OBESITY AS A POTENTIAL MODIFIER FOR PERIODONTAL INFECTION PATHOGENESIS

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PART 1

The relationship between periodontal disease and the presence of metabolic disorders among adults in the Atherosclerosis Risk in Communities (ARIC) study

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THE RELATIONSHIP BETWEEN PERIODONTAL DISEASE AND THE PRESENCE OF METABOLIC DISORDERS AMONG ADULTS IN THE ATHEROSCLEROSIS RISK IN COMMUNITIES (ARIC) STUDY

Abstract

AIM: To examine the association between periodontal disease and two metabolic disorders (obesity, defined by Body Mass Index (BMI) and impaired fasting glucose (IFG)), using a cross-sectional observational study design.

MATERIALS AND METHODS: Participants (n= 6780) who had a periodontal exam were selected from the Atherosclerosis Risk in Communities (ARIC) study. The Dental ARIC also consisted of collection of gingival crevicular fluid (GCF), oral plaque and serum. Level of disease was either classified by the CDC-AAP definition or Biofilm Gingival Interface (BGI) classification.

RESULTS: CRP and IL-6 was statistically significant in any level of disease in obese patients (*P* <0.0001). D8Iso (*P*= 0.04) and sICAM-1 (*P*= 0.04) were significant in participants classified with moderate level disease (CDC-AAP) while sCD14 (*P*= 0.06) approached significance. No significant differences were observed between groups for all tested GCF mediators suggesting a more important systemic inflammatory component rather than local tissue effect. Increased odds for gingivitis (OR 1.94, 1.27-2.95) and BGI-P3 disease (OR 2.28, 1.49-3.50) was identified for patients that were non-obese and diabetic. Also, an increased likelihood for gingivitis and BGI-P3 was found in obese

patients that were euglycemic (OR 1.39, 1.07-1.80 and OR 1.67, 1.27-2.19, respectively) or had diabetes (OR 1.81, 1.24-2.64 and OR 2.31, 1.57-3.39, respectively).

CONCLUSION: Important associations between diabetes and periodontal disease and obesity and periodontal disease were reaffirmed. For the first time, increased likelihood for gingivitis was identified in both patients with diabetes and an obese BMI. Reaffirmation of the critical role of CRP and IL-6 on systemic inflammatory burden was shown in an obese population. Prevention and management of obesity and diabetes should be adjunctive treatment approaches to improving overall oral health.

INTRODUCTION

The prevalence of obesity, defined as a Body Mass Index (BMI) $\geq$ 30.0 kg/m^2 (Keys et al. 1972; Mora et al. 2005), has dramatically increased in children and adults both in the United States and throughout the world. The National Health Examination Survey I (NHES I) study (1960-1962) reported that the age-adjusted (18-74 years old) prevalence of adult obesity in the U.S. was 13.4% (Flegal et al. 2002; Ogden et al. 2006). In 2007-2008, the National Health and Nutrition Examination Survey (NHANES) reported that the age-adjusted prevalence of obesity nearly tripled to 33.8% (95% confidence interval [CI], 31.6%-36.0%) overall, 32.2% (95% CI, 29.5%-35.0%) among men, and 35.5% (95% CI, 33.2%-37.7%) among women (Flegal et al. 2010).

Obesity has been recognized as an important risk factor for numerous chronic adult diseases, including non-insulin dependent diabetes mellitus (NIDDM), hyperlipidemia, hypertension, arteriosclerosis and cardiovascular and cerebrovascular diseases (Kopelman 2000). Recently, increased interest in the link between obesity and oral health, has suggested that obesity is also associated with periodontitis (Perlstein and Bissada 1977; Saito et al. 1998, 2001, 2005; Al-Zahrani et al. 2003; Wood et al. 2003; Della Vecchia et al. 2005; Genco et al. 2005). Adipose tissue secretes hormones and cytokines that are intimately involved in inflammatory processes suggesting that similar pathways may be involved in the pathophysiology of obesity, diabetes and periodontitis (Pischon et al. 2007) (Figure 1). If obesity is a true risk factor for periodontal disease, the

associations among periodontal disease, obesity and diabetes mellitus must be very complex because each is known to be associated with the other.

FIGURE 1

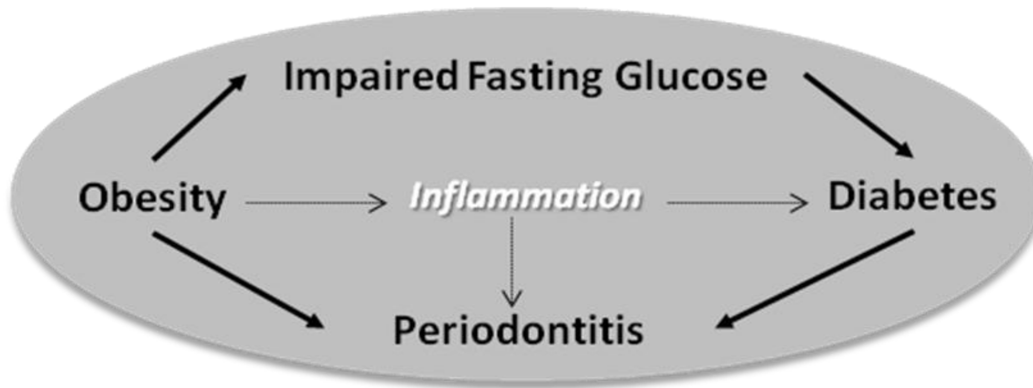

Figure 1: Relationships among obesity, diabetes and periodontal disease.

Atherosclerosis Risk in Communities (ARIC) Study

The ARIC study is a prospective investigation, supported by the National Heart Lung and Blood Institute, of the etiology and natural history of atherosclerosis and clinical cardiovascular disease in four US communities (Jackson, Mississippi; Washington County, Maryland; suburban Minneapolis, Minnesota; and Forsyth County, North Carolina). A sample of 15,792 community-dwelling residents aged 45 to 64 years at baseline took part in the evaluation of cardiovascular risk factors and their sequelae (The ARIC Investigators, 1989). The Dental ARIC (DARIC), a cross-sectional, ancillary study funded by the National Institute of Dental and Craniofacial Research, was conducted during ARIC Visit 4 in 1996 to 1998. The aims of the study were to determine the prevalence, extent and severity of periodontal conditions in the dentate ARIC population and to describe the associations between those conditions and prevalent coronary heart disease, carotid artery intima-media thickness, presence of carotid artery lesions, and atherosclerosis risk factors. DARIC consisted of an oral examination, collection of gingival crevicular fluid, oral plaque, serum and interviews (Beck et al. 2001).

The implications of obesity for cardiovascular disease have been well established with evidence from the ARIC study (Folson et al. 1991; Yatsuya et al. 2010). The relationship between periodontal disease and obesity in these subjects has yet to be investigated. The potential association of this increasingly common systemic disease highlights the possible benefit of identifying a modifiable risk factor related to oral health that may lead to overall health.

BACKGROUND

Obesity and its Association to Metabolic Disorders

An increased BMI is associated with an increase in the size and number of adipocytes (Mohamed-Ali et al. 1997). Research in the field of obesity has determined that adipose tissue is an endocrine organ (Ahima et al. 2000) and a key participant of the immune system (Wellen et al. 2005). It can secrete free fatty acids (Boden et al. 2003), inflammatory cytokines (Hotamisligil et al. 1996; Fain et al. 2004), adipokines (leptin, adiponectin, resistin) (Zhang et al. 1994; Maeda et al. 1996; Kusminski et al. 2005) and plasminogen activator inhibitor type-1 (Shimomura et al. 1996). Reports have shown that production of these factors is more pronounced in obese individuals (Weisberg et al. 2003; Bouloumié et al. 2005). Possibly due to these changes, a low-grade chronic systemic inflammation is observed.

In comparison to people classified as having a normal BMI, obese people have a greater than 10-fold increased risk of developing NIDDM (Field et al. 2001; Pischon et al. 2007). Similarly, obesity has long been recognized as an important determinant of hypertension. Obese people have a 5 times higher risk of hypertension (Wolf et al. 1997) and a 1.5-fold increased risk for cardiovascular disease (Wilson et al. 2002).

Periodontal Disease and Systemic Inflammation

Periodontal disease, a chronic infection and inflammatory condition that affects tooth-supporting tissues, may cause local and systemic inflammatory events. Systemic

dissemination of the periodontopathogens or their products may induce bacteremia or endotoxemia, which in turn stimulates and increases levels of serum inflammatory markers (Wu et al. 2000). Evidence suggests that periodontal diseases can induce or perpetuate an elevated systemic chronic inflammatory state, as reflected in increased serum CRP, IL-6, and fibrinogen levels seen in many people with periodontitis (D'Aiuto et al. 2004; Loos et al. 2000). Nishimura and coworkers (2000) showed that serum TNF-α concentrations were elevated in periodontitis patients as compared to periodontally healthy subjects. Those periodontal patients with elevated TNF-α serum levels had reduced levels after periodontal therapy. In addition, two analyses using NHANES III data reveal that obesity measures significantly correlated with periodontal disease severity in adults (Della Vecchia et al. 2005; Genco et al. 2005).

Association between Obesity and Periodontal Disease

The first paper on the relationship between obesity and periodontal disease was published as early as 1977. An experimental ligature-induced periodontitis model was used in Zucker rats (Perlstein and Bissada, 1977). Greater alveolar bone resorption was seen in obese animals as compared with non-obese rats. Further, the response to bacterial plaque accumulation, periodontal inflammation and destruction was more severe in obese animals (Perlstein and Bissada, 1977).

This association has largely been supported by epidemiological studies in Brazilian, Japanese and US populations. Saito et al. was the first to find an association between obesity and periodontal disease in humans (Saito et al. 1998). A periodontal assessment of 241 healthy Japanese by applying the community periodontal index of

treatment needs (CPITN), led to determine that the relative risk for periodontitis in overweight persons (BMI 25-29.9 kg/m^2) was 3.4 and 8.6 in obese persons, after adjustment for age, gender, oral hygiene status and smoking (Saito et al. 1998). In fact, one study suggested that obesity is the second strongest risk factor, after smoking, for periodontal disease (Nishida et al. 2005). Studies have also reported that fat distribution is a significant consideration in the association with periodontitis. High upper body obesity and high total body fat were correlated with a higher risk of periodontal disease, compared with normal weight (Saito et al. 2001). NHANES III data demonstrated that waist-to-hip ratio, BMI, fat-free mass, and log sum of subcutaneous fat significantly correlated with periodontal disease (Wood et al. 2003). Further analysis of the NHANES III data shows that BMI positively correlated with severity of periodontal attachment loss and that this relationship was modifiable by insulin resistance (Genco et al. 2005).

Diabetes and Periodontal Disease

Several well-characterized adipokines are essentially the same as pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Obese individuals have elevated production of TNF- α and IL-6 and these increases are also important in the pathogenesis of insulin resistance (Festa et al. 2003). In addition, increased levels of inflammatory cytokines lead to increased C-reactive protein (CRP) production in the liver, which may impact insulin resistance as well (Gabay et al. 1999). Recruited macrophages, in co-operation with adipocytes, secrete large quantities of proinflammatory cytokines, leading to the formation of a vicious inflammatory cycle (Nishimura et al. 2000).

Impaired Fasting Glucose and Periodontal Disease

Impaired fasting glucose (IFG) refers to a condition in which the fasting blood glucose is elevated above normal levels but is not high enough to be classified as diabetes mellitus. It is a pre-diabetic state and has been associated with insulin resistance and increased risk of cardiovascular pathology. IFG can possibly progress to NIDDM. More recently, studies have begun to analyze the relationship between periodontal disease and the past development of glucose intolerance (Saito et al. 2004). Saito and colleagues assessed a group of 961 community-dwelling Japanese subjects aged 40-79 years and subsequently examined their glucose tolerance test results from 10 years earlier. A total of 415 subjects were identified with normal glucose tolerance, of which over the course of a decade, 72 developed glucose intolerance and nine subjects developed diabetes. In a multivariate analysis, it was concluded that impaired glucose tolerance was significantly associated with deeper periodontal pockets (Saito et al. 2004).

Previous epidemiological studies assessing the association between obesity and periodontal disease have been able to relate an obese BMI to increased risk for periodontal disease based solely on BMI measurement and adipose tissue distribution. This study will contribute novel findings regarding the associations of systemic inflammatory markers in gingival crevicular fluid and serum concerning DARIC participants categorized as non-obese and obese according with their periodontal status. To date, no other study with such an impressive number of study participants has been able to study the relationship between obesity and periodontal disease at the inflammatory level.

AIM

The proposed study examined the association between periodontal disease and two metabolic disorders (obesity defined as a BMI of $\geq$30kg/m^2; IFG defined as a fasting plasma glucose level from 5.6mmol/L (100 mg/dL) to 6.9 mmol/L (125 mg/dL)), using a cross-sectional observational study design.

HYPOTHESIS

The investigators hypothesized that both obesity and IFG (outcome variables) were associated with periodontal disease (exposure variable). Furthermore, it was presumed that the association between obesity and periodontitis was likely mediated through systemic inflammation and both these associations were likely modified by the extent of periodontal destruction.

