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PERIODONTAL MICROORGANISMS, OBESITY, CHRONIC INFLAMMATION, AND TYPE 1 DIABETES

by

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DEDICATION

I would like to dedicate this dissertation to my family and friends. The completion of this work could not have been possible without the direct financial and emotional support of my father and mother who provided unlimited guidance and encouragement through the most stressful time and insisted on calling me “Dr.” ever since I received that acceptance letter. I would like to express my deepest appreciation to my sister and brother who believed in me even at times where I did not believe in myself and quitting was a desirable option.

I would like to dedicate this to all my friends who supported me throughout my studies and were there for me and bought me a drink when I whined too much.

Thank y’alls for your continuous support.

Cheers!

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Many thanks to my committee members who provided great guidance and encouragement. Dr. Merchant, my mentor, promoted my critical thinking as an epidemiologist and encouraged me to come up with my own research questions. Dr. Zhang helped me get a better grasp of advanced statistical methods and was always willing to provide guidance. Dr. Hazlett greatly enhanced my knowledge through her critical feedback on epidemiologic principles and provided great advisement on academic logistics. Dr. Wadwa and Dr. Morrato provided very thorough guidance on clinical aspects of epidemiology.

ABSTRACT

Periodontal disease is a low-grade chronic inflammation in the tissues surrounding the teeth caused by multiple, mostly gram-negative pathogens. It is associated with diabetes, obesity, and chronic inflammation. The specific roles that periodontal microorganism play in these conditions are not well-studied. Hereby, we explored how periodontal bacteria from sub gingival plaque clustered in youth with and without type 1 diabetes, and how such patterns related to body-mass-index percentile (BMI percentile), C-reactive protein (CRP) and adiponectin.

Cross-sectional data were collected from 105 youth with type 1 diabetes and 71 without diabetes. Participants were between 12 and 19 years of age receiving care at the Barbara Davis Center in Colorado, 2009-2011. Counts of 41 oral-bacteria from sub gingival-plaque were obtained using DNA-DNA hybridization, and grouped using cluster-analysis. Standardized-mean counts of each organism were computed and summed to get microbial-scores per cluster. A subset (n=101, 54 with type 1 diabetes) underwent dental examinations at the University of Colorado, School of Dental Medicine clinic. Participants were 15-years old on average; 51% were female; 73% non-Hispanic white; 37% overweight; the average diabetes duration was 8 years. About 48% brushed their teeth twice/day; 12% flossed once/day; 47% visited a dentist in the past 6 months.

Bacterial clusters were identified and named after Socransky's color-coded complexes as 'blue-other', 'orange-blue', 'orange-red', and 'yellow-other'. Individuals with and without type 1 diabetes had similar microbial composition. Cases of type 1 diabetes ranking in the highest tertile of CRP were older, female, had higher Hemoglobin-A1c (HbA1c) and glucose levels, brushed their teeth at least twice a day but did not floss at all. Those in the highest tertile of adiponectin were similar. Gingival condition was similar across the tertiles of CRP and adiponectin. Cluster scores were not significantly different; however, overweight participants had qualitatively lower scores for clusters 2 and 3 than normal participants. Clusters of periodontal microorganisms were associated with CRP and adiponectin after accounting for potential confounders.

The oral composition of microorganisms was similar among youth with and without type 1 diabetes. Normal and overweight youth with type 1 diabetes had similar profiles too. This may be due to young age of participants, relatively short type 1 diabetes duration, regular medical care, and low level of periodontal disease. CRP was positively-related to the 'orange-blue' cluster and adiponectin was negatively-related to the 'Blue-Other ' cluster.

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LIST OF ABBREVIATIONS

AGE ..... Advanced glycosylation endproducts

BMI ..... Body mass index

CAL ..... clinical attachment loss

CEJ ..... Cementoenamel junction

CRP ..... C-reactive protein

DAISY ..... The Diabetes and Autoimmunity Study in the Young

DNA ..... Deoxyribonucleic acid

HbA1c ..... Hemoglobin A1c

HIV ..... Human immunodeficiency virus

NHANESIII ..... National Health and Nutrition Examination Survey III

NHW ..... Non-Hispanic white

PAI-1 ..... Plasminogen activator inhibitor-1

PPD ..... periodontal pocket depth

TEDDY ..... The Environmental Determinants of Diabetes in the Young

TNF-α ..... Tumor necrosis factor alpha

WC ..... Waist circumference

WHR ..... waist-to-hip ratio

WHtR ..... waist-to-height ratio

CHAPTER 1

INTRODUCTION

BACKGROUND

Recently, researching the role of periodontal disease in obesity gained momentum in the literature(Darveau, 2010; Goodson, Groppo, Halem, & Carpino, 2009; Gregor & Hotamisligil, 2011; Teeuw, Gerdes, & Loos, 2010), as well as the effect of periodontal microorganisms on markers of systemic-inflammation(D'Aiuto et al., 2006; Miyashita et al., 2012; A. Pejcic, L. Kesic, & J. Milasin, 2011b). Goodson et al., explored the association between obesity and 40 different oral microorganisms and reported that a subset of them were associated with weight gaining. They even postulated three hypothesized mechanisms by which periodontal microorganisms may contribute to that(Goodson et al., 2009).

Other studies found debatable association between periodontal disease and several metabolic risk factors, however they all assessed periodontal disease by the known clinical methods (clinical attachment loss, periodontal pocket depth, and radiographic bone loss) without looking into the periodontal microbial composition(Beck et al., 2005; Feng & Weinberg, 2006; Humphrey, Fu, Buckley, Freeman, & Helfand, 2008; E. Lalla et al., 2007a, 2007b; Loesche & Grossman, 2001). A randomized-controlled-trial showed that intensive periodontal treatment reduced systemic inflammation and

improved lipid profile(Wadwa et al., 2010). Moreover, when clinical periodontal disease ascertainment was coupled with profiling of periodontal microorganisms, the relationship between periodontal disease and obesity became substantially stronger.

In a recent study, Desvarieux et al., identified certain species of periodontal microorganisms that were associated with thickening of the intima media of the carotid artery. They suggested that cardiovascular diseases could be predicted by the current composition of the microorganisms that are associated with periodontal disease(Beck et al., 2005; Desvarieux et al., 2005; Mustapha, Debrey, Oladubu, & Ugarte, 2007). Cani et al., proposed a mechanism that links obesity to the composition of intestinal flora and identified a lipopolysaccharide produced by certain gram-negative bacteria to be a factor triggering the onset of obesity and insulin-resistance. Hence, obesity can be predicted, or prevented, by the gastrointestinal microbial profile(Cani et al., 2007). Ley et al., found that certain bacterial species of the gut, Fermicutes, were more common among those who were obese(Ley, Turnbaugh, Klein, & Gordon, 2006). The gastrointestinal micro flora has metabolic capabilities as diverse as its constituents. Evidence is building up in favor of the positive relationship between micro flora (gastrointestinal and oral) and obesity.

Current clinical methods of periodontal disease diagnosis-clinical attachment loss (CAL), periodontal pocket depth (PPD), and radiographic bone loss-evaluate the impact of periodontal disease without taking into consideration its microbial composition, and hence may not be ideal in assessing the systemic effects of periodontal

disease(Albandar, 2007; Burt, Research, & Therapy Committee of the American Academy of, 2005). In the case of non-advanced stages of periodontal disease, these clinical assessment measures, become more complex to determine(Burt et al., 2005), especially since conditions such as gingival bleeding can be reversed by good oral hygiene(Honkala & Freeman, 1988). Therefore, the likelihood of underestimating the association between periodontal bacterial profile with both obesity and markers of chronic inflammation cannot be ruled out(Humphrey et al., 2008).

AIMS AND RESEARCH QUESTIONS

The overall objective of the study in hand is to deliver rigorous epidemiologic indication of the constitution of oral bacterial profile of youth with or without type 1 diabetes and its relationship with anthropometric and biochemical indicators of obesity and chronic inflammation.

Aim 1: To identify clusters of periodontal microorganisms found in dental plaque among youth with and without type 1 diabetes.

Research question 1.1: How do periodontal microorganisms found in dental plaque cluster among youth with type 1 diabetes?

Research question 1.2: How do the periodontal bacterial clusters, from research question 1.1, among youth with type 1 diabetes compare to/differ from those without type 1 diabetes?

Research question 1.3: How do periodontal bacterial clusters, from in research question 1.1, compare to/differ from the color-coded complexes as defined by Socransky et al. (red, orange, yellow, green, and blue)?

Research question 1.4: How do periodontal bacterial clusters, from research question 1.1, compare to/differ from the functionally-classified clusters by Desvarieux et al. (health, putative, and etiologic)?

Aim 2: To identify and quantify the relationship between the previously-identified bacterial clusters, from research question 1.1, and an anthropometric measure of adiposity [Body Mass Index (BMI) z-scores] among youth with and without type 1 diabetes.

Research question 2.1: Are clusters of periodontal microorganisms, from research question 1.1, related to BMI percentiles in youth with and without type 1 diabetes?

Research question 2.2: Are clusters of periodontal microorganisms, from research question 1.1, related to BMI percentiles in youth with and without type 1 diabetes, after adjusting for oral hygiene and health?

Research question 2.3: Are clusters of periodontal microorganisms, from research question 1.1, related to BMI percentiles in youth with type 1 diabetes, after adjusting for diabetes control?

Aim 3: To identify and quantify the relationship between the previously-identified bacterial clusters and markers of chronic inflammation among youth with and without type 1 diabetes.

Research question 3.1: Are clusters of periodontal microorganisms, from research question 1.1, related to plasma CRP and adiponectin levels in youth with and without type 1 diabetes?

Research question 3.2: Are clusters of periodontal microorganisms, from research question 1.1, related to plasma CRP and adiponectin levels in youth with and without type 1 diabetes, after adjusting for oral hygiene and health?

Research question 3.3: Are clusters of periodontal microorganisms, from research question 1.1, related to plasma CRP and adiponectin in youth with type 1 diabetes, after adjusting for diabetes control?

SIGNIFICANCE

Looking into periodontal bacterial composition and its association with measures of adiposity and markers of systemic-inflammation was advantageous because: 1) it was possible to detect the organisms even when CAL, PPD, and radiographic bone loss levels were low(Demmer, Papapanou, Jacobs, & Desvarieux, 2010; Suda et al., 2004); 2) it was biologically relevant and plausible(Boutaga, van Winkelhoff, Vandenbroucke-Grauls, & Savelkoul, 2005; Li & Hotamisligil, 2010; Socransky & Haffajee, 2005); and 3) it was reliably measured(Teles, Haffajee, & Socransky, 2008).

The innovative features of the presented research are the use of a novel biologically-relevant tool of assessing periodontal microbial profile and its conduct among youth with type 1 diabetes. This will help elucidate another layer to the complexity of the relationship between periodontal disease and systemic manifestations. The comparison of such a measure under the specified research questions will provide the scientific society with new insights to these conditions in an under-studied, population group of youth with type 1 diabetes.

CHAPTER 2

LITERATURE REVIEW

TYPE 1 DIABETES

Diabetes is a disease in which the body does not have strict control over the level of sugar in the blood. There are mainly 2 types of diabetes: Type 1 and Type 2. Type 1 diabetes is characterized by the inability of the pancreatic beta-cells to produce enough insulin (a hormone that down regulates blood glucose). It mainly occurs in children under 20 years of age, and less commonly in adults. Type 1 diabetes occurs for no known reasons, but it is hypothesized that genetic and/or environmental factors contribute to its causation("Life with T1D,").

In the US, among adolescents less than 20 years old, 215,000 (prevalence = 0.26%, adjusted for age) had diabetes (both types) in 2010. Out of all cases of diabetes only 5% were type 1. The prevalence of type 1 diabetes among 0-19 year-olds in the US was 1.7/1000. Type 2 diabetes is more common among 10-19 year-olds who have a family history of diabetes and are overweight. It is less common among Whites and most common among American Indians. Among 15-19 years-old American-Indians the prevalence of type 2 diabetes ranged from 4.5/1000 to 50.9/1000. Being a rare disease, prevalence of diabetes (type 1 and type 2) is not available for all ethnic/racial groups

and might be under-reported("Children and Diabetes — More Information,"). Between 2001 and 2009 the prevalence of type 1 diabetes increased by 30% in most age-groups, ethnic/racial groups, and both sexes. In 2001 the prevalence was 1.48/1000 (4958/3.34 million youth) and increased to 1.93/1000 (6666/3.4million youth) in 2009(Dabelea et al., 2014). The SEARCH for Diabetes in Youth reported 15,600 new cases of type 1 diabetes, 2002-2005. The incidence of type 1 diabetes was higher among those who were <10 years old (19.7/100,000) than those who were 10-19 years old (18.6/100,000). The incidence of type 1 diabetes was highest among Whites; (24.8/100,000) among those who were < 10 years old and (22.6/100,000) among those who were 10-19 years old("Children and Diabetes — More Information," ; "FAST FACTS ON DIABETES,"). Other than race and age, factors driving such incidence of type 1 diabetes may include genetic predisposition, environmental factors, and exogenous factors. Different genetic loci have been identified to contain genes that influence type 1 diabetes. The most studied gene is IDDM1. It encodes a mutated Human Leukocyte Antigen (HLA) that contributes to genetic factors influencing type 1 diabetes. , however, genetic factors are not enough to trigger the onset of the disease. Environmental factors are also suggested to contribute to the development of type 1 diabetes. It is suggested that a dietary antigen could be involved in this process. Moreover, there appears to be a difference in the gut micro flora of those who clinically manifest type 1 diabetes than those who do not. This may be an environmental factor that contributes to the initiation of type 1 diabetes. Exogenous factors such as viruses are also suspected to contribute to the development of type 1 diabetes although there is no comprehensive assessment of this hypothesis

and mechanism. However, a couple of studies, The Diabetes and Autoimmunity Study in the Young (DAISY) and The Environmental Determinants of Diabetes in the Young (TEDDY), aimed at looking into environmental factors as well as exogenous factors driving the incidence of type 1 diabetes such as infectious agents, eating habits, and psychological stress(Hagopian et al., 2006).

The complications of type 1 diabetes are many, but the most important and common are ketoacidosis, hypoglycemia, nephropathy, neuropathy, and adverse cardiovascular complications such as stroke, myocardial infarction, angina, and atherosclerosis(Dean L, 2004; Filippi & von Herrath, 2008; Knip & Simell, 2012).

- Ketoacidosis: the most common complication of type 1 diabetes. It is the result of breaking down fat, instead of sugar, in the absence of insulin. This causes the accumulation of ketones in the blood stream leading to systemic toxicity.
- Hypoglycemia: results from eating too little, intense physical activity, or too much insulin. It may lead to losing consciousness.
- Cardiovascular complications: stroke, myocardial infarction, angina, and atherosclerosis.
- Nephropathy: destruction of kidney nephrons leading to kidney failure in its more advanced stages.
- Neuropathy: affecting > 60% of those with type 1 diabetes. It can lead to weakness and even loss of sensation in the extremities.

- Retinopathy: the most common complication. It is characterized by the destruction of the blood capillaries in the retina of the eyes. Most patients with type 1 diabetes show some form of this complication over time and in about 20-30% it progresses to its severe form.

