017). The role of miRNAs in the pathobiology of periodontal diseases has also been recently explored (Kebschull & Papapanou, 2015), demonstrating specific roles of miRNAs in regulating gene expression and affecting different cellular and molecular pathways in periodontal homeostasis and disease. They act in the innate and adaptive immune responses and in the response to several stressors such as bacterial products and inflammatory mediators.

miRNAs are non-coding, single-stranded, ~22-base RNA sequences that have been shown to play critical roles in post-transcriptional regulation through their ability to bind to complementary untranslated mRNA sequences, generally resulting in gene silencing (Bartel, 2004). Since the function of miRNAs was established (Calin et al., 2002; Pasquinelli et al., 2000), these molecules have concerned the attention of researchers due to their impact on the regulation of biological functions, such as cell development, proliferation, differentiation, metabolism and apoptosis (Ambros, 2004; He & Hannon, 2004).

Previous studies have shown the relationship between miRNAs and human diseases such as developmental abnormalities (Kloosterman, Lagendijk, Ketting, Moulton, & Plasterk, 2007), muscular diseases (Eisenberg, Eran, & Nishino, 2007), cardiovascular disorders (Ikedo, Kong, & Lu, 2007) and cancer (Croce & Calin, 2005). Moreover, abnormal expression of miRNAs may lead to earlier disease progression (Kloosterman et al., 2007).

Despite the relevance of miRNAs in several inflammatory diseases (Lindsay, 2008; Nakasa et al., 2008; Pauley et al., 2008), only limited data are available on the function of miRNAs in the gingival tissues and their role in the pathogenesis of human periodontitis. Some studies have previously demonstrated a different pattern of miRNAs expression between healthy and inflamed periodontal tissues, in addition to an overexpression in diseased tissues (Lee et al., 2011; Xie, Shu, Jiang, Liu, & Zhang, 2011). To the best of our knowledge, there are no studies comparing the miRNAs expression profiles between CP and AP. Consequently, there is a lack of knowledge on how these expression profiles may lead to a better understanding of the pathogenesis and the intriguing differences in CP and AP clinical manifestations. Therefore, the aim of the present study was to compare the inflammation-related miRNAs expression profiles between CP- and AP-diseased gingival tissues.

## MATERIAL AND METHODS

### 2.1 Study design and study sample

The present investigation was an observational cross-sectional study, and STROBE guidelines were followed when appropriate. The study was conducted according to the ethical principles of the Helsinki Declaration and was approved by the Institutional Internal Review Board and the Ethics Research Committee of the Federal University of Minas Gerais (CAAE-58396416.8.0000.5149). All participants were informed of the aims of the study and signed an informed written consent form prior to entering the study. All rights of the participants were preserved at all times.

Participants were selected at the Clinics of Periodontology from the School of Dentistry, Federal University of Minas Gerais. During the period of data collection, from July/2015 to March/2016, ~230 individuals sought periodontal care in the unit of the study where they were determined to be eligible. Individuals were invited to participate in this study according to their accessibility and availability to the dental care routine. Among them, possible participants were excluded if (a) presented any history of systemic disease which could influence the course of periodontitis such as diabetes, cardiovascular diseases, rheumatoid arthritis and chronic respiratory diseases; (b) were under hormone replacement therapy at the time of study; (c) were pregnant or lactating women; (d) presented any history of systemic antibiotic usage ≤6 months prior to the study or long-term treatment with non-steroidal anti-inflammatory drugs; (e) any history of periodontal treatment; (f) were smokers or former smokers; and (g) were alcohol dependent. Only never smokers and occasional alcohol users were included.

The convenience sample from the present study comprised eighteen individuals from a multiethnic group, of both sexes, and divided into two groups: chronic (CP, n = 9) or aggressive (AP, n = 9) periodontitis. All data were submitted to the GEO platform (GSE116209).

### 2.2 Periodontal examinations

Periodontal status was assessed using periodontal probing depth (PD), clinical attachment level (CAL) and bleeding on probing (BOP). All examinations were performed with a manual periodontal probe (PCPUNC-15, Hu-Friedy, Chicago, IL, USA) by a trained periodontist. In order to evaluate intra-examiner agreement, measurements of PD and CAL were performed prior to the beginning of the study.
in five individuals (not included in the sample) and repeated within a 1-week interval. These procedures were also repeated 6 months after the beginning of the study in other five different individuals. Intra-examiner agreement revealed kappa values higher than 0.85 and intraclass correlation coefficients higher than 0.80.

