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MicroRNA-146a and microRNA-155 as novel crevicular fluid biomarkers for periodontitis in nondiabetic and type 2 diabetic patients

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Abstract

Aim: Recent studies point at the crucial role of epigenetic mechanisms in the development of multifactorial diseases such as periodontitis and diabetes mellitus (DM) type 2. In addition, circulatory microRNAs (miRs) have emerged as novel biomarkers for various diseases. Aim of this study was to investigate the levels of miR-146a and miR-155 and superoxide dismutase (SOD) activity in gingival crevicular fluid (GCF) of periodontitis patients with (CPDM) and without (CP) DM type 2 as well as in periodontally healthy, control groups (PHDM and PH, respectively).

Material and methods: miR modulation was analyzed using quantitative real-time PCR while SOD activity was measured spectrophotometrically.

Results: The upregulation of miR-146a and miR-155 was observed in CP and CPDM patients baseline while the levels decreased after six weeks of the non-surgical therapy to the levels comparable to PH and PHDM, respectively. Expression levels of miRs positively correlated with SOD activity. Levels of miR-146a were higher in PHDM compared to PH patients. Multivariate analysis revealed that levels of miR-146a and miR-155 were significantly associated with periodontitis when adjusting for age and gender.

Conclusions: miR-146a and miR-155 may be consider as possible novel biomarkers for periodontitis in nondiabetic and type 2 diabetic patients.

Clinical Relevance

Scientific rationale for the study: miRs as epigenetic regulators are appreciated as key players in the development of multifactorial diseases such as periodontitis and DM and represent an attractive new class of specific biomarkers. No previous study reported miRs in GCF from patients with periodontitis and DM.

Principal findings: Expression levels of miR-146a and miR-155 were significantly increased in GCF of periodontitis patients with/without DM type 2 relative to healthy while decreased after non-surgical periodontal therapy.

Practical implications: Present results imply that miR-146a and miR-155 in GCF could be potential biomarkers for periodontitis in nondiabetic as well as type 2 diabetic patients

Introduction

Periodontal disease is a set of bacteria-induced inflammatory diseases reflecting disturbance in interactions between microbials and host immunological responses. It has been reported that diabetes mellitus (DM) represents established risk factor for periodontitis but also, periodontitis adversely affect glycemic control in patients with DM contributing to the development of diabetic complications (Mealey & Oates 2006). Moreover, it has been shown that periodontal treatment could stabilize and even improve serum glucose levels in patients with DM (Noor et al.

2015, Sima & Glogauer 2013, Teeuw et al. 2010)

The mounting evidence suggests that interrelationship between DM and periodontal disease, based on intracellular level, reflects vicious circle between oxidative stress and inflammation (Monea et al. 2014, Patil et al. 2016). However, the question rises which entities could be common regulators of these pathological processes, at the molecular level. In the light of

recent appreciation of epigenetics as one of the key player in the development of multifactorial diseases such as DM and periodontal disease (Ling & Groop 2009, Keating et al. 2016, Martins et al. 2016, Larsson et al. 2015), microRNAs (miRs), might acting as epigenetic regulators, draw an attention . miRs are small, non-coding RNAs that can suppress expression of a variety of genes thereby orchestrating a number of processes, such as host cell immune responses (Moffatt & Lamont 2011) including antibacterial (Li et al. 2016) and antiinflammatory as well as antioxidative (Hulsmans et al. 2011). It is noteworthy that circulating miRs (in serum, saliva and other body fluids), due to their stability and distinctive function, have an advantage of being biomarkers compared with other biomarkers such as proteins or bacterial products (Kim et al. 2015). It is noteworthy that salivary miRs have been evaluated as diagnostic biomarkers for oral squamous cell carcinomas (Zahran et al. 2015). On the other side, since miR activity and function could be modified in patients by pharmacological modulation, they are considered to be attractive therapeutic targets in a variety of diseases, among them DM micro - and macrovascular complications (van Rooij et al. 2012). In this research we focused on the two immunomodulatory miRs, miR-146a and miR-155, which are co-induced in many cell types responding to microbial lipopolysaccharide, facilitating a negative feedback control of NFkB target genes which encode various mediators of inflammation (Taganov et al. 2006, Ma et al. 2011) and involved in oxidative stress by targeting superoxide dismutase (SOD) (Ji et al. 2013). Noteworthy, periodontitis patients had higher gingival tissue levels of miR-146a accompanied with the reduced levels of inflammatory cytokines than healthy (Motedayyen et al. 2015). We aimed to quantify expression levels of miR-146 and miR-155 in gingival crevicular fluid obtained from nondiabetic and type 2 diabetic periodontitis patients, at baseline and after non-surgical periodontal treatment in order to examine their association with periodontal status as well as oxidative stress.