Factors affecting the development of these complications can also worsen the outcomes if ignored or not taken seriously("Diabetes Complications," ; Filippi & von Herrath, 2008);. however, patients with type 1 diabetes can significantly reduce the risk of complications and improve health outcomes by:

- Adhering to their healthcare plan: checking blood glucose regularly, taking their medication and/or the right dose of insulin on time, and scheduling regular clinical check-ups.
- Exercising regularly: promotes a controlled level of blood glucose.
- Eating healthy and nutritious foods : understanding how different foods affect the control of blood glucose helps maintain a balanced diet and helps in keeping a controlled level of blood sugar.
- Having a social support system: a key factor in motivating affected individuals in carrying-on normal life activities and increasing their quality-of-life.

Insulin therapy remains the most significant treatment of type 1 diabetes(Atkinson, Eisenbarth, & Michels, 2014). Long-acting insulin provides a baseline level of control and short-acting insulin (usually taken before meals and proportional to the amount of carbohydrates in the meal) provides a rapid control of blood glucose

level. With the advancement of medical technologies, the management of this condition includes the use of mechanical devices such as blood glucose monitors and insulin pumps, as well as insulin analogues(Hirsch, 2009). These devices are able to continuously sense the level of glucose in the blood and administer the right amount of insulin needed to maintain normal levels of glucose, enabling individuals with type 1 diabetes to reach a desirable level of long-term glucose control and is usually measured as HbA1c<7.5%.

After being diagnosed with type 1 diabetes, some individuals retain the ability to produce insulin in very low levels, and thus is very important in maintaining those secretions since they are associated with better health outcomes than other forms of exogenous insulin(Steffes, Sibley, Jackson, & Thomas, 2003).

PERIODONTAL DISEASE

Chronic generalized periodontitis is a low-grade inflammation of the gum and the surrounding and supporting tissue of the teeth in general, not localized. It starts as gingivitis which is characterized by redness, swelling, and increased susceptibility to bleeding of the gum, but causing no discomfort. It starts due to inadequate oral hygiene, but regresses with proper professional treatment and home-care(Periodontology). If untreated, gingivitis progresses to periodontitis. It is the most common form of periodontitis occurring in adults and children. It is also associated with the presence of plaque and different microbial configurations(Periodontology., 2001). In the advanced stages chronic generalized periodontitis can lead to separation of gum from the teeth and the formation of periodontal pocket and eventually tooth-loss, if not treated(Feng &

Weinberg, 2006; Loesche & Grossman, 2001; Socransky, Haffajee, Cugini, Smith, & Kent, 1998).

Turesky modification of Quigley Hein Plaque Index (MHQ)(Quigley & Hein, 1962) is the most widely used measure clinical measure of plaque index in research. MHQ is a measure of comprehensive plaque formation on all the teeth. After all teeth have been assessed on a 0-5 scale the total score is divided by the total number of tooth surfaces to get the MHQ. Teeth are assessed according to the following scale:

0 No plaque.

1 Separate spots of plaque at gingival margin.

2 Thin continuous band of plaque at gingival margin (≤ 1mm).

3 Continuous band of plaque covering up to 33% of tooth surface.

4 Continuous band of plaque covering 33-66% of tooth surface.

5 Tooth is mostly covered with plaque >66% of tooth surface.

Gingival Index is a common measure of gingival condition based on color and bleeding(Loe, 1967; Mankodi et al., 2005). Teeth are assessed according to the following scale:

0 Normal gingiva.

1 Mild inflammation: Slight change in color, slight edema; no bleeding on probing.

2 Moderate inflammation: Redness, edema, and glazing; bleeding upon probing.

3 Severe inflammation: Marked redness and edema; ulceration; tendency to spontaneous bleeding.

Gingival crevice is the gap between the gingiva and the surface of the tooth (enamel or cementum) (Periodontology., 2001). As periodontal diseases progresses this gap (fissure) starts widening and deepening forming a periodontal pocket. Among healthy individuals the gingival crevice completely surrounds the tooth and measures 3mm in depth at most; in this case the periodontal pocket depth (PPD) and the gingival crevice have the same measurement and clinical attachment loss (CAL) does not exist (PD≤3mm, CAL=0). In affected individuals the deepening of the pocket depth due to separation of the gum and supporting tissue from the teeth is referred to clinical attachment loss. CAL and PPD are essentially measured in the same manner, the only difference being the reference point of measurement. CAL (mm) is the defined as the distance from the cementoenamel junction (CEJ) to the base of the periodontal pocket while PPD (mm) is defined as the distance from the gingival margin to the base of the periodontal pocket. Both CAL and PPD are measured with a labeled periodontal probe(Arora, Weuve, Schwartz, & Wright, 2009; Turesky, Gilmore, & Glickman, 1970).

PERIODONTAL MICROORGANISMS

Oral microbiota has been studied since the 17th century and its composition has been under exploration ever since the first examination by Van Leeuwenhoec in

1683(Tal, 1980). Organization of oral microbes had been identified since the 1970's(Listgarten, Mayo, & Tremblay, 1975). Assessment of sub gingival plaque by DNA-probes yielded a deeper understanding of co-occurrence of different oral bacteria(Ali, Skaug, Nilsen, & Bakken, 1994; Gmur, Strub, & Guggenheim, 1989; Simonson, Robinson, Pranger, Cohen, & Morton, 1992). In more recent studies, technological development of DNA-DNA hybridization lead to characterization of the famous color-coded complexes (red, orange, yellow, green, and purple complexes) of bacteria by Socransky and colleagues(Socransky et al., 1998). The red complex included *P. gingivalis*, *T. forsythia*, and *T. denticola*. The orange complex comprised *P. intermedia*, *P. nigrescens*, *P. micros*, *F. nuc. vincentii*, *F. nuc. nucleatum*, *F. nuc. polymorphum*, *F. periodonticum*, *C. gracilis*, *C. rectus*, *E. nodatum*, *C. showae*, and *S. constellatus*. *S. mitis*, *S. oralis*, *S. sanguis*, *S. gordonii*, and *S. intermedius*, made-up the yellow complex, while *E. corrodens*, *C. gingivalis*, *C. sputigena*, and *C. ochracea* made-up the green complex. The purple complex consisted of *V. parvula* and *A. odontolyticus*. The blue complex consisted of different *Actinomyces* species. Members of the red complex were highly correlated with pocket depth. *P. gingivalis*, *B. forsythus*, and *T. denticola* increased in count were related to deeper pocket depths. Moreover, sites where all 3 species were present had greatest mean pocket depth. All members of the orange complex showed a positive correlation with pocket depth, too. In another study Socransky and Haffajee found that *A. actinomycetemcomitans, P. gingivalis and T. forsythia* were highly correlated with periodontal disease status and progression. *F.nucleatum subsp. vincentii, C. rectus* and *P. intermedia* were also more prevalent in periodontitis. At early stages of periodontal

disease, species of the blue, purple, green, and yellow complexes start to colonize the surface of the teeth. Then species of the orange complex take over, bridging the early colonizers with species of the red complex at more advanced stages of the disease (Socransky & Haffajee, 2002).

In a more recent study Desvarieux *et al.*, defined three groups of bacteria according to their association with oral health and related them to subclinical carotid atherosclerosis. They defined the (1) etiologic group (*Porphyromonas gingivalis*, *Tannerella forsythensis*, *Actinobacillus actinomycetemcomitans*, and *Treponema denticola*); (2) putative group (*Prevotella intermedia*, *Fusobacterium nucleatum*, *Micromonas micros*, *Campylobacter rectus*, and *Eikenella corrodens*); and (3) healthy-condition group (*Veillonella parvula* and *Actinomyces naeslundii*). They reported a significant association between the overall periodontal bacterial burden and thickness of the intima media of the carotid artery. Moreover, higher burden of the etiologic group was associated with increased atherosclerosis as well as increased count of white blood cells as a result of inflammation(Desvarieux et al., 2005).

PERIODONTAL DISEASE AND TYPE 1 DIABETES

The relation between periodontal disease and diabetes has been studied very well for several decades(Mealey, 2006). A meta-analysis concluded that patients with diabetes, both types, had more severe levels, but not necessarily higher prevalence, of periodontal disease than those without diabetes(Khader, Dauod, El-Qaderi, Alkafajei, & Batayha, 2006). A recently published systematic review suggested that periodontal diseases might affect diabetes outcomes, however it prompted the need for high-quality

follow-up studies to determine that(Borgnakke, Ylostalo, Taylor, & Genco, 2013). The literature suggests that diabetes is a risk factor for both, gingivitis and periodontitis(Papapanou, 1996). In a review of the directionality of association between diabetes and periodontal disease, Taylor concluded that the association was bidirectional(G. W. Taylor, 2001). Meenawat et al. reported that Type 1 diabetes impacted periodontal disease severity and progression and was associated with higher bleeding index corresponding to more severe periodontal inflammation caused by bacteria in dental plaque. They also reported higher levels of periodontal inflammation among those with poorer diabetes control. Greater attachment loss was reported among those with T1D(Meenawat et al., 2013). Diabetes can influence periodontal disease by several mechanisms through vascular abnormalities, immune cells dysfunction, abnormal synthesis of collagen, and genetic predisposition(Oliver & Tervonen, 1994). In the presence of hyperglycemia, proteins of the basement membrane undergo non-enzymatic glycation(Oliver & Tervonen, 1994) and the inflamed gums increase the production of pro-inflammatory cytokines such as Tumor Necrosis Factor- α (TNF-α). As advanced glycation end products increase, the secretion of pro-inflammatory cytokines increase as well. This could be one way in which diabetes promotes periodontal destruction(Kiran, Arpak, Unsal, & Erdogan, 2005). Diabetes exaggerates the innate immune responses in the presence of periodontal disease which explains its increased severity. Moreover, periodontal disease tends to exaggerate the immune response and in turn accelerate the nephropathy and macrovascular complications of diabetes(Nishimura, Iwamoto, & Soga, 2007). Type 1 diabetes was

shown to be associated with higher susceptibility to periodontal disease when compared to individuals without diabetes. Poorer metabolic control was related to increased inflammation and longer duration of diabetes was related to increased attachment loss. Even after adjusting for plaque index individuals with type 1 diabetes were more susceptible to developing periodontal disease(Ajita, Karan, Vivek, S, & Anuj, 2013).

Evidence suggests that type 1 diabetes is related to increased susceptibility for developing periodontal disease. Poor metabolic control, in addition to environmental factors such as smoking and poor oral hygiene was related to increased periodontal destruction(Poplawska-Kita et al., 2014). Moreover, periodontal pathogens (*T. forsythia* and *T. denticola*) were the most abundant microorganisms in sub gingival plaque among individuals with type 1 diabetic and correlated with poorer metabolic control(Schara, Skaleric, Seme, & Skaleric, 2013). Type 1 diabetes could contribute to increased pathogenesis of periodontal disease by interfering with the immune system in the presence of advanced glycation products through increased insulin resistance, vascular complications, and enhanced growth of periodontal pathogens, prompting the importance of controlling periodontal disease and thus achieving better metabolic control(E. Lalla & Papapanou, 2011). Although dental plaque was a main risk factor for patients with progressive periodontal disease, the severity of such condition was more evident among individuals with type 2 diabetes than those with diabetes type 1(Pranckeviciene, Siudikiene, Ostrauskas, & Machiulskiene, 2014).

PERIODONTAL DISEASE AND OBESITY

Periodontal disease has been shown to be highly associated with obesity. Several studies have found significant correlation between periodontal disease and obesity. In a Japanese population 20-59 years old, the adjusted relative risk of periodontitis was 3.4 among those who were overweight, and 8.6 among the obese, and for every 5% increase in body fat the risk increased by 30%(Saito, Shimazaki, & Sakamoto, 1998). Gorman et al., found that among periodontitis-free men, the hazard for developing periodontitis was more than 40% higher in those who were obese when compared to their leaner counterparts, as assessed by both BMI and waist-to-height ratio (WHtR)(Gorman et al., 2012). Another prospective study found significant association between periodontal disease and obesity even among non-diabetic individuals and never-smokers for all measures of adiposity (BMI, waist circumference (WC), and Waist-to-Hip Ratio (WHR)). Elevated hazard ratios for developing periodontitis was observed among those who were obese or had high WC or WHR(Jimenez, Hu, Marino, Li, & Joshipura, 2012). In a recently-published meta-analysis of 57 observational studies the prevalence odds of obesity was 33% higher for those who have periodontitis, across different populations from all around the world(Chaffee & Weston, 2010). Al-Zharani et al. reported an association between measures of obesity and periodontal disease among younger adults, using data from the National Health and Nutrition Examination Survey III (NHANES III)(Al-Zahrani, Bissada, & Borawskit, 2003). Wood et al. found correlations between measures of obesity (BMI, WHR) and measures of periodontal disease (CAL, PD)(Wood, Johnson, & Streckfus, 2003).

Goodson et al. proposed 3 pathways that are hypothesized to link periodontal bacteria to obesity. The first hypothesized mechanism suggests that certain oral bacteria may contribute to an increased efficiency in fat storage in such a way that keeping diet and exercise constant an increase of 100cal/day could add about 10lbs of fat per year. The second hypothesized mechanism is through the control of appetite through controlling leptin (satiety-stimulating and fat-metabolizing hormone) and ghrelin (hunger-stimulating hormone) which in turn control food intake. A third hypothesized mechanism is through the up-regulation of systemic inflammation markers (TNF-α) and down-regulation of adiponectin, by some unknown pathway, which leads to increased insulin-resistance. This, in turn, decreases the storage of energy in the form of glycogen and increases the storage of energy in the form of fat(Goodson et al., 2009).

A recent novel study by Goodson et al.,(Goodson et al., 2009) looked at the difference in median percentage of 40 different bacterial species between overweight and non-overweight individuals. The bacteria they looked at were members of 6 phyla: Fermicutes, Bacteroidetes, Fusobacteria, Actinobacteria, and Spirochaetae. They found that the median percentage difference was greater than 2%, for 7 species, among overweight individuals compared to those who were not. More than 98% of those who were overweight could be identified by just S. noxia > 1.05%, according to the classification tree topology technique, with 98.4 sensitivity and 80.2 specificity(Steinberg & Colla, 1997).

The oral cavity is inhabited by more than 700 species of bacteria(Paster et al., 2001). It is dominated by Fermicutes (76%), Bacteroidetes (6%), Actinobacteria (10%), Fusobacteria (3%), Proteobacteria (4%), and Spirochetes (<1%). The gastrointestinal flora is similar in composition to the oral flora, but different in proportions. It is dominated by Fermicutes, then Bacteroidetes, Actinobacteria, and Proteobacteria. However, the gut is richer in Fermicutes and Bacteroidetes, but poorer in Fusobacteria and Proteobacteria than the mouth(Koren et al., 2011). Members (*Selenomonas noxia*, *Actinomyces gerencseriae*, *Actinomyces naeslundii*, *Neisseria mucosa*, *Fusobacterium periodonticum*, *Fusobacterium nucleatum ss vincentii*, and *Prevotella melaninogenica*) of the 4 main phyla (Fermicutes, Bacteroidetes, Actinobacteria, and Fusobacteria) comprising the oral microbiota were found to be highly associated with obesity(Goodson et al., 2009).

DiBaise et al., observed a positive association between gastrointestinal bacterial profile and overweight. The mechanism they proposed is that the gut flora facilitates the extraction of energy from food, and facilitates its storage in adipose tissue for later use. Certain signaling molecules present in the gut microbiota enhance factors that link inflammation to metabolic syndrome(DiBaise, Young, & Vanderhoof, 2006).