CP was defined as a slowly progressive disease; thick deposits of plaque and calculus often present on affected teeth, and the amount of destruction was consistent with the presence of local factors. AP diagnosis was based on the following common features: usually affecting clinically healthy individuals except for periodontitis, amounts of microbial deposits (biofilm and calculus) were inconsistent with the severity of periodontal destruction, rapid attachment or bone loss, localized to permanent first molars and incisors and/or other permanent teeth (American Academy of Periodontology, 2015).

2.3 | Samples collection

Gingival tissue samples, containing both epithelial and connective tissues, were obtained during initial periodontal therapy. Tissue samples were collected from the deeper periodontal pocket of each individual with a Gracey curette and then stored in Eppendorfs containing RNA stock solution (RNA Later®, Sakura Finetek, Torrance, CA, USA) at −80°C.

2.4 | RNA extraction

Total RNA was obtained using the miRNA isolation kit (Ambion mirVana™ miRNA Isolation Kit, Life Technologies, Vilnius, LT, USA) according to the manufacturer’s instructions. Tissue samples were macerated and then placed in a 1.5 ml Eppendorf containing 1 ml of lysis/binding buffer for each 0.1 g of tissue. After homogenization, 1/10 of miRNA homogenate additive volume was added and incubated on ice for 10 min. Afterwards, the same volume of lysis/binding buffer of acid phenol: chloroform was added. The contents were mixed for 60 s and centrifuged for 5 min at 10,000 x g. Supernatant solutions were then transferred to another Eppendorf with the addition of 1.25 of the volume of 100% ethanol. Hence, the solutions were placed in filter cartridge, according to the manufacturer’s protocol for total RNA extraction.

In the sequence, samples were quantified (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA) and RNA quality was assessed (Bioanalyzer 2100 RNA 6000 Nano Kit, Agilent Technologies, Waldbronn, Germany). RNA with an A260/A280 ratio ≥1.8 and an RNA integrity number (RIN) between 6.5 and 10 were the quality parameters pursued for starting material (Fleige & Pfaffl, 2006).

2.5 | cDNA synthesis and miRNAs expression profile

RNA samples obtained from the extraction procedure were digested with DNase (Invitrogen Life Technologies, Carlsbad, CA, USA). Then, 1.5 μg of RNA from each sample was used for reverse transcription reaction (Superscript First-Strand Synthesis Kit, Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol.

Each sample was divided and processed in duplicate (Megaplex™ primer pools A & B and miRNA reverse transcription kit, Applied Biosystems). cDNA resulting from the previous step was amplified before the real-time PCR (Megaplex™ PreAmp pools A & B primer pool and TaqMan® PreAmp master mix solution, Applied Biosystems). Ameliorated cDNA was then diluted and added to PCR mix (TaqMan® OpenArray® Real-Time PCR master mix, Applied Biosystems) on a 384-well plaque. Following this, plaques containing miRNA panel (TaqMan® OpenArray® Human MicroRNA Panel, Applied Biosystems) were loaded (AccuFill™ system, Applied Biosystems) and then real-time PCRs were performed (QuantStudio™ 12K Flex Real-Time PCR System, Applied Biosystems). The analysis of the results was performed with statistical software (Applied Biosystems® Analysis Software, v.1.0), assuming global normalization and correction of Benjamini and Hochberg for false-positive findings in the volcano plot analysis (Benjamini & Hochberg, 1995).

Minimal fold change threshold was defined as 2, and statistical significance was established at p ≤ 0.05 (Student t test). Expression profiles of 754 miRNAs were assessed (TaqMan® OpenArray® Human MicroRNA Panel, Applied Biosystems) in all tissue samples.

2.6 | Statistical analysis

Statistical analysis was performed after the exclusion of two outlier samples. Periodontal parameters of study groups were compared through the Mann-Whitney test. miRNAs expression profiles were compared between CP (n = 8) and AP (n = 8) groups. All analyses were conducted with statistical software (Applied Biosystems® Analysis Software, v.1.0), and global normalization was used. Statistical significance was established at p ≤ 0.05 (Student t test), adopting the correction of Benjamini and Hochberg for false-positive findings (Benjamini & Hochberg, 1995).

