Systemic Inflammation, Obesity, and Single Nucleotide Polymorphisms Impact on Gingival Inflammation: A Clinical Pilot Study.

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ABSTRACT

Background

Our primary goal is to investigate the cross-sectional relationship between systemic and gingival inflammation and assess the feasibility of future investigations. We asked whether an association exists between a) inflammatory markers and measures of adiposity, b) C-reactive protein (CRP) levels in serum and gingival crevicular fluid (GCF), and c) selected single nucleotide polymorphisms (SNPs) in CRP genes with GCF CRP.

Methods

We recruited 15 normocentric participants and 15 obese participants of predominantly European descent. We obtained anthropometric and clinical measurements, performed periodontal exams, and collected GCF and blood samples

from the participants. CRP and select cytokine levels in participant GCF were determined using immunoassays. Six SNPs associated with CRP levels were genotyped.

Results

GCF CRP levels differed significantly between obese vs normocentric participants ($P = 0.0096$). GCF CRP levels correlated moderately with BMI ($r = 0.46$; $P = 0.01$) and waist circumference ($r = 0.49$; $P = 0.0062$). Departure from Hardy-Weinberg was noted only for SNP rs4129267 ($P = 0.019$). GCF CRP levels associated with genotype for 2 of the SNPs (rs4129267, $P = 0.034$ and rs4420638, $P = 0.032$).

Conclusions

Investigations regarding potential relationships between

systemic and gingival inflammation using similar methods are warranted in larger cohorts. Correlations were observed between inflammatory markers and measures of adiposity and CRP levels in serum and GCF. Our data also suggests an association between SNPs in CRP genes and CRP levels in GCF warranting further investigation.

There is potential for genetic variation to impact the expression of oral inflammatory markers. Improved understanding of the systemic-oral dynamic will provide insight for the pathogenesis of chronic inflammatory conditions and facilitate development of diagnostic markers and novel treatment strategies.

+ Cytokine(s); Biomarkers; C-Reactive Protein; Genetics; Inflammation.

Introduction

C-reactive protein (CRP) is a nonspecific marker of systemic inflammation.¹⁻³ The half-life of serum CRP is approximately 19 hours, and its elevation is primarily governed by pathologically derived cytokines. As such, CRP expression has been directly correlated with the severity of concomitant pathology.^{1,2} As examples of its systemic influence, elevations in the concentrations of CRP are related to increased risk for myocardial infarction, ischemic stroke, peripheral arterial disease, ischemic heart disease mortality, diabetes, insulin resistance, impaired insulin secretion, dyslipidemia, and accelerated atherosclerosis.^{1,2,4-6} To highlight one example of systemic inflammation, serum CRP expression has been proposed as a predictor for the onset and/or worsening of diabetes. This has led to the hypothesis that CRP may have an etiological role with diabetic states by acting to promote insulin resistance.⁶⁻¹¹

To better understand a local disease such as chronic periodontitis, we must characterize how systemic inflammatory conditions relate to local disease. Chronic periodontitis is a highly prevalent inflammatory disease of bacterial origin with host-mediated destruction of the dental supporting structures. Statistically significant elevations in CRP concentrations have been noted in chronic periodontitis.¹² Systemic CRP expression is positively correlated with increased severity of periodontal disease and has been observed to diminish upon periodontal therapy.¹³

Compelling evidence exists for an association between chronic periodontitis and factors such as obesity, overweight, weight gain, and waist circumference, though most authors indicate that a causal relationship has yet to be definitively investigated.¹⁴⁻¹⁷ Similar to chronic periodontitis, obesity is highly prevalent in the United States and is a serious public health concern.¹⁸ Obesity correlates with low-grade systemic inflammation, attributable to the pro-inflammatory nature of adipose tissue.^{8,19}

One hypothesized association between chronic periodontitis and obesity is that inflammation leads to oxidative stress and endothelial dysfunction.²⁰ Further, increased Body Mass Index (BMI) has been associated with the severity and extent of periodontitis, nearly to the extent that smoking influences periodontitis.^{20,21} Both periodontitis disease activity and measures of adiposity have been shown to be associated with inflammatory markers in the Gingival crevicular fluid (GCF), a unique exudate in the gingival sulcus surrounding teeth. However, results have been mixed, and an understanding of how patterns of inflammatory biomarkers in GCF present in different scenarios remains incomplete.^{16,17,22}

We must also consider that inflammation may be influenced by genetic variation. Genome-wide association studies (GWAS) reported associations between single nucleotide polymorphisms (SNPs) and serum CRP levels in individuals.^{24,25} This suggests there may be genetic/epigenetic underpinnings for CRP levels that should be considered for

inflammatory states. SNPs are variations seen at a single location in the genome that may manifest in particular differences between individuals. However, little is known about the influence that CRP SNPs may have on the expression of this molecule in oral tissues. Interestingly, gingival tissues do not produce CRP messenger RNA (mRNA), suggesting that the presence of CRP in the periodontium likely originates from serum exudate.²⁶ Thus, we propose that analyzing the GCF and its relationship to systemic inflammatory markers will provide insight in the host response mechanism of chronic periodontitis.

This study is a clinical pilot investigating the feasibility of future research regarding possible oral manifestations of systemic inflammation. Our intention is to inform researchers on protocol development, workability, sample size considerations, and preliminary insights on outcomes.

In this study, we investigate 3 aims with the goal of characterizing factors that influence GCF inflammatory biomarkers, including measures of adiposity, serum inflammatory biomarkers, and genetic variation. These aims include (1) concentrations of pro-inflammatory and anti-inflammatory markers in GCF were determined and analyzed for differences between obese and normocentric individuals, (2) serum and GCF CRP concentrations were determined and assessed for differences, and (3) DNA samples were genotyped to evaluate SNPs in CRP genes and variations in phenotype (GCF CRP concentrations).