PERIODONTAL DISEASE, CRP, AND ADIPONECTIN

In a west-European study of older men, 60-70 years old, and after a 10-years follow-up period found a significant association between periodontitis and elevated levels of CRP, also a significant association was detected between high serum CRP and tooth loss(Linden, McClean, Young, Evans, & Kee, 2008). Similar finding were also

reported by Chitsazi et al. they found a positive correlation between measures of periodontal disease (CAL and PD) and elevated levels of serum CRP(Mohammad Taghi Chitsazi, 2008). A controlled clinical trial reported a significant decrease in serum CRP following periodontal treatment of obese and normal-weight individuals with periodontal diseases(Al-Zahrani & Alghamdi, 2012). A Serbian study reported a significantly higher level of serum CRP among individuals with periodontal disease. Those having high measures of CAL had higher levels of CRP (>5 mol/L). The presence of periodontal pathogens was related to higher CRP levels and poor periodontal condition(Pejcic et al., 2011b).

A recently published study found that the level of circulating adiponectin was highly influenced by the presence of periodontal disease and obesity up regulated TNF-α, an inflammatory marker(Zimmermann, Bastos, Dias Goncalves, Chambrone, & Duarte, 2013). Among individuals with type 2 diabetes, periodontal treatment was found to reduce serum inflammatory markers and increase serum adiponectin levels, even under suboptimal diabetes control(W. L. Sun et al., 2011). The results of the latter study confirmed those of an earlier one by Matsumoto et al(Matsumoto et al., 2009). A study in an animal model explored the effect of periodontal disease on adiponectin receptors (AdipoR1 and AdipoR2). The expression of these receptors was found to be lower in those who have severe periodontal disease when compared to those who were healthy(Yamaguchi et al., 2010).

As described by Gregor and Hotamisligil, obesity-induced inflammation was called ‘metaflammation’ since it involves the systemic interference of specialized immune cells. ‘Metaflammation’ can result in reduced insulin effect through the production of inflammatory kinases(Gregor & Hotamisligil, 2011). In another study, Hotamisligil described the effect of organelle stress, the endoplasmic reticulum, on systemic inflammation(Hotamisligil, 2010). Several other studies found that higher levels of serum inflammatory mediators like C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1) are associated with increased risk for developing type 2 diabetes(Festa, D'Agostino, Tracy, Haffner, & Insulin Resistance Atherosclerosis, 2002; Pradhan, Manson, Rifai, Buring, & Ridker, 2001; Vozarova et al., 2002), and were found to have higher levels in obese individuals in both, animal and human models(Pickup & Crook, 1998; Shoelson, Lee, & Goldfine, 2006). One of the proposed mechanisms by which oral bacteria inhibits insulin-sensitivity is by decreasing the production of adiponectin(Goodson et al., 2009). Moreover, insulin-resistance is no longer associated to just diabetes, but also infectious diseases such as Human Immunodeficiency Virus (HIV) and hepatitis C(Bahtiyar, Shin, Aytaman, Sowers, & McFarlane, 2004; Pao, Lee, & Grunfeld, 2008; Sidiropoulos, Karvounaris, & Boumpas, 2008), and systemic diseases such as sepsis(Clowes et al., 1978; Wichterman, Chaudry, & Baue, 1979) and rheumatoid arthritis(Sidiropoulos et al., 2008). Studies have shown that insulin-resistance among obese individuals was highly associated with the secretion of inflammatory cytokines(Hotamisligil, Arner, Caro, Atkinson, & Spiegelman, 1995; Hotamisligil et al., 1996; Hotamisligil, Shargill, & Spiegelman, 1993), especially among those who have

diabetes(Baker et al., 2006; Bloom, 1969; Fearnley, Vincent, & Chakrabarti, 1959; Ogston & McAndrew, 1964; Van Cromphaut, Vanhorebeek, & Van den Berghe, 2008).

PERIODONTAL MICROORGANISMS, TYPE 1 DIABETES, CRP, AND ADIPONECTIN

Among adults with type 1 diabetes, the most abundant microbes found in dental plaque were *Fusobacterium nucleatum* (77.8%), *Capnocytophaga* species (66.7%), and *E. corrodens* (33.4%); *A. actinomycetemcomitans* was identified 40.7% more frequently among those without diabetes. *F. nucleatum* and *Capnocytophaga spp.* were associated with poorer HbA1c control(Sakalauskiene et al., 2014). Another study found that *T. forsythia* (48%) and *T. denticola* (31%) were the most abundant microbes found in sub gingival plaque of adults with type 1 diabetes, followed by *P. gingivalis* (26%), *P. intermedia* (9%), and *A. actinomycetemcomitans* (7%); *T. denticola* and *T. forsythia* were associated with higher HbA1c levels(Schara et al., 2013). Higher serum antibodies against *P. gingivalis* and *A. actinomycetemcomitans* were associated with atherosclerosis of the coronary artery among adults with type 1 diabetes(Colhoun et al., 2008). A Japanese study reported that in adults with type 1 diabetes, *P. gingivalis* and duration of diabetes greatly influenced the progression of periodontal disease(Takahashi et al., 2001). Patients with type 1 diabetes were found to have comparable antibody levels to periodontal microbes as well as similar infection patterns when compared to those without diabetes. Serum antibody titer against *E. nodatum* was significantly higher among those with diabetes. Similar, yet statistically insignificant, pattern was observed for *A. naeslundii*(E. Lalla, Kaplan, et al., 2006).

A recently published study reported a positive correlation adiponectin and peripheral neuropathy among youth with type 1 diabetes, but not related to the thickness of intima media of the carotid artery or other cardiovascular risk factors(Sherief, Amr, Adly, & Gharib, 2014). In another study adiponectin levels were not significantly different among youth with type 1 diabetes when compared to those without diabetes, however higher CRP levels were detected among those with diabetes(Goksen, Levent, Kar, Ozen, & Darcan, 2013). A study in south India reported a significantly higher level of adiponectin among youth with type 1 diabetes after adjusting for age and sex, when compared to a control group without diabetes(Solomon & Varadarajan, 2013). Similarly, among a sample of American youth and adults with type 1 diabetes, adiponectin was found to be significantly higher than those without diabetes(E. Lalla, Kaplan, et al., 2006). P. gingivalis and A. actinomycetemcomitans were found to be independently associated with CRP(A. Pejcic, L. J. Kesic, & J. Milasin, 2011a). Periodontal pathogens may contribute to systemic conditions and inflammation. Similar results were shown by Dye et al., among participants of the National Health and Nutrition Examination Survey III(Dye, Choudhary, Shea, & Papapanou, 2005).

GAPS IN KNOWLEDGE

Obesity and periodontal disease are complex systemic diseases. The relationship between them is well-documented in the literature but the underlying mechanisms between them are under-investigated(Ylostalo, Suominen-Taipale, Reunanen, & Knuuttila, 2008). The direction of the association between periodontal disease and obesity is not known, and might not be even possible to design a study to determine it.

Whether periodontal disease affects lipid metabolism or hunger/satiety or obesity affects the susceptibility to periodontal disease is under investigation. Although evidence favors an association between periodontitis and both, obesity and markers of inflammation, the physiologic mechanism behind that is understudied(Chaffee & Weston, 2010). In this study we shed some light on how periodontal microorganisms influence the relationship between periodontal disease and, obesity and chronic inflammation, among youth with type 1 diabetes.

CHAPTER 3

CLUSTERS OF ORAL BACTERIA IN DENTAL PLAQUE AMONG YOUTH WITH TYPE 1 DIABETES[1]

[1] Georges J. Nahhas, R. Paul Wadwa, Elaine H. Morrato, Jiajia Zhang, Lonnie Johnson, Franziska Bishop, Ricardo Teles, Linda J. Hazlett, David M. Maahs, Anwar T. Merchant. . In preparation for submission to the Journal of Periodontology.

ABSTRACT

We aimed to explore differences in oral-bacterial profile between youth with and without type 1 diabetes.

Data were collected from 105 youth with type 1 diabetes and 71 without diabetes. Counts of 41 oral-bacteria from sub gingival-plaque were obtained by DNA-DNA hybridization and grouped using cluster-analysis. Standardized-mean counts of each organism were computed and summed to get microbial-counts per cluster. A subset (n=101, 54 with type 1 diabetes) underwent dental examinations at the University of Colorado, School of Dental Medicine clinic.

Participants were 15-years old on average; 51% were female; 73% were white; the average diabetes duration was 8.6-years. About 48% brushed their teeth twice/day; 12% flossed once/day; 93% visited a dentist in the last year. Four mutually-exclusive clusters were identified. Individuals with and without type 1 diabetes had similar microbial composition ('blue-other' cluster: 6% versus 9%, 'orange-blue' cluster: 43% versus 42%, 'orange-red' cluster: 35% versus 32%, and 'yellow-other' cluster: 16% versus 17%, $p>0.05$ for all comparisons). 'orange-blue' cluster contained microorganisms associated with gingival-bleeding. 'orange-red' cluster contained microorganisms associated with periodontal-disease. 'yellow-other' cluster contained mostly microorganisms unassociated with periodontal disease.

The distribution of microorganisms was similar among youth with and without type 1 diabetes receiving regular medical and dental care.

INTRODUCTION

Oral microbiota has been studied since the 17th century ever since the first examination by Van Leeuwenhoec in 1683(Tal, 1980). Attempts to organize oral microbes were reported in the 1970's(Listgarten et al., 1975), and recent assessment of sub gingival plaque by DNA-probes enhanced identification and quantification of different oral microbes(Ali et al., 1994; Gmur et al., 1989; Simonson et al., 1992). This was a major step forward that led Socransky and colleagues to characterize empirically-formed groups of oral microbes related to periodontal disease in adults into color-coded complexes (red, orange, yellow, green, purple and blue)(Socransky et al., 1998). The red and orange complexes were found to be closely related to each other and distantly related to the yellow and green complexes. Moreover, members of the red complex were rarely found in the absence of members from the orange complex. Evidence suggests that colonization of sites by members of the orange complex precede the colonization by members of the red complex. The red and orange complexes were both found to be highly associated with periodontal disease as measured by increasing pocket depth, and members of the red complex were all associated with bleeding on probing(Socransky et al., 1998).

In a more recent study, Desvarieux *et al.*, reported that pre-specified groups of selected oral microbes were related to subclinical atherosclerosis. They organized the microbes in the following way: (1) etiologic group based on presence of these microbes in lesions of both periodontal disease and atherosclerotic plaque(Gunaratnam, Smith,

Socransky, Smith, & Haffajee, 1992; Socransky et al., 1994); (2) putative group which is thought to be putatively associated with periodontal disease(Socransky et al., 1994); and (3) healthy-condition group(Desvarieux et al., 2005) which has been found to be associated with healthy periodontal condition(Gunaratnam et al., 1992). However, these groups did not include all the oral microbes that are commonly associated with oral health.

The association between diabetes and periodontal disease is well established(Al-Shammari et al., 2006; Chapple, Genco, & Working group 2 of joint, 2013; Dakovic & Pavlovic, 2008; E. Lalla et al., 2007b; Mealey & Ocampo, 2007). , but its relation with oral microbes is understudied (E. Lalla, Kaplan, et al., 2006; Mashimo, Yamamoto, Slots, Park, & Genco, 1983). This paper provides new and original insight into a previously under-explored area extending this research into adolescents, among whom such relationships are less known. We therefore studied the relation between oral microbes and type 1 diabetes. The primary aim of this study was to identify groups of oral microbes found in dental-plaque among youth with type 1 diabetes using cluster analysis. The secondary aim was to compare the distribution of the oral microbial groups identified in youth with type 1 diabetes to those without.

METHODS

Study Participants

A nested-case-control study was conducted among participants of a cohort studying cardiovascular risk factors among youth with and without type 1 diabetes(Maahs et al., 2011; Specht et al., 2013). From 2009 to 2011, data were available from 176 youth, aged 12-19 years including 105 participants with type 1 diabetes ("cases") who were patients at the Barbara Davis Center for Childhood Diabetes in Aurora, Colorado and 71 participants without type 1 diabetes ("controls"). Youth with type 1 diabetes were diagnosed by islet cell antibody or provider clinical diagnosis and had type 1 diabetes for 5 years or more. Subjects were excluded if they had any history of abnormal cardiac anatomy or arrhythmia, if they had smoked or had caffeine in the 8 hours preceding the study visit, or if they were ever diagnosed with any diabetes other than type 1. Controls were recruited by community advertisement or from the pool of friends of the participants. Siblings and first-degree relatives of youth with diabetes were also excluded. Individuals were additionally excluded if they needed the administration of antibiotics for prophylaxis before dental procedures, as part of dental treatment, or if they had received antibiotic treatment in the 30 days preceding the study visit, since its use alters the oral microbes. All participants signed a consent or assent (if <18 years old). The Colorado Multiple Institutional Review Board and the University of South Carolina Institutional Review Board reviewed and approved this study.

Demographics and Clinical Measures

Information on age, sex, race, and diabetes duration and medications were collected via standardized questionnaires. Measurements of HbA1c were obtained by the DCA Advantage by Siemens (Princeton, New Jersey) at the Children's Hospital Colorado's main clinical lab. All participants visited the clinic after an overnight fast. BMI z-scores were calculated and defined as normal (< 85^{th} percentile), overweight (≥85^{th} but ≤95^{th} percentile), and obese (>95th percentile).

Oral Health Measures

Information on oral health knowledge and attitudes, oral hygiene behavior, and use of dental care was collected on a sub-sample (54 cases and 47 controls) by a separate questionnaire adapted from the National Survey of Children's Health, the Medical Expenditure Panel Survey, and the CDC Periodontal Surveillance Survey(Orlando et al., 2010). All Questionnaires were completed prior to the oral exam in order to minimize the influence of knowledge and/or response bias (Morrato et al., 2014).

Assessment of oral health included measurements of using a University of North Carolina color-coded periodontal probe [Hu-Friedy]: (1) Plaque Index: indication of short-term oral hygiene [0 (none) - 3 (abundance)](Silness & Loe, 1964); (2) Calculus Index: indication of long-term oral hygiene[0 (none) - 3 (abundance)]; and (3) Gingival Index: indication of general gingival inflammation [0 (normal color) - 3 (spontaneous bleeding at the gingival margin)](Loe & Silness, 1963).

Plaque Collection

Sub gingival plaque samples were taken prior to periodontal examination, from all study participants (n=176), using individual sterile Gracey curettes from the mesial aspect of the first permanent molar tooth in 2 randomly chosen quadrants. Samples were placed in separate Eppendorf tubes containing 0.15 ml TE (10mM Tris-HCl, 1mM EDTA, pH 7.6) to which 0.10 ml of 0.5M NaOH (freshly prepared) was added(W. Sun et al., 2010). They were sent by mail(Morita et al., 2010; Paraskevas, Huizinga, & Loos, 2008; Snell-Bergeon et al., 2010) to the Forsyth Institute, Boston, where they were evaluated for counts of 41 periodontal microbes using DNA-DNA hybridization(Romano et al., 2001; Socransky et al., 2004; Socransky et al., 1994).

Both sub-gingival plaque samples from each individual were pooled and lysed. Then DNA was loaded in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA, USA). After fixation, the membrane was placed in a Miniblotter 45 (Immunetics), with the lanes of DNA orthogonal to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to multiple sub gingival species were hybridized in individual lanes of the Miniblotter. Then, membranes were washed at high stringency, and DNA probes were detected using antibody to digoxigenin conjugated with alkaline phosphatase and chemifluorescence. Signals were detected using AttoPhos substrate (Amersham Life Science, Arlington Heights, IL, USA) and read by a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA), a computer-linked instrument that reads the intensity of the fluorescence signals resulting from the probe-

target hybridization. Signals were then converted to absolute counts by comparison with the standards on the same membrane (A. T. Merchant, Pitiphat, Ahmed, Joshipura, & Haffajee, 2003; Romano et al., 2001).

