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Exposure to *Porphyromonas gingivalis* and Modifiable Risk Factors Modulate Risk for Early Diabetic Retinopathy

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Received: October 29, 2020
Accepted: January 6, 2021
Published: February 16, 2021

Keywords: microbiota; oral cavity; periodontitis; *Porphyromonas gingivalis*; retina; diabetic retinopathy; Third National Health and Nutrition Examination Survey (NHANES III)

Citation: Chiu C-J, Chang M-L, Kantarci A, Van Dyke TE, Shi W. Exposure to *Porphyromonas gingivalis* and modifiable risk factors modulate risk for early diabetic retinopathy. Trans Vis Sci Tech. 2021;10(2):23, https://doi.org/10.1167/tvst.10.2.23

Purpose: We hypothesized that exposure to *Porphyromonas gingivalis* (*Pg*) increases the risk for early diabetic retinopathy (DR) and that the risk can be modulated.

Methods: We identified 116 early DR cases, and 116 non-DR controls were selected randomly by frequency matching for age, sex, race, and education from the US Third National Health and Nutrition Examination Survey. DR was assessed using non-mydriatic fundus photographs and graded by trained graders using the Modified Airlie House Classification scheme and the Early Treatment for Diabetic Retinopathy Study severity scale. Serum *Pg* immunoglobulin G (IgG) antibody (Ab) was measured in enzyme-linked immunosorbent assay units. Logistic regression was used to relate serum *Pg* IgG Ab levels to the risk for early DR.

Results: Per tenfold increase in *Pg* IgG Ab levels, there was an over 60% increased risk for early DR (odds ratio = 1.64; 95% confidence interval, 1.36–1.97), and a linear trend was noted for the estimated probabilities of early DR at various *Pg* IgG Ab levels (P for trend = 0.0053). The analysis also suggested that moderate alcohol consumption (less than 12 drinks in the past 12 months; P for interaction = 0.0003) and maintaining a normal serum glycated hemoglobulin level (HbA1c $\leq$ 5.7%; P for interaction $<$ 0.0001) helped reduce the *Pg*-related DR risk.

Conclusions: The increased *Pg*-related DR risk could be alleviated by managing alcohol consumption and maintaining a normal blood glucose level.

Translational Relevance: Findings from this study provide new directions for developing novel therapeutics and prevention strategies for DR.

Introduction

The global prevalence of diabetes mellitus is predicted to increase dramatically in the coming decades, from an estimated 382 million in 2013 to 592 million by 2035.[1] Patients with diabetes suffer many life-limiting and life-threatening complications, including macrovascular-related stroke, ischemic heart disease, and peripheral vascular disease and/or microvascular-related retinopathy, neuropathy, nephropathy, and periodontal disease.[2] Periodontitis is a frequent complication that affects up to 70% of the type 2 diabetic population.[3] As the most common microvascular complication of diabetes,[4] diabetic retinopathy (DR) is a burgeoning problem globally. The condition currently affects almost 100 million people worldwide. Estimates for 1990 and 2010 suggest that DR-related visual impairment and blindness increased by 64% and 27%, respectively.[5] DR falls into two broad categories: the earlier stage of nonproliferative diabetic retinopathy (NPDR) and the advanced stage of proliferative diabetic retinopathy. Current therapeutic paradigms for DR are invasive and destructive and focus on arresting the advanced stages of DR without curing the disease. Therefore, although there is an unmet need for noninvasive, nondestructive, longer lasting treatment options (because prevention

is better than cure), early prevention strategies that address multiple risk factors are particularly needed for DR.[6]

Mucosal surfaces, including the oral mucosa, are colonized by a complex and dynamic microbial ecosystem that has important implications in human health and disease.[7,8] *Porphyromonas gingivalis* (*Pg*) is an asaccharolytic anaerobe frequently associated with periodontal disease.[9] In the oral cavity, this Gram-negative anaerobe colonizes the gingival sulcus in low numbers in health and comprises a significant proportion of the microbiota of the periodontal pocket in disease, contributing to a multispecies microbiome that eventually results in alveolar bone loss.[10] Recent studies suggest that, despite its low abundance, *Pg* may play a key role in periodontitis by contributing to a shift in the commensal microbiota and dysbiosis.[7] *Pg* elicits a systemic inflammatory response resulting in elevated levels of various inflammatory mediators.[11] This *Pg*-related systemic inflammation has been suggested to increase the risk for several systemic diseases, such as atherosclerosis,[12–14] rheumatoid arthritis,[15,16] neurodegenerative diseases,[17–20] and diabetes,[21] providing a vivid example of how disturbances in the commensal microbiome can impact other aspects of human health and disease in sites remote from the site of colonization. In addition, recent studies have identified *Pg* in the brain of Alzheimer's disease patients and demonstrated that oral *Pg* infection in mice resulted in brain colonization and increased production of a major component in amyloid plaques,[22,23] suggesting that *Pg* could access and spread into the human neural system and induce neuroinflammatory damages.