Material and methods

Study population

Ninety-six subjects (44 males and 52 females, mean age 44.13±12.46), chosen among individuals with periodontitis (n= 48) and periodontally healthy controls (n=48) undergoing dental examination were included in this study. The study details were fully explained to the participants and the written consents were obtained. The study protocol was approved by the Ethical Research Committee of the School of Dental Medicine, University of Belgrade, Serbia (ethics approval № 36/3).

Subjects were allocated in one of the four groups: CP (24 healthy individuals with chronic periodontitis), CPDM (24 patients with chronic periodontitis in association with DM type 2), PH (24 healthy individuals with clinically healthy periodontium) or PHDM group (24 patients with clinically healthy periodontium in association with DM type 2). The sample size calculation was based on data of the previous studies on acute increase in miR-146a in inflamed periodontal tissue (Xie et al. 2011) and DM (Rong et al. 2013). A total number of 96 participants (24 subjects per group) were needed to demonstrate a large difference (effect size-0.4) in GCF levels of miRs between groups with the test power of 90% and the alpha probability of 0.05. Calculation was done using ANOVA for independent groups (statistical software package GPower 3.1).

Inclusion criteria for subjects in PH group and CP group were as follows: ≥18 years of age, self-reported good general health, ≥ 20 erupted teeth excluding third molars, no periodontal treatment in previous six months. Periodontally healthy participants that were included in this study had to have no gingival inflammation (gingival index, GI=0), and probing depth ≤ 3 mm in every site (Himani et al. 2014). Participants with moderate or severe chronic

periodontitis had to have at least four sites with a probing pocket depth (PPD) of ≥ 4 mm along with the presence of attachment loss of ≥ 3 mm, and GI≥ 1 (Armitage 2004).
The enrolled diabetic patients were diagnosed with type 2 DM for at least two years and did not suffer with any additional systematic diseases. Only well controlled diabetic patients who had exhibited HbA1c levels of < 8% were eligible for the study (Ribeiro et al. 2011).
Exclusion criteria for all participants were as follows: 1) smoking 2) presence of any other systemic diseases 3) the use of immunosuppressants, antibiotics, antiinflammatory drugs in the last six months 4) dietary supplementations 5) pregnancy or lactation.

Clinical outcome variables

Using a periodontal probe (PCPUNC 15, Hu Friedy, Chicago, USA), full mouth periodontal evaluation in six sites per tooth (mesiobuccal, midbuccal, distobuccal,mesiolingual, midlingual and distolingual), were done. The following clinical parameters were recorded: 1) probing pocket depth (PPD) - distance between the gingival margin and the bottom of the sulcus/pocket 2) clinical attachment level (CAL) - distance between the cemento - enamel junction and the depth at which the probe met resistance 3) bleeding on probing (BOP) (Ainamo & Bay 1975) - measured 15 seconds after probing and recorded as presence (1) or absence (0) 4) gingivitis index (GI) (Loe & Silness 1963) and 5) plaque index (PI) (Silness & Loe 1964).
The clinical parameters were recorded by the well experienced periodontist (NNJ). Intraexaminer calibration was performed twice, before and during the study to ensure intraexaminer consistency and reproducibility achieving Cohen's Kappa of over 0.75 for each index. Patients received hygiene instruction, scaling and root planning with ultrasonic device (Cavitron® Ultrasonic Inc., Long Island City, NY, USA) and hand instruments (Gracey

curretes, Hu-Friedy® Manufacturing Inc., Chicago, IL, USA), under local anesthesia, quadrant by quadrant, within two weeks. The periodontal therapy was carried out by the same clinician (NR). The clinical measurements were recorded and GCF samples were taken from the sampling site at baseline and 6 weeks after the periodontal treatment.