Statistical Analysis

The sample mean and standard deviation of each microorganism were obtained. Standard-normal z-scores were generated by subtracting the sample-mean of each microorganism from each observation and then dividing the whole quantity by the sample standard deviation of the respective microorganism. This was performed separately for cases and controls.

Cluster analysis is a statistical procedure dividing observation into mutually-exclusive clusters, in which each cluster is composed of individual elements sharing some similarities. We used the ward's distance to measure the distance among two oral microbes in the cluster analysis, and applied it to the standard-normal z-scores of 41 oral microbes. Note, this method minimizes within-cluster variation and uses the within-cluster sum of squares as a measure of homogeneity. Then, we specified the number of clusters to 4, a priori (Figure 3.1.). Since our primary objective was to explore how these oral microbes cluster in youth with diabetes and compare such clustering to controls, cluster analysis was performed among cases of diabetes only.

Standardized scores were generated by dividing the counts of each microorganism by its respective standard deviation. Standardized scores for each microbe were summed for all participants by diabetes status. Dominance of each

microbe was determined by dividing its sum of summary score by the total sum of summary scores separately.

RESULTS

Participants were age 15 years on average; 49% were males; 73% were non-Hispanic white (NHW), 5% were black, and 22% were of other races. The mean BMI percentile was significantly different ($p = 0.004187$) between cases (70.2%) and controls (58.6%). There was a significant difference in the frequency of dental visits ($p = .0162$) between youth with type 1 diabetes and those without. Clinical and demographic characteristics are described in Table 3.1. Youth with type 1 diabetes were mainly treated by insulin injections (59%); 14% had an HbA1c <7.5%. Of the subset of participants for whom data on oral hygiene practice were available (n=101, 54 cases), the majority of cases (54%) and controls (75%) brushed their teeth more than once a day. Dental flossing was less frequent among cases of diabetes (46%) than youth without type 1 diabetes (62%), however, these differences did not reach statistical significance. Percent bleeding sites on probing was more common among cases than controls (20.8% versus 17.9) but this difference was not statistically significant (p-value=0.3083).

The 41 oral microbes were classified into 4 mutually-exclusive clusters using cluster analysis, a data-driven classification method, and named in concordance with the nomenclature of the clusters identified by Socransky and colleagues (Socransky et al., 1998). Cluster 1 (Blue-Other) contained 4 microorganisms and was dominated by a

member of the *Actinomyces* species (blue complex). Cluster 2 (Orange-Blue) contained most of the microorganisms that are found in the orange complex, which is known to be associated with gingival bleeding, and the green complex, which is not associated with any sort of periodontal condition. Cluster 3 (Orange-Red) contained all members of the red cluster, *P. gingivalis*, *T. forsythia*, and *T. denticola* which are known to be associated with periodontal disease and some members of the orange and yellow complexes. Cluster 4 (Yellow-Other) contained most of the microorganisms found in the yellow complex, which is not associated with periodontal disease. Total and cluster counts of microorganisms were similar for cases of type 1 diabetes and controls (Table 3.2.).

Comparing standardized scores of oral microbes classified according to the clusters defined by Socransky(Socransky et al., 1998), youth with and without type 1 diabetes had very similar proportions for the different clusters, 9%, for both groups, for the red complex, 37% versus 35% for the orange complex, 17% versus 16% for the yellow complex, 13% versus 12% for selected members of the green complex, and 9%, for both groups, for the purple complex. Probes for detection of 2 members, *Actinobacillus actinomycetemcomitans* serotype a and *Campylobacter concisus*, of the green complex were not available. The proportion of *S. noxia* was about 2%. The total standardized microbial count for the available microbes was 3020 million among youth with type 1 diabetes and 2698 million among controls.

Comparing standardized scores of oral microbes classified according to the groups defined by Desvarieux *et al*.(Desvarieux et al., 2005), youth with and without

type 1 diabetes had very similar proportions for the different groups. The group of etiologic microbes made up 27% versus 30%, the group of putative microbes made up 58% versus 54% among youth with type 1 diabetes versus controls, respectively. The group of health-associated microbes made up 15% versus 16% for cases of type 1 diabetes and controls, respectively.

DISCUSSION

We identified 4 mutually exclusive clusters in youth with type 1 diabetes that were derived from 41 commonly occurring oral microbes. The distribution of oral microbes was very similar among youth with and without type 1 diabetes in this population of mostly non-Hispanic white, normal-weight youth, who had regular health check-ups.

Few studies have examined youth with diabetes. Al-Khabbaz *et al*.(Al-Khabbaz, Al-Shammari, Hasan, & Abdul-Rasoul, 2013) found that among youth 4-14 years old, those who had type 1 diabetes had higher level of gingival inflammation and plaque accumulation when compared to those without diabetes. A follow-up study of youth with type 1 diabetes(E. Lalla, Cheng, et al., 2006) found that the level of periodontal destruction is related to the level of metabolic control (E. Lalla, Kaplan, et al., 2006). Another study of youth with type 1 diabetes, that is comparable to our sample, found a low prevalence of periodontal disease among this population, however, those who had elevated HbA1c were at higher risk of developing early signs of periodontal disease(Morrato et al., 2014).

The composition of the periodontal microbial profile has been shown to be highly affected by hyperglycemia (Makiura et al., 2008)' (Suarez, Alvarez, de Bernal, & Collazos, 2013). This is in line with findings from previously published research by our group (A. W. Wilson et al., 2013). Merchant et al. found that patients with and without diabetes have similar distribution of oral microbes and that etiologic and putative microbes were inversely associated with the frequency of tooth-brushing and flossing(A. T. Merchant et al., 2014b). In another study we reported the absence of any association between oral microbial profile and markers of cardiovascular disease among youth with diabetes(A. T. Merchant et al., 2014a). In a preliminary study, we found a similar distribution of oral microbes was reported among youth with and without diabetes; however, a negative correlation was found between microbes comprising cluster 3 and oral-hygiene practices(Nahhas et al., 2014).

Socransky and Haffajee grouped oral microorganisms among adults and named them by comparing individuals with and without periodontal disease (Socransky et al., 1998). Desvarieux *et al*.(Desvarieux et al., 2005) grouped oral microbes based on their association with cardiac atherosclerosis. They defined 3 groups of microbes that were etiologically, putatively, and protectively associated with the formation of vascular plaque. No differences in the microbial composition of the etiologic, putative, and protective groups were found between youth with type 1 diabetes when compared to those without. Our study differs from both of the formerly-reported study in that the clustering is data-forced and that we looked at how these clusters form youth with type 1 diabetes.

One possible reason for not observing a difference in oral bacterial profile among youth with and without diabetes could be the low level of periodontal disease in this population (A. W. Wilson et al., 2013). This may be because the participants received regular medical and dental care and reported relatively good oral hygiene practices. Dental treatment and oral hygiene practices can positively impact periodontal disease and the composition of oral microbial flora (E. Lalla, Kaplan, et al., 2006; Mandell, Dirienzo, Kent, Joshipura, & Haber, 1992; A. Merchant, Pitiphat, Douglass, Crohin, & Joshipura, 2002; A. T. Merchant et al., 2012).

The current study had several advantages. First the sample size is larger than most other studies evaluating the relationship between clustering of oral microorganisms in youth with diabetes. Second, the inclusion of a comparison group of youth without type 1 diabetes helped characterize the similarities with the periodontal microbial profile of youth with diabetes. Third, counts of 41 oral microbes found in dental-plaque were available for evaluating their clustering in the presence of diabetes and comparing their distribution among a control group without type 1 diabetes. Fourth, we implemented cluster analysis to determine 4 mutually-exclusive clusters of oral microorganisms in the presence of type 1 diabetes similar to what was done by Socransky and colleagues to determine clusters of oral bacteria in adults with periodontal disease(Socransky, Smith, & Haffajee, 2002). Our study also had some limitations. First, our sample was mostly non-Hispanic white, however this is a reflection of higher prevalence of type 1 diabetes in this population and this was not unexpected (Group et al., 2006; Pettitt et al., 2014). Second, it was not possible to evaluate the

temporal sequence between type 1 diabetes status and oral microbes because of the case-control design. Third, an empirical approach used to group oral microorganisms among youth with type 1 diabetes because there is no gold-standard method reported in the literature. For this reason our results need to be verified in other populations.

In summary, the distributions of oral microbes were similar in individuals with type 1 diabetes and those without diabetes in this sample of youth who received regular medical and dental care, had low level of periodontal disease. It may be possible for youth with type 1 diabetes to maintain oral health similar to those without diabetes.

Table 3.1. Baseline population characteristics by diagnosis of Type 1 Diabetes.

	Non-Diabetic Controls N (%) (n = 71)	Type 1 Diabetes Cases N (%) (n = 105)
Sex		
Male	33 (46)	53 (50)
Female	38 (54)	52(50)
Race		
Non-Hispanic White	50 (70)	78 (74)
Black	4 (6)	4 (7)
Other	17 (24)	17 (19)
Dental visit in the past 24 months[$]		
No	23 (32)	53 (50)
Yes	48 (68)	52 (50)
Diabetes treatment		
Injection	--	62 (59)
Pump	--	43 (41)
HbA1c		
< 7.5%	--	15 (14)
7.5 – 9.5%	--	57 (54)
> 9.5%	--	33 (32)

Brushing teeth	**n = 47**	**n = 54**
once a day or less	12 (26)	25 (46)
>1, but less than 2x/day	9 (19)	6 (11)
2x/day or more	26 (55)	23 (43)

Flossing teeth	**n = 47**	**n = 54**
None	18 (38)	29 (54)
< once a day	22 (47)	20 (37)
once a day or more	7 (15)	5 (9)

	Mean (SD) **(n = 71)**	**Mean (SD)** **(n = 105)**
Age (years)	15.3 (2.0)	15.3 (2.2)
BMI percentile (%)	58.6 (28.1)	70.2 (22.2)
HbA1c (%)	--	9.0 (1.5)
Glucose mg/dL	--	194.91 (96.0)
Duration of diabetes (years)	--	8.7 (3.2)
Mean calculus index	0.1 (0.1)	0.1 (0.1)
Mean plaque index	0.5 (0.4)	0.1 (0.3)
Mean gingival condition	0.7 (0.2)	0.7 (0.2)

Table 3.2. Proportion of standardized scores of organisms in dental plaque of youth by Cluster and diagnosis of Type 1 Diabetes.

	Non-Diabetic Controls (n = 71)		Type 1 Diabetes Cases (n = 105)	
	Sum	**%**	**Sum**	**%**
Blue-Other Cluster				
A. actinomycetemcomitans	74	3	43	1
E. nodatum	32	1	37	1
E. saburreum	89	3	49	2
T. socranskii	46	2	44	1
Total %	**241**	**9**	**173**	**6**
Orange-Blue Cluster				
A gerencseriae	74	3	64	2
A israelli	82	3	70	2
A naeslundii	69	3	71	2
A oris	89	3	103	3
C gingivalis	46	2	64	2
C gracilis	55	2	75	2
C ochracea	80	3	96	3

E corrodens	66	2	93	3
F nuc nucleatum	72	3	84	3
F nuc polymorphum	76	3	78	3
F nuc vincentii	59	2	74	2
F periodonticum	81	3	89	3
P intermedia	48	2	59	2
P melaninogenica	74	3	73	2
P nigrescens	34	1	57	2
S noxia	60	2	63	2
V parvula	67	2	79	3
Total %	**1135**	**42**	**1294**	**43**
Orange-Red Cluster				
A odontolyticus	108	4	130	4
C rectus	48	2	94	3
C showae	52	2	89	3
C sputigena	47	2	58	2
P acnes	75	3	78	3

P gingivalis	70	3	92	3
P micra	72	3	79	3
S constellatus	72	3	78	3
S intermedia	64	2	73	2
S mitis	83	3	96	3
S mutans	67	2	57	2
T denticola	54	2	71	2
T forsythia	55	2	61	2
Total %	**867**	**32**	**1055**	**35**

Yellow-Other Cluster

G morbillorum	82	3	49	2
L bucallis	62	2	67	2
N mucosa	69	3	70	2
S anginosus	69	3	84	3
S gordonii	53	2	73	2
S oralis	67	2	80	3
S sanguinis	52	2	76	3

Total %	**455**	**17**	**498**	**16**
Overall Total	**2698**	**100**	**3020**	**100**

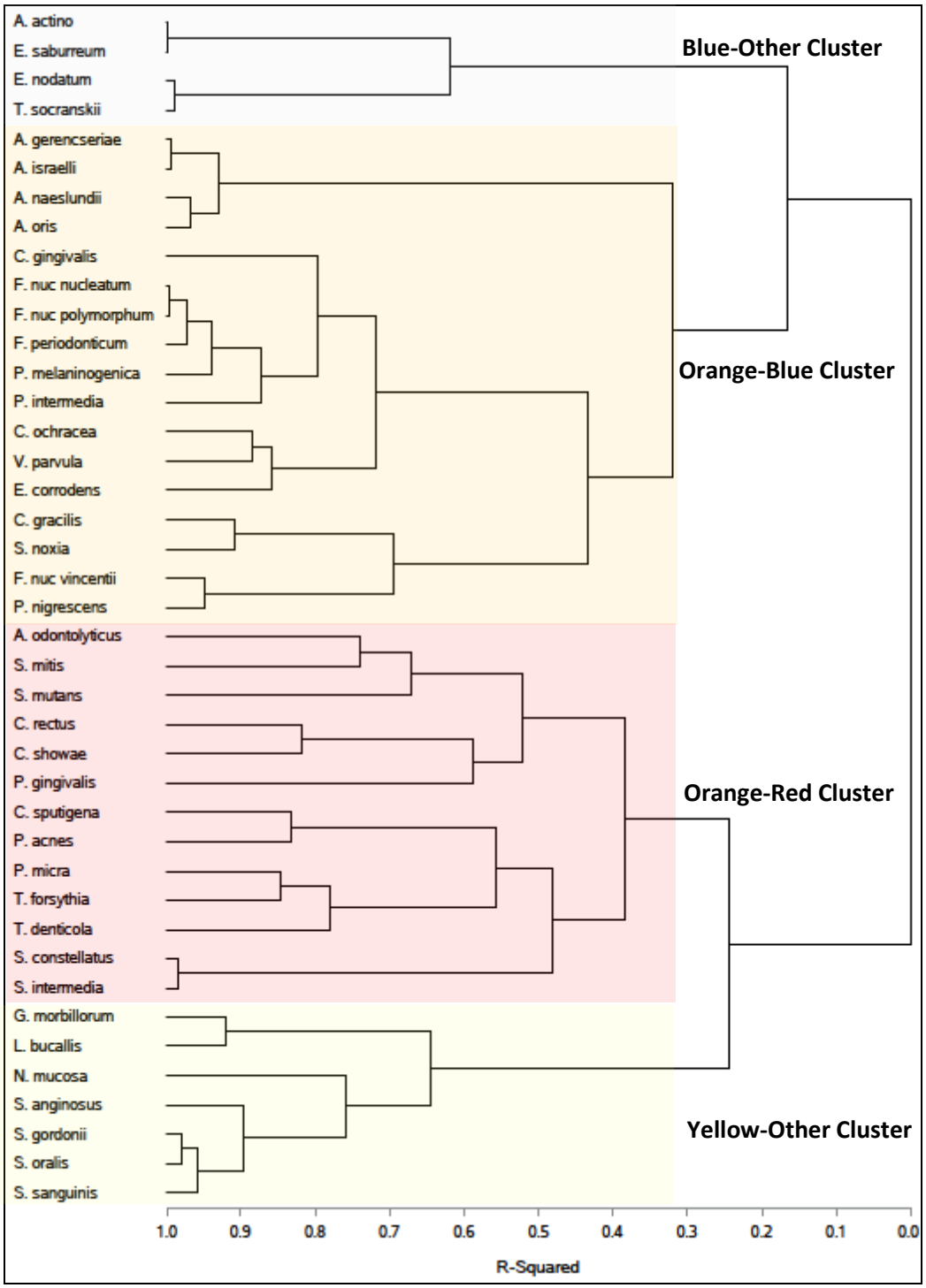

Figure3.1. Dendrogram of cluster-analysis tree showing 4 mutually-exclusive clusters of 41 oral microbes among youth with type 1 diabetes. Clusters were formed according to Ward's method and 4 clusters were determined a priori.