Our previous studies suggested a significant association between a *Pg*-dominant microbiota and increased diabetes-related mortality.[24] We therefore hypothesized that infection of *Pg* increases the risk for DR. In this study, we tested this hypothesis by relating serum immunoglobulin G (IgG) antibody (Ab) to *Pg* to the risk for early DR in a case-control study from a representative sample of the US population.

Methods

Study Cohort

The Third National Health and Nutrition Examination Survey (NHANES III) was performed between 1988 and 1994 by the National Center for Health Statistics (NCHS) of the US Centers for Disease Control and Prevention (CDC). It is a cross-sectional nationwide health survey of non-institutionalized US residents 2 months of age and older using a stratified multistage probability sampling design to sample a representative cohort of the US general population. Of the 39,695 individuals included in the NHANES III survey, 33,994 (85.6%) participated in home interviews, which were used to collect data on demographic characteristics, socioeconomic status, family medical history, current medical conditions, and use of medications. All individuals who participated in a home interview were invited to visit a mobile examination center (MEC) for a medical examination, which consisted of a physical examination, gradable fundus photography, and collection of blood and urine samples for laboratory testing. Therefore, the fundus photographs and blood for IgG testing in this study were collected at the same time. A total of 16,575 participants 20 years of age or older were examined in the MEC.[25]

This study involved only the secondary data analysis of existing US national databases that are publicly available and have been de-identified. This research qualified for exemption of institutional review board human subjects approval under 45 CFR 46.101(b) (4) as specified by the Federal Regulations for Protection of Human Research Subjects. Thus, this is an exempt study and there was no need for institutional review board approval from our institutions.

Case and Control Definition

In NHANES III, DR was assessed in 9737 adults 40 years of age and older using non-stereoscopic, color 45° photographs centered between the optic nerve and the macula. The camera used was a Canon CR4-45NM non-mydriatic fundus camera (Canon Inc., Tokyo, Japan), which incorporates an infrared video camera to allow photographs to be taken in a darkened examination room without the use of dilating drops, allowing for dilation of the pupil, usually to 6 mm to 10 mm in diameter.[26] Trained graders at the University of Wisconsin Ophthalmic Epidemiology Reading Center used the Modified Airlie House Classification scheme and the Early Treatment for Diabetic Retinopathy Study severity scale to grade the photographs.[27,28]

Out of ~8000 eligible participants, we identified 116 early DR cases. We focused on the early stages because we consider early prevention to be the best strategy for this irreversible, visual-impairment disease when it progresses into later stages. Early DR included hard exudates, soft exudates, intraretinal microvascular abnormalities without microaneurysms, hemorrhages only without microaneurysms, microaneurysms only, and early or moderate NPDR. Among the persons not displaying signs of DR, we selected 116 matched controls (case:control = 1:1) by random selection using frequency matching in age, race, sex, and education.

Serum *Pg* IgG Antibody

The Ab measurement in the NHANES III was performed at the Forsyth Institute, Boston MA. Detailed experimental methods and a panel of oral bacterial strains used to prepare whole-cell antigenic extracts for determining the levels of IgG Ab by means of the "checkerboard" immunoassay can be found in the CDC, NCHS, and NHANES III Data Documentation.[29,30] To assess the level of Ab to *Pg*, a mixed suspension of *Pg* comprised of ATCC strains 33277 and 53978 (ATCC, Manassas, VA) was used. The serum levels of IgG Ab were reported in enzyme-linked immunosorbent assay units (EU).

Statistical Methods

Because the distribution of serum *Pg* IgG Ab levels is positively skewed, before further analysis was performed the *Pg* IgG Ab variable was $\log_{10}$ transformed to an approximately normal distribution.[24] This $\log_{10}$(IgG Ab) variable was used in further analyses.

The following were covariates in our analyses: age, sex, race, education level, smoking status, alcohol consumption status, including alcohol drinker status (non-drinkers vs. drinkers) and alcohol drinking status among drinkers (moderate drinking, less than 12 drinks in the past 12 months; excessive drinking, at least 12 drinks in the past 12 months), body mass index (BMI, computed from weight and height; kg/m^2), duration of diabetes, insulin use, hypertension, serum levels of C-reactive protein (CRP), two clinical periodontal measurements (mean number of tooth sites that bled on probing [mBOP] and mean clinical attachment loss [mCAL]), and glycated hemoglobin (HbA1c; Diabetes Control and Complications Trial percent [DCCT%]). Note that high HbA1c status was defined as HbA1c > 5.7 DCCT%, and normal HbA1c status was defined as HbA1c $\leq$ 5.7 DCCT%.[31]

Descriptive statistics for these covariates between cases and controls were calculated. To determine significance of differences, analysis of variance (ANOVA) for comparison of means of continuous variables and χ^2 tests for categorical variables were used. We also examined the correlations among serum *Pg* IgG Ab levels and these covariates using Pearson's correlation coefficient (r), Wilcoxon–Mann–Whitney tests, or Kruskal–Wallis tests, as appropriate.