Gingival crevicular fluid (GCF) sampling

Baseline, GCF samples were collected from two representative sites per participant, 24 hours after the clinical measurements were recorded. At post-treatment visit, GCF sampling was done prior the periodontal examination, but at same visit. Representative sites in periodontally healthy participants were mesiovestibular aspect of the right upper central incisor and right upper first molar. In patients with periodontitis, the two sites demonstrating the highest values of PPD and positive BOP were considered as representative. Before the sampling procedure, supragingival biofilm was gently removed from the sampling site, isolated with cotton rolls and dried. Sterile paper strip, Periopaper® (Pro-Flow Corp., Amityville, NY, USA) was inserted into the gingival sulcus or periodontal pocket until mild resistance was felt, and left in place for 30 seconds. Strips contaminated by saliva or blood were excluded. Using a device for impedance measurement (Periotron 6000, ProFlow), the sampled fluid volume was measured, and the paper strips were pulled and inserted in sterile plastic tubes (Eppendorf®) containing 250µl RNAse free water. The samples were immediately frozen and stored at -70˚C until analyses.

miR expression measurements

Total RNA was extracted by using miRCURY™ RNA Isolation Kits – Biofluids (Exiqon A/S, Vedbaek, Denmark), according to the manufacturer's instructions.

For the reaction of reverse transcription and quantitative real-time PCR (qRT-PCR) we have used 10 ηg of total RNA, TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA), Taqman Universal master mix, No UNG amperase, and specific assays: hsa-miR-146a (ID:000468); hsa- miR-155(ID: 002623) and RNU6B (ID:001093), using standard temperature conditions. All reactions were run on Real Time PCR 7500 (Applied Biosystems, Foster City, California, USA). Expression levels of miRs were presented in relative units, and normalized to RNU6B, as the endogenous control. Comparison of RNU6B values between the groups showed no significant difference ($p>0.05$, Mann-Whitney test)

As a calibrator reference (1X sample), the sample with the lowest relative quantity (RQ) value was used, and data were analyzed with the 7500 System SDS Software (Applied Biosystems, Foster City, California, USA), by comparative ΔΔCt method.

SOD activity measurements

SOD activity (%) in GCF was measured by SOD determination kit (Sigma-Aldrich, USA) according to manufacturer instructions.

Statistical analysis

Results were expressed as mean ± SD (clinical parameters) or as median values. Patients characteristics were compared with nonparametric tests: Mann-Whitney for continuous and cross tables were constructed for dichotomous variables. Differences between groups in clinical parameters, miRs expression levels and activity of SOD were compared by one way ANOVA and Bonferroni post hoc test. The Spearman and Pearson correlation tests were used to asses association between clinical variable (PPD) and SOD activity with miRs expression levels, respectively. Multiple linear regression analysis was used with the relative

expression of miRs as dependent and periodontitis, age and gender as independent variables. In order to evaluate accuracy of the miR-146a and miR-155 in differentiating healthy from moderate to severe periodontitis patients, the receiver operating characteristic (ROC) curves were constructed by calculating the area under the curve (AUC) and identifying cut-off points to estimate the highest sensitivity and specificity as assessed by Youden's index. Calculations were performed using software package (SPSS 18.0, IBM Statistics, USA) and differences were considered significant at $p<0.05$

Results

Characteristics of the subjects

The mean age of participants was significantly higher in the periodontitis groups, both in nondiabetic and type 2 diabetic, in comparison with the groups of periodontally healthy participants. There were no significant differences between gender distribution within and between the groups. Patients showed moderate overweight with no differences in mean BMI between the groups observed (Table 1). The full mouth and sample sites PPD, CAL, BOP, GI, and PI were statistically greater, reflecting moderate periodontitis (CDC/AAP classification, Page and Eke 2007) in the CP and CPDM groups compared to periodontally healthy groups (PH and PHDM) ($p<0.01$),