CHAPTER 4

ORAL MICROBIAL PROFILE AND ADIPOSITY IN YOUTH WITH TYPE 1 DIABETES[2]

[2] Georges J. Nahhas, R. Paul Wadwa, Elaine H. Morrato, Jiajia Zhang, Lonnie Johnson, Franziska Bishop, Ricardo Teles, Linda J. Hazlett, David M. Maahs, Anwar T. Merchant. In preparation for submission to the Journal of Dental Research.

ABSTRACT

We explored the association between clusters of oral microorganisms and adiposity in youth with type 1 diabetes and a comparison group without type 1 diabetes.

Dental plaque data from 105 youth with type 1 diabetes and 68 without type 1 diabetes (< 20 years old) were collected at the Barbara Davis Center in Colorado, 2009-2011. Samples were assessed by DNA-DNA hybridization for counts of 41 microbes. Microorganisms were grouped into four mutually exclusive clusters. Microbial counts were standardized and cluster summary scores were calculated by adding-up standardized scores of all microbes within each cluster. BMI z-scores were defined as normal (<85%) or overweight (≥85%).

On average, participants were 15 years old, mostly non-Hispanic white (72%), with about equal proportions of males to females. The average duration of type 1 diabetes was 8 years. The prevalence of overweight (24%) and obesity (9%) was low in both comparison groups. Overweight and normal weight youth brushed more than once a day (65% versus 63%) and flossed at least once weekly (55% versus 53%) and measures of oral health (calculus, gingival, and plaque indices) were similar in both groups. Periodontal disease was not very common in this sample. On average, bleeding on probing was present in 58% of the teeth examined corresponding to 19% of the total sites probed, and a total of 34% had clinical attachment loss >2mm. BMI percentile was

significantly associated with the 'blue-other' cluster in both groups, however the association was greater in type 1 diabetes.

There was an observed difference in the oral microbial profile between normal weight and overweight youth with low level of periodontal disease. However, further research is needed to confirm if overweight youth who receive regular dental care can maintain an oral microbial profile similar to normal weight youth.

INTRODUCTION

Periodontal disease has been shown to be associated with obesity(Saito et al., 1998), (Gorman et al., 2012), (Jimenez et al., 2012). In a recently-published meta-analysis of 57 observational studies the prevalence odds of obesity was 33% higher for those who have periodontitis, across different populations from all around the world(Chaffee & Weston, 2010). Obesity induces low-grade chronic systemic inflammation, and is associated with many chronic diseases such as type 2 diabetes, cardiovascular disease, respiratory diseases, and even some cancers. It is also associated with elevated levels of markers of systemic inflammation in youth(Valle et al., 2005). It is hypothesized that cytokines secreted by adipose tissue promote systemic inflammation that drives the pathogenesis of metabolic abnormalities which are a result of obesity(Fontana, Eagon, Trujillo, Scherer, & Klein, 2007). Chronic low grade systemic inflammation leads to glycemia and contributes to insulin resistance in adults(McLaughlin et al., 2002) as well as in youth(Visser, Bouter, McQuillan, Wener, & Harris, 2001). Chronic hyperglycemia lead to the formation of advanced glycosylation endproducts (AGE) most famous of

which is hemoglobin A1c (HbA1c)(Brownlee, Cerami, & Vlassara, 1988). AGE tends to accumulate in organs which leads to complications of diabetes including nephropathy, neuropathy, and vascular atherosclerosis(Jakuš & Rietbrock, 2004). AGE also accumulates in gingival tissue of individuals with diabetes(Schmidt et al., 1996) leading to destruction of periodontal connective tissue and periodontal bone resorption(Reynolds & Meikle, 1997). This inflammatory reaction becomes aggravated in the presence of periodontal pathogens(Evanthia Lalla, Lamster, Drury, Fu, & SCHMIDT, 2000) such as *P. gingivalis*(J. J. Taylor, Preshaw, & Lalla, 2013).

T. forsythia was present in higher concentrations in those who were obese but did not have periodontitis, increasing their risk for developing periodontal disease(Haffajee & Socransky, 2009). *T. denticola* and *T. forsythia* from sub-gingival plaque were also found to be significantly associated with elevated HbA1c levels in type 1 diabetes(Schara et al., 2013).

Although periodontitis is associated with obesity, the related microbiology is understudied**(Chaffee & Weston, 2010)**,**(Goodson et al., 2009)**, particularly among youth populations. In this paper we explored the association between clusters of oral microorganisms and adiposity in youth with type 1 diabetes.

METHODS

Study Population

Cross-sectional data was available from 173 individuals attending the Barbara Davis Center for Childhood Diabetes, Aurora, Colorado, 2009-2011(Specht et al., 2013).

There were 105 individuals who had type 1 diabetes ("cases") and 68 without diabetes ("controls"). Participants with type 1 diabetes had been diagnosed for at least 5 years by islet cell antibody or provider diagnosis, and were 12-19 years old at entry into the study. Individuals who had smoked or consumed caffeine in the 8 hours prior to their study-visit were excluded. Participants were also excluded if they had a previous diagnosis of any type of diabetes other than type 1, including gestational diabetes, if they had history of cardiac arrhythmia or abnormal cardiac anatomy, or if they required the administration of any type of antibiotics within 30 days preceding their visit. Siblings and first-degree relatives of participants with type 1 diabetes were also excluded. Controls were selected either from the friends of the participants or from the community by advertisement. Participants signed a consent form prior to enrollment; those who were under 18 years of age signed an assent. The study was reviewed and approved by and the University of South Carolina Institutional Review Board and the Colorado Multiple Institutional Review Board.

Sub-gingival Plaque Assays

Sub-gingival plaque samples (2 per participant) were collected(W. Sun et al., 2010) in Colorado and mailed(Morita et al., 2010; Paraskevas et al., 2008; Snell-Bergeon et al., 2010) to the Forsyth Institute, Boston for evaluation of counts of 41 different bacterial species, by DNA-DNA hybridization(Romano et al., 2001; Socransky et al., 2004; Socransky et al., 1994). Intensity of fluorescence signaling was determined and converted to absolute counts(A. T. Merchant et al., 2003; Romano et al., 2001).

Study Variables

Demographics that were collected included information on age, sex, race, dental insurance status, and frequency of dental visits. Information on diabetes duration and treatment was also available for 'cases' of type 1 diabetes. Measurements of HbA1c and blood glucose were obtained at the Children's Hospital Colorado's main clinical laboratory.

Information on oral hygiene practices (frequency of brushing and flossing) was collected by means of a questionnaire. Information on oral health status (calculus index, gingival index, plaque index, bleeding on probing, and pocket depth) was collected during a clinical dental examination, at the same hospital. Oral-health and hygiene data were available for a sub-sample (n=99).

Anthropometric measures were measured clinically during a routine physical examination by a trained professional. Height, with shoes removed, was measured by a wall-mounted stadiometer, and weight was measured by a Detecto scale (Detecto, Webb City, Missouri). BMI z-scores were calculated and defined as obese (≥95th percentile), overweight (85th-95th percentile), or normal (< 85th percentile).

Microorganisms (n = 41) were divided into 4 groups as defined by Nahhas et al.(Nahhas et al., 2014) The four groups were named 'orange-blue', 'yellow-other', 'blue-other', and 'orange-red' clusters. Counts of each of the 41 different microbes were standardized by dividing the count by the sample's standard deviation. Standardization was performed separately for cases of type 1 diabetes and controls without type 1

diabetes. Cluster scores were defined as the cumulative summation of each cluster's constituting microbes.

Statistical Analyses

All analyses were done using SAS 9.3 (SAS Institute, Cary, NC). The threshold for statistical significance was fixed at 5%. Frequencies and percentages were reported for categorical covariates; means and standard deviations were reported for continuous covariates. The outcomes were the 4 cluster scores, separately, and BMI percentile was the exposure. Generalized linear regression modeling with a 'repeated' statement was utilized with the negative-binomial distribution, and the log link. There were two measurements of plaque from two different sites for each individual and hence two cluster scores were calculated for each participant. The first model was adjusted for the other clusters. The second model was additionally adjusted for race (non-Hispanic white; other) and dental insurance (yes; no/don't know). The third was run among cases of type 1 diabetes only and additionally adjusted for diabetes duration in years. The fourth model was run on a subsample from which oral hygiene and condition data were available and was adjusted for the other clusters, race, dental insurance, dental visits within the past 6 months (yes; no), frequency of brushing (once a day or less; >1, but less than 2x/day; 2x/day or more), and frequency of daily flossing (None; < once a day; once a day or more).

RESULTS

In this sample, the mean age was 15 years and the proportion of male/female participants was about equal. Of those who had type 1 diabetes, 30% were overweight (BMI z-score [85^{th}-95^{th}] percentiles) and 7% were obese (BMI z-score≥95^{th} percentile); of those without type 1 diabetes 15% were overweight and 12% were obese. The majority was non-Hispanic white (74% of cases and 69% of controls). Controls were significantly more likely to have dental insurance coverage (60%) than cases of type 1 diabetes (40%) ($p = 0.0091$) and they were more likely to have visited the dentist within the past 6 months, 59% versus 40% ($p = 0.0154$) respectively. Among individuals with type 1 diabetes, the mean level of glucose was higher in those who were overweight (204mg/dL) when compared to those who were normal weight (189 mg/dL) ($p = 0.4455$) and treatment by insulin injection was more common than insulin pump ($p = 0.4181$). The mean level of HbA1c ($p = 0.5823$) was similar, 8.9% for overweight versus 9% for normal weight (Table 4.1.).

Measures of oral hygiene and health were available for a sub-sample of 54 youth with type 1 diabetes and 45 without type 1 diabetes. Tooth brushing more than once daily was common among cases of type 1 diabetes (54%) and controls (76%) ($p = 0.1094$). Flossing at least once weekly was not as common among cases of type 1 diabetes (46%) as controls (62%) ($p = 0.2602$). Overweight and normal weight youth both brushed more than once a day (65% versus 63%) and flossed at least once weekly (55% versus 53%). Measures of oral health (calculus, gingival, and plaque indices) were

similar in overweight and normal weight youth (Table 4.2.). Periodontal disease was not very common in this sample. On average, bleeding on probing was present in 58% of the teeth examined corresponding to 19% of the total sites probed, and a total of 34% had clinical attachment loss >2mm.

The microbial composition of each cluster is shown in Figure3.1. There was a significant positive association between BMI percentile and the 'blue-other' cluster. Among cases of type 1 diabetes BMI percentile was significantly related to the 'blue-other' cluster and remained significant after additional adjustment for duration of diabetes. This relationship did not change in magnitude after additional adjustment for oral hygiene variables, but it did not reach statistical significance. Among controls without type 1 diabetes BMI percentile was also significantly and positively associated with the 'blue-other' cluster (Table 4.3.).

DISCUSSION

There was a significant association between BMI percentile and oral microbial profile among youth with and without type 1 diabetes with low prevalence of overweight and obesity. A recent national study reported an obesity prevalence of 17% among adolescents under 20 years of age(Ogden, Carroll, Kit, & Flegal, 2014). In stratified analyses the 'blue-other' cluster score was higher in normal weight compared with overweight individuals after adjustment for type 1 diabetes, race, dental insurance, frequency of dental visits, brushing, and flossing in addition to the other three clusters. Such low rates of overweight and obesity could be related to the type of youth who

volunteered for this study or to Colorado's overall low rates of overweight and obesity. These rates are more in line with those reported by the 2009 Youth Risk Behavior Survey.

Goodson *et al.* showed that there is a positive association between salivary bacterial profile and overweight(Goodson et al., 2009). They found that members of the 'blue-other' cluster had were more prevalent in obesity than normal weight. *A. actinomycetemcomitans* was about four times higher among overweight individuals and *T. socranskii* was not even detected in normal weight individuals compared to those who were overweight. A follow-up study of youth with type 1 diabetes by Lalla *et al.*, found that the level of periodontal destruction is related to the level of metabolic control(E. Lalla, Kaplan, et al., 2006). Al-Zahrani et al. reported that measures of obesity were positively associated with periodontal disease among younger adults, using data from NHANES III(Al-Zahrani et al., 2003). Wood et al. found correlations between measures of obesity (BMI, waist-to-height ratio) and measures of periodontal disease (CAL, PD)(Wood et al., 2003). Members of the 4 main phyla (Fermicutes, Bacteroidetes, Actinobacteria, and Fusobacteria) comprising the oral saliva microbiota were found to be highly associated with obesity among obese women(Goodson et al., 2009). A recent Brazilian study from a population-based birth cohort examining the association between obesity and periodontal disease found that gingivitis was related to obesity, mediated by oral hygiene and inflammation. They also reported a cumulative effect of obesity on calculus, a measure of oral hygiene(de Castilhos et al., 2012).

Several pathways were hypothesized in which periodontal microorganisms may contribute to obesity. First, oral microbes could cause an increased efficiency in food metabolism. In this case, the slightest increased in energy intake could lead to an exaggerated gain in weight(Goodson et al., 2009). Second, oral microbes could increase the level of ghrelin (an appetite stimulating hormone) and decrease the level of leptin (a satiety hormone that is involved in regulating the mass of adipose tissue)(Pischon et al., 2007), which in turn would lead to an increase in weight(Goodson et al., 2009). Third, through increased inflammation, oral microbes can disrupt the endocrine function of the adipose tissue which in turn would cause an imbalance in glucose homeostasis, which may lead to increased weight(Gregor & Hotamisligil, 2011).

Studies in animal models and humans have shown that the gastrointestinal flora has a critical role in maintaining digestion(Berg, 1996; Falk, Hooper, Midtvedt, & Gordon, 1998; Macfarlane & Macfarlane, 1997; Neu, Douglas-Escobar, & Lopez, 2007), detoxification of carcinogens(Nicholson et al., 2012; Sekirov, Russell, Antunes, & Finlay, 2010) and drug metabolism and biotransformation(Bjorkholm et al., 2009; I. D. Wilson & Nicholson, 2009). DiBaise *et al.*, observed a positive association between gastrointestinal bacterial profile and overweight and suggested gut flora facilitates the extraction of energy from food, and facilitates its storage in adipose tissue(DiBaise et al., 2006). There is sufficient evidence to suggest that the gut microbiota heavily contributes to the development of obesity (Li & Hotamisligil, 2010).