To evaluate the association between *Pg* IgG Ab levels and early DR risk, logistic regression models were fitted by controlling for selected covariates. We used a hierarchical strategy in our model construction to examine the confounding effects from the covariates. Starting from an age-adjusted model (Model 1), we stepwise included the other covariates: Model 2 additionally adjusted for sex, race, education, and BMI; Model 3 additionally adjusted for smoking history and alcohol drinker/drinking status; Model 4 additionally adjusted for serum CRP and HbA1c levels, and hypertension history; and Model 5 additionally adjusted for the two clinical periodontitis measurements, mBOP and mCAL. We also used Model 5 to calculate the estimated probabilities for early DR at various *Pg* IgG Ab levels. For Models 3, 4, and 5, we did two sets of analyses (see footnotes in Table 3); one adjusted for alcohol drinker status (non-drinkers vs. drinkers) and the second adjusted for alcohol drinking status among drinkers (moderate drinking, less than 12 drinks in the past 12 months; excessive drinking, at least 12 drinks in the past 12 months). Forty-one non-drinkers, including 19 controls and 22 cases, were excluded from the analyses for alcohol drinking status (moderate vs. excessive). In the analysis for the first case, the smoking histories for the 41 non-drinkers were included in the analysis. In the analysis for the second case, the 41 non-drinkers were excluded from the analysis.

We further evaluated whether or not the effect of serum *Pg* IgG Ab levels on early DR risk varies by the status of the seven modifiable risk factors, including education levels, BMI, smoking history, alcohol drinker/drinking status, serum HbA1c status, mBOP, and mCAL. Seven models were constructed; in each model, an interaction term between serum *Pg* IgG Ab levels and the modifiable risk factor of interest was added to Model 5.

All analyses were performed using the SAS 9.4 SURVEY procedures (SAS Institute Inc., Cary, NC), which take into account the complex sampling design used in NHANES III to calculate unbiased point estimates, such as means and odds ratios (ORs), standard errors (SEs), and confidence intervals (CIs). We used $P < 0.05$ to denote statistical significance, and all tests were two-sided.

Results

Because our controls were matched with cases for age, sex, race, and education, it is not surprising that the distributions of these four covariates were not significantly different between cases and controls (Table 1). The distribution for BMI; serum levels of CRP, HbA1c, and *Pg* IgG Ab; and the two clinical periodontitis measurements, mBOP and mCAL, were not significantly different, either. However, cases

Table 1. Comparison of Characteristics of DR Cases Versus Frequency-Matched Controls

Characteristics	Controls* ($n = 116$)	Cases* ($n = 116$)	$P^{\dagger}$
Age (yr), mean (SE)	58.4 (0.17)	58.4 (0.15)	0.99
Male sex, *n* (%)	62 (48.3)	62 (47.2)	0.18
Race, *n* (%)			0.10
Non-Hispanic white	49 (80.8)	49 (82.9)	
Non-Hispanic black	37 (14.7)	37 (12.1)	
Hispanic	30 (4.5)	30 (5.0)	
Education, *n* (%)			0.23
<12 yr	57 (33.3)	57 (34.5)	
12 yr	41 (42.3)	41 (39.2)	
>12 yr	18 (24.4)	18 (26.3)	
BMI (kg/m^2), mean (SE)	27.6 (0.06)	28.3 (0.12)	0.50
Smoking history, *n* (%)			<0.0001
Non-smoker	50 (45.5)	47 (31.4)	
Former smoker	37 (35.1)	37 (44.0)	
Active smoker	29 (19.4)	32 (24.6)	
Alcohol drinking status (at least 12 drinks in the past 12 months), *n* (%)			
Non-drinker	19 (6.2)	22 (8.1)	<0.0001
No (moderate drinking)	47 (22.2) (50.7‡)	44 (15.5) (37.0‡)	
Yes (excessive drinking)	50 (21.6) (49.3‡)	50 (26.4) (63.0‡)	<0.0001‡
Serum CRP level (mg/dL), mean (SE)	0.44 (0.001)	0.56 (0.022)	0.43
Serum HbA1c (DCCT%), mean (SE)	5.62 (0.012)	5.66 (0.011)	0.82
Serum HbA1c category, *n* (%)			
≤5.7% (normal)	64 (61.68)	70 (67.75)	
5.7%–6.5% (prediabetic)	48 (35.80)	33 (20.04)	0.0005§
>6.5% (diabetic)	4 (2.52)	13 (12.21)	<0.0001‖
Ever diagnosed with diabetes or diabetic eye diseases, *n* (%)			
No	116 (100)	116 (100)	
Yes	0 (0)	0 (0)	1.00
Ever diagnosed with hypertension, *n* (%)			
No	69 (67.49)	59 (52.10)	
Yes	47 (32.51)	57 (47.90)	<0.0001
Serum $\log_{10}$(*Pg* IgG Ab) (EU), mean (SE)	2.32 (0.018)	2.59 (0.01)	0.12
mBOP, mean (SE)	0.044 (0.0009)	0.026 (0.001)	0.11
mCAL, mean (SE)	0.70 (0.006)	0.64 (0.017)	0.68

*For categorical variables, sample sizes are raw numbers, and the percentages are weighted for the sampling design used in the NHANES III study.