MATERIALS AND METHODS

Study Population

The study cohort was selected from ARIC study participants attending Visit 4. Inclusion criteria were stipulated: (1) participants were aged ≥40 years at Visit 1, (2) participants must have been dentate and had both dentate status information and a periodontal examination at Visit 4, (3) participants must have had BMI measurements at Visit 4, and (4) participants must have had a serum sample available at Visit 4 in order to measure systemic inflammatory markers. No exclusion criteria were defined.

Cases were defined as patients presenting with periodontal disease. The presence of periodontal disease was determined based on the Centers for Disease Control and Prevention-American Academy of Periodontology (CDC-AAP) definitions for disease (Page and Eke, 1997) and the Biofilm-Gingival Interface (BGI) classification of disease (Offenbacher et al. 2007). Non-cases were defined as patients without periodontal disease and were identified from the same study population.

Variables of Interest

Obesity: Obesity status was determined by the Body Mass Index (BMI) whereby $\geq 30 kg/m^2$ is classified as obese. All participants with a BMI of $<30 kg/m^2$ were clustered into one category labeled as non-obese while all those with a BMI $\geq 30 kg/m^2$ were labeled as obese.

Impaired fasting glucose (IFG): IFG (or impaired fasting glycemia) was determined using the World Health Organization (WHO) definition of fasting plasma glucose level from 5.6 mmol/L (100 mg/dL) to 6.9 mmol/L (125 mg/dL). Euglycemic or normal fasting glucose is from 3.9 mmol/L (70 mg/dL) to 5.5 mmol/L (99 mg/dL), whereas a person is considered to have diabetes when testing on more than one occasion as $\geq$7.0 mmol/L ($\geq$126 mg/dL).

Periodontal status: Periodontal status was assessed according to the AAP-CDC definition of periodontal disease (Table 1A) and the BGI classification of periodontal disease (Table 1B) which does not incorporate attachment level measurements.

TABLE 1A & 1B

Table 1A. CDC-AAP Definition of Periodontal Disease

Disease Category	Clinical Criteria
Severe periodontitis	≥2 interproximal sites with clinical attachment loss (CAL) ≥6mm (not on the same tooth) AND ≥1 interproximal sites with probing depths (PD) ≥5mm
Moderate periodontitis	≥2 interproximal sites with clinical attachment loss (CAL) ≥4mm (not on the same tooth) OR ≥2 interproximal sites with probing depths (PD) ≥5mm (not on the same tooth)
No or mild periodontitis	Neither "moderate" nor "severe" periodontitis

Table 1B. BGI Classification of Periodontal Disease

Disease Category	Clinical Criteria
P3	subjects have one or more PD ≥4mm with extent BOP >50%
P2	one or more PD ≥4mm with extent BOP 10-50%
P1	one or more PD ≥4mm with extent BOP<10%
Gingivitis	subjects have all PD ≤3mm and extent BOP ≥10%
Healthy	subjects with all PD ≤3mm and extent BOP <10%

Systemic inflammatory markers: The following markers of systemic inflammation were measured in serum samples from Visit 4: high-sensitivity interleukin (IL)-6, high sensitivity C-reactive protein (CRP), and soluble intercellular adhesion molecule (sICAM)-1. All three markers have been demonstrated to be valid markers of acute systemic inflammation or infection. In addition, 8-PGF2α (d8iso) and a soluble form of CD14 (sCD14) were also assessed.

Other Potential Confounders: The following variables were also examined as potential confounders of the association between obesity and periodontal disease (or IFG and periodontal disease): race and ethnicity, trial center, gender, age, diabetes, smoking (5-levels), hypertension, education (3-levels), and income (3-levels). In addition, marital status, alcohol intake and dental care were also considered. These variables were collected by the parent ARIC study during Visit 4.

Statistical Methods

Descriptive statistics were performed using chi-square analysis and t-tests. Generalized logit regression will be used to examine the association between obesity or IFG among subjects without diabetes and periodontal disease status. The exposure variables, which are obesity or impaired fasting glucose (IFG), will be examined as dichotomous variables (e.g., non-obese vs. obese; IFG vs. euglycolic). Multiple logistic regression models will be used to control for confounding. The effects of interaction on periodontal status will be explored including potential interactions between IFG and obesity. In the generalized logistic models, each non-reference category was contrasted

with the reference category. In this case, each combination of obesity and diabetes status was contrasted with the group that was both non-obese and non-diabetic group.

RESULTS

Study population demographics

The study sample included 6780 subjects, recruited from four study centers, with a mean age of 62.7 years in the non-obese group and 61.8 years in the obese group (Table 2). A greater proportion of non-obese people presented to Visit 4 as non-diabetic and non-hypertensive in comparison to the obese study population ($P < 0.0001$). In addition, the majority of non-obese subjects had an advanced education level and high income in comparison to the obese group ($P < 0.0001$). A statistically significant difference between current heavy smokers and current drinkers was found between the groups with the majority in the non-obese group ($P < 0.0001$). The majority of non-obese study participants were currently under the care of a dentist (68.1%), sought routine dental care (70.9%) and their last examination was less than one year ago (69.5%). All factors were statistically significant in comparison to the obese participants ($P < 0.0001$).

TABLE 2

Table 2. Relationship of Study Variables to Obesity in the ARIC Study Visit 4 (N=6780)

	Non-Obese N* (%)		**Obese** N* (%)		***P*-value**
Race/Center					<0.0001
Jackson, MS	574	(50.6%)	560	(49.4%)	
Washington County, MD	1116	(65.9%)	577	(34.1%)	
Minneapolis, MN	1483	(68.6%)	672	(21.2%)	
Forsyth County, NC					
NC African-American	88	(64.7%)	48	(35.3%)	
NC Caucasian	1244	(77.1%)	369	(22.9%)	
Gender					0.002
Female	2403	(65.3%)	1276	(34.7%)	
Male	2135	(68.9%)	966	(31.2%)	
Mean Age (StdDev)	62.7	(5.71)	61.8	(5.41)	<0.0001
Married	3599	(67.8%)	1713	(32.3%)	0.005
Un-Married	924	(63.9%)	523	(36.1%)	0.005
Education					<0.0001
Basic	534	(58.4%)	381	(41.6%)	
Intermediate	1958	(67.2%)	957	(32.8%)	
Advanced	2041	(69.4%)	900	(30.6%)	
Income					<0.0001
Low	987	(60.3%)	650	(39.7%)	
Medium	1634	(67.9%)	774	(32.1%)	
High	1754	(70.8%)	725	(29.3%)	
Diabetes mellitus†					<0.0001
Yes	422	(45.1%)	514	(54.9%)	
No	4094	(70.6%)	1704	(29.4%)	
Hypertension‡					<0.0001
Yes	1270	(55.2%)	1032	(44.8%)	
No	3250	(73.0%)	1200	(27.0%)	
Smoking					<0.0001
Never	2047	(64.6%)	1121	(35.4%)	
Former Light	1006	(68.8%)	457	(31.2%)	
Former Heavy	706	(63.7%)	403	(36.3%)	
Current Light	118	(71.1%)	48	(28.9%)	

Current Heavy	486	(78.9%)	130	(21.1%)	
					<0.0001
Current Drinker					
Yes	2589	(71.0%)	1059	(29.0%)	
No	1937	(62.2%)	1175	(37.8%)	
Under care of dentist					<0.0001
No	415	(57.6%)	306	(42.4%)	
Yes	4097	(68.1%)	1923	(31.9%)	
Routine dental care					<0.0001
Regular DDS Utilization	3499	(70.9%)	1437	(29.1%)	
Episodic DDS Utilization	1016	(56.1%)	794	(43.9%)	
Last dental visit					<0.0001
< 1year	3643	(69.5%)	1600	(30.5%)	
≥ 1year	869	(58.1%)	627	(41.9%)	

*Totals not equal to N due to missing values.

†Fasting blood glucose ≥126 mg/dL, nonfasting ≥200 mg/dL, or self-reported history of diabetes, or evidence of diabetes medications.

‡Systolic ≥140 or diastolic ≥90 mmHg.

Periodontal measures of disease and obesity

A statistically significant difference between periodontal health and obesity was found in this population (Table 3). A greater percentage of non-obese participants had healthy periodontium while a greater proportion of obese patients presented with severe disease according to both the CDC-AAP definition for periodontal disease ($P = 0.02$) and BGI classification ($P < 0.0001$). A greater percentage of sites with probing depths (PD) ≥4mm and attachment loss (AL) ≥3mm was found in obese patients and this difference was statistically significant ($P < 0.0001$). Similarly, a greater proportion of obese subjects presented with higher scores for percentage of bleeding on probing, Gingival Index (GI) score ≥1 and a Plaque Index (PI) score ≥1 ($P < 0.0001$). However, when mean extent of PD>4mm, AL>3mm and BOP was adjusted for plaque score, the associations were no longer significant.

TABLE 3

Table 3. Relationship of Periodontal Measures of Disease and Obesity in the ARIC Study Visit 4 (N=6731)

Case Definitions	Non-Obese N* (%)		Obese N* (%)		*P*-value
CDC-AAP Definition					0.02
Severe	760	(16.7%)	421	(18.7%)	
Entry	1852	(40.8%)	941	(41.9%)	
Healthy or Mild	1926	(42.4%)	880	(39.2%)	
BGI Classification					<0.0001
P3	538	(11.8%)	340	(15.1%)	
P2	1767	(38.9%)	925	(41.2%)	
P1	894	(19.7%)	323	(14.4%)	
Gingivitis	675	(14.8%)	349	(15.5%)	
Healthy	664	(14.6%)	305	(13.6%)	
Mean (StdDev) Extent PD ≥4mm	6.92	(11.3)	8.27	(12.5)	<0.0001
Mean (StdDev) Extent AL ≥3mm	22.5	(22.8)	24.8	(23.8)	<0.0001
Mean (StdDev) Extent BOP	24.1	(22.9)	28.0	(25.0)	<0.0001
Mean (StdDev) Extent GI ≥1	30.3	(38.2)	43.2	(42.7)	<0.0001
Mean (StdDev) Extent PI ≥1	38.2	(36.9)	51.5	(39.4)	<0.0001

*Totals not equal to N due to missing values; column percents presented.

Serum and GCF inflammatory mediators and obesity

Statistically significant differences in valid markers of acute systemic inflammation or infection were found. Notably, CRP (*P* <0.0001), IL-6 (*P* <0.0001) and sICAM-1 (*P* = 0.02) were increased in the obese group (Table 4). Mean log serum mediators were stratified by the CDC-AAP definition and BGI classification of periodontal disease. CRP and IL-6 reached significance in all levels of disease suggesting that increased levels of these pro-inflammatory cytokines with obesity is independent of periodontal status and a true representation of systemic inflammation. In the CDC-AAP moderate periodontitis group, D8Iso (*P*= 0.04), sICAM-1 (*P*= 0.04) were significant along with CRP and IL-6 (data not shown).

GCF was collected from 179 participants to assess various inflammatory mediators (PGE2, IL-1ra, IL-1B, 1L-4, IL-5, IL-6, IL-8, 1L-10, TNFα, gCSF, MIP1a, MIP1b, RANTES, MCP1, IFNγ, IL-17, ENA78). No significant differences were observed between groups for all tested.

TABLE 4

Table 4. Mean Log Serum Mediators (StdDev) by Obese Status in the ARIC Study

		Non-Obese (StdDev)	**Obese (StdDev)**	***P*-value**
CRP	(N=5596)	0.55 (0.42)	0.75 (0.44)	<0.0001
IL-6	(N=5527)	0.37 (0.24)	0.46 (0.24)	<0.0001
D8Iso	(N=4893)	3.69 (1.87)	3.78 (1.85)	0.09
sICAM-1	(N=5516)	2.34 (0.33)	2.37 (0.34)	0.02
sCD14	(N=1292)	6.19 (0.32)	6.17 (0.29)	0.14

Periodontal measures of disease, obesity and diabetic status

In a generalized logit model, where the reference group had both non-obese and non-diabetic participants, the odds ratio was calculated for likelihood of having periodontal disease. Using the CDC-AAP definition of periodontal disease, a significant odds ratio was found in participants with moderate and severe levels of disease who were non-obese and diabetic (moderate level OR=1.64, 95%CI 1.30-2.08; severe level OR=2.04, 95%CI 1.54-2.69), and obese and diabetic (moderate level OR=1.39, 95%CI 1.13-1.71; severe level OR=1.62, 95%CI 1.26-2.10) (Table 5a). Obese participants had an increased likelihood for severe level of disease (OR 1.24, 95%CI 1.04-1.49) despite being non-pre-diabetic and non-diabetic. An increased odds for gingivitis was identified when using the BGI classification for participants that were non-obese and diabetic. In a fully adjusted model, an increased likelihood for gingivitis was found in obese participants that were euglycemic or had diabetes (Table 5b). Increased risk for BGI-P3 disease was found in patients that were non-obese and had diabetes and for all levels of glycemic control in the obese participants.