3 | RESULTS

The mean age of individuals in the CP group was 49.6 ± 8.1 years (range: 27–45). A total of 44% (n = 4) and 33% (n = 3) of individuals were males in CP and AP groups, respectively. Periodontal clinical parameters of study groups are shown in Table 1.

According to miRNA microarray comparison analysis including 754 human miRNAs, no statistically significant differences were observed between CP and AP groups (Figure 1). Samples from CP and AP groups presented similar miRNA expression profile, as shown in Figure 2. The most overexpressed miRNAs in both groups were hsa-miR-1274b, hsa-let-7b-5p, hsa-miR-24-3p, hsa-miR-19b-3p, hsa-miR-720, hsa-miR-126-3p, hsa-miR-17-3p and hsa-miR-21-3p.
| Periodontal parameters | Aggressive periodontitis (AP) | Chronic periodontitis (CP) | p*
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<tr>
<td>Number of teeth</td>
<td>24.7 ± 2.4 (21–28)</td>
<td>22.9 ± 5.9 (9–28)</td>
<td>0.895</td>
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<tr>
<td>% of sites with BOP</td>
<td>28.8 ± 20.5 (3.0–62.0)</td>
<td>33.8 ± 17.5 (10.0–50.0)</td>
<td>0.537</td>
</tr>
<tr>
<td>Mean PD (mm)</td>
<td>3.0 ± 1.0 (1.9–5.0)</td>
<td>2.3 ± 0.5 (1.6–3.0)</td>
<td>0.537</td>
</tr>
<tr>
<td>% of sites with PD from 3 to 4 mm</td>
<td>29.7 ± 6.6 (25.0–34.4)</td>
<td>24.0 ± 7.6 (17.0–32.0)</td>
<td>0.400</td>
</tr>
<tr>
<td>% of sites with PD from 5 to 6 mm</td>
<td>12.7 ± 11.3 (3.7–38.9)</td>
<td>7.4 ± 5.1 (1.0–14.13)</td>
<td>0.297</td>
</tr>
<tr>
<td>% of sites with PD ≥7 mm</td>
<td>6.6 ± 4.5 (1.0–13.9)</td>
<td>4.9 ± 4.2 (1.0–13.9)</td>
<td>0.387</td>
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<tr>
<td>Mean CAL</td>
<td>3.38 ± 0.9 (2.3–4.8)</td>
<td>2.8 ± 0.6 (1.9–4.0)</td>
<td>0.162</td>
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<tr>
<td>% of sites with CAL from 3 to 4 mm</td>
<td>34.5 ± 7.9 (21.6–51.3)</td>
<td>32.1 ± 14.3 (15.4–58.3)</td>
<td>0.666</td>
</tr>
<tr>
<td>% of sites with CAL from 5 to 6 mm</td>
<td>13.7 ± 12.1 (2.1–38.0)</td>
<td>9.1 ± 6.0 (2.9–19.4)</td>
<td>0.489</td>
</tr>
<tr>
<td>% of sites with CAL ≥7 mm</td>
<td>12.1 ± 11.4 (1.0–39.8)</td>
<td>5.1 ± 4.4 (1.0–13.9)</td>
<td>0.077</td>
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Note. BOP: bleeding on probing; CAL: clinical attachment level; PD: probing depth.

*Mann–Whitney test.

**TABLE 1** Comparison of periodontal clinical parameters between groups CP and AP groups

**FIGURE 1** Volcano plot of the miRNA microarray results assuming aggressive periodontitis (AP) as a reference group in comparison with chronic periodontitis (CP) group. Horizontal dashed line represents p = 0.05. Vertical red lines delimit fold change values between 0.5 and 2
BIOINFORMATICS

RIKEN FANTOM5 (de Rie et al., 2017) databases were accessed in order to find elevated miRNAs expressed by specific cell types (Supporting Information Table S1). Then, Gene Ontology databases were accessed in order to find miRNA functions and describe the possible roles of elevated miRNA on the causative process of both types of periodontitis (Supporting Information Table S2). All data were submitted and approved to GEO (GSE 116209).

DISCUSSION

By affecting young systemically healthy individuals with severe destruction, rapid rate of progression and no compatible amount of local factors, AP has been intriguing researchers over the past decades, especially because its diagnosis and treatment could represent a challenge for the dental professionals. On the other hand, CP is more predictable and easier to manage. However, it is relevant to consider that both diseases are periodontitis, thus having many aspects in common.