Mean PPD and CAL values were significantly higher in periodontitis patients with type 2 DM compared with nondiabetic periodontitis patients, while PI, GI, and BOP were not notably greater. All patients responded well to the non-surgical therapy as shown by decreased values of periodontal inflammation parameters (Table 1).

miR-146a and miR-155 expression levels in GCF of nondiabetic and type 2 diabetic subjects

miR-146a levels were higher in type 2 diabetic compared to nondiabetic periodontally healthy patients . In CP and CPDM patients, miR-146a and miR-155 levels were significantly enhanced before non-surgical therapy while the levels decreased after the therapy to the levels comparable to periodontally healthy patients (Figure 1). Multiple linear regression analyses revealed that periodontitis contributed significantly to the GCF expression levels of miR-146a and miR-155, when adjusting for age and gender (Table 2). There were no significant correlations between BMI values and investigated miRs (Supplementary material). The accuracy of miR-146a and miR-155 as GCF biomarkers of periodontitis is illustrated with ROC curves (Figure 2) and their respective values (Table 3). The ROC curves for both miRs in nondiabetic and type 2 diabetic patients showed a high accuracy for CP since AUC≥ 0.9. miR-146a showed the best performance with a sensitivity of 100% , a specificity of 96% , at a cutoff point of 4.11 au (arbitrary units) in nondiabetic CP patients. In DM type 2 patients, miR-155 showed better performance compared to miR-146a with a sensitivity of 92%, a specificity of 100% at cut off point of 3.56 au (Table 3). miR-146a showed strong positive correlation with PPD in both nondiabetic and type 2 diabetic periodontitis patients (Spearman r= 0.676 and r= 0.641, respectively, $p< 0.01$). Also, miR-155 showed significant positive correlation with PPD in the group of nondiabetic and type 2 diabetic periodontitis patients (Spearman r= 0.623 and r= 0.645, respectively, $p< 0.01$) .

SOD activity

Activity of major antioxidant enzyme, SOD, is significantly enhanced in CP and CPDM patients baseline and decreased six weeks after non-surgical therapy to the levels comparable to periodontally healthy (Figure 3). There are strong positive correlations of measured SOD

activity with miR-146a (Pearson r = 0.735, R^2= 0.540, p< 0.001 in CP and Pearson r = 0.552, R^2= 0.304, p < 0.001 in CPDM patients) and miR-155 (Pearson r = 0.693, R^2= 0.480, p< 0.01 in CP and r = 0.623, R^2= 0.388, p< 0.001 in CPDM patients).

***In silico* analysis**

We used ComiR, a web-based software for the combinatorial prediction of mutual targets for miR-146a and miR-155 (Coronnello & Benos, 2013). ComiR calculates the probability of mRNAs to be targeted by a set of miRNAs. ComiR results for the two examined miRNAs from our study (expression levels input data), showed that SOD2 mRNA has the high probability to be recognized (equal abundance = 0.924) suggesting that miR-146a and miR-155 in combination may have exceptionally strong probability to silence SOD2 transcription.

Discussion

Research based on miRs involvement in periodontal disease is in its infancy. This is, to our knowledge, the first study investigating potential of circulatory miRs, obtained in GCF, as biomarkers of periodontal disease. We have quantified miRs expression in GCF because it represents an inflammatory exudate that reflects an ongoing processes in inflamed periodontium more reliably than saliva or serum. miRs persist in body fluids as circulatory and exosomal (Zhang et al. 2015) and participate in intercellular communication and immune regulatory function (Mittelbrunn and Sanchez-Madrid, 2012). Xie et al. showed that miR-146a and miR-155 are present in periodontal tissues (Xie et al. 2011), and since activation of macrophages is strongly dependent on their presence (Jablonski et al. 2016, O'Connell et al. 2007), it could be assumed that inflammatory cells along with periodontal tissue cells and systemic circulation represent possible sources of these miRNAs in GCF.

Present results show that in nondiabetic and type 2 diabetic periodontitis patients, miR-146a and miR-155 show significantly higher levels compared with periodontally healthy and that non-surgical periodontal therapy significantly reduced expression levels of both miRs in GCF reflecting the reduction of periodontal inflammation observed clinically. Considering the higher age of subjects in the periodontitis groups relative to the periodontally healthy subjects, we used multiple regression analyses adjusted for age in order our results to be applicable to younger patients, also.