†ANOVA was used for statistical tests of significance for continuous variables, and the Wald χ^2 test was used for all other categorical measures.

‡Forty-one non-drinkers, including 19 controls and 22 cases, were excluded from the analysis.

§HbA1c > 5.7% vs. HbA1c ≤ 5.7%.

‖HbA1c ≤ 5.7% vs. 5.7 <HbA1c ≤ 6.5% vs. HbA1c > 6.5%.

tended to be in the normal category (HbA1c ≤ 5.7% vs. HbA1c > 5.7%; $P = 0.0005$) or the diabetic category (HbA1c > 6.5% vs. HbA1c ≤ 5.7% and 5.7 < HbA1c ≤ 6.5%; $P < 0.0001$) more than controls. Smoking history and alcohol drinking status were significantly different (both $P < 0.0001$), and cases tended to be in higher categories for both exposures. Notably, none of our subjects had been diagnosed as either diabetic or having diabetic eye diseases. Cases were more likely to have a hypertension history than controls ($P < 0.0001$).

Table 2. Bivariate Associations Between Serum *Pg* IgG Ab Concentrations and Covariates

Continuous Covariates	*n*	Pearson's *r* with $\log_{10}$(*Pg* IgG Ab) (EU)	*P*
Age (yr)	232	–0.20	0.002
BMI (kg/m^2)	232	0.17	0.012
Serum CRP level (mg/dL)	232	0.08	0.23
Serum HbA1c (DCCT%)	232	0.052	0.43
mBOP	232	0.32	<0.0001
mCAL	232	0.33	<0.0001
Categorical Covariates	***n***	**Median (IQR) for $\log_{10}$(*Pg* IgG Ab) (EU)**	***P****
Sex			
Male	124	2.50 (1.95–3.12)	0.06
Female	108	2.38 (1.78–2.92)	
Race			
Non-Hispanic white	98	2.12 (1.80–2.83)	<0.0001
Non-Hispanic black	74	2.94 (2.50–3.47)	
Hispanic	60	3.14 (2.82–3.49)	
Education			
<12 yr	114	2.63 (1.91–3.18)	
12 yr	82	2.38 (1.62–2.97)	0.19
>12 yr	36	2.14 (1.96–2.77)	
Smoking history			
Non-smoker	97	2.76 (2.04–3.09)	
Former smoker	74	2.02 (1.85–2.77)	0.48
Active smoker	61	2.48 (1.75–3.19)	
Alcohol drinking status (at least 12 drinks in the past 12 months)			
Non-drinkers	41	2.55 (1.91–3.08)	0.42
No (moderate drinking)	91	2.28 (1.75–2.92)	0.22[†]
Yes (excessive drinking)	100	2.19 (1.95–3.12)	
Ever diagnosed with hypertension			0.18
No	104	2.04 (1.85–2.82)	
Yes	128	2.66 (1.91–3.34)	

IQR, interquartile range.

[*] *P* values were obtained by Wilcoxon two-sample test for variables with two categories and from the Kruskal–Wallis test for variables with three or more categories.

[†] Forty-one non-drinkers were excluded from the analysis.

In our bivariate analysis (Table 2), age ($r = –0.20$; $P = 0.002$) was inversely correlated with serum *Pg* IgG Ab levels, whereas BMI ($r = 0.17$; $P = 0.12$), mBOP ($r = 0.32$; $P < 0.0001$), and mCAL ($r = 0.33$; $P < 0.0001$) were positively correlated. Males ($P = 0.06$) and Hispanics ($P < 0.0001$) tended to have higher levels of serum *Pg* IgG Ab. However, education level, smoking status, alcohol drinking status, hypertension history, and serum levels of CRP and HbA1c were not significantly correlated with serum *Pg* IgG Ab levels.