This is the first report on miRNA expression profiles comparing CP and AP. In the present study, miRNA microarrays were performed to compare the expression of 754 inflammatory-related miRNAs and no statistical difference was found when CP and AP groups were compared. Current knowledge shows that some studies had examined the expression of miRNAs in inflamed gingival tissues in comparison with healthy gingival tissues (Kalea et al., 2015; Lee et al., 2011; Na et al., 2016; Ogata et al., 2014; Perri, Nares, Zhang, Barros, & Offenbacher, 2012; Stoecklin-Wasmer et al., 2012; Xie et al., 2011).

It was reported that 17 miRNAs were overexpressed and 22 underexpressed in inflamed gingival tissues (Ogata et al., 2014). The three most overexpressed miRNAs were hsa-miR-150, hsa-miR-223 and hsa-miR-200b. The three most underexpressed miRNAs were hsa-miR-379, hsa-miR-199a-5p and hsa-miR-214. Similar investigations showed that five miRNAs (hsa-miR-126, hsa-miR-190, hsa-miR-20a, hsa-miR-32 and hsa-miR-362-3p) (Xie et al., 2011) and six miRNAs (hsa-let-7a, hsa-let-7c, hsa-miR-130a, hsa-miR-301a, hsa-miR-520d and hsa-miR-548a) (Lee et al., 2011) were greatly up-regulated in inflamed gingival tissues when compared with healthy gingival tissues. Up-regulation of miR-223 was also observed in another investigation with similar methodology (Stoecklin-Wasmer et al., 2012). Despite some differences in ethnicity and experimental methods between studies, hsa-miR-223 was demonstrated to be overexpressed in inflamed gingiva in all of the reports. This miRNA is involved in many types of cancer, inflammatory and autoimmune diseases, as well as other pathological processes such as leukaemia, rheumatoid arthritis and cardiovascular disease (Eyholzer et al., 2010; Sugatani & Hruska, 2007). Gingival tissues from individuals with periodontitis were also recently investigated, and an increased expression of miR-128, miR-34a and miR-381, and a decreased expression of miR-15b, miR-211, miR-372 and miR-656 were observed (Na et al., 2016). Although these previous studies showed many
differences between miRNAs expression profiles in healthy and diseased individuals, our investigation observed no difference between AP and CP groups, possibly because groups of diseased subjects were compared.

Among the most commonly expressed miRNAs in AP e CP groups, the overexpression of hsa-let-7b-5p, hsa-miR-17-3p and hsa-miR-19b-3p is in accordance with previous studies (Lee et al., 2011; Luan et al., 2018; Xie et al., 2011). hsa-let-7b has been proved to be a pivotal regulator of cell differentiation (Peter, 2009). In humans, the let-7 family is composed of 13 members that are located on nine different chromosomal loci (Rough & Slack, 2008). hsa-let-7b is involved in the regulation of stem cell activities including stem cell maintenance or direct differentiation into lineage cells via downregulation of certain target genes (Lee, Han, Kwon, & Lee, 2016). It was demonstrated that hsa-let-7b-5p suppressed the osteogenic differentiation capacity of stem cells (Wang et al., 2018). hsa-miR-17-3p also influenced bone metabolism through the suppression of BMPR2 (Larabee, Coia, Jones, Cheung, & Gallicano, 2015).

The expression of hsa-miR-126-3p seemed to downregulate the inflammatory response (Tang, Wang, Shao, Wang, & Zhu, 2017), although findings of the present study showed its overexpression in both diseased groups. These findings are in agreement with other studies (Luan et al., 2018; Stoecklin-Wasmer et al., 2012; Xie et al., 2011). Interestingly, expression of hsa-miR-126-3p showed to be up-regulated in gingival crevicular fluid of periodontitis subjects (Saito et al., 2017). Authors stated that gingival crevicular fluid from individuals with periodontitis may present a particular miRNA profile when compared to healthy individuals and, as supported by other studies (Ogata et al., 2014; Xie et al., 2011), expression patterns of miRNAs could be used to differentiate healthy and diseased individuals. In this manner, miRNA detection in gingival crevicular fluid may serve as a novel diagnostic tool (Luan et al., 2018). hsa-miR-21-3p and hsa-miR-720-3p were also found to be overexpressed in periodontal individuals by others authors (Kalea et al., 2015; Lee et al., 2011; Luan et al., 2018), though the overexpression of hsa-miR-1274b was in disagreement with a recent study (Xie et al., 2011). No previous investigation showed an association between hsa-miR-19b-3p and hsa-miR-24-3p with periodontitis.