TABLE 5A

Table 5a. Logistic Regression Models of the Crude Associations between Obesity, Glycemic Control with Periodontitis Case Status According to Euglycemic, IFG and Diabetes Status

	Non-Obese (OR 95%CI)*		**Obese (OR 95%CI)***		
	IFG	**Diabetic**	**Euglycemic**	**IFG**	**Diabetic**
***CDC-AAP Definition*†**					
Moderate	1.23 (0.97-1.56)	**1.64 (1.30-2.08)**	1.12 (0.98-1.29)	1.18 (0.93-1.48)	**1.39 (1.13-1.71)**
Severe	1.27 (0.94-1.73)	**2.04 (1.54-2.69)**	**1.24 (1.04-1.49)**	1.32 (0.98-1.76)	**1.62 (1.26-2.10)**
***BGI Classification*††**					
Gingivitis	0.97 (0.66-1.43)	**2.09 (1.40-3.12)**	1.15 (0.90-1.45)	1.31 (0.90-1.89)	1.35 (0.96-1.91)
P1	0.79 (0.55-1.14)	1.17 (0.78-1.77)	0.851 (0.67-1.07)	0.64 (0.43-0.95)	0.73 (0.51-1.05)
P2	0.91 (0.66-1.25)	**1.48 (1.03-2.13)**	1.21 (1.00-1.48)	1.09 (0.79-1.50)	1.18 (0.88-1.59)
P3	1.23 (0.83-1.82)	**2.95 (1.97-4.39)**	**1.38 (1.07-1.76)**	**1.67 (1.15-2.44)**	**2.16 (1.54-3.03)**

* Reference group= non-obese, non-pre-diabetic, non-diabetic subjects
† Reference group= health-mild periodontitis
†† Reference group= healthy

TABLE 5B

Table 5b. Logistic Regression Models of the Fully Adjusted Associations between Obesity, Glycemic Control with Periodontitis Case Status According to Euglycemic, IFG and Diabetes Status

	Non-Obese (OR 95%CI)*		Obese (OR 95%CI)*		
	IFG	**Diabetic**	**Euglycemic**	**IFG**	**Diabetic**
***CDC-AAP Definition*†**					
Moderate	1.10	**1.58**	**1.23**	1.21	**1.50**
	(0.85-1.41)	**(1.23-2.03)**	**(1.06-1.43)**	(0.94-1.55)	**(1.19-1.89)**
Severe	0.96	**1.65**	**1.41**	1.24	**1.59**
	(0.68-1.35)	**(1.21-2.26)**	**(1.16-1.73)**	(0.90-1.72)	**(1.19-2.14)**
***BGI Classification*†**					
Gingivitis	0.83	**1.94**	**1.39**	1.48	**1.81**
	(0.55-1.26)	**(1.27-2.95)**	**(1.07-1.80)**	(0.99-2.21)	**(1.24-2.64)**
P1	0.82	1.24	0.98	0.71	1.02
	(0.55-1.22)	(0.79-1.94)	(0.76-1.26)	(0.46-1.10)	(0.67-1.53)
P2	0.83	**1.58**	**1.55**	1.23	**1.70**
	(0.58-1.17)	**1.08-2.32)**	**(1.25-1.92)**	(0.86-1.74)	**(1.21-2.37)**
P3	0.88	**2.28**	**1.67**	**1.56**	**2.31**
	(0.57-1.36)	**(1.49-3.50)**	**(1.27-2.19)**	**(1.03-2.38)**	**(1.57-3.39)**

* Reference group= non-obese, non-pre-diabetic, non-diabetic subjects
† Reference group= health-mild periodontitis
†† Reference group= healthy

DISCUSSION

Periodontitis has long been considered a chronic inflammatory disease instigated by mostly anaerobic Gram-negative bacterial insult leading to gingival inflammation and bone resorption (Socransky and Haffajee, 1992). The classic model of periodontal disease has been altered due to emerging evidence that indicates systemic diseases may also influence the cellular and molecular mechanisms involved in periodontal pathogenesis. Numerous epidemiological studies have assessed these relationships between periodontal disease and systemic diseases. Of interest, associations between obesity and periodontitis have been demonstrated in population studies (Saito et al. 1998, 2001, 2004; Genco et al. 2005; Nishida et al. 2005). To our knowledge, the current available literature fails to demonstrate underlying biological mechanisms that may further elucidate the relationship between these chronic inflammatory diseases. We sought to examine the association between periodontal disease and metabolic disorders like obesity and IFG while also providing information on systemic and GCF inflammatory mediator production.

The periodontal status of DARIC study participants was classified using two different methods. The CDC-AAP definition (Page and Eke, 1997), which records probing depths and attachment loss, classifies patients as healthy-mild periodontitis, moderate periodontitis and severe periodontitis. It uses clinical measures alone to determine a patient's periodontal disease level whereas the BGI classification system was created by considering clinical measures of probing depth and BOP as those measures relate to microbial, inflammatory and host-response data for these subjects (Offenbacher

et al. 2007). With this classification, patients are categorized not only by clinical measurements but clustered according to biologic phenotypes.

Serum and GCF was collected at ARIC Visit 4 to detect important markers of acute systemic inflammation or infection. Increased levels of CRP, IL-6 and sICAM-1 were detected in obese DARIC participants and statistically significant differences were present compared to the non-obese subset of patients. We sought to determine if increased mediator production was present at a local and/or systemic level thus implicating local tissue effects due to periodontal disease or systemic inflammatory burden from an obese status. In so doing, detection of mediators was stratified by periodontal status (CDC-AAP definition or BGI classification). Overall, CRP and IL-6 remained significant in any level of disease in obese patients and only participants classified with moderate level (CDC-AAP) approached significance with other mediators such as D8Iso (P= 0.04), sICAM-1 (P= 0.04) and sCD14 (P= 0.06). The evidence from this study therefore suggests that important pro-inflammatory mediators like CRP and IL-6 have increased production solely due to obesity status and periodontal disease severity does not significantly alter these levels or levels of other mediators.

These findings reaffirm discoveries from previous studies regarding the importance of CRP and IL-6. IL-6 production has been linked to periodontal disease destruction by activating osteoclasts and promoting osteoclastic bone resorption (Yamamoto et al. 1997). CRP is produced by the liver in response to inflammatory factors produced and released by adipocytes (Lau et al. 2005). Recent results from a pilot study in the Periodontitis and Vascular Events (PAVE) Study indicated the importance of obesity on CRP levels (Offenbacher et al. 2009). An earlier study on the ARIC

population (Slade et al. 2003) showed that extensive periodontal disease and BMI were jointly associated with increased CRP levels. In our study, CRP levels in the obese population compared to normal weight remained significant even in patients with healthy-mild periodontitis.

No significant differences were observed in GCF measurements according to BMI or glycemic control. This may suggest that the local effect of glycemia may not be as important as previously thought in this study population. Conversely, Lalla et al. (2001) implied that the receptor for advanced glycation end products (RAGE), expressed on endothelium and mononuclear phagocytes, plays a crucial role in oral infection and inflammatory responses in a hyperglycemic state or diabetes. Similarly, diabetes has been shown to modulate gene expression in the gingival tissues of patients with chronic periodontitis (Duarte et al. 2007). Increased mRNA levels for IL-1B, IL-1ra, IL-6, IL-8, IFN-γ and RANKL were found in those gingival tissues, while IL-10 and osteoprotegerin expressed was decreased in diseased tissues. In this study, GCF was collected in a much smaller subset of participants (n=179) and this alone may make it difficult to assess any true differences between groups.

The association between obesity status (non-obese or obese), glycemic control (euglycemic, IFG or diabetic) and periodontal disease (CDC-AAP or BGI classification) was assessed. The role of glycemic control was shown in this study. Generally, participants with diabetes had increased odds for gingivitis and for CDC-AAP severe level or BGI-P3 level of disease, regardless of BMI. For the first time, it was demonstrated that participants with diabetes have increased risk for gingivitis as well as

periodontal disease (Figure 2). Interestingly, non-obese patients that had impaired fasting glucose did not present with an increased risk for any level of periodontal disease.

FIGURE 2

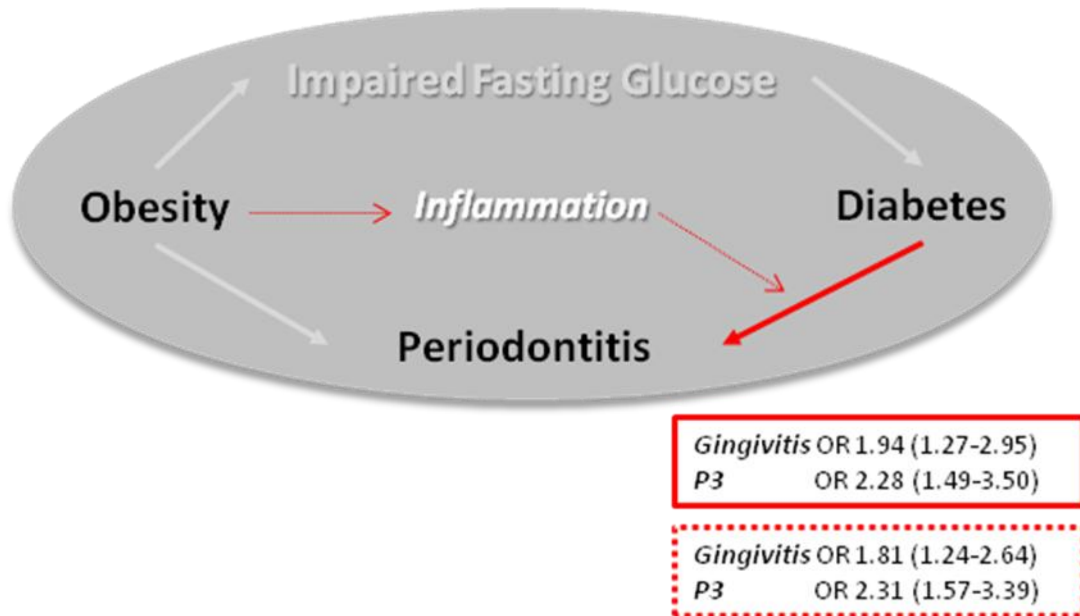

Figure 2: The association between diabetes, obesity and periodontal disease: *the estimated odds of gingivitis and P3 (BGI) in non-obese diabetics (solid red) and obese diabetic (dashed red) DARIC participants.*

In addition, it was shown that euglycemic obese participants have an increased risk for gingivitis (adjusted OR 1.39, 1.07-1.80) and CDC-AAP severe level or BGI-P3 level of periodontal disease (adjusted OR 1.67, 1.27-2.19) (Figure 3). It remains important to recognize that this increased risk was in the presence of normal glucose levels suggesting that the systemic inflammatory burden of obesity alone may contribute to this increased risk. Data from participants in the NHANES III study similarly present an adjusted odds ratio for having periodontal disease in obese subjects of 1.76 (1.18-2.61) (Al-Zahrani et al 2003). While the study participation was impressive in NHANES III (n=13 665), their definition for periodontal disease and half-mouth periodontal examinations likely underestimated disease presence.

The biological mechanisms that relate obesity and periodontitis largely remain unclear but we have shown consistently in this study increased and statistically significant differences in CRP and IL-6 production in the obese participants compared to the non-obese. Potentially contributing further to disease pathogenesis are the secretion of adipokines (leptin, adiponectin) and pro-inflammatory cytokines (TNF-α, plasminogen activator inhibitor 1, in addition to IL-6) secreted from adipose tissue and having paracrine and endocrine functions (Furugen et al. 2010). Toll-like receptor 4 (TLR4) present in adipocytes can either be stimulated by LPS or free fatty acids and subsequently enhances production of TNF-α and Il-6 which also contribute to systemic insulin resistance (Song et al. 2006).

FIGURE 3

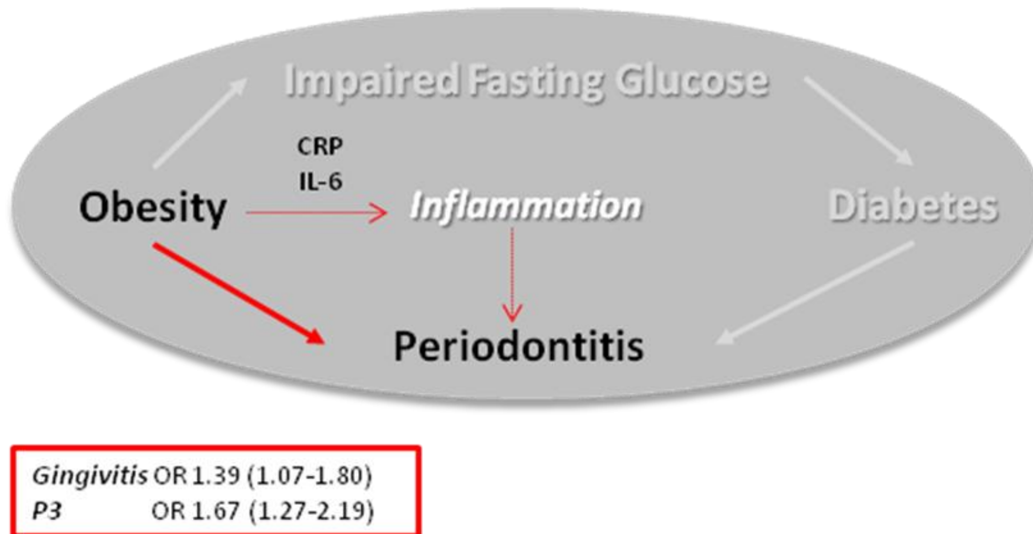

Figure 3: The association between obesity and periodontal disease: *the estimated odds of gingivitis and P3 (BGI) in obese non-pre-diabetic and non-diabetic DARIC participants.*

In the crude model (Table 5a) and the fully adjusted model (Table 5b), the P1 level participants did not show any increased risk for disease regardless of BMI or glycemic control. The level of plaque removal and better home care exemplified by P1 patients indicates that periodontal disease prevention is achievable despite other systemic influences that may increase risk for disease, namely obesity and diabetes.