Next, using logistic regression analysis, we evaluated our primary interest in the association between serum *Pg* IgG Ab levels and risk for early DR (Table 3). Except for Model 5, the ORs per tenfold increase of *Pg* IgG Ab levels in every higher hierarchical model were similar, which conferred over 30% increased risk for early DR. The difference in OR between Model 4 and Model 5 was over 20%, which is much higher than the OR difference (~10%) between any other two neighbor models in Table 3, implying a significant confounding effect from either one of the two periodontal

Table 3. Logistic Analysis Relating Serum *Pg* IgG Ab Levels to Risk for Early DR

Serum *Pg* IgG Level (EU)	Adjusted OR (95% CI)* Model 1	Model 2	Model 3	Model 4	Model 5
Per tenfold increase	1.51 (1.41–1.62)	1.58 (1.46–1.70)	1.76 (1.59–1.95)†	1.71 (1.54–1.89)†	2.06 (1.80–2.36)†
			1.43 (1.26–1.63)‡	1.33 (1.13–1.57)‡	1.64 (1.36–1.97)‡
P for trend	<0.0001	<0.0001	<0.0001†	<0.0001†	<0.0001†
			<0.0001‡	<0.0001‡	<0.0001‡

*Model 1 was adjusted for age; Model 2 is Model 1 additionally adjusted for sex, race, education, and BMI; Model 3 is Model 2 additionally adjusted for smoking history and alcohol consumption status; Model 4 is Model 3 additionally adjusted for serum CRP and HbA1c levels and hypertension diagnosis; and Model 5 is Model 4 additionally adjusted for two clinical periodontitis measurements, mBOP and mCAL.

†Adjusted for alcohol drinker status (non-drinkers vs. drinkers).

‡Adjusted for alcohol drinking status among drinkers (moderate drinking, less than 12 drinks in the past 12 months; excessive drinking, at least 12 drinks in the past 12 months); 41 non-drinkers, including 19 controls and 22 cases, were excluded from the analysis.

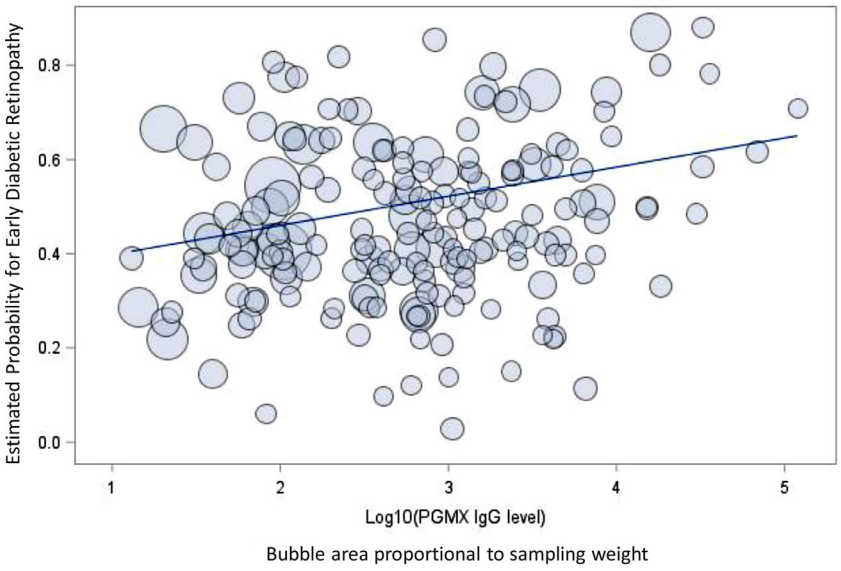

Figure 1. Logistic regression was used to calculate estimated probabilities for early DR at various *Pg* IgG Ab levels. The model was adjusted for age, sex, race, education, BMI, smoking history, alcohol drinking status, serum CRP and HbA1c levels, hypertension, mBOP, and mCAL. Forty-one non-drinkers were excluded from the analysis. The *Pg* IgG Ab levels were $\log_{10}$ transformed. The estimated probabilities for early DR at various *Pg* IgG Ab levels and the fitted linear regression line are shown (regression coefficient $\beta = 0.06192$; *P* for trend = 0.0053).

measurements, mBOP or mCAL, or from both. Further analyses indicated that mBOP was responsible for the majority of the confounding. All five models showed a significant trend ($P < 0.0001$) in the relationship between serum *Pg* IgG Ab levels and early DR risk. The estimated probabilities from Model 5 for *Pg*-related early DR risk at various *Pg* IgG Ab levels and the fitted linear regression line among drinkers are shown in Figure 1 (regression coefficient $\beta = 0.06192$; *P* for trend = 0.0053).