Individual differences exist regarding the degree of susceptibility to destructive periodontal diseases. Moreover, clinical differences between chronic and aggressive forms might be explained by other mechanisms rather than the miRNA expression profiles. Host response (Puri, Chhokra, Dodwad, & Puri, 2015), bacterial aspects (Van der Velden, 2017) and other genetic variables (Offenbacher et al., 2016) may be an answer to clarify the differences between these two forms of periodontitis. However, it is important to highlight that other recent studies have questioned a real difference between the chronic and aggressive forms of periodontitis (Van der Velden, 2017). A recent systematic review examined the profile of cytokines in the gingival crevicular fluid and concluded that the current weight of evidence is not sufficient to prove a difference between AP and CP (Duarte et al., 2015). Moreover, a microbiological study on bacterial screening of Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis in individuals with CP or AP concluded that the detection of these known periodontopathogenic bacteria is not able to discriminate different forms of periodontitis (Nibali et al., 2012). No statistical differences were found in neutrophil function related to chemotaxis, phagocytosis, superoxide production and adhesion (Fehily et al., 2001).

Recently, the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) proposed a new classification scheme for periodontal diseases and conditions. In this new system, based on the inconsistency of the scientific evidence to supports specific differences in the physiopathology, the forms of the disease previously recognized as “chronic” or “aggressive” were now grouped under a single category named “periodontitis” (Tonetti, Greenwell, & Kornman, 2018). However, it is interesting to emphasize that an extensive literature review was performed and showed that there is, indeed, phenotypic differences between the chronic and aggressive forms that include age of onset, location of initial lesions and rates of progression (based on limited exposure because of age). Although both forms of periodontitis are the primary result of an inflammatory response to the bacterial challenge, there is also great evidence suggesting microbiologic, immunologic, pathophysiological and genetic differences between these two “forms” of periodontitis. In this manner, a better comprehension of the events that lead to different clinical outcomes in both “aggressive” and “chronic” periodontitis needs a more precise definition of the initial events (Fine, Patil, & Loos, 2018). These issues point to the need for additional robust studies directed towards the identification of genetic, microbiologic and host markers that can improve the diagnosis.

The diagnosis of periodontitis has traditionally been assessed through clinical parameters such as probing pocket depth, clinical attachment level and radiographic evaluations (Page & Eke, 2007). Even though those parameters are still in use, they have limitations in identifying patients with high susceptibility to periodontitis progression. Hence, molecular methods appear to be a promising and reliable method for the diagnosis of periodontal disease. Therefore, it is reasonable to assume that the number of investigations aimed to prove the important role of miRNAs in periodontal pathogenesis is increasing, and the results are still vastly different probably due to methodological divergences. Furthermore, since host response and disease expression are time-related, important issues on the pathogenesis will not be determined until time to infection and disease is considered (Fine et al., 2018).

Some limitations must be pointed in the present study. Sample size and convenience sample in the study may impact external validity of the results. Hence, additional investigations in different and larger samples should contribute in validating the present results. Moreover, further studies should be performed to confirm the potential influence of miRNA expression profiles in periodontitis in order to use periodontitis-related miRNAs as new diagnostic markers and therapeutic targets. Findings from the present study could provide subsidies for further analysis of miRNAs in periodontal disease.
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AUTHOR CONTRIBUTION

S. A. Amaral, T. S. Pereira and J. A. R. Brito conducted all clinical and laboratorial stages, data analysis and manuscript preparation; L. O. Miranda Cota was the general supervisor of the study, contributing to data analysis and interpretation, manuscript preparation and revision; R. S. Gomez was the co-supervisor, contributing to data interpretation and manuscript revision. S. C. Cortelli, J. R. Cortelli and F. O. Costa contributed to manuscript preparation. L. O. Miranda Cota, R. S. Gomez and F. O. Costa designed the study. All authors read and approved the final manuscript version.

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SUPPORTING INFORMATION

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

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