The principle limitation of this study is its cross-sectional design. Causality therefore cannot be implied and only an association between obesity and periodontal disease or diabetes and periodontal disease was shown. Future studies should have longitudinal designs to determine if obesity is a risk factor for periodontitis. Secondly, dental history information was collected only at Visit 4. It was probable that study participants lost their teeth several years prior to attending the Visit 4 examination. Bias may have been introduced as a consequence of assuming that all edentulous patients lost their teeth at the time dental history was obtained. Patients included in DARIC had to have complete dental and periodontal examination information at Visit 4. This may introduce bias due to differential loss to follow-up prior to Visit 4 with respect to dentate status. Additionally, the only measure of obesity status in this study was BMI. Certainly participants who may have presented with a large muscle mass and high height to weight ratio would have been incorrectly categorized as obese despite possibly having low body fat. Other anthropometry measures like waist circumference and waist to hip ratio would have strengthened the finding of an obese status. Future investigations may seek to incorporate more measures of obesity status. Nevertheless, this study has many strengths namely, a large study population was recruited and data regarding other potential confounders is available from the parent study. It also should be noted that study subjects

were not patients, but were originally selected during Visit 1 to be representative of their community.

In conclusion, we found important associations between diabetes and periodontal disease as well as obesity and periodontal disease. For the first time, we showed that increased likelihood for gingivitis to be present in both subjects with diabetes and an obese BMI. Reaffirmation of the critical role of CRP and IL-6 on systemic inflammatory burden was shown in an obese population. These results further elucidate the importance of relationships between oral and systemic disease. Prevention and management of obesity and diabetes should be considered as adjunctive treatment approaches to improving overall oral health.

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PART 2

Differential expression of microRNA species in obese patients as a potential modifier of periodontal infection pathogenesis

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DIFFERENTIAL EXPRESSION OF MICRORNA SPECIES IN OBESE PATIENTS AS A POTENTIAL MODIFIER OF PERIODONTAL INFECTION PATHOGENESIS

Abstract

Obesity is an established risk factor for periodontitis but our understanding of the molecular pathogenesis of obesity as it relates to periodontal disease pathogenesis is unknown. Many microRNA (miRNA) species that post-transcriptionally modulate the expression of genes linked to regulation of metabolism are abnormally expressed in obesity and it is possible that they impact periodontal pathogenesis. This is important given the recent evidence implicating specific miRNA species as modifiers of inflammatory pathways.

AIMS: The overall goal is to integrate genetic, bioinformatic and epigenetic approaches in order to identify miRNAs and their collaborative networks involved in obesity as a modifier of periodontal infection pathogenesis. The specific aims include: (1) to determine if diseased periodontal tissues biopsied from patients with periodontal disease express unique miRNAs compared to biopsies obtained from non-diseased, healthy subjects; and (2) to determine whether Body Mass Index (BMI) will influence the miRNA expression patterns in biopsies from diseased periodontal tissues as compared to periodontal biopsy samples obtained from non-diseased, non-obese subjects.

METHODS: Total RNA was extracted (miRNeasy, Qiagen) from gingival biopsy samples collected from 24 subjects: 12 non-obese (BMI $<30kg/m^2$) patients and 12 obese (BMI $\geq 30kg/m^2$) patients, each group with 6 periodontally healthy sites and 6 chronic periodontitis sites. miRNA expression patterns were assessed using a human genome RT^2 miRNA PCR array (SABiosciences) which contained probes for 88 of the most abundantly expressed and best characterized miRNA sequences in the human miRNA genome. Data obtained was analyzed using four miRNA databases to identify potential target genes of important miRNAs expressed in this study.

RESULTS: Eleven unique miRNAs were identified (miR-15a, -18a, -22, -30d, -30e, -103, -106b, -130a, -142-3p, -185, -210), whereby the majority were up-regulated in the periodontally disease and obese group. Sixty-nine different genes with miRNA target sequences were identified which included proinflammatory cytokines/chemokines, proteins involved in collagen formation, glucose and lipid metabolism.

CONCLUSION: The miRNAs identified in this study may play a crucial role in the pathogenesis of periodontal infection modified by obesity as well as provide a basis for further investigations regarding the effect of obesity on periodontal pathogenesis. In doing so, these miRNA may represent novel therapeutic targets.

INTRODUCTION

Obesity has reached epidemic proportions globally in adults and children alike, and is a major contributor to the global burden of chronic illness and disability (Mealey and Ocampo 2007). The chronic imbalance between food intake and energy expenditure leads to obesity (Clément 2006). Research has shown that a multitude of gene polymorphisms can determine a person's susceptibility to weight gain (Adair and Prentice 2004). Gene expression of these key susceptibility genes can be influenced by epigenetic and environmental factors (Emilsson et al. 2008). Transcription factors and microRNAs (miRNAs) are the most abundant regulatory systems. Evidence from *in vitro* studies and more recently, from human studies, have identified specific miRNAs that are associated with obesity.

The role of miRNAs in adipocyte development and lipid metabolism has been investigated in mouse models and on cultured human preadipocytes. Convincing evidence from these studies has indicated that miR-27a, -103 and -143 are important in adipocyte differentiation (Esau et al. 2004; Lin et al. 2009; Kim et al. 2009; Xie et al. 2009). Expression of miR-27 is capable of suppressing PPARγ and C/EBPα, two very important early regulators of adipogenesis (Lin et al. 2009; Kim et al. 2009). miR-143 expression was up-regulated 3.3-fold in obese mice with similar increases in adipocyte differentiation markers peroxisome proliferator-activated receptor-γ (PPARγ) and adipocyte fatty acid-binding protein (aP2) (Takanabe et al. 2008). Using microarray analysis, an up-regulation of miR-335 was detected in obese mice. miR-122 was

determined to be an important regulator of cholesterol and fatty-acid metabolism (Esau et al. 2006).

miRNA expression in human adipose tissue was first studied by Klöting et al. (2009) and significant correlations between expression of miRNA-17-5p, -132, -99a, -134, 181a, -145, -197 and adipose tissue morphology, metabolic parameters (ie. HbA1c, fasting plasma glucose) and leptin, adiponectin and IL-6 levels. More recently, another human study showed that miR-519d was up-regulated in an obese population and its predicted target was peroxisome proliferator-activated receptor-α (PPARA) (Martinelli et al. 2010).

Obesity is an established risk factor for periodontitis (Saito et al. 2001, 2005; Al-Zahrani et al. 2003; Wood et al. 2003; Lundin et al. 2004; Della Vecchia et al. 2005; Genco et al. 2005; Nishida et al. 2005) but our understanding how obesity modifies periodontal disease pathogenesis at the molecular level is unknown. Many miRNA species that post-transcriptionally modulate the expression of genes regulating metabolism are abnormally expressed in obesity. Recently, some of these miRNA species have been identified as possible modifiers of inflammatory pathways (Emilsson et al. 2008; Iliopoulos et al. 2008). More specifically, inhibition of miR-22 causes up-regulation of PPARA and BMP7, blocked the inflammatory process through inhibition of IL1B and promoted repair in osteoarthritic chondrocytes (Iliopoulos et al. 2008).

Data regarding potential miRNA regulation of periodontal pathogenesis, a chronic inflammatory disease, is very limited. Only one published animal report (Nahid et al. 2011) is available whereby *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* infected ApoE (-/-) mice showed remarkable miR-146a expression

and negative correlation with inflammatory cytokine TNF-α production. This preliminary animal study sheds light on the possible direct or indirect modulatory role of miRNAs on alteration of chronic periodontal pathology. To date, there is no available published data studying the differential expression of miRNAs in obese humans with periodontal disease. Findings from this study may enable us to discover key miRNA species involved in mediating the effect of obesity on periodontal pathogenesis. In so doing, we can target these miRNA for therapeutic interventions.

AIM

The overall goal is to integrate genetic, bioinformatic and epigenetic approaches in order to identify miRNAs and their collaborative networks involved in obesity as a modifier of periodontal infection pathogenesis.

The specific aims include:

(1) to determine if diseased periodontal tissues biopsied from patients with periodontal disease express unique miRNAs compared to biopsies obtained from non-diseased, healthy subjects; and

(2) to determine whether BMI will influence the miRNA expression patterns in biopsies from diseased periodontal tissues as compared to periodontal biopsy samples obtained from non-diseased, non-obese subjects.

HYPOTHESIS

It is hypothesized that there will be converging and diverging miRNA expression patterns between gingival tissues obtained in obese patients with healthy periodontium vs obese patients with periodontal disease. Possible target genes for these altered miRNA profiles will be associated with inflammatory gene expression of PPARγ, NFκB and IL-6.

MATERIALS AND METHODS

Study Participant Selection and Gingival Tissue Biopsies

This study was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. A total of 24 participants, aged between 18 and 65 years, provided informed consent and were enrolled into this cross-sectional case-control study (Appendix A: Supplementary Materials and Methods). Height and weight measurements were collected on all recruited subjects in order to assess Body Mass Index (BMI). Participants were divided into two groups based on BMI. Group 1 consisted of 12 non-obese subjects (BMI <30 kg/m^2) and was further divided into two subsets: six patients with healthy periodontium and 6 patients with chronic periodontitis. Group 2 consisted of 12 obese subjects (BMI ≥ 30 kg/m^2) and was similarly divided into two subsets: six patients with healthy periodontium and 6 patients with chronic periodontitis. Overall, four main groups of participants were identified: (1) non-obese and periodontally healthy (n=6), (2) non-obese and periodontally diseased (n=6), (3) obese and periodontally healthy (n=6), and (4) obese and periodontally diseased (n=6). Identified subjects with a healthy periodontium or chronic periodontitis were ineligible to participate if they had chronic systemic diseases which also had oral manifestations, active infectious systemic diseases or significant oral damage, gross oral pathology or severe unrestored caries, needed prophylactic antibiotics or were treated with antibiotics for any condition within 1 month prior to the periodontal examination or, were taking medication known to affect

the periodontium (e.g., phenytoin, calcium antagonists, cyclosporin, coumadin, non-steroidal anti-inflammatory drugs, aspirin >81mg) within one month of the examination.

Comprehensive periodontal examinations were performed on all participants and probing depths (PDs), clinical attachment level (CAL) and bleeding on probing (BOP) was recorded at six sites per tooth. In patients categorized as having a healthy periodontium, a biopsy was taken from an interproximal site with a PD $\leq$4mm, no BOP and no radiographic evidence of bone loss. Sites biopsied from patients with chronic periodontitis had a PD $\geq$5mm, BOP and radiographic evidence of bone loss. Gingival biopsy samples were immediately placed in RNAlater® (Applied Biosystems/Ambion Inc., Austin, TX) overnight at 4°C and stored at -80ºC.

RNA Isolation and Quantitative Real-Time Reverse Transcription-PCR

Total RNA, including small RNAs, was isolated from the collected gingival tissue samples using a TissueLyser LT and miRNeasy Mini Kit (Qiagen, Valencia, CA). RNA was quantified using the NanoDrop (Thermo Scientific, Wilminngton, DE) and quality and purity was assessed following isolation using the 2100 Bioanalyzer (Agildent, Foster City, CA). Based on these variables, the best 20 samples (n=5 per group based on BMI and periodontal status) were used for further analysis. Briefly, a volume of 600ng total RNA was used for reverse transcription reactions to generate complementary DNA (cDNA) using the RT^2 miRNA First Strand Kit (SABiosciences, Frederick, MD) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with 100µL of synthesized cDNA, 1275µL 2x RT2 SYBR Green PCR Master Mix and 1175µL of ddH_2O in a 7500 Sequence Detection System (ABI Prism, Applied

Biosystems, Carlsbad, CA). MicroRNA expression patterns were assessed using a human genome RT^2 miRNA PCR Array (Catalogue no. MAH-001A, SABiosciences, Frederick, MD) which contained probes for 88 of the most abundantly expressed and best characterized miRNA sequences in the human miRNA genome and 8 additional housekeeping genes (Appendix B: Array Layout).

miRNA qRT-PCR Microarray Analysis and Target Selection

SNORD48 (Accession Number: NR_002745) and RNU6-2 (Accession Number: NR_002752) were used for normalization. Amplification parameters, conditions and melting curves were set by the manufacturer. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and results were reported as fold regulation. miRNAs with a *P*-value <0.05 were selected for further investigation. *In silico* analysis was performed using microCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), TargetScan Release 5.1 (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and microRNA.org Release August 2010 (http://www.microrna.org/microrna/home.do) databases for computationally predicted targets of each of the statistically significant differentially expressed human miRNA. To ensure that true targets were identified for each miRNA, they were identified in 3 out of 4 database systems utilized.