The results from our interaction analysis indicated that *Pg*-related early DR risk significantly varied by alcohol drinking status among drinkers (moderate vs. excessive drinking; interaction $\beta = 0.45$; *P* for interaction = 0.0003), serum HbA1c category (interaction $\beta = 0.45$; *P* for interaction < 0.0001), and mBOP (interaction $\beta = -3.44$; *P* for interaction = 0.0003). Our analysis also indicated that *Pg*-related early DR risk did not significantly vary by alcohol drinker status (non-drinkers

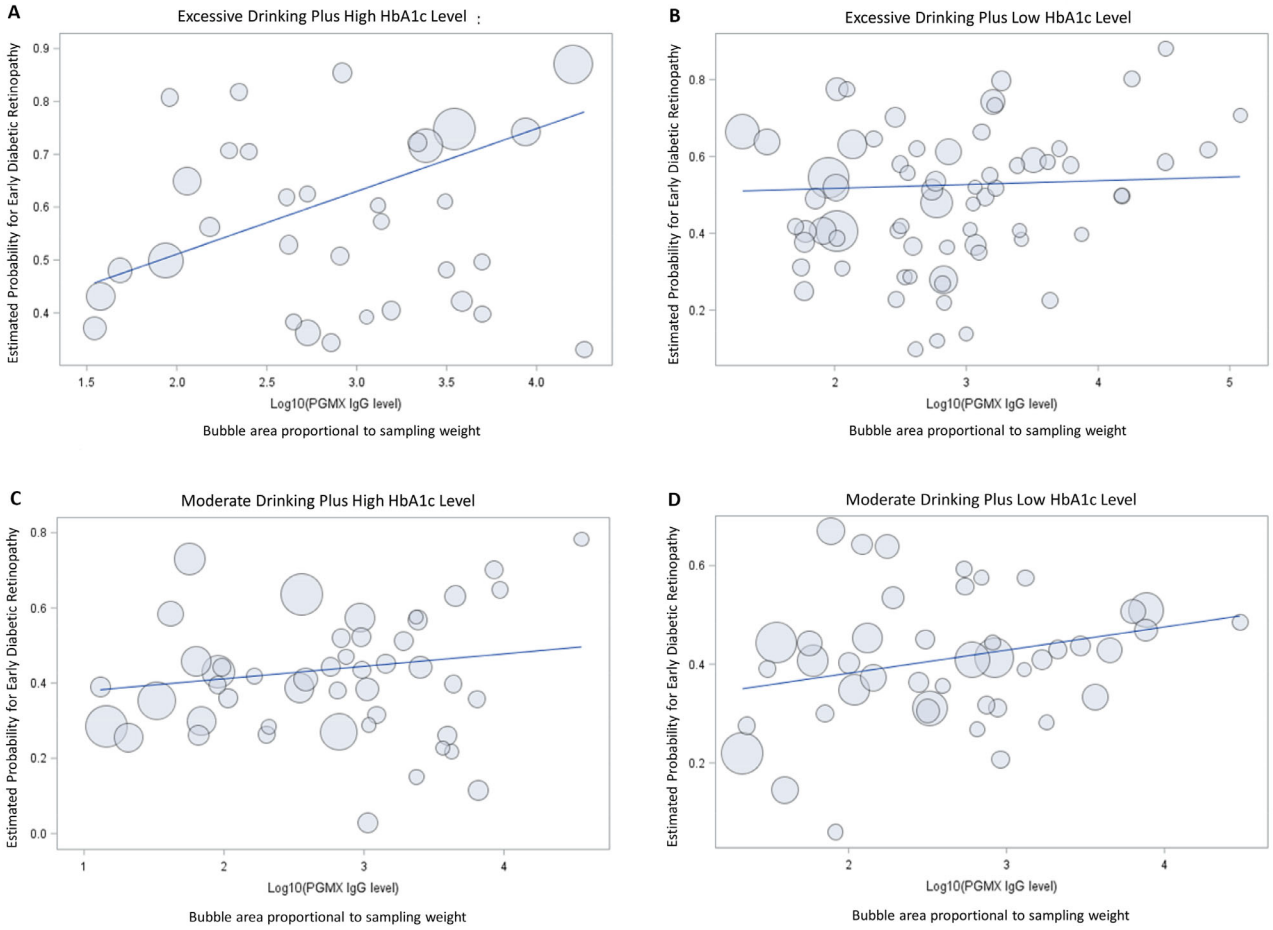

Figure 2. *Pg*-related early DR risk significantly varied by the four combinations of alcohol drinking status (moderate or excessive drinking) and serum HbA1c status (high, HbA1c > 5.7 DCCT%; normal, HbA1c ≤ 5.7 DCCT%) (interaction $\beta = 0.27$; P for interaction < 0.0001). Shown are the estimated probabilities and fitted linear regression lines for (**A**) excessive drinking and high HbA1c ($\beta = 0.11882$; P for trend < 0.0001) vs. (**B**) excessive drinking and normal HbA1c ($\beta = 0.00985$; P for trend < 0.0001) vs. (**C**) moderate drinking and high HbA1c ($\beta = 0.03317$; P for trend < 0.0001) vs. (**D**) moderate drinking and normal HbA1c ($\beta = 0.04695$; P for trend < 0.0001). PGMX, a mixed suspension of *Pg* ATCC strains 33277 and 53978.