Levels of both, miR-146a and miR-155 could differentiate between healthy and moderate to severe periodontitis patients with high accuracy based on ROC curves analysis pointing at better performance of miR-146a in nondiabetic, and miR-155 in type 2 diabetic patients. In the previous studies, Motedayyen et al. showed upregulation of miR-146a in the gingiva of patients with chronic periodontitis while Xie et al. showed upregulation of miR-146a and downregulation of miR-155 levels in human inflamed gingival tissues collected during periodontal flap surgery, and suggested a complex role of these miRs in gingival inflammation (Motedayyen et al. 2015, Xie et al. 2011). Also, Nahid et al. reported that periodontal pathogens induced *in vitro* persistent miR-146a and less frequent miR-155 expression in the mice maxilla and spleen (Nahid et al. 2011). The complexity of the miR-146 and miR-155 actions in inflammation has been elegantly analyzed in work by Schulte et al. showing differential activation of miR-146 and miR-155 in murine macrophages (Schulte et al. 2013). Namely, the authors found that upon LPS stimulation, miR-146 and miR-155 act in two- tier manner: miR-146 is firstly activated at low LPS doses while miR-155 is gradually induced to full expression and represents limiter of proinflammatory gene expression when the miR-146-dependent anti-inflammatory effect has been breached. In order to investigate regulatory roles of these two miRs in periodontitis patients we used *in silico* analysis (Coronnello & Benos, 2013). The obtained results showed that these two miRs

in combination may have exceptionally strong probability to regulate SOD2 gene expression, suggesting miR-146a and miR-155 action in periodontal disease may involve regulation of oxidative stress. Namely, SOD2 is an mitochondrial antioxidant enzyme but mainly, all three types of SOD act in the same manner, by catalyzing neutralization of superoxide anions to oxygen and H_2O_2. In the present study, we measured SOD activity which reflects ROS generation, and found it to be highly positively correlated with miR-146a and miR-155 expression levels in both, nondiabetic and type 2 diabetic periodontitis patients. Indeed, in *in vitro* experiments (PC 12 cell line) showed that H_2O_2, the product of SOD activity, induced expression of miR-146a (Ji et al. 2013) which then feedback-represses SOD2 gene expression. Moreover, in the present study, reduction of inflammation in periodontal tissues after non-surgical therapy decreases levels of SOD activity as well as miR-146a and miR-155 expressions. Very recent study showed that circulatory miR-21, miR-23a and miR-222 regulate expression of SOD2, enzyme described as a cell protector in the diabetic cardiomyopathy (Dubois-Deruy et al. 2017, Shen et al. 2006).

In GCF of type 2 diabetic patients with no signs of periodontal disease we found higher miR-146a expression levels compared to nondiabetic periodontally healthy subjects. In line with this, Rong et al (2013) found upregulated plasma circulatory levels of miR-146a in DM type 2 patients and suggested that expression of circulating miR-146a could hold predictive value for DM type 2 (Rong et al. 2013). Moreover, it seems that upregulation of miR-146a in DM exerts a protective effect by downregulating NF-κB's target genes such as TNF alpha thereby reducing inflammation (Ye & Steinle 2016, Bhatt et al. 2016). In the present study, periodontitis patients with DM type 2, although with well controlled glycemia, showed more severe and extensive periodontal inflammation. Usually, such findings were explained by increased inflammatory response to bacteria in DM (Graves et al. 2007) but mechanisms are not elucidated. The present results showing no difference in miR-146a expression in CPDM