RESULTS

Study Population Demographics

The mean participant age in this study population varied from 39.4 to 49.4 years and a large standard deviation is present in each category due to the wide age range of study participants included in the final analysis (18 to 64 years old) (Table 1). Smoking status of all patients was recorded. The majority were categorized as never smokers (60%), 35% considered themselves to be ex-smokers while only one participant in the non-obese and periodontally diseased subset presented as a current smoker. None of the participants had diabetes. Participants in each subset were not matched according to age, gender or race. The participants in the non-obese groups had mean BMIs of 24.8 and 25.3 which is categorized as normal (BMI 18.5-24.9kg/m^2) and slightly overweight (BMI 25-29.9kg/m^2). The participants in the obese subsets had mean BMIs which would be categorized as obese (BMI 30-34.9kg/m^2) and severely obese (BMI 35-39.9kg/m^2). Periodontal diagnosis was determined according to the criteria previously stipulated.

TABLE 1

Table 1: Study participant demographics according to periodontal status and BMI (N=20, N=5/group)

	Healthy Periodontium		Periodontitis	
Variables	**Non-Obese N (%)**	**Obese N (%)**	**Non-Obese N (%)**	**Obese N (%)**
Mean Age (StdDev)	39.4 (10.0)	49.4 (15.4)	46.6 (16.7)	48.8 (13.3)
Gender				
Female	4 (80%)	2 (40%)	3 (60%)	3 (60%)
Male	1 (20%)	3 (60%)	2 (40%)	2 (40%)
Race				
Caucasian	5 (100%)	4 (80%)	2 (40%)	3 (60%)
African-American	0	1 (20%)	3 (60%)	0
Other	0	0	0	2 (40%)
Mean BMI (StdDev)	24.8 (2.8)	34.6 (3.7)	25.3 (2.3)	36.1 (5.6)

Differential miRNA Expression in Participants with Periodontitis

A comparison between participants with a healthy periodontium (and non-obese) and participants with periodontitis (either non-obese or obese) indicated that there was an up-regulation of 10 miRNAs (N=5/group, $P \leq 0.05$) (Figure 1). Generally, the fold regulation varied from 1.5 fold to 6.4 fold. The majority of miRNA expressed in the periodontitis group reached statistical significance in those that were also obese. miR-30e was the only unique miRNA to reach statistical significance in the periodontitis and non-obese group. miR-106b reached a high level of statistical significance ($P \leq 0.01$) in the periodontitis participants regardless of their BMI classification suggesting a significant role in periodontitis pathogenesis. Nevertheless, the fold regulation was greater (6.4 fold) for the participants who were obese compared to the non-obese participants (4.9 fold) possibly implying an additive effect of an obese status.

An assessment of the influence of periodontal status on miRNA expression in an obese population revealed that only two miRNA were differentially expressed and up-regulated in diseased gingiva compared to healthy gingiva. miR-185 (3.0 fold, P=0.03) and miR-106b (2.5 fold, P= 0.02) expression was most significant in comparison to obese participants with a healthy periodontium. These same miRNA also reached statistical significance when compared to non-obese participants with a healthy periodontium however, the fold regulation was greater (miR-106b: 6.4 fold, P=0.001 and miR-185: 3.5 fold, P=0.025). The quantitative differences in expression of miR-106b and miR-185 may be due to an underlying effect of obesity-related systemic inflammation.

FIGURE 1

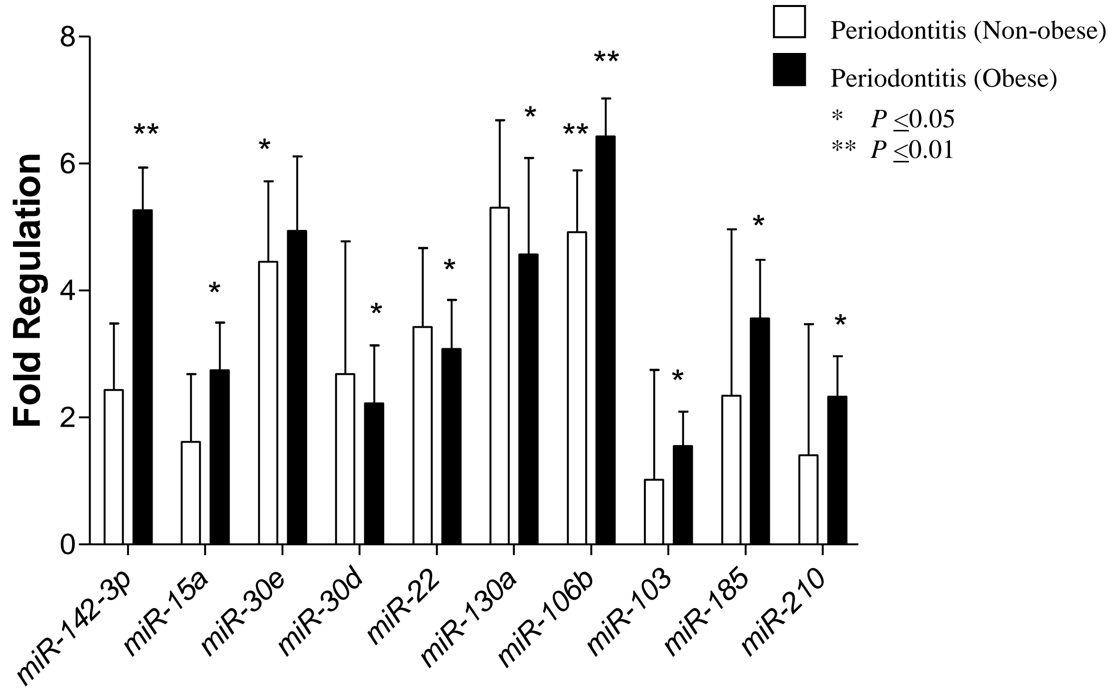

Figure 1: Fold regulation of miRNA species observed in periodontitis participants according to BMI compared to participants with healthy periodontium (non-obese).

Differential miRNA Expression between Non-obese and Obese Participants

Using a similar control group, a comparison was made between participants who were non-obese (with a healthy periodontium) to participants that presented with an obese BMI (and either periodontally healthy or had periodontitis). An up-regulation was seen in 11 miRNA (N=5/group, $P \leq 0.05$) (Figure 2). Fold up-regulation was similar to those observed when assessing differences in periodontal status. Overall, considerable up-regulation of numerous miRNA is observed in the participants who were obese and presented with periodontal disease.

miR-18a was uniquely expressed in obese participants with a healthy periodontium. mR-106b expression did not reach statistical significance for the obese participants with healthy periodontium. Rather, within this sub-cohort, it was uniquely expressed in obese participants with periodontitis. This further strengthens the supposition that miR-106b may play an important role in periodontal infection pathogenesis.

FIGURE 2

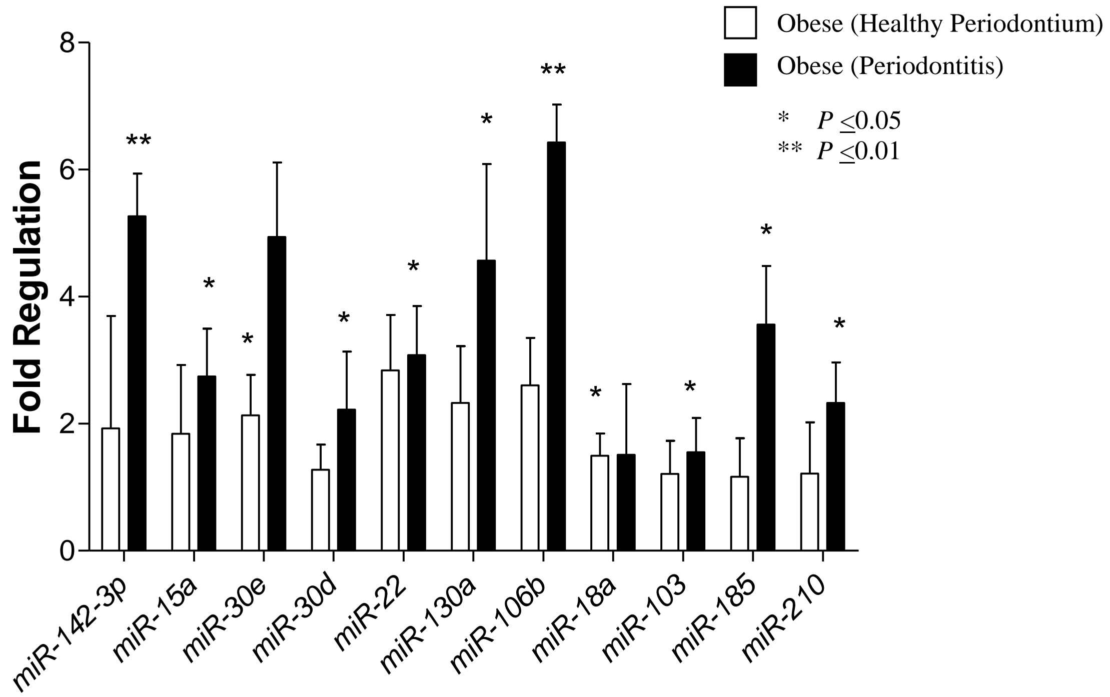

Figure 2: Fold regulation of miRNA species observed in obese participants according to periodontal status compared to non-obese participants (healthy periodontium).

Overall, 11 miRNA were identified with significant up-regulation in this study cohort (Table 2). Nine of these were expressed in the obese group with periodontal disease. This may suggest an additive effect of both chronic inflammatory conditions.

Predicted mRNA Targets

mRNA targets linked to immunology, inflammation and metabolic disorders by GO Biological Process Term were determined for these 11 miRNA using four available miRNA databases. Predicted targets were considered validated if present in three out of four miRNA databases. Sixty-nine different genes with miRNA target sequences were identified for the 11 miRNA differentially expressed in this study (Appendix C: Predicted Genes and GO Terms). When including the targets validated in two out of three databases, a total of 139 different genes were identified (listed in boxed parentheses). The potential targets include key cytokines involved in systemic inflammation: members of the interleukin family (IL1A, IL1F9, IL31 [IL15, IL17B, IL1F8, IL20]) and receptors (IL13RA1, IL27RA [IL9R, IL10RB, IL1RL1, IL21R]), interferon family (IFNA16, IFNA21 [IFNA1, IFNA2, IFNK]) and receptor (IFNAR2, IRF2, IRF5), tumor necrosis factor superfamily (TNF, TNFAIP3, TNFSF8, TNFSF14 [TNFSF9]) and receptors (TNFRSF10A, TNFRSF10D, TNFRSF13B [TNFRSF13C]), and a suppressor of cytokine signaling (SOCS1); chemokines [CCL1, CCL5, CCL19]; proteins involved in collagen formation and fibroblast growth factor (COL9A3, COL13A, COL13A1, FGF21); receptor molecules involved in metabolism (IRS1, PEX3, PEX13, PPARG, PPARGC1B [PPARD, LRP1B, LRP2, LRP6, LRP8, VLDLR]); adhesion molecule (ICAM4); and

proteases or molecules associated with bone metabolism (BAMBI, CASP6, MMP21 [MMP3, MMP10, MMP13, MMP16, OSTF1]).

TABLE 2

Table 2: Comparison of statistically significant differential miRNA expression according to periodontal status and BMI.

PERIODONTAL STATUS **Healthy Periodontium** *compared to* **Periodontitis**		**BMI STATUS** **Non-obese** *compared to* **Obese**
Periodontitis (Non-obese)	Periodontitis Obese	Obese (Healthy Periodontium)
miR-30e		miR-30e
miR-106b	miR-106b **	miR-18a
	miR-130a	
	miR-142-3p **	
	miR-15a	
	miR-30d	
	miR-22	
	miR-103	
	miR-185	
	miR-210	

** $P \leq 0.01$

DISCUSSION

It is well established that obesity leads to numerous morbidities such as cardiovascular and cerebrovascular disease and is associated with increased mortality from cardiovascular events and malignancies. Several miRNAs have also already been implicated with the pathogenesis of certain human diseases. As an impetus to discovering new therapeutic targets for adipogenesis, new discoveries have been made regarding certain miRNAs and their central role in adipocyte differentiation, metabolic functions and insulin resistance (Heneghan et al. 2009). Conversely, few reports indicate the role of miRNAs in periodontal disease pathogenesis. A single experimental polymicrobial periodontitis model in mice showed increased miR-146a expression (Nahid et al. 2011) but no further human reports exist. Therefore, the effect of miRNA transcriptional repression or mRNA cleavage on periodontal pathogenesis, especially in the presence of obesity, is largely unknown.