The gastrointestinal flora is similar in composition to the oral flora, but different in proportions. It is dominated by Fermicutes, followed by Bacteroidetes, Actinobacteria, and Proteobacteria. However, the gut is richer in Fermicutes and Bacteroidetes, but poorer in Fusobacteria and Proteobacteria than the mouth(Koren et al., 2011). Members (*Selenomonas noxia, Actinomyces gerencseriae, Actinomyces naeslundii, Neisseria mucosa, Fusobacterium periodonticum, Fusobacterium nucleatum ss vincentii, and Prevotella melaninogenica*) of the 4 main phyla (Fermicutes, Bacteroidetes, Actinobacteria, and Fusobacteria) comprising the oral microbiota were found to be highly associated with obesity(Goodson et al., 2009).

Our study had some limitations. First, it was not possible to determine the direction of the relationship between BMI percentiles and clusters scores. Second, the prevalence of obesity and periodontal disease were low in this sample which might explain the null relationship. Third, we could not find a gold-standard grouping of oral microorganisms among youth with type 1 diabetes against which to validate the clusters. Fourth, participants were mostly non-Hispanic white which limits the generalizability of our results to other races and to the general population.

On the other hand, this study had several strengths. First, to our knowledge, this study had information on the largest number of microorganisms (41) and number of youth participants with type 1 diabetes. Second, we looked at BMI percentiles which are more relevant to this age group than non-standardized BMI measure. Moreover we used the continuous form of the variable eliminating the limitations of categorization.

Third, we used a novel procedure (cluster analysis) to look at data-driven groups free of apriori-determined restrictions. Fourth, we looked at the comprehensive oral microbial profile by considering all the clusters together as one entity comprising the exposure.

In conclusion, we observed differences in the oral microbial profile between normal weight and overweight youth with low level of periodontal disease. The 'blue-other' cluster, which was dominated by *A. actinomycetemcomitans* was significantly and positively correlated to BMI percentile. This relationship was significant in both comparison groups, but higher among those with type 1 diabetes. Overweight youth who receive regular dental care can maintain an oral microbial profile similar to normal weight youth. Further research is needed to examine the relationship between oral microbial profile and adiposity, especially in the presence of type 1 diabetes. The difference may be greater in youth with type 2 diabetes where the prevalence of obesity is likely to be higher and the underlying metabolic mechanisms different.

Table 4.1. Population characteristics by Type 1 Diabetes and weight.

	Controls without diabetes		Cases of Type 1 Diabetes	
Selected Characteristics	**Normal weight**	**Overweight**	**Normal weight**	**Overweight**
	N = 50	**N = 18**	**N = 66**	**N = 39**
Sex / n (%)				
Male	24 (48)	6 (33)	36 (55)	17 (44)
Female	26 (52)	12 (67)	30 (45)	22 (56)
Race / n (%)[€]				
Other	11 (22)	10 (56)	13 (20)	14 (36)
Non-Hispanic White	39 (78)	8 (44)	53 (80)	25 (64)
Dental Insurance / n (%)				
No/Don't know	22 (44)	5 (28)	37 (56)	26 (67)
Yes	28 (56)	13 (72)	29 (44)	13 (33)
Dental visit in the past 6 months / n (%)				
No	21 (42)	7 (39)	39 (59)	24 (62)
Yes	29 (58)	11 (61)	27 (41)	15 (38)
Diabetes treatment / n (%)				
Injection			37 (56)	25 (64)
Pump			29 (44)	14 (36)
HbA1c / n (%)				
< 7.5%			7 (11)	8 (21)
7.5 – 9.5%			39 (59)	18 (46)
> 9.5%			20 (30)	13 (33)
Age in years / mean (SD)	15.3 (2)	15.5 (2.1)	15.2 (2.2)	15.4 (2.2)

Glucose mg/dL / mean (SD)	82.2 (6.3)	83.9 (8.5)	189.4 (95.6)	204.3 (96.9)
HbA1c % / mean (SD)	5.3 (0.2)	5.2 (0.3)	9 (1.5)	8.9 (1.5)
Diabetes Duration in years / mean (SD)			8.8 (3.2)	8.3 (3)

Normal weight was defined as < 85th percentile

Overweight was defined as ≥ 85th percentile

€: χ^2 test for controls < 0.05

Table 4.2. Oral hygiene and dental measures characteristics by Type 1 Diabetes and weight for a subsample (N = 99).

	Normal weight N = 68	Overweight N = 31
Cases of Type 1 Diabetes / n (%)	36 (53)	18 (58)
Brushing teeth / n (%)		
once a day or less	25 (37)	11 (35)
>1, but less than 2x/day	9 (13)	5 (16)
2x/day or more	34 (50)	15 (48)
Flossing teeth / n (%)		
None	32 (47)	14 (45)
< once a day	30 (44)	11 (35)
once a day or more	6 (9)	6 (19)
Calculus Index / mean (SD)	0.1 (0.1)	0.1 (0.1)
Gingival Index / mean (SD)	0.7 (0.2)	0.7 (0.2)
Plaque Index / mean (SD)	0.6 (0.3)	0.6 (0.3)
No. of teeth with bleeding / mean (SD)	7.9 (4.1)	7.8 (4.2)
Mean probing depth in mm / mean (SD)	2 (0.2)	2 (0.2)

Normal weight was defined as < 85th percentile

Overweight was defined as ≥ 85th percentile

Table 4.3. Estimate of BMI% coefficient for models stratified by type 1 diabetes.

Cluster	Model	Control	type 1 diabetes
		β (SE)	β (SE)
Blue-Other	**1**	0.007 (0.003)*	0.0079 (0.0062)
	2	0.0062 (0.0026)*	0.0124 (0.0058)*
	3	--	0.0121 (0.0056)*
	4	0.0073 (0.0026)*	0.0123 (0.0065)
Orange-Blue	**1**	-0.0002 (0.0028)	-0.0008 (0.0043)
	2	-0.0017 (0.0025)	0.0026 (0.0035)
	3	--	0.0028 (0.0036)
	4	0.0007 (0.003)	0.0025 (0.004)
Orange-Red	**1**	0.0019 (0.0016)	-0.0007 (0.0028)
	2	0.0017 (0.0017)	-0.0006 (0.0027)
	3	--	-0.0007 (0.0027)
	4	-0.0002 (0.0021)	-0.0042 (0.0031)
Yellow-Other	**1**	-0.0026 (0.0025)	0.0031 (0.0041)
	2	-0.0029 (0.0026)	0.0044 (0.0043)
	3	--	0.004 (0.0042)
	4	-0.0040 (0.0028)	0.0051 (0.0045)

Model 1: adjusted for the other clusters.

Model 2: adjusted for the other clusters, race, and dental insurance status.

Model 3: adjusted for the other clusters, race, dental insurance status, and diabetes duration.

Model 4: adjusted, additionally to model 2, for frequency of dental visits, brushing, and flossing.

*: p-value (2-sided < 0.05)

CHAPTER 5

ORAL MICROBIAL PROFILE AND MARKERS OF CHRONIC INFLAMMATION IN YOUTH WITH TYPE 1 DIABETES[3]

[3] Georges J. Nahhas, R. Paul Wadwa, Elaine H. Morrato, Jiajia Zhang, Lonnie Johnson, Franziska Bishop, Ricardo Teles, Linda J. Hazlett, David M. Maahs, Anwar T. Merchant. In preparation for submission to Diabetes Care.

ABSTRACT

Identify the correlation between clusters of periodontal microorganisms found in dental plaque of youth with type 1 diabetes and markers of chronic inflammation (C-reactive protein and adiponectin) compared to that among youth without type 1 diabetes.

Cross-sectional data were available from 165 youth, 12-19 years old, (99 with type 1 diabetes, 66 controls) receiving care at the Barbara Davis Center for Childhood Diabetes, Aurora, Colorado, 2009-2012. Forty-one periodontal microbes from sub gingival plaque were grouped into 4 clusters using cluster analysis. CRP and adiponectin were regressed on each cluster in a multivariable negative-binomial regression.

Individuals with type 1 diabetes in the highest tertile of CRP were more likely to be older, female, with higher HbA1c level. Cases in the highest tertile of adiponectin were more likely to be female, having dental insurance, and not be overweight. Gingival and oral health conditions were similar across the tertiles of CRP and adiponectin. CRP was related to the cluster which was dominated by members of Socransky's orange complex which is associated with gingivitis. Adiponectin was inversely associated with a cluster that was dominated by *A. actinomycetemcomitans*.

Clusters of periodontal microorganisms were associated with CRP and adiponectin after accounting for potential confounders. This suggests that specific microorganisms may have different effects on inflammatory markers.

INTRODUCTION

C-reactive protein (CRP) has been positively associated with tooth loss, clinical attachment loss, pocket depth, and periodontal microorganisms(Pitiphat, Savetsilp, & Wara-Aswapati, 2008) (Linden et al., 2008) (Mohammad Taghi Chitsazi, 2008). Elevated CRP is associated with *P. gingivalis* and *A. actinomycetemcomitans* in dental plaque(Pejcic et al., 2011a). Moreover, CRP is associated with several systemic conditions such as obesity, high lipid levels, diabetes, and cardiovascular disease(Gomes-Filho et al., 2011). A controlled clinical trial reported a significant decrease in serum CRP following treatment of periodontal diseases even among obese individuals(Al-Zahrani & Alghamdi, 2012).

Adiponectin, an adipokine that regulates immune and inflammatory responses and is secreted by adipose tissue (Brochu-Gaudreau et al., 2010; Lago, Dieguez, Gomez-Reino, & Gualillo, 2007), has been linked to chronic inflammation and periodontal disease, especially in the presence of obesity(Chaffee & Weston, 2010; Preshaw, Foster, & Taylor, 2007; Suvan, D'Aiuto, Moles, Petrie, & Donos, 2011). The presence of periodontal disease increases levels of TNF-α(Zimmermann et al., 2013); adiponectin counteracts the action of *P. gingivalis*, and is negatively associated with periodontal bacteria, and more particularly with *P. gingivalis* and *T. denticola*, which are main periodontal pathogens(Kraus et al., 2012).

Periodontal microorganisms such as *A. actinomycetemcomitans, P. gingivalis and T. forsythia* are highly associated with periodontal disease status and progression;

F.nucleatum subsp. vincentii, C. rectus and *P. intermedia* are highly prevalent in periodontitis(Socransky & Haffajee, 2002). The relation between periodontal disease and markers of chronic inflammation is well documented(D'Aiuto et al., 2006; Miyashita et al., 2012; Pejcic et al., 2011b). Such markers can be either mediators or effect modifiers of the relation between periodontal disease and systemic diseases(Y. H. Choi et al., 2014). Mediators of pro-inflammation can be increased in the presence of periodontal disease in type 1 diabetes. CRP was found to be highly associated with advanced periodontal disease in a cohort of Spanish adults with type 1 diabetes(Llambes et al., 2012). Adiponectin was also reported to be higher in individuals who have type 1 diabetes, when compared to controls without type 1 diabetes of similar periodontal status(E. Lalla, Kaplan, et al., 2006).

The roles that periodontal microorganisms play in relation to CRP and adiponectin are not well-known, particularly in youth with type 1 diabetes. In this paper we aimed at identifying the correlation between clusters of periodontal microorganisms found in dental plaque of youth with type 1 diabetes and markers of chronic inflammation (CRP and adiponectin); and compared that among youth without type 1 diabetes.

METHODS

Study Sample

Data were available cross-sectionally from 165 youth, 12-19 years old, (99 cases with type 1 diabetes, 66 controls without type 1 diabetes) receiving regular care at the

Barbara Davis Center for Childhood Diabetes, Aurora, Colorado, 2009-2012(Maahs et al., 2011; Specht et al., 2013). Cases of type 1 diabetes had been diagnosed for at least 5 years based on provider clinical diagnosis or islet cell antibody. Controls were recruited from either the community by advertisement, or from the pool of the participants' friends. Individuals were excluded if they had history of abnormal cardiac anatomy or arrhythmia, had smoked or had caffeine in the 8 hours preceding the study visit, were ever diagnosed with any diabetes other than type 1, if they had any treatment involving antibiotic medication within the preceding 30 days, or if they were first-degree relatives of participating youth with type 1 diabetes. All participants signed a consent form, or an assent (if <18 years old) and approval was granted by the South Carolina Institutional Review Board and the Colorado Multiple Institutional Review Board.

Sub-gingival plaque quantification

Each participant provided 2 samples of sub-gingival plaque, which were collected in Colorado, placed in a fixing solution, and mailed(Morita et al., 2010; Paraskevas et al., 2008; Snell-Bergeon et al., 2010) to the Forsyth Institute, Boston for quantification. Counts of 41 bacterial species were evaluated by DNA-DNA hybridization(Romano et al., 2001; Socransky et al., 2004; Socransky et al., 1994). Intensity of fluorescence signaling was determined and converted to absolute counts(A. T. Merchant et al., 2003; Romano et al., 2001).

Independent variables

CRP and adiponectin were the two main independent variables of interest measured in mg/dL.

Dependent variables

Four mutually-exclusive clusters of 41 periodontal microorganisms were defined as the main dependent variables, separately. The clusters (Figure3.1.) were named as 'blue-other', 'orange-blue', 'orange-red', and 'yellow-other' according to the complexes as defined by Socransky(Socransky & Haffajee, 2002). The formation of the clusters and calculation of the cluster scores were performed as described by Nahhas et al(Nahhas et al., 2014). Standardized scores were obtained for each of the 41 microorganisms by dividing its count, for each observation, by its respective sample's standard deviation. Standardization was performed separately for cases of type 1 diabetes and controls. Standardized cluster scores were defined as the cumulative sum of standardized scores of all individual microorganisms within each cluster.

Covariates

Information was available on sex, race (non-Hispanic white, other), age, BMI percentile, frequency of dental visits (within the past 6 months yes, no), and having dental insurance (yes, no/don't know). Also available was type of diabetes treatment (pump, injection) and duration in years. HbA1c (<7.5, 7.5 – 9.5, >9.5%) and blood glucose levels were available from the main laboratory at the Children's Hospital Colorado.

Oral hygiene practices over the past 7 days were ascertained via a standardized structured questionnaire and categorized: brushing(once a day or less, >1 but <2x/day, 2x/day or more) and flossing (none, <1x/day, ≥1x/day) were collected, from a sub-sample (cases=50; controls=44). Clinical data on general oral health (plaque index, gingival index, calculus index, and bleeding on probing) was collected through clinical dental assessment. Also, information on clinical attachment loss and pocket depth were collected from a random bilateral quadrants assessment(Morrato et al., 2014; Orlando et al., 2010).

Statistical Analyses

We used SAS 9.3 (SAS Institute, Cary, NC) to conduct all statistical analyses. The level of significance was fixed at 5%. Categorical covariates were reported as frequencies and percentages, and continuous covariates were reported as means and standard deviations.

Multivariable generalized linear regression modeling was performed using a log-linear model in 'proc genmod' with 'repeated' statement to account for the 2 measures, per person, for each of the main independent variables. The negative-binomial distribution was used with the 'log' link. Model 1 was adjusted for the other clusters; model 2 was adjusted for the other clusters, race, and dental insurance; model 3 was adjusted for the other clusters, race, dental insurance, and diabetes duration; and model 4 was adjusted for the other clusters, race, dental insurance, diabetes duration, dental insurance, brushing, and flossing.

Covariance structures with the lowest absolute value of Quasi-Information Criterion (QIC) were used for the 4 different outcomes; the 'unstructured' covariance matrix was used for modeling the 'blue-other' and 'yellow-other' clusters and 'independent' matric to model the 'orange-blue' and 'orange-red' clusters.