vs. drinkers; interaction $\beta = 0.03$; P for interaction = 0.64). Further analysis showed that *Pg*-related early DR risk significantly varied for the four combinations of alcohol drinking status (moderate vs. excessive drinking) and serum HbA1c status (high, HbA1c > 5.7 DCCT% vs. normal, HbA1c ≤ 5.7 DCCT%) (interaction $\beta = 0.27$; P for interaction < 0.0001). We also calculated the estimated probabilities and plotted the four fitted linear regression lines for excessive drinking and high HbA1c ($\beta = 0.11882$; P for trend < 0.0001) (Fig. 2A) vs. excessive drinking and normal HbA1c ($\beta = 0.00985$; P for trend < 0.0001) (Fig. 2B) vs. moderate drinking and high HbA1c ($\beta = 0.03317$; P for trend < 0.0001) (Fig. 2C) vs. moderate drinking and normal HbA1c ($\beta = 0.04695$; P for trend < 0.0001) (Fig. 2D).

Discussion

People with diabetes have increased risk for periodontitis and caries, and oral (periodontal) inflammation negatively impacts glycemic control by contributing to systemic inflammation in both diabetic and non-diabetic people.[2,3,32] Although several studies have related clinical periodontitis to DR,[33–36] little is known about the relationship between oral microbes and eye health, and, importantly, their interaction with the host immune response has not been investigated in the relationship. In this study, we showed that serum *Pg* IgG Ab levels were positively associated with the risk for early DR, adding to the accumulating evidence that, through immune responses, microbes in

the human body may have an impact on tissues and organs remote from their original habitat. Interestingly, we also found that moderate alcohol intake and normal HbA1c levels alleviated this *Pg*-related risk for early DR, suggesting that the *Pg*-related early DR risk could be attenuated by managing multiple modifiable risk factors for DR.

Previous studies have suggested that serum Ab is a better measure than clinical periodontitis for exposure to oral microbes.[37] The findings indicated that all Ab levels were significantly and strongly associated with carriage of the corresponding pathogens, but only weakly with the presence or number of teeth with periodontitis. It was also noted that individual Ab levels and the numbers of corresponding bacteria in saliva showed a positive association, independent of the severity of periodontitis. For many years, the Ab response to oral bacteria, particularly IgG, has been regarded as a reliable surrogate for systemic exposure to the organisms.[37] Interestingly, translocated oral bacteria, including *Pg*, directly impact the gut microbiome and possibly immune defense, resulting in IgG responses to translocated microbial antigens.[38,39] These findings suggest that the origin of serum IgG Ab to oral microbes is not limited to the oral cavity.

The complement classic pathway is activated when complement component C1q binds to the IgG constant region (Fcγ) attached to microbe surface antigens.[40] The pathway leads to targeted lysis of the pathogenic surface through the assembly of membrane-penetrating pores known as the membrane attack complex (MAC). Interestingly, it has been well documented that the generation of undesirable quantities of MAC plays a significant role in the pathogenesis of DR.[41–44] However, although it has been demonstrated that the inflammatory and immune response triggered by *Pg* has not only local but also systemic effects,[13,14,16,20,21,37,45] the remote effects of complement activation induced by *Pg* on the retina remain to be determined. On the other hand, recent studies have indicated that, even in IgA-dominated tissues, such as oral mucosa, commensal–IgG Fcγ may cross-link Fcγ receptors (FcγRs) on mononuclear phagocytes (MNPs), inducing IL-1β production, and that the MNP FcγR active:inhibitory (FcγR:FcγRIIB) ratio determines the magnitude of type 17 immunity and local inflammation.[46] Importantly, type 17 immunity plays an important role in maintaining mucosal barriers and contributing to pathogen clearance at mucosal surfaces, and it is implicated in autoimmune disorders. Furthermore, the loss of T helper 17 cell populations at mucosal surfaces has been linked to local inflammation and microbial translocation, leading to chronic inflammation at remote sites. These findings suggest that the IgG Ab levels are independent of clinical periodontitis and that serum *Pg* IgG Ab indicates a systemic response to this periodontal disease-associated pathogenic bacterium, lending biological support to our approach to study serum IgG Ab levels instead of the microbe per se.

Recent work has conceptualized DR as a disease of the neurovascular unit.[6] In addition to the component vascular cells (endothelial cells and pericytes), diverse retinal neuronal cell types, macroglial elements (Müller cells and astrocytes), and microglia, the neurovascular concept also suggests the importance of additional cell types, such as retinal pigment epithelium (RPE) and immune cells. Importantly, as diabetes progresses, the retina exhibits multiple elements of chronic, subclinical inflammation, including immune cell activation and production of inflammatory molecules, which play a pivotal role in modulating the constituent cells of the neurovascular unit and driving the progression of DR.[6,47,48] This new concept of DR pathogenesis lends further support to our strategy of using immune-related biomarkers in epidemiological studies on DR.