The miRNA predicted targets observed in this study focused primarily on key aspects of periodontal pathogenesis and obesity: inflammatory cytokines, immune regulation, bone and glucose metabolism. We found an up-regulation of miR-30e in the obese (and periodontally healthy) patient group. Klöting et al. (2009) is the only report to have found increased expression of miR-30e in subcutaneous adipose tissue and suggested its potential involvement in the association between adipose tissue dysfunction and the development of obesity related disorders like non-insulin dependent diabetes mellitus (NIDDM). Conversely, up-regulation of miR-30e was also observed in the non-

obese and periodontally diseased subset of patients. To date, no published studies have described an association between miR-30e and periodontal disease or inflammatory modulation. Nevertheless, the assumption then may be that the similarity between the obese and periodontally healthy subset and the non-obese and periodontally diseased subset is an underlying inflammatory process which may be related to miR-30e up-regulation. While none of the study participants had diabetes, a predicted target for miR-30e was IRS1, an insulin receptor substrate. Interestingly, expression of miR-30e was not significant in the subset of patients with both an obese status and periodontal disease and expression of nine other miRNAs was more impressive.

Up-regulation of miR-103 was observed in participants with periodontal disease and an obese BMI. An up-regulation of miR-103 (Xie et al. 2009) and miR-143 (Esau et al. 2004; Kajimoto et al. 2006) was observed during adipogenesis. Xie et al. (2009) were the first to provide cell culture and animal model evidence that miR-103 has a critical role in adipose biology, whereby presence of miR-103 in pre-adipocytes accelerated adipogenesis. Much of the predicted targets found for miR-103 were only validated in 2 out of 3 miRNA databases. Represented in this group were members of complement-related genes (C1QB, C1QL3), growth factors (FGF12, FGFR2, FIGF), interferon (IFNK), interleukins (IL1F8, IL20) and insulin-like peptide INSL5 (Appendix C). While miR-143 expression was not found in this study, it is significantly associated with elevated body weight and mesenteric fat weight in mice fed a high-fat diet. Adipocyte differentiation markers like PPARγ, aP2 and plasma leptin levels were intimately correlated with miR-143 expression levels (Takanabe et al. 2008).

Similarly, an interesting association between an inflammatory disease and BMI was found by Iliopoulos and colleagues (2008). Human osteoarthritic cartilage was obtained and compared it with normal cartilage. Among the most important differences, specific miRNAs (miR-22, miR-103) and proteins (PPARA, BMP7, IL1B) were highly correlated with Body Mass Index (BMI). Furthermore, miR-22 was found to regulate PPARA and BMP7 expression while inhibition of miR-22 causes up-regulation of PPARA and BMP7, blocked the inflammatory process through inhibition of IL1B and promoted repair in osteoarthritic chondrocytes (Iliopoulos et al. 2008). Our study also found a significant up-regulation in miR-22 expression in the obese and periodontally diseased group compared to healthy/non-obese controls. *In silico* analysis did not find similar predicted targets as demonstrated in the aforementioned study. However, computationally-derived mRNA targets still related to metabolic pathways (APOA5, IGFBP4) and inflammation (IL13RA1, IL1F9, IL27RA, IL31, IRF5, TNFRSF10D, TNFRSF13B) (Appendix C).

No unique miRNAs were found in the periodontally diseased (and non-obese) study subset. Instead, miR-30e expression was shared as previously mentioned with the periodontally healthy but obese group. In addition, expression of miR-106b was shared with the periodontally diseased and obese group with the most impressive fold regulation (6.4-fold) and highest level of statistical significance. Much of the literature implicating miR-106b in disease pathogenesis is related to carcinogenesis and cell cycle regulation. For the first time, miR-106b predicted targets in this study suggest that it is related to inflammation and bone metabolism pathways. In particular, prostaglandin E2 receptor (PTGER3) and tumor necrosis factor superfamily members (TNFSF14, TNFRSF10A)

were potential targets (Appendix C). PGE2 and TNF are established markers of gingival inflammation within gingival crevicular fluid (Adrianakaja et el. 2009). Confirmation of these *in silico* findings could be performed by performing a functional assay using a luciferase reporter construct to assess, for instance, alterations in PGE2 or TNF expression. Furthermore, *in vitro* over-expression and knockdown studies can be initiated and finally knockout mice may be generated to validate these findings.

Other important miRNAs (miR-15a, -18a, -30d, -130a, -142-3p, -185, -210) were expressed uniquely in the periodontally disease and obese subset. Similarly, the predicted targets related to immune regulation, collagen metabolism and insulin and cholesterol metabolism (Appendix C). The impressive number of miRNAs up-regulated in this group potentially suggests an additive effect of inflammatory burden of these two chronic diseases. A small study population was used for this proof-of-concept study but larger cohorts will be necessary to further validate these findings. In addition, *in silico* findings must be validated using a luciferase reporter transfection model, *in vitro* over-expression and knockdown studies may be initiated, followed by generation of knockout mice.

In conclusion, 11 miRNAs were differentially expressed in subsets of patients with either healthy or diseased gingiva or either non-obese or obese BMI. Nine of these miRNA were expressed by participants with both periodontal disease and an obese BMI. Several predicted targets were identified for these miRNA, many of which are implicated in immune and inflammatory pathways and metabolic processes (glucose and lipid-related). The miRNAs identified in this study may play a crucial role in the pathogenesis of periodontal infection modified by obesity as well as provide a basis for further

investigations regarding the effect of obesity on periodontal pathogenesis. In doing so, these miRNA may represent novel therapeutic targets.

APPENDIX A

SUPPLEMENTARY MATERIALS AND METHODS

Institutional Review Board, University of North Carolina at Chapel Hill.

IRB Study Number 07-0749

Clinical Study Design

This was a cross-sectional case-control study in which clinical parameters and biological samples were collected from both obese and non-obese subjects that presented with a healthy periodontium or chronic periodontitis. The study was conducted in a single masked or blinded manner such that all laboratory assessments were performed without knowledge of the subjects' BMI or periodontal status.

Human Subjects

A total of 24 subjects were recruited from the GO Health Center and the Graduate Periodontology clinic at the University of North Carolina - School of Dentistry. Height and weight measurements were collected on all recruited subjects in order to assess Body Mass Index (BMI). Participants were divided into two groups based on BMI. Group one consisted of 12 non-obese subjects (BMI <30 kg/m^2) and was further divided into two subsets: six patients with healthy periodontium and 6 patients with chronic periodontitis. Group 2 consisted of 12 obese subjects (BMI ≥ 30 kg/m^2) and was similarly divided into two subsets: six patients with healthy periodontium and 6 patients with chronic periodontitis. In both groups, patients had a tissue sample collected (either during crown

lengthening, extraction, or periodontal procedure appointment). The tissue was subsequently used for evaluation of miRNA expression profile. Additionally, a comprehensive periodontal examination was performed at the time of biopsy collection along with gingival crevicular fluid and plaque sample collection. Smoking and diabetes information was collected and examined post-hoc for evidence of significant effect modification.

The inclusion criteria for study participation included: (1) male or female subjects between the ages of 18-65 years (inclusive) and in good general health, (2) must have been able and willing to follow study procedures and instructions, (3) must have been able to read, understand and sign an informed consent form, (4) must have presented with at least 20 teeth in the functional dentition, excluding third molars. Of the total 24 recruited participants, half must have presented with chronic periodontitis as determined by two calibrated examiners during the periodontal examination. Such subjects exhibited periodontal pocketing (≥5 mm and Bleeding on Probing) and severe alveolar bone loss. Control subjects must have presented with periodontal health as determined by two calibrated examiners during the periodontal examination. Such subjects exhibited no evidence of periodontal pocketing (PDs ≤4mm), no bleeding on probing at the site of the biopsy or radiographic bone loss.

Patients were ineligible based on these exclusion criteria: (1) individuals who had a chronic disease and presented with oral manifestations, (2) individuals who exhibited gross oral pathology, (3) treatment with antibiotics for any medical or dental condition within 1 month prior to the screening examination, (4) chronic treatment (i.e., two weeks or more) with any medication known to affect periodontal status (e.g., phenytoin, calcium

antagonists, cyclosporin, coumadin, non-steroidal anti-inflammatory drugs, aspirin >81mg) within one month of the screening examination, (5) ongoing medications initiated less than three months prior to enrollment, (6) subjects with clinically significant organ disease including impaired renal function, or any bleeding disorder, (7) subjects with active infectious diseases such as hepatitis, HIV or tuberculosis, (8) severe unrestored caries, or any condition that is likely to require antibiotic treatment over the trial, including the need for prophylactic antibiotics.

Clinical Study Methods

All study participants had the following procedures performed on them at a single visit with the restriction that all biological specimens were also collected on the same day.

- Study personnel obtained medical history and demographics related to the patient
- Patient height and weight were recorded on site
- Patient vital signs (blood pressure and pulse) were recorded
- Two calibrated dental examiner performed oral and periodontal screening examinations
- The dental examiner collected 16 gingival crevicular fluid samples from the interproximal surfaces of the molars and premolars for future inflammatory mediator analyses making sure to include the biopsy site
- The dental examiner collected subgingival plaque sample(s) from the site(s) of the biopsy(ies) for future microbial DNA analyses

- The dental examiner scored all teeth for probing depth, percent bleeding on probing and clinical attachment level
- For healthy subjects, 1 gingival biopsy sample (gingival epithelium and connective tissue) per subject was collected at the time of a scheduled crown extension procedure, extraction, or voluntarily in the Go Health Clinic. The sample was obtained with a 4 mm punch biopsy blade or scalpel blade. This biopsy site selected within the subject was the site of the crown extension procedure, or at the interproximal papillae with a probing depth ≤4 mm.
- For subjects with chronic periodontitis, two gingival biopsy samples were obtained at the time of a scheduled periodontal flap procedure. Both samples were obtained with a 4 mm punch or scalpel blade. One sample was obtained from a severe inflamed periodontitis site (probing depth ≥5mm) and the other was obtained from an adjacent, minimally inflamed disease-free site (probing depth ≤4 mm). The latter sample was stored for future studies and not analyzed in this study.

Clinical Periodontal Assessments

Clinical examiners were calibrated prior to commencement of the study for training of study procedures.

Clinical parameters assessed in the periodontal examination included probing depth, bleeding on probing, and clinical attachment level. Clinical parameters were measured using a manual University of North Carolina (UNC-15) periodontal probe. For periodontitis and periodontally healthy subjects, these parameters were measured at six

periodontal sites per tooth (i.e., mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) and at all teeth (including third molars). Probing depth (PD) was defined as the linear distance from the gingival margin to base of the pocket. If a PD reading fell between two millimeter readings, the rule was to round down and the lower of the two readings was recorded. Presence or absence of bleeding to manual probing was recorded as a dichotomous variable where 0 indicated that there was no bleeding within 10 seconds after probing and 1 indicated that bleeding was produced within 10 seconds after probing. The clinical attachment level (CAL) was defined as the linear distance from the cemento-enamel junction to base of the pocket. If a CAL reading fell between two millimeter readings, the rule was to round down and the lower of the two readings was recorded.

General Assessments

Blood pressure was recorded for all patients using an automated blood pressure machine. BMI was collected for each participant based on recorded height and weight measurements taken at the time of examination. BMI categories include: underweight <18.5 kg/m^2, normal weight 18.5-24.9 kg/m^2, overweight 25-29.9 kg/m^2, obesity ≥ 30 kg/m^2. For the purposes of this study, participants were initially either categorized as non-obese (<30 kg/m^2) or obese (≥ 30 kg/m^2).