RESULTS

In this sample of 165 youth, there were 99 cases of type 1 diabetes and 66 controls without type 1 diabetes. The mean age was 15.3 years. The proportion of males to females was about 1:1 in both groups. The majority was non-Hispanic white (73%), normal weight (68%), did not visit the dentist in the past 6 months (53%), and did not know/have dental insurance (53%). The mean glucose level was 192 mg/dL and that of HbA1c was 9% among cases of type 1 diabetes and they were mostly treated by insulin injection (62%). The mean duration of type 1 diabetes was 8.7 years.

Among cases of type 1 diabetes, individuals in the highest tertile of CRP were mostly female (67%), of older age (mean = 16.3 years) and higher HbA1c (mean = 9.6%) than those in the lowest tertile (33% female, mean age 14.8 years, and mean of 8.3% for HbA1c). Cases of type 1 diabetes in the highest tertile of adiponectin were more likely to be female (67%), having dental insurance (52%), have visited the dentist within the past 6 months (52%), and lean (82%). Those in the lowest tertile of adiponectin were mostly males (72%), less likely to have dental insurance (21%) or have visited the dentist within the past 6 months (24%), yet only 58% were not overweight (Table 5.1.). Controls in the highest tertile of CRP were mostly similar to those in the lowest tertile except that they

were more likely to be overweight (55%) than those on the lowest tertile (10%). Controls in the highest tertile for adiponectin were not different from those in the lowest teritle.

Data on oral health and hygiene was available from a sub-sample of 50 cases of type 1 diabetes and 44 controls without type 1 diabetes. Both groups brushed their teeth at least twice a day and flossed at least once a week. Of the individuals in the highest tertile of CRP 9% flossed once a day or more, 34% flossed less than once per day. Of those in the lowest tertile of CRP 17% flossed once daily or more and 53% flossed less than once a day. Tooth brushing and measures of gingival conditions and oral health were similar across the tertiles of CRP and tertiles of CRP and adiponectin (Table 5.2.).

Table 5.3 shows the regression coefficient of CRP and adiponectin, separately, on each of the 4 clusters, stratified by type 1 diabetes status. Model 1 was adjusted for the other clusters; model 2 was adjusted for the other clusters, sex, age, and race; model 3 was adjusted for the other clusters, sex, age, race, dental insurance, and dental visits; and model 4 was adjusted for the other clusters, sex, age, race, dental insurance, dental visits, and diabetes duration and run only among cases of type 1 diabetes. There was a significant positive association between the log score of the 'orange-blue' cluster and CRP among youth with type 1 diabetes adjusted for the other clusters (β=0.1291, 95% CI: 0.0388, 0.2193). The relationship remained significant and of similar magnitude, after several adjustments including the fully-adjusted model (β=0.0991, 95% CI: 0.0388, 0.2193) and the diabetes only model (β=0.1007, 95% CI: 0.0017, 0.1997). There was also

a significant negative association between the log score of the 'blue-other' cluster and adiponectin adjusting for the other clusters, sex, age, race, dental insurance, and dental visits (β=-0.042, 95% CI: -0.0725, -0.0115), as well as among individuals with type 1 diabetes only (β=-0.0418, 95% CI: -0.0731, -0.0105) after additionally adjusting for duration of diabetes.

DISCUSSION

Clusters of periodontal microorganisms were associated with CRP and adiponectin after accounting for potential confounders. CRP was positively associated with the 'orange-blue' cluster, which was dominated by members of the orange complex that are precursors of the red complex and associated with early stages of periodontal disease(Socransky et al., 1998), among youth with type 1 diabetes. Adiponectin on the other hand was inversely associated with the 'blue-other' cluster, containing early colonizers of dental plaque, among youth with type 1 diabetes.

High levels of serum inflammatory mediators, like CRP and plasminogen activator inhibitor-1 (PAI-1), were associated with increased risk for developing type 2 diabetes(Festa et al., 2002; Pradhan et al., 2001; Vozarova et al., 2002), and were higher among obese individuals in both, animal and human models (Pickup & Crook, 1998; Shoelson et al., 2006). Inflammation can result in reduced insulin effect through the production of inflammatory kinases(Gregor & Hotamisligil, 2011)' (Hotamisligil, 2010). Insulin-resistance is no longer associated to just diabetes, but also infectious diseases(Bahtiyar et al., 2004; Pao et al., 2008; Sidiropoulos et al., 2008), and systemic

diseases(Clowes et al., 1978; Wichterman et al., 1979) (Sidiropoulos et al., 2008). Studies have shown that insulin-resistance among obese individuals was highly associated with the secretion of inflammatory cytokines(Hotamisligil et al., 1995; Hotamisligil et al., 1996; Hotamisligil et al., 1993), especially among those who have diabetes(Baker et al., 2006; Bloom, 1969; Fearnley et al., 1959; Ogston & McAndrew, 1964; Van Cromphaut et al., 2008). One of the proposed mechanisms by which oral bacteria inhibits insulin-sensitivity is by decreasing the production of adiponectin. Tumor necrosis factor-α (TNFα) is a pro-inflammatory cytokine produced in periodontal disease. It decreases insulin sensitivity and inhibits the production of anti-inflammatory cytokines such as adiponectin. By doing so, periodontal microbes interfere with basic metabolic pathways and affect systemic outcomes(Goodson et al., 2009).

P. gingivalis, which is a major periodontal pathogen associated with periodontal disease, increases the synthesis of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8. Adiponectin exerts a counter effect on the action of *P. gingivalis*, and induces the production of anti-inflammatory cytokines as IL-10(Deschner, Eick, Damanaki, & Nokhbehsaim, 2014). Kraus et al., showed that adiponectin prevented the action of *P. gingivalis,* decreased cell viability and increased proliferation and differentiation, suggesting that adiponectin may play an important role in the prevention of pocket epithelium formation in periodontal disease(Kraus et al., 2012). Furthermore, adiponectin inhibited the *A. actinomycetemcomitans* LPS-stimulated inducible nitric oxide synthase expression and nitric oxide production as well as the *A.*

actinomycetemcomitans LPS/receptor activator of NFκB ligand-induced osteoclast formation in a murine macrophage-like cell line(Yamaguchi et al., 2007).

Among individuals without diabetes, Kern et al. reported a decreased level of adiponectin mRNA in those who were obese. Also a significant inverse correlation (r = −0.47) was found between the expression level of adiponectin mRNA and TNF-α mRNA(Kern, Di Gregorio, Lu, Rassouli, & Ranganathan, 2003). A review by Ouchi et al. suggested that adiponectin had an anti-inflammatory effect through mediating immune responses of TNF-α-secreting cells. Moreover, adiponectin could be one factor linking obesity to various vascular diseases(Ouchi, Kihara, Funahashi, Matsuzawa, & Walsh, 2003). Stefan et al. reported that among children 10 years old percent-body-fat was independently correlated with plasma adiponectin levels, cross-sectionally(Stefan et al., 2002). In our sample, we found a negative correlation between BMI percentile and adiponectin level among cases of type 1 diabetes (Spearman's r=-0.3, p=0.0025).

Studies have reported a relation between the presence of periodontal pathogens and higher CRP levels and poor periodontal condition(Pejcic et al., 2011b). *P. gingivalis* was positively associated with high CRP levels in the presence of periodontal disease. Contrary to that, *A. actinomycetemcomitans* was not associated with elevated CRP(Pejcic et al., 2011a). On the other hand Miyashita et al., did not find any association between titers of antibodies to *P. gingivalis* and CRP in a Japanese adult population(Miyashita et al., 2012). Teles et al., reported no associations between serum analytes, including adiponectin, and microbial parameters, however they did find an association between elevated levels of adipokines and both sex and BMI. Moreover,

microbiological parameters were improved following periodontal therapy(Teles, Teles, Martin, Socransky, & Haffajee, 2012).

A longitudinal study (CARDIA) found that those who had repeatedly high level of CRP (10 mg/L) were more likely to be obese(Ishii et al., 2012). Park et al. reported a significantly high level of serum CRP, TNF-alpha, and IL-6 (inflammatory cytokines) with high BMI, WC, hip circumference, and waist-to-hip ratio(Park, Park, & Yu, 2005). Farouhi et al. reported that in in South Asians and Europeans visceral adiposity was a key factor in promoting chronic inflammation(Forouhi, Sattar, & McKeigue, 2001). A recently published metananalysis of 51 cross-sectional studies showed that BMI, WC, and WHR were all associated with elevated levels of CRP(J. Choi, Joseph, & Pilote, 2013). In the presented study the prevalence of overweight was low and that of obesity was even lower. Moreover, this sample of youth had generally low level of periodontal disease. Thus, it is not unusual to observe such low levels of CRP (mean=1.1mg/dL, SD=0.5).

Some limitations related to the presented study were, first, the temporal relationship of the levels of BMI percentiles, CRP, and adiponectin with bacterial cluster scores could not be determined due to the cross-sectional design. Follow-up studies designed to monitor the changes of oral microbial pattern in relation to BMI percentile, CRP, and adiponectin are needed to determine the temporality of such relationships. Second, the prevalence of periodontal disease and the levels of serum CRP were both low in this sample which could have decreased the power to gain significance. We hypothesize that in a sample with higher levels of periodontal disease the association between oral microbial clusters and markers of adiposity and inflammation would be

stronger and more shifted towards the 'orange-red' cluster, which contains pathogens associated with advanced stages of periodontal disease. Third, the majority of youth were non-Hispanic white which limits the generalizability of such findings to other races; however this could be a reflection of the dominantly non-Hispanic white population of Colorado. Fourth, there was no gold-standard classification of periodontal microorganisms among youth with type 1 diabetes and low levels of periodontal disease to validate our results against, but we hope that this study provides a preliminary framework for future research on oral microbial clusters. Fifth, the low levels of periodontal disease and overweight could be related to the type of youth that would volunteer for such study (selection bias), however, to control for that we compared the results to a control group from taken at convenience from the same hospital or from friends of patients with type 1 diabetes, assuming it is a representative sample of the source population. Sixth, we compared 4 different outcomes to BMI percentile, CRP, and adiponectin without adjusting for multiple comparisons, since there is no known method of adjustment for such comparisons. Adjustment methods for sub-group analysis such as Bonferroni would not be applicable, and other methods such as false-discovery rate would not be efficient.

The strengths of this study were, first, we used an empirical method of clustering periodontal microorganisms (cluster analysis). This process is data-driven and free of any apriori restriction or categorization. This also limited multiple comparisons of the different microbes to just 4 clusters that were mutually-exclusive. Second, we looked at the comprehensive microbial profile by adjusting for all other clusters at all times,

limiting the detection of sporadic and unadjusted associations with bacterial clusters. Third, we presented data on a large number of periodontal microorganisms (41 species) from a sample of 105 youth with type 1 diabetes; this is one of the largest studies conducted among youth with type 1 diabetes and such a wide range of microbial species. Fourth, we standardized all counts of microorganisms which eliminated baseline differences. Fifth, expenses of this study were limited since it was ancillary to two other follow-up studies on was related to oral hygiene and the other to markers of cardiovascular disease. Sixth, the study participants were taken from the Barbara Davis Center which is specialized for childhood diabetes and serves a wide geographic range and large source population.

In summary, CRP was related to periodontal microbial profile, particularly to the 'orange-blue' cluster, which was dominated by members of the orange complex that are known to be associated with early periodontal disease, in youth with type 1 diabetes. Adiponectin, on the other hand, was inversely associated with the 'blue-other' cluster that was dominated by *A. actinomycetemcomitans*, among youth with type 1 diabetes. This suggests that specific microorganisms may have different effects on inflammatory markers in youth with type 1 diabetes.

Table 5.1. Population characteristics by CRP and adiponectin tertiles stratified by type 1 diabetes.

	Controls without type 1 diabetes				Cases of Type 1 Diabetes			
	CRP (mg/dL)		Adiponectin (µg/ml)		CRP (mg/dL)		Adiponectin (µg/ml)	
	Tertile 1	Tertile 3	Tertile 1	Tertile 3	Tertile 1	Tertile 3	Tertile 1	Tertile 3
Selected Characteristics	**N = 21**	**N = 22**	**N = 22**	**N = 22**	**N = 33**	**N = 33**	**N = 33**	**N = 33**
Sex / n (%)[¥‡]								
Male	11 (52)	6 (27)	12 (55)	9 (41)	22 (67)	11 (33)	23 (70)	11 (33)
Female	10 (48)	16 (73)	10 (45)	13 (59)	11 (33)	22 (67)	10 (30)	22 (67)
Race / n (%)								
Other	5 (24)	10 (45)	8 (36)	4 (18)	10 (30)	12 (36)	8 (24)	8 (24)
Non-Hispanic White	16 (76)	12 (55)	14 (64)	18 (82)	23 (70)	21 (64)	25 (76)	25 (76)
Dental Insurance / n (%)[‡]								
No/Don't know	11 (52)	8 (36)	11 (50)	6 (27)	20 (61)	21 (64)	26 (79)	16 (48)
Yes	10 (48)	14 (64)	11 (50)	16 (73)	13 (39)	12 (36)	7 (21)	17 (52)
Dental visit in the past 6 months / n (%)[‡]								
No	10 (48)	11 (50)	11 (50)	7 (32)	19 (58)	21 (64)	25 (76)	16 (48)

Yes	11(52)	11 (50)	11 (50)	15 (68)	14 (42)	12 (36)	8 (24)	17 (52)
Overweight[€‡]								
No (< 85th percentile)	19 (90)	10 (45)	16 (73)	18 (82)	25 (76)	19 (58)	19 (58)	27 (82)
Yes (≥ 85th percentile)	2 (10)	12 (55)	6 (27)	4 (18)	8 (24)	14 (42)	14 (42)	6 (18)
Diabetes treatment / n (%)								
Injection	--	--	--	--	22 (67)	19 (58)	17 (52)	22 (67)
Pump	--	--	--	--	11 (33)	14 (42)	16 (48)	11 (33)
HbA1c / n (%)[¥]								
< 7.5%	--	--	--	--	8 (24)	2 (6)	6 (18)	4 (12)
7.5 – 9.5%	--	--	--	--	23 (70)	14 (42)	18 (55)	16 (48)
> 9.5%	--	--	--	--	2 (6)	17 (52)	9 (27)	13 (39)
Age in years / mean (SD) [€¥]	14.2 (1.2)	16 (1.9)	15.6 (2)	15.1 (2.4)	14.8 (1.9)	16.3 (2.1)	15.8 (1.9)	15.2 (2.2)
Glucose mg/dL / mean (SD)	--	--	--	--	170.3 (97.1)	197.1 (83)	172.2 (86.9)	201.5 (76.7)
HbA1c % / mean (SD) [¥]	--	--	--	--	8.3 (1.1)	9.6 (1.3)	8.8 (1.2)	9.3 (1.6)
Diabetes Duration in years / mean (SD)	--	--	--	--	8.8 (3.3)	8.7 (3.7)	9.1 (3.4)	8.2 (3.6)

€: X^2 test for CRP among controls < 0.05

¥: X^2 test for CRP among cases of type 1 diabetes < 0.05

‡: X^2 test for Adiponectin among cases of type 1 diabetes < 0.05

Table 5.2. Oral hygiene and dental measures characteristics by CRP and adiponectin tertiles.