compared to CP suggest complex engagement of miR-146a in immunomodulation depending on a different physiopathological context (periodontitis and/or DM) and point at importance of miR-155 co-involvement in CPDM. Schulte et al. showed that as a consequence of differential regulation, upregulated miR-146 under the invasion of bacteria may augment the initial LPS threshold required for miR-155 activation (Schulte et al. 2013). In such context, it seems possible that upregulated miR-146a in type 2 diabetic patients observed presently, when exposed to periodontal pathogens may postpone the anti-inflammatory effect of miR-155 resulting in augmented inflammation in periodontal tissues. On the other hand, there is a proposition that circulatory miRs, released in the body fluids could be taken up by neighbor or distant endothelial or immune cells acting as local or systemic communication tools (Ling & Groop 2009, Zhang et al. 2015). Under the diabetic condition, hyperglycemia or inflammation exert epigenetic alterations in host tissues which in turn change circulating miRs profiles and disseminate incorrect epigenetic signature to neighbor or distant cells, a phenomenon defined as epigenetic damage transmission (Prattichizzo et al. 2016). Therefore, it seems reasonable to assume that alteration of miR-146a expression in GCF of type 2 diabetic patients could affect the inflammatory milleu of neighbour tissues resulting in more severe periodontal damage in DM patients.

Limitation of the present study was that we didn't evaluate serum miR-146a levels in order to investigate whether GCF miR-146a levels may reflect its systemic levels in type 2 diabetic patients. However, based on previous (Rong et al. 2013 and Baldeón et al. 2014) as well as on our present study, it may be possible to resume that obesity and/or dyslipidemia status, the stage of type 2 diabetes as well as an associate inflammatory events affect circulatory miR-146a expression and therefore further investigation is needed in order to qualify miR-146a as reliable biomarker of type 2 DM.

It is noteworthy that recent studies showed that host immune reactions against periodontal bacteria could promote the development of atherosclerosis associated with diabetic vascular disease (Aarabi et al. 2015, Reyes et al. 2013). The underlying mechanism proposed include activation of T helper17- inducing pathways involved in atherosclerotic plaque formation (Cai et al. 2014). Having in mind that periodontal bacteria may activate T helper17 by upregulating miR-155 (Du et al. 2014), it seems reasonable to speculate that downregulation of miR-155, achieved by non-surgical periodontal therapy as shown presently, may be beneficial in reducing risk for vascular disease development.

In conclusion, the present study is the first to provide evidence that GCF expression levels of both miR-146a and miR-155, could be considered as potential biomarkers for periodontal disease in nondiabetic and type 2 diabetic periodontitis patients. Furthermore, we found the association of these gene regulators with oxidative stress mechanisms. Further elucidation of the detailed miRNA-146a and miRNA-155 mechanisms involved in the periodontal inflammation, especially in the setting of DM could expand the knowledge of the pathogenesis of periodontal disease and enable development of improved treatment strategies.

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***Table* 1. Demographic characteristics and clinical parameters before and after treatment in the study groups**

Group	Mean age and BMI	Sex M/F		Before treatment PPD (mm)	CAL (mm)	PI	GI	BOP (%)	After treatment PPD (mm)	CAL (mm)	PI	GI
CPDM N=24	54.9 25.45	10/14	Full mouth	4.79 ± 0.45#	5.16 ± 1.32#	2.38 ± 0.28#	2.35 ± 0.43#	72.5 ± 8.3#	3.15 ± 0.35‡	4.37± 1.18‡	0.46± 0.24‡	0.67±
			Sampled sites	4.71 ± 0.33#*	6.17 ± 0.16#*	2.25 ± 0.36#	2.55 ± 0.50#	100.00 ± 0.00#	3.11 ± 0.51‡	4.12 ± 1.43‡	0.36± 0.46‡	0.51±
CP N=24	54.7 27.31	13/11	Full mouth	4.42 ± 0.24**	4.92 ± 0.11**	2.21±0.18**	2.36±0.43**	76.5 ± 8.7**	3.26 ± 0.49‡	4.19 ± 1.23‡	0.50± 0.35‡	0.86±
			Sampled sites	4.33 ± 0.22**	5.85 ± 0.35**	2.16±0.49**	2.58±0.39**	100.00 ±0.00**	2.34 ± 0.28‡	4.11 ± 0.85‡	0.39± 0.43‡	0.54±
PHDM N=24	33.2 26.93	10/14	Full mouth	2.43 ± 0.42	2.40 ± 0.40	0.15 ± 0.10	0.18 ± 0.07	9.6 ± 4.3				
			Sampled sites	2.25 ± 0.23	0.63 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00				
PH N=24	33.4 26.37	11/13	Full mouth	2.17 ± 0.30	2.24 ± 0.45	0.18 ± 0.08	0.16 ± 0.08	8.7 ± 5.4				
			Sampled sites	1.77 ± 0.24	0.48 ± 0.38	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00				