APPENDIX B

ARRAY LAYOUT

RT^2 microRNA PCR Array Human miFinder (MAH-001A) (SABiosciences, Frederick, MD)

	1	2	3	4	5	6	7	8	9	10	11	12
A	miR-142-5p	miR-16	miR-142-3p	miR-21	miR-15a	miR-29b	let-7a	miR-126	miR-143	let-7b	miR-27a	let-7f
B	miR-9	miR-26a	miR-24	miR-30e	miR-181a	miR-29a	miR-124	miR-144	miR-30d	miR-19b	miR-22	miR-122
C	miR-150	miR-32	miR-155	miR-140-5p	miR-125b	miR-141	miR-92a	miR-424	miR-191	miR-17	miR-130a	miR-20a
D	miR-27b	miR-26b	miR-146a	miR-200c	miR-99a	miR-19a	miR-23a	miR-30a	let-7i	miR-93	let-7c	miR-106b
E	miR-101	let-7g	miR-425	miR-15b	miR-28-5p	miR-18a	miR-25	miR-23b	miR-302a	miR-186	miR-29c	miR-7
F	let-7d	miR-30c	miR-181b	miR-223	miR-320a	miR-374a	let-7e	miR-151-5p	miR-374b	miR-196b	miR-140-3p	miR-100
G	miR-103	miR-96	miR-302b	miR-194	miR-125a-5p	miR-423-5p	miR-376c	miR-195	miR-222	miR-28-3p	miR-128	miR-302c
H	miR-423-3p	miR-185	miR-30b	miR-210	SNORD48	SNORD47	SNORD44	U6	miRTC	miRTC	PPC	PPC

APPENDIX C

TARGET GENES AND GO TERMS

Identified in 3 out of 4 databases

miRNA	Gene	Description	GO Term
miR-22	APOA5	Apolipoprotein A-V precursor (Apolipoprotein A5) (ApoAV) (ApoA-V) (Regeneration-associated protein 3).	cholesterol homeostasis
miR-106b	BAMBI	BMP and activin membrane-bound inhibitor homolog precursor (Putative transmembrane protein NMA) (Non-metastatic gene A protein).	transforming growth factor beta receptor signaling pathway
miR-15a	BMX	Cytoplasmic tyrosine-protein kinase BMX (EC 2.7.10.2) (Bone marrow tyrosine kinase gene in chromosome X protein) (Epithelial and endothelial tyrosine kinase) (ETK) (NTK38).	apoptosis
miR-106b	CASP6	Caspase-6 precursor (EC 3.4.22.59) (CASP-6) (Apoptotic protease Mch-2)	apoptosis
miR-106b	CCL1	Small inducible cytokine A1 precursor (CCL1) (T lymphocyte-secreted protein I-309)	chemotaxis
miR-106b	CD68	Macrosialin precursor (GP110) (CD68 antigen)	
miR-106b	CFLAR	CASP8 and FADD-like apoptosis regulator precursor (Cellular FLICE-like inhibitory protein) (c-FLIP) (Caspase-eight-related protein) (Casper) (Caspase-like apoptosis regulatory protein) (CLARP) (MACH-related inducer of toxicity) (MRIT) (Caspase homolog)	apoptosis
miR-30d **miR-130a**	CHST1	Carbohydrate sulfotransferase 1 (EC 2.8.2.21) (Keratan sulfate Gal-6 sulfotransferase) (KSST) (KSGal6ST) (KS6ST) (Galactose/N-acetylglucosamine/N-acetylglucosamine 6-O-sulfotransferase 1) (GST-1).	inflammatory response

miR-30d	CHST2	Carbohydrate sulfotransferase 2 (EC 2.8.2.-) (N-acetylglucosamine 6-O-sulfotransferase 1) (GlcNAc6ST-1) (Gn6ST) (Galactose/N-acetylglucosamine/N-acetylglucosamine 6-O-sulfotransferase 2) (GST-2).	inflammatory response
miR-30e	COL9A3	Collagen alpha-3(IX) chain precursor.	axon guidance
miR-30e	COL13A	Collagen alpha-1(XIII) chain (COLXIIIA1).	cell-cell adhesion
miR-30d	COL13A1	Collagen alpha-1(XIII) chain (COLXIIIA1).	cell-cell adhesion
miR-22	CSF1R	Macrophage colony-stimulating factor 1 receptor precursor (EC 2.7.10.1) (CSF-1-R) (Fms proto-oncogene) (c-fms) (CD115 antigen).	signal transduction
miR-30e	CTHRC1	Collagen triple helix repeat-containing protein 1 precursor (NMTC1 protein).	cell migration
miR-30e	FCER1G	High affinity immunoglobulin epsilon receptor gamma-subunit precursor (FceRI gamma) (IgE Fc receptor gamma-subunit) (Fc-epsilon RI-gamma).	leukocyte migration
miR-15a	FCRL2	Fc receptor-like protein 2 precursor (FcR-like protein 2) (FcRL2) (Fc receptor homolog 2) (FcRH2) (SH2 domain-containing phosphatase anchor protein 1) (Immunoglobulin receptor translocation-associated 4 protein).	cell-cell signaling
miR-30e	FCN1	Ficolin-1 precursor (Ficolin-A) (Ficolin-alpha) (M-ficolin) (Collagen/fibrinogen domain-containing protein 1).	opsonization
miR-30e	FGF21	Fibroblast growth factor 21 precursor (FGF-21).	cell-cell signaling
miR-106b	FGL2	Fibroleukin precursor (Fibrinogen-like protein 2) (pT49)	signal transduction
miR-106b	FNDC3A	Fibronectin type-III domain-containing protein 3a.	cell-cell adhesion
miR-18a	FRS2	Fibroblast growth factor receptor substrate 2 (FGFR substrate 2) (Suc1-associated neurotrophic factor target 1) (SNT-1) (FGFR-signaling	fibroblast growth factor receptor signaling

		adaptor SNT).	pathway
miR-130a	GADD45A	Growth arrest and DNA-damage-inducible protein GADD45 alpha (DNA- damage-inducible transcript 1) (DDIT1).	apoptosis
miR-142-3p	HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate (Protein pp110)	signal transduction
miR-106b	ICAM4	Intercellular adhesion molecule 4 precursor (ICAM-4) (Landsteiner-Wiener blood group glycoprotein) (LW blood group protein) (CD242 antigen)	cell adhesion
miR-30e	IFNA16	Interferon alpha-16 precursor (Interferon alpha-WA).	defense response
miR-30e	IFNA21	Interferon alpha-21 precursor (Interferon alpha-F) (LeIF F).	defense response
miR-30e	IFNAR2	Interferon-alpha/beta receptor beta chain precursor (IFN-alpha-REC) (Type I interferon receptor) (IFN-R) (Interferon alpha/beta receptor 2).	cytokine-mediated signaling pathway
miR-22	IGFBP4	Insulin-like growth factor-binding protein 4 precursor (IGFBP-4) (IBP-4) (IGF-binding protein 4).	inflammatory response
miR-30e	IL1A	Interleukin-1 alpha precursor (IL-1 alpha) (Hematopoietin-1).	inflammatory response
miR-30d	IL1AQ	Interleukin-1 alpha precursor (IL-1 alpha) (Hematopoietin-1).	N/A
miR-106b	ILDR1	immunoglobulin-like domain containing receptor 1	N/A
miR-22	IL13RA1	Interleukin-13 receptor alpha-1 chain precursor (IL-13R-alpha-1) (IL-13RA-1) (CD213a1 antigen).	cell surface receptor linked signaling pathway
miR-22	IL1F9	Interleukin-1 family member 9 (IL-1F9) (Interleukin-1 homolog 1) (IL-1H1) (Interleukin-1 epsilon) (IL-1 epsilon) (IL-1-related protein 2) (IL-1RP2).	cell-cell signaling
miR-22	IL27RA	Interleukin-27 receptor subunit alpha precursor (IL-27R-alpha) (WSX-1) (Type I T-cell cytokine receptor) (TCCR) (Protein CRL1).	immune response
miR-22	IL31	Interleukin-31 precursor (IL-31).	N/A
miR-18a	IRF2	Interferon regulatory factor 2 (IRF-2).	interfereon-gamma-

			mediated signaling pathway
miR-22	IRF5	Interferon regulatory factor 5 (IRF-5).	interfereon-gamma-mediated signaling pathway
miR-30d miR-30e	IRS1	Insulin receptor substrate 1 (IRS-1).	glucose homesostasis
miR-106b	ITFG1	T-cell immunomodulatory protein precursor (Protein TIP) (Integrin alpha FG-GAP repeat-containing protein 1)	integral to membrane
miR-106b	LIAS	Lipoic acid synthetase, mitochondrial precursor (Lip-syn) (Lipoate synthase).	inflammatory response
miR-106b	LILRB1	Leukocyte immunoglobulin-like receptor subfamily B member 1 precursor (Leukocyte immunoglobulin-like receptor 1) (LIR-1) (Immunoglobulin- like transcript 2) (ILT-2) (Monocyte/macrophage immunoglobulin-like receptor 7) (MIR-7) (CD85j antigen).	regulation of immune response
miR-142-3p	LRP1B	Low-density lipoprotein receptor-related protein 1B precursor (Low-density lipoprotein receptor-related protein-deleted in tumor)	protein transport
miR-15a	LRP6	Low-density lipoprotein receptor-related protein 6 precursor.	bone remodeling
miR-130a	LRP8	Low-density lipoprotein receptor-related protein 8 precursor (Apolipoprotein E receptor 2).	cytokine-mediated signaling pathway
miR-106b	MAP3K11	Mitogen-activated protein kinase kinase kinase 11 (EC 2.7.11.25) (Mixed lineage kinase 3) (Src-homology 3 domain-containing proline- rich kinase).	cell death
miR-106b	MAPK9	Mitogen-activated protein kinase 9 (EC 2.7.11.24) (Stress-activated protein kinase JNK2) (c-Jun N-terminal kinase 2) (JNK-55).	Toll signaling pathway
miR-130a	MET	Hepatocyte growth factor receptor	signal

		precursor (EC 2.7.10.1) (HGF receptor) (Scatter factor receptor) (SF receptor) (HGF/SF receptor) (Met proto-oncogene tyrosine kinase) (c-Met).	transduction
miR-30e	MMD	Monocyte to macrophage differentiation protein (Progestin and adipoQ receptor family member XI).	cytolysis
miR-30e	MMP21	Matrix metalloproteinase-21 precursor (EC 3.4.24.-) (MMP-21).	proteolysis
miR-15a	NFATC3	Nuclear factor of activated T-cells, cytoplasmic 3 (NF-ATc3) (NFATc3) (T cell transcription factor NFAT4) (NF-AT4) (NFATx)	regulation of transcription
miR-185	NFATC3	Nuclear factor of activated T-cells, cytoplasmic 3 (NF-ATc3) (NFATc3) (T cell transcription factor NFAT4) (NF-AT4) (NFATx)	regulation of transcription
miR-30d	NFIB	Nuclear factor 1 B-type (Nuclear factor 1/B) (NF1-B) (NFI-B) (NF-I/B) (CCAAT-box-binding transcription factor) (CTF) (TGGCA-binding protein).	regulation of transcription, DNA-dependent
miR-106b	PBK	T-lymphokine-activated killer cell-originated protein kinase (EC 2.7.12.2) (T-LAK cell-originated protein kinase) (PDZ-binding kinase) (Spermatogenesis-related protein kinase) (SPK) (MAPKK-like protein kinase) (Nori-3).	protein phosphorylation
miR-30e	PEX3	Peroxisomal biogenesis factor 3 (Peroxin-3) (Peroxisomal assembly protein PEX3).	protein import into peroxisome membrane
miR-15a	PEX13	Peroxisomal membrane protein PEX13 (Peroxin-13).	fatty-acid alpha-oxidation
miR-142-3p	PGM1	Phosphoglucomutase-1 (EC 5.4.2.2) (Glucose phosphomutase 1) (PGM 1).	carbohydrate metabolic process
miR-130a	PPARG	Peroxisome proliferator-activated receptor gamma (PPAR-gamma).	lipid homeostasis
miR-30d	PPARGC1B	Peroxisome proliferator-activated receptor gamma coactivator 1-beta (PPAR gamma coactivator-1beta) (PGC-1-related estrogen receptor alpha coactivator) (PPARGC-1-beta) (PGC-1-beta).	regulation of transcription

miR-106b	PTGER3	Prostaglandin E2 receptor, EP3 subtype (Prostanoid EP3 receptor) (PGE receptor, EP3 subtype) (PGE2-R)	signal transduction
miR-30d miR-30e	SOCS1	Suppressor of cytokine signaling 1 (SOCS-1) (JAK-binding protein) (JAB) (STAT-induced STAT inhibitor 1) (SSI-1) (Tec-interacting protein 3) (TIP-3).	cytokine-mediated signaling pathway
miR-130a	STARD13	StAR-related lipid transfer protein 13 (StARD13) (START domain-containing protein 13) (46H23.2) (Deleted in liver cancer protein 2) (Rho GTPase-activating protein).	signal transduction
miR-18a	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3 (Putative DNA-binding protein A20) (Zinc finger protein A20).	negative regulation of inflammatory response OR signal transduction
miR-130a	TNF	Tumor necrosis factor precursor (TNF-alpha) (Tumor necrosis factor ligand superfamily member 2) (TNF-a)	chronic inflammatory response to antigenic stimulus
miR-106b	TNFRSF10A	Tumor necrosis factor receptor superfamily member 10A precursor (Death receptor 4) (TNF-related apoptosis-inducing ligand receptor 1) (TRAIL receptor 1) (TRAIL-R1) (CD261 antigen).	activation of NK-kappaB-inducing kinase activity
miR-22	TNFRSF10D	Tumor necrosis factor receptor superfamily member 10D precursor (Decoy receptor 2) (DcR2) (TNF-related apoptosis-inducing ligand receptor 4) (TRAIL receptor 4) (TRAIL-R4) (TRAIL receptor with a truncated death domain) (CD264 antigen).	signal transduction
miR-22	TNFRSF13B	Tumor necrosis factor receptor superfamily member 13B (Transmembrane activator and CAML interactor) (CD267 antigen).	B cell homeostasis
miR-30e	TNFSF8	Tumor necrosis factor ligand superfamily member 8 (CD30 ligand) (CD30- L) (CD153 antigen).	signal transduction

miR-106b	TNFSF14	Tumor necrosis factor ligand superfamily member 14 (Herpesvirus entry mediator-ligand) (HVEM-L) (CD258 antigen) [Contains: Tumor necrosis factor ligand superfamily member 14, membrane form; Tumor necrosis factor ligand superfamily member 14, soluble form]	immune response
miR-106b	VLDLR	Very low-density lipoprotein receptor precursor (VLDL receptor) (VLDL- R).	cholesterol metabolic process