	CRP (mg/dL)		Adiponectin (μg/ml)	
	Tertile 1	**Tertile 3**	**Tertile 1**	**Tertile 3**
Selected Characteristics	**N = 30**	**N = 32**	**N = 23**	**N = 37**
Cases of Type 1 Diabetes / n (%)	16 (53)	18 (56)	10 (43)	21 (57)
Brushing teeth / n (%)				
once a day or less	7 (23)	11 (34)	7 (30)	13 (35)
>1, but less than 2x/day	7 (23)	5 (16)	3 (13)	8 (22)
2x/day or more	16 (53)	16 (50)	13 (57)	16 (43)
Flossing teeth / n (%)[€]				
None	9 (30)	18 (56)	12 (52)	17 (46)
< once a day	16 (53)	11 (34)	8 (35)	15 (41)
once a day or more	5 (17)	3 (9)	3 (13)	5 (14)
Calculus Index / mean (SD)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)
Gingival Index / mean (SD)	0.7 (0.2)	0.7 (0.1)	0.7 (0.2)	0.7 (0.2)
Plaque Index / mean (SD)	0.6 (0.3)	0.6 (0.3)	0.6 (0.3)	0.6 (0.3)
No. of teeth with bleeding / mean (SD)	7.8 (4.0)	8.0 (4.3)	8.2 (4.0)	7.4 (4.6)
Mean probing depth in mm / mean (SD)	2.0 (0.3)	1.9 (0.2)	1.9 (0.2)	1.9 (0.2)

€: X^2 test for CRP tertiles < 0.05

Table 5.3. Adjusted multivariable regression coefficient of CRP and adiponectin on 4 empirically-formed clusters of periodontal microorganisms found in dental plaque of youth.

		CRP log(mg/dL)		Adiponectin log(μg/ml)	
Cluster	**Model**	**Control**	**type 1 diabetes**	**Control**	**type 1 diabetes**
		β (SE)	**β (SE)**	**β (SE)**	**β (SE)**
Blue-Other	**1**	0.177 (0.1352)	0.1187 (0.0731)	-0.0142 (0.0168)	-0.0158 (0.0131)
	2	0.1456 (0.1665)	0.1076 (0.0738)	-0.0191 (0.015)	-0.0222 (0.0142)
	3	0.2235 (0.1479)	0.0924 (0.0659)	-0.0186 (0.0113)	**-0.042 (0.0156)***
	4	--	0.0873 (0.0651)	--	**-0.0418 (0.0159)***
Orange-Blue	**1**	0.0192 (0.0836)	**0.1291 (0.046)***	0.006 (0.0097)	0.0125 (0.0164)
	2	0.0193 (0.0947)	**0.1172 (0.0527)***	0.0091 (0.0115)	0.0142 (0.0145)
	3	-0.0024 (0.0965)	**0.0991 (0.0502)***	0.0044 (0.0106)	-0.0087 (0.0124)
	4	--	**0.1007 (0.0505)***	--	-0.009 (0.0122)
Orange-Red	**1**	-0.0256 (0.0521)	-0.0445 (0.0423)	0.0064 (0.0064)	0 (0.008)
	2	0.0308 (0.0533)	-0.048 (0.0459)	0.0125 (0.0072)	-0.0047 (0.0082)
	3	0.0181 (0.0523)	-0.0475 (0.0472)	0.0137 (0.0075)	-0.0014 (0.0089)

	4	--	-0.0471 (0.0468)	--	-0.0013 (0.0089)
Yellow-Other	**1**	-0.0924 (0.0544)	-0.0384 (0.0508)	-0.0011 (0.0089)	-0.0024 (0.0133)
	2	-0.109 (0.0565)	-0.0585 (0.0476)	0.0029 (0.0078)	-0.0146 (0.0124)
	3	-0.1109 (0.058)	-0.048 (0.0461)	-0.0014 (0.007)	-0.0215 (0.0151)
	4	--	-0.0471 (0.049)	--	-0.0215 (0.0145)

Model 1: Adjusted for the other clusters.

Model 2: Adjusted for the other clusters, sex, age, and race.

Model 3: Adjusted for the other clusters, sex, age, race, dental insurance, and dental visits.

Model 4: Adjusted for the other clusters, sex, age, race, dental insurance, dental visits, and diabetes duration.

*: p-value (2-sided < 0.05)

CHAPTER 6

DISCUSSION

Periodontitis is a low-grade, chronic, gram-negative infection of the gum affecting 1 in 2 American adults (Eke et al., 2012). As plaque(Rana et al., 2010), an aggregation of bacteria adhering to the surface of teeth, forms, toxins produced by the inhabiting bacteria start destroying the connective tissue between the teeth and the gum, causing separation of gum from the teeth and the formation of periodontal pocket(Feng & Weinberg, 2006). It is associated with diabetes, both types, obesity, cardiovascular diseases, chronic inflammation, and other systemic diseases(D'Aiuto et al., 2006; Mustapha et al., 2007). However the role that periodontal microorganism play in these conditions is not well-studied(Feng & Weinberg, 2006; E. Lalla, Kaplan, et al., 2006). In this study we explored how periodontal bacteria from sub gingival plaque clustered in youth with and without type 1 diabetes, and related their pattern of clustering to body mass index percentile (BMI%) as well as C-reactive protein (CRP) and adiponectin, which are markers of chronic inflammation.

Cross-sectional data were collected from 105 youth with type 1 diabetes and 71 without diabetes. Participants were between 12 and 19 years of age receiving care at the Barbara Davis Center in Colorado, 2009-2011. Counts of 41 oral-bacteria from

sub gingival-plaque were obtained by DNA-DNA hybridization and grouped using cluster-analysis. Standardized-mean counts of each organism were computed and summed to get microbial-counts per cluster, stratified by diabetes status. A subset (n=101, 54 with type 1 diabetes) underwent dental examinations at the University of Colorado, School of Dental Medicine clinic. BMI z-scores were defined as normal (<85%) or overweight (≥85%). CRP and adiponectin were divided into tertiles.

Participants were 15-years old on average; 51% were female; 73% non-Hispanic white; 37% overweight; the average diabetes duration was 8 years. About 48% brushed their teeth twice/day; 12% flossed once/day; 47% visited a dentist in the past 6 months. Bacterial clusters were identified and named as 'blue-other', 'orange-blue', 'orange-red', and 'yellow-other', according to Socransky's color-coded complexes.

Youth with type 1 diabetes had very similar periodontal bacterial profile to those without type 1 diabetes. The four empirically-identified clusters had similar proportions. The three groups identified by Desvarieux et al. were also not different among youth with and without type 1 diabetes. Looking at the complexes identified by Socransky, youth with and without type 1 diabetes were not different in terms of microbial composition in sub gingival dental plaque. Clusters of periodontal microorganisms were associated with CRP and adiponectin after accounting for potential confounders. CRP was positively associated with the 'orange-blue' cluster, which was dominated by members of the orange complex that are precursors of the red complex and associated with early stages of periodontal disease(Socransky et al., 1998), among youth with type

1 diabetes. Adiponectin on the other hand was inversely associated with the 'blue-other' cluster among youth with type 1 diabetes.

High levels of serum inflammatory mediators, like CRP and plasminogen activator inhibitor-1 (PAI-1), were associated with increased risk for developing type 2 diabetes(Festa et al., 2002; Pradhan et al., 2001; Vozarova et al., 2002), and were higher among obese individuals in both, animal and human models (Pickup & Crook, 1998; Shoelson et al., 2006). Inflammation can result in reduced insulin effect through the production of inflammatory kinases(Gregor & Hotamisligil, 2011)' (Hotamisligil, 2010). Insulin-resistance is no longer associated to just diabetes, but also infectious diseases(Bahtiyar et al., 2004; Pao et al., 2008; Sidiropoulos et al., 2008), and systemic diseases(Clowes et al., 1978; Wichterman et al., 1979) (Sidiropoulos et al., 2008). Studies have shown that insulin-resistance among obese individuals was highly associated with the secretion of inflammatory cytokines(Hotamisligil et al., 1995; Hotamisligil et al., 1996; Hotamisligil et al., 1993), especially among those who have diabetes(Baker et al., 2006; Bloom, 1969; Fearnley et al., 1959; Ogston & McAndrew, 1964; Van Cromphaut et al., 2008). One of the proposed mechanisms by which oral bacteria inhibits insulin-sensitivity is by decreasing the production of adiponectin(Goodson et al., 2009). Tumor necrosis factor-α (TNFα) is a pro-inflammatory cytokine produced in periodontal disease. It decreases insulin sensitivity and inhibits the production of anti-inflammatory cytokines such as adiponectin. By doing so, periodontal microbes interfere with basic metabolic pathways and affect systemic outcomes(Goodson et al., 2009).

P. gingivalis, an organism associated with periodontal disease and its systemic effects, increases the synthesis of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8. Adiponectin exerts a counter effect on the action of *P. gingivalis*, and induces the production of anti-inflammatory cytokines as IL-10(Deschner et al., 2014). Kraus et al., showed that adiponectin prevented the action of *P. gingivalis,* decreased cell viability and increased proliferation and differentiation, suggesting that adiponectin may play an important role in the prevention of pocket epithelium formation in periodontal disease(Kraus et al., 2012). Furthermore, adiponectin inhibited the *A. actinomycetemcomitans* LPS-stimulated inducible nitric oxide synthase expression and nitric oxide production as well as the *A. actinomycetemcomitans* LPS/receptor activator of NFκB ligand-induced osteoclast formation in a murine macrophage-like cell line(Yamaguchi et al., 2007).

Among individuals without diabetes, Kern et al. reported a decreased level of adiponectin mRNA in those who were obese. Also a significant inverse correlation ($r = -0.47$) was found between the expression level of adiponectin mRNA and TNF-α mRNA(Kern et al., 2003). A review by Ouchi et al. suggested that adiponectin had an anti-inflammatory effect through mediating immune responses of TNF-α-secreting cells. Moreover, adiponectin could be one factor linking obesity to various vascular diseases(Ouchi et al., 2003). Stefan et al. reported that among children 10 years old percent-body-fat was independently correlated with plasma adiponectin levels, cross-sectionally(Stefan et al., 2002). In our sample, we found a negative correlation between BMI percentile and adiponectin level (Spearman's r=-0.3, p=0.0015).

The presence of periodontal pathogens was related to higher CRP levels and poor periodontal condition(Pejcic et al., 2011b). *P. gingivalis* was positively associated with high CRP levels in the presence of periodontal disease. Contrary to that, *A. actinomycetemcomitans* was not associated with elevated CRP(Pejcic et al., 2011a). On the other hand Miyashita et al., did not find any association between titers of antibodies to *P. gingivalis* and CRP in a Japanese adult population(Miyashita et al., 2012). Teles et al., reported no associations between serum analytes, including adiponectin, and microbial parameters, however they did find an association between elevated levels of adipokines and both sex and BMI. Moreover, microbiological parameters were improved following periodontal therapy(Teles et al., 2012).

A longitudinal study (CARDIA) found that those who had repeatedly high level of CRP (10 mg/L) were more likely to be obese(Ishii et al., 2012). Park et al. reported a significantly high level of serum CRP, TNF-alpha, and IL-6 (inflammatory cytokines) with high BMI, WC, hip circumference, and waist-to-hip ratio(Park et al., 2005). Farouhi et al. reported that in in South Asians and Europeans visceral adiposity was a key factor in promoting chronic inflammation(Forouhi et al., 2001). A recently published metananalysis of 51 cross-sectional studies showed that BMI, WC, and WHR were all associated with elevated levels of CRP(J. Choi et al., 2013). In the presented study the prevalence of overweight was low and that of obesity was even lower. Moreover, this sample of youth had generally low level of periodontal disease. Thus, it is not unusual to observe such low levels of CRP (mean=1.1mg/dL, SD=0.5).

Some limitations related to the presented study were, first, the temporal relationship between levels of CRP or adiponectin and bacterial cluster scores could not be determined due to the cross-sectional design. Second, the prevalence of periodontal disease and the levels of serum CRP were both low in this sample which could have decreased the power to gain significance. Third, the majority of youth were non-Hispanic white which limits the generalizability of such findings to other races. Fourth, there was no gold-standard classification of periodontal microorganisms to validate our results against. The strengths of this study were, first, we used an empirical method of clustering periodontal microorganisms (cluster analysis) which is free of a priori restriction of groups. Second, we looked at the comprehensive microbial profile by adjusting for all other clusters at all times. Third, we presented data on a large number of periodontal microorganisms (41 species) from a sample of youth with type 1 diabetes. Fourth, we standardized all counts of microorganisms which eliminated any baseline difference among them and among the cluster summary scores.

In summary, youth with and without type 1 diabetes had similar periodontal microbial profile of sub gingival dental plaque. CRP was related to periodontal microbial profile, particularly to the 'orange-blue' cluster, which was dominated by members of the orange complex that are known to be associated with early periodontal disease, in youth with type 1 diabetes. Adiponectin, on the other hand, was inversely associated with the 'blue-other' cluster that was dominated by *A. actinomycetemcomitans*, among youth with type 1 diabetes. This suggests that specific microorganisms may have different effects on inflammatory markers in youth with type 1 diabetes. Here, we could

see an association of systemic makers of disease with periodontal pathogens that colonize the oral cavity during early stages of periodontal disease, such as members of the blue and orange complexes. It is known that periodontal disease raises the level of CRP(Shojaee et al., 2013) and that its treatment, even at the early stage of gingivitis, reduces markers of systemic inflammation(Alzahrani et al., 2013). Hence, it is important that dentists and pediatricians stress the benefits of daily oral hygiene practice and regular dental check-ups in identifying early stages of periodontal disease, even gingivitis, in reducing the systemic level of CRP(Ajwani, Mattila, Narhi, Tilvis, & Ainamo, 2003).

The role that the oral microbial components have on health in general has not been thoroughly studied. Studies conducted in human and animal models evaluated mainly the effect of gastrointestinal microbiome than the periodontal microbiome. How different dietary habits, host conditions, and environmental triggers influence the periodontal microbiome must be thoroughly studied. Would differences in periodontal microbiome cause obesity or are caused by obesity, or is it a bidirectional relationship? Does overweight/obesity identify with a certain periodontal microbiome profile and not another? How does diabetes in youth affect such a relationship and are such mechanisms different between type 1 diabetes and type 2 diabetes? What environmental factors come in play and interfere with a microbiome shift in case of obesity? In case this shift happens, would it be significant enough clinically to cause a weight shift? More importantly, can it be clinically detected to prevent overweight and obesity? Moreover, would manipulation of the periodontal microbiome lead to

decreased systemic inflammation and lean weight? Clearly, there are many questions to be asked so we can better understand the role of periodontal microorganisms in regulating metabolic interactions of the host environment.

Although this is an understudied field, the future implications that can emerge from further studies can be of significant clinical importance. A better understanding of the periodontal microbiome may lead to individualized dietary recommendations that could reduce, or even prevent, obesity and chronic inflammation(Kau, Ahern, Griffin, Goodman, & Gordon, 2011). Modulation of microbial composition could be possible through the use of probiotics, prebiotics, and antibiotics. Moreover, it might serve as an early diagnostic or screening tool for systemic diseases that are too early to detect clinically and call for a preventive action. Such advancement in the knowledge of periodontal microbes could lead to a more personalized treatment(Nicholson, Holmes, & Wilson, 2005).

The periodontal microbiome is a very complex and dynamic system that adapts to different exposures and interacts with its surrounding environment throughout our daily routine. In this study we discussed the importance of the periodontal microbiome in adiposity and chronic inflammation in a group of youth with type 1 diabetes. certain periodontal microorganisms were associated with overweight and markers of chronic inflammation. The advances of epidemiologic and biomedical sciences are providing us with better tools to study and better understand the role that periodontal microbes exert on health and disease and prompt novel diagnostic tools for health management.

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