PPD, probing pocket depth; CAL, clinical attachment level; BOP, bleeding on probing; PI, plaque index; GI, gingivitis index

Data are expressed as the mean ± standard deviation. ‡ $p< 0.01$ before vs after non-surgical treatment.** $p< 0.01$ CP compared to PH .# $p<0.05$ CPDM compared to PHDM *$p<0.05$ CP compared to CPDM

***Table* 2. Multiple linear regression analysis of the association between miR-146a and miR-155 expression in gingival crevicular fluid (GCF) and periodontitis, adjusted for age and gender in nondiabetic and type2 diabetic patients**

Dependent variable	Independent variable	Estimate	SE	p value	95% CI
miR-146a	Periodontitis	0.950	0.806	< **0.001**	3.629 to 6.879
	Age	-0.118	0.032	0.422	-0.090 to 0.039
	Gender	-0.071	0.426	0.363	-1.251 to 0.467
miR-155	Periodontitis	0.953	0.792	< **0.001**	2.444 to 5.637
	Age	-0.286	0.031	0.132	-0.112 to 0.015
	Gender	-0.160	0.419	0.112	-1.522 to 0.166
miR-146a (diabetic)	Periodontitis	-0.882	1.111	< **0.001**	-6.518 to -2.041
	Age	-0.174	0.046	0.454	-0.127 to 0.058
	Gender	0.075	0.527	0.485	-0.691 to 1.433
miR-155 (diabetic)	Periodontitis	-1.013	0.939	< **0.001**	-6.983 to -3.199
	Age	-0.211	0.039	0.269	-0.121 to 0.035
	Gender	0.063	0.445	0.473	-0.575 to 1.220

***Table* 3. Accuracy of miR-146a and miR-155 as biomarkers of chronic periodontitis in nondiabetic and type 2 diabetic patients**

Biomarker	AUC	Cutoff	Sensitivity	1-Specificity	Youden`s index
miR-146a (CP)	0.999	4.11	1.00	0.04	0.96
miR-146a (CPDM)	0.952	5.45	0.88	0.04	0.83
miR-155 (CP)	0.929	2.70	0.96	0.21	0.75
miR-155 (CPDM)	0.988	3.56	0.92	0.00	0.92

AUC- area under the curve; CP- chronic periodontitis; CPDM- chronic periodontitis associated with diabetes mellitus type 2;

Figure 1. Relative expression levels of microRNA-146a (miR-146a) and microRNA-155 (miR-155) in GCF samples. Boxplots represent levels of miR-146a and miR-155 in GCF of periodontal healthy: nondiabetic (PH) and type 2 diabetic (PHDM) as well as in patients with periodontitis: nondiabetic (CP) and type 2 diabetic (CPDM) patients, baseline and six weeks after non-surgical therapy.

* $p< 0.05$ and ** $p< 0.01$ compared to PH; $^{\#}$ $p< 0.05$ compared to PHDM patients;

‡$p< 0.01$ before vs after non-surgical periodontal therapy.

Figure 2. The receiver operating characteristic (ROC) curves for differentiating healthy from chronic periodontitis patients by using expression levels of microRNA-146a (miR-146a) in nondiabetic (A) and type 2 diabetic (B) patients and of microRNA-155 (miR-155) in nondiabetic (C) and type 2 diabetic (D) patients obtained by logistic regression analysis.

Figure 3. The activity of superoxide dismutase (SOD) in GCF samples. Boxplots represent the SOD activity in GCF of periodontal healthy: nondiabetic (PH) and type 2 diabetic (PHDM) as well as in patients with periodontitis: nondiabetic (CP) and type 2 diabetic (CPDM) patients, baseline and after non-surgical therapy.

** $p< 0.01$ compared to PH;

$^{\#}p< 0.01$ compared to PHDM patients;

‡ $p< 0.01$ baseline vs after non-surgical periodontal therapy.