Identified in 2 out of 3 databases

miRNA	Gene	Description	GO Term
miR-30d	ADAM22	ADAM 22 precursor (A disintegrin and metalloproteinase domain 22) (Metalloproteinase-like, disintegrin-like, and cysteine-rich protein 2) (Metalloproteinase-disintegrin ADAM22-3).	proteolysis
miR-130a	ADAM23	ADAM 23 precursor (A disintegrin and metalloproteinase domain 23) (Metalloproteinase-like, disintegrin-like, and cysteine-rich protein 3) (MDC-3).	proteolysis
miR-30d	ADAMTS6	ADAMTS-6 precursor (EC 3.4.24.-) (A disintegrin and metalloproteinase with thrombospondin motifs 6) (ADAM-TS 6) (ADAM-TS6).	proteolysis
miR-185	AGER	Advanced glycosylation end product-specific receptor precursor (Receptor for advanced glycosylation end products).	inflammatory response
miR-130a	CAPRIN2	C1q domain containing 1 isoform 2	negative regulation of translation
miR-103	C1QB	Complement C1q subcomponent subunit B precursor.	complement activation
miR-30d	C1QL1	C1q-related factor precursor (Complement component 1 Q subcomponent- like 1).	locomotory behavior
miR-103	C1QL3	Complement C1q-like protein 3 precursor.	N/A

miR-106b	CCL5	Small inducible cytokine A5 precursor (CCL5) (T-cell-specific RANTES protein) (SIS-delta) (T cell-specific protein P228) (TCP228)	cellular defense response
miR-30d	CCL19	Small inducible cytokine A19 precursor (CCL19) (Macrophage inflammatory protein 3 beta) (MIP-3-beta) (EBI1-ligand chemokine) (ELC) (Beta chemokine exodus-3) (CK beta-11).	inflammatory response
miR-106b	CCS	Copper chaperone for superoxide dismutase (Superoxide dismutase copper chaperone).	removal of superoxide radicals
miR-106b	CD1B	T-cell surface glycoprotein CD1b precursor (CD1b antigen).	immune response
miR-210	CD22	B-cell receptor CD22 precursor (Sialic acid-binding Ig-like lectin 2) (Siglec-2) (Leu-14) (B-lymphocyte cell adhesion molecule) (BL-CAM).	cell adhesion
miR-103	CD28	T-cell-specific surface glycoprotein CD28 precursor (TP44).	humoral immune response
miR-30d	CD99	CD99 antigen precursor (T-cell surface glycoprotein E2) (E2 antigen) (Protein MIC2) (12E7).	cell adhesion
miR-103	CHST11	Carbohydrate sulfotransferase 11 (EC 2.8.2.5) (Chondroitin 4-O-sulfotransferase 1) (Chondroitin 4-sulfotransferase 1) (C4ST) (C4ST-1) (C4S-1).	carbohydrate biosynthetic process
miR-30d	COL9A3	Collagen alpha-3(IX) chain precursor.	axon guidance
miR-210	CR1	Complement receptor type 1 precursor (C3b/C4b receptor) (CD35 antigen).	innate immune response
miR-106b	CSF2RA	Granulocyte-macrophage colony-stimulating factor receptor alpha chain precursor (GM-CSF-R-alpha) (GMR) (CD116 antigen) (CDw116).	N/A
miR-185	CSF2RB	Cytokine receptor common beta chain precursor (GM-CSF/IL-3/IL-5 receptor common beta-chain) (CD131 antigen) (CDw131).	signal transduction
miR-142-3p	CXADR	Coxsackievirus and adenovirus receptor precursor (Coxsackievirus B- adenovirus receptor) (hCAR)	leukocyte migration

		(CVB3-binding protein) (HCVADR).	
miR-106b	DIS3L	DIS3 mitotic control homolog (S. cerevisiae)-like	N/A
miR-185	EBI3	Interleukin-27 beta chain precursor (IL-27B) (Epstein-Barr virus-induced gene 3 protein) (EBV-induced gene 3 protein).	cytokine-mediated signaling pathway
miR-130a	EDG1	Sphingosine 1-phosphate receptor Edg-1 (Sphingosine 1-phosphate receptor 1) (S1P1).	positive regulation of positive chemotaxis
miR-130a	F3	Tissue factor precursor (TF) (Coagulation factor III) (Thromboplastin) (CD142 antigen).	positive regulation of positive chemotaxis
miR-103	FAT4	FAT tumor suppressor homolog 4	cell adhesion
miR-30d	FCER1G	High affinity immunoglobulin epsilon receptor gamma-subunit precursor (FceRI gamma) (IgE Fc receptor gamma-subunit) (Fc-epsilon RI-gamma).	leukocyte migration
miR-185	FCRL3	Fc receptor-like 3 precursor	N/A
miR-22	FFAR3	Free fatty acid receptor 3 (G-protein coupled receptor 41).	protein coupled receptor protein signaling pathway
miR-103	FGF12	Fibroblast growth factor 12 (FGF-12) (Fibroblast growth factor homologous factor 1) (FHF-1) (Myocyte-activating factor).	cell-cell signaling
miR-103	FGFR2	Fibroblast growth factor receptor 2 precursor (EC 2.7.10.1) (FGFR-2) (Keratinocyte growth factor receptor 2) (CD332 antigen).	bone morphogenesis
miR-142-3p	FLT1	Vascular endothelial growth factor receptor 1 precursor (EC 2.7.10.1) (VEGFR-1) (Vascular permeability factor receptor) (Tyrosine-protein kinase receptor FLT) (Flt-1) (Tyrosine-protein kinase FRT) (Fms-like tyrosine kinase 1).	intracellular receptor mediated signaling pathway
miR-103	FIGF	Vascular endothelial growth factor D precursor (VEGF-D) (c-fos-induced growth factor) (FIGF).	induction of positive chemotaxis
miR-30d	GLCCI1	Glucocorticoid-induced transcript 1	N/A

		protein.	
miR-15a	GLS2	Glutaminase liver isoform, mitochondrial precursor (EC 3.5.1.2) (GLS) (L-glutamine amidohydrolase) (L-glutaminase).	glutamine metabolic process
miR-130a	HLA-DOA	HLA class II histocompatibility antigen, DO alpha chain precursor (MHC class II antigen DOA) (MHC DZ alpha) (MHC DN-alpha).	immune response
miR-142-3p	HSD17B4	Peroxisomal multifunctional enzyme type 2 (MFE-2) (D-bifunctional protein) (DBP) (17-beta-hydroxysteroid dehydrogenase 4) (17-beta-HSD 4) (D-3-hydroxyacyl-CoA dehydratase) (EC 4.2.1.107) (3-alpha,7- alpha,12-alpha-trihydroxy-5-beta-cholest-24-enoyl-CoA hy	oxidation-reduction process
miR-106b	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3 (IFIT-3) (IFIT-4) (Interferon-induced 60 kDa protein) (IFI-60K) (ISG-60) (CIG49) (Retinoic acid-induced gene G protein) (RIG-G).	cytokine-mediated signaling pathway
miR-22	IKBKG	NF-kappa-B essential modulator (NEMO) (NF-kappa-B essential modifier) (Inhibitor of nuclear factor kappa-B kinase subunit gamma) (IkB kinase subunit gamma) (I-kappa-B kinase gamma) (IKK-gamma) (IKKG) (IkB kinase-associated protein 1) (IKKAP1) (FIP-3).	immune response
miR-18a	IFNA1	Interferon alpha-1/13 precursor (Interferon alpha-D) (LeIF D).	defense response
miR-18a	IFNA2	Interferon alpha-2 precursor (Interferon alpha-A) (LeIF A).	inflammatory response
miR-103	IFNK	Interferon kappa precursor (IFN-kappa).	inflammatory response
miR-103	IL1F8	Interleukin-1 family member 8 (IL-1F8) (Interleukin-1 eta) (IL-1 eta) (FIL1 eta) (Interleukin-1 homolog 2) (IL-1H2).	immune response
miR-22	IL1RL1	Interleukin-1 receptor-like 1 precursor (ST2 protein).	immune response
miR-130a	IL15	Interleukin-15 precursor (IL-15).	immune response

miR-18a	IL9R	Interleukin-9 receptor precursor (IL-9R) (CD129 antigen).	signal transduction
miR-18a	IL10RB	Interleukin-10 receptor beta chain precursor (IL-10R-B) (IL-10R2) (Cytokine receptor family 2 member 4) (Cytokine receptor class-II member 4) (CRF2-4) (CDw210b antigen).	inflammatory response
miR-18a	IL17B	Interleukin-17B precursor (IL-17B) (Cytokine-like protein Zcyto7) (Neuronal interleukin-17-related factor) (Interleukin-20) (IL-20).	inflammatory response
miR-103	IL20	Interleukin-20 precursor (IL-20) (Four alpha helix cytokine Zcyto10).	regulation of inflammatory response
miR-30d	IL21R	Interleukin-21 receptor precursor (IL-21R) (Novel interleukin receptor).	natural killer cell activation
miR-106b	IL27RA	Interleukin-27 receptor subunit alpha precursor (IL-27R-alpha) (WSX-1) (Type I T-cell cytokine receptor) (TCCR) (Protein CRL1).	immune response
miR-103	INSL5	Insulin-like peptide INSL5 precursor (Insulin-like peptide 5) [Contains: Insulin-like peptide INSL5 B chain; Insulin-like peptide INSL5 A chain].	biological process
miR-15a	INSR	Insulin receptor precursor (EC 2.7.10.1) (IR) (CD220 antigen) [Contains: Insulin receptor subunit alpha; Insulin receptor subunit beta].	positive regulation of glycolysis
miR-30e **miR-185**	LDLR	Low-density lipoprotein receptor precursor (LDL receptor).	cholesterol homeostasis
miR-185	LDLRAD1	low density lipoprotein receptor A domain containing 1	N/A
miR-130a	LIPI	Lipase member I precursor (EC 3.1.1.-) (Membrane-associated phosphatidic acid-selective phospholipase A1-beta) (mPA-PLA1 beta) (LPD lipase).	lipid catabolic process
miR-130a	LRP2	Low-density lipoprotein receptor-related protein 2 precursor (Megalin) (Glycoprotein 330) (gp330).	lipid metabolic process
miR-18a	LTBR	Tumor necrosis factor receptor superfamily member 3 precursor (Lymphotoxin-beta receptor) (Tumor necrosis factor receptor 2-	signal transduction

		related protein) (Tumor necrosis factor C receptor).	
miR-18a	MASP1	Complement-activating component of Ra-reactive factor precursor (EC 3.4.21.-) (Ra-reactive factor serine protease p100) (RaRF) (Mannan-binding lectin serine protease 1) (Mannose-binding protein-associated serine protease) (MASP-1) (Serine protease 5)	proteolysis
miR-130a	MBD4	Methyl-CpG-binding domain protein 4 (EC 3.2.2.-) (Methyl-CpG-binding protein MBD4) (Methyl-CpG-binding endonuclease 1) (Mismatch-specific DNA N-glycosylase).	DNA damage response, signal transduction resulting in induction of apoptosis
miR-18a	MMP3	Stromelysin-1 precursor (EC 3.4.24.17) (Matrix metalloproteinase-3) (MMP-3) (Transin-1) (SL-1).	proteolysis
miR-130a	MMP10	Stromelysin-2 precursor (EC 3.4.24.22) (Matrix metalloproteinase-10) (MMP-10) (Transin-2) (SL-2).	proteolysis
miR-130a	MMP13	Collagenase 3 precursor (EC 3.4.24.-) (Matrix metalloproteinase-13) (MMP-13).	proteolysis
miR-142-3p	MMP16	Matrix metalloproteinase-16 precursor (EC 3.4.24.-) (MMP-16) (Membrane-type matrix metalloproteinase 3) (MT-MMP 3) (MTMMP3) (Membrane-type-3 matrix metalloproteinase) (MT3-MMP) (MT3MMP) (MMP- X2).	proteolysis
miR-18a	NKIRAS1	NF-kappa-B inhibitor-interacting Ras-like protein 1 (I-kappa-B-interacting Ras-like protein 1) (Kappa B-Ras protein 1) (KappaB-Ras1).	I-kappaB kinase/ NF-kappaB cascade
miR-130a	OSTF1	Osteoclast-stimulating factor 1.	signal transduction
miR-106b	PLAUR	Urokinase plasminogen activator surface receptor precursor (uPAR) (U- PAR) (Monocyte activation antigen Mo3) (CD87 antigen).	chemotaxis
miR-185	PGLYRP3	Peptidoglycan recognition protein I-	defense

		alpha precursor (Peptidoglycan recognition protein intermediate alpha) (PGRP-I-alpha) (PGLYRPIalpha) (Peptidoglycan recognition protein 3).	response to Gram-positive bacterium
miR-106b	PPARD	Peroxisome proliferator-activated receptor delta (PPAR-delta) (PPAR-beta) (Nuclear hormone receptor 1) (NUC1) (NUCI).	adipose tissue development
miR-30d	TNFSF9	Tumor necrosis factor ligand superfamily member 9 (4-1BB ligand) (4- 1BBL).	immune response
miR-142-3p	TNFRSF13C	Tumor necrosis factor receptor superfamily member 13C (B cell-activating factor receptor) (BAFF receptor) (BAFF-R) (BLyS receptor 3) (CD268 antigen).	regulation of immune response

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