In a recent in vitro study, Arjunan et al.[49] characterized *Pg* invasion in the human RPE cells, including vacuolar/cytosolic localization and prolonged survival by autophagy evasion within the RPE cells. These findings lend further support to the accumulating evidence that *Pg* may access and spread in the neural system.[19] Although infection has been suggested as a potential risk factor for DR and *Pg* has been related to several human metabolic disorders,[50–53] our study provides the first epidemiological evidence showing a significant association between *Pg* infection and risk for early DR.

In our interaction analysis, we found that, although either alcohol drinking status or serum HbA1c status had a synergistic action with serum *Pg* IgG Ab levels (interaction $\beta > 0$), the effect of the combined action between mBOP and serum *Pg* IgG Ab levels is less than the sum of their individual effects (interaction $\beta < 0$). This phenomenon was not surprising, because it is probably due to the overlapping information provided by mBOP and serum *Pg* IgG Ab levels, both of which are related to periodontitis. Our confounding analysis indicating that the association between serum *Pg* IgG Ab levels and early DR risk was confounded by mBOP (Table 3) also provides support to this antagonistic interaction. Notably, although moderate drinking conferred lower *Pg*-related early DR risk than excessive drinking, non-drinkers did not gain additional benefit from not drinking alcohol. Furthermore, compared with either consuming moderate alcohol or maintaining a normal serum HbA1c level alone (Fig. 2B, 2C), our data suggest that their combined effect (Fig. 2D),

probably through eating a low-glycemic-index Mediterranean diet,[54–68] confers the lowest *Pg*-related early DR risk. Indeed, randomized nutritional intervention studies have demonstrated benefit of such a diet, including shifting the *Pg*-driven microbiota by decreasing the relative abundance of periodontopathogenic bacterial in the saliva and increasing levels of *Streptococcus cristatus*, which has been reported to be an antagonistic taxon inhibiting *Pg* gene expression.[69–72] It is also noteworthy that recent studies have found that prenylated flavonoids, which are present in wine and beer,[73,74] suppress gingipains, *Pg* growth, and biofilm formation.[75] Furthermore, studies have suggested that *Pg* and related dysbiosis contribute to the development of insulin resistance and poor glycemic control.[76–78] Novel therapeutics and prevention strategies for DR may be developed from further mechanistic studies on these *Pg*-related mechanisms.

We found that Hispanics had higher serum levels of *Pg* IgG Ab (Table 2). Studies have also indicated that Hispanics have a higher risk of DR and that the increased risk could be attributed to both genetic background and environmental exposures, including diet.[79] Taking these data together, race was a potential confounder in the association of our interest; that is, between serum levels of *Pg* IgG Ab and risk for DR, we therefore adjusted race in our multivariable regression analyses. However, more research is needed to investigate the independent role of genetic background and diet in the association between serum levels of *Pg* IgG Ab and risk for DR.

The strengths of this study include its being a matched case–control study in a representative cohort of the US population, standardized collection of risk factor information, and photographic grading of maculopathy to minimize the influence of confounding factors and misclassifications. The cross-sectional nature of this study limits its strength in defining temporality; however, serum *Pg* IgG Ab is considered to reflect chronic exposure,[18] and the average age and prevalence of *Pg* infection are much younger and higher, respectively, than those for DR.[80–83] Furthermore, this study involved only secondary data analysis of existing U.S. national databases that are publicly available and have been de-identified. The NHANES III performed only *Pg* IgG measurement in the blood without measuring *Pg* IgA. It would be interesting to examine the association with *Pg* IgA Ab in future studies. In light of the importance of complement activation in *Pg* pathogenesis, complement factors, such as C1q, C2b, and C3a, should also be included in future studies. In this study cohort, we focused our interest on early DR because there were only a limited number of advanced DR cases which did not allow for meaningful analysis. Further study should investigate the associations with different categories of DR. We recognized that oral hygiene or socioeconomic factors (access to medical care) could be confounding factors in our analysis and therefore adjusted for education level in our multivariable regression analysis. However, residual confounding could still be an issue and remains to be further investigated.

In conclusion, we demonstrated a novel association between exposure to *Pg* and risk for early DR. Although the causality and detailed mechanism warrant further study, our findings may result in a significant impact on the therapeutic and prevention strategies for DR by virtue of the high prevalence of *Pg* infection in the human population.

Acknowledgments

Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views or policies of the US Centers for Disease Control and Prevention, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government. The funding sources had no role in the design and conduct of the study; the collection, analysis, and interpretation of the data; or the preparation, review, or approval of the manuscript.

Supported by grants from the National Institutes of Health (NEI R21EY028209, C-JC; NIDCR DE025020, TEVD).

Disclosure: **C.-J. Chiu,** None; **M.-L. Chang,** None; **A. Kantarci,** None; **T.E. Van Dyke,** None; **W. Shi,** None

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