Methods

Population

A combined sample size of 30 subjects was chosen, with an attempt to balance power considerations and cost constraints given the pilot nature of the study. Two groups of 15 individuals, predominately of European descent, were defined by BMI. Individuals with BMI > 30 defined our obese group, and individuals with a BMI range of 18.5 to 25.55 defined our normocentric group. Study

participants (18 years and older) were recruited using the university mass email system. The following criteria were used to exclude respondents: pregnant or nursing, taking hormone replacement therapy, missing more than 15 teeth, having a history of aggressive periodontitis, or being a current smoker (as smoking may affect CRP levels or predispose to diseases that impact CRP).²⁷ Age and gender balance were sought by enrolling individuals consecutively within each of 5 age categories (18–29, 30–39, 40–49, 50–59, 60+) until each category was filled. In the event of a deficit of subjects in certain age groups, we sought to maintain age balance in the obese and normocentric groups. We obtained informed consent from participants and adhered to the Institutional Review Board (IRB) approved protocol (IRB ID# 201307772).

Clinical Procedures

During the appointment, height (in meters), weight (in kilograms), and waist circumference (in centimeters) were determined for each subject. BMI was calculated using the formula $[\text{Weight} / (\text{Height})^2]$. Casual blood glucose level was measured using a point-of-care device (One Touch Ultra 2 Blood Glucose Meter, USA). Blood pressure was measured in duplicate in each arm.

The oral exam began by assessing plaque index as described by Silness and Löe (from 0 [none] to 3 [an abundance]). Gingival crevicular fluid (GCF) was collected using paper strips (Pro-flow Inc, Amityville, NY) from existing teeth (but not restored implants), as described by Elangovan et al.^{28,29} Briefly, Periopaper® strips were gently placed into the mucosal crevice around 1 tooth in each quadrant in 4 sites (mesiobuccal, distobuccal, mesiolingual, and distolingual) for 30 seconds per site. Teeth 3, 13, 19, and 29 were chosen. If any of these were missing, the next valid tooth anterior to the missing one was selected. To avoid localized confounders, these sites were predetermined to allow a more generalized investigation,

rather than selecting the most severe sites following periodontal examination.

After 30 seconds, the strips were removed and the volume of fluid collected in each strip was measured using a calibrated device (Periotron®, Oraflow). In cases of visible contamination with blood, the strip was discarded, and a new site was sampled. After measuring and confirming the adequateness of the volume, the 4 paper strips from each tooth were pooled in 0.01M sodium phosphate buffer (pH 7.2) using pyrogen-free water containing protease inhibitors (Complete, Mini Protease Inhibitor Cocktail Tablets; Roche Applied Science, Indianapolis, IN, USA), mixed, and centrifuged at 16,100 RCF (13,200 RPM) (Centrifuge 5415D, Brinkmann Instruments, Inc., Westbury, NY) for 5 minutes at 24°C. The strips and supernatants were saved and stored at -80°C for later use.

Following GCF collection, a full-mouth periodontal exam was performed. Probing depths, gingival recession, bleeding on probing, and gingival indices (from 0 [normal gingiva] to 3 [severe inflammation]) were determined using a Hu-Friedy calibrated periodontal probe (Hu-Friedy Corp, Chicago IL) and recorded.

Determination of Pro-Inflammatory Cytokines and CRP

The concentrations of IL-1 β , IL-8, IL-10, TNF- α , and CRP were determined in each GCF sample using multiplexed fluorescent bead-based immunoassays (Millipore, Billerica, MA) in the Luminex 100 IS Instrument (Luminex, Austin, TX) as described by Elangovan et al.^{28,29} 25.0 μ l of supernatant was added to antihuman, multicytokine magnetic beads (MILLIPLEX immunoassay, Millipore, Billerica, MA) and incubated at 4°C for 18.0 hours. Unbound material was removed by aspiration using a magnetic plate washer (ELx405TS, BioTek, Winooski, VT USA), anti-human multi-cytokine biotin reporter was added, and the reactions were incubated at room temperature for 1.5 hours in the dark. Streptavidin phycoerythrin was then

added and the plates were incubated at room temperature for an additional 30 minutes. The plates were washed twice in buffer, suspended in sheath fluid, and then were read (Luminex model 100 IS, Austin, TX). Standard curves for each cytokine were prepared from 3.2 to 10,000 pg/mL and concentrations of chemokines and cytokines in each sample were interpolated from standard curves (xPonent v3.1, Luminex, Austin, TX; MILLIPLEX Analyst v5.1, Millipore, Billerica, MA). Concentrations were reported as nanograms or picograms per 30 seconds, with the 30 seconds representing the length of time for collection of every sample from the sulcus.

Blood was collected for serum and DNA (Clinical Research Unit, University of Iowa Hospitals and Clinics). Serum CRP was assessed with a 0.5 mg/dL threshold for detection (Pathology Laboratory, University of Iowa Hospitals and Clinics).

Genotyping

DNA was processed from blood using the QuickGene-610 L processing DNA whole blood kit L (Kurabo, Osaka, Japan). The complete protocol for processing can be accessed via the Murray Lab (genetics@uiowa.edu). We measured the DNA concentration for all the samples using Qubit that allows for measuring double stranded DNA and a 2 μ g/l was available for genotyping. For each sample, XY genotyping was carried out (1 X chromosome and 1 Y chromosome marker) as part of the quality control before genotyping. We selected 6 single nucleotide polymorphisms (SNPs) with minor allele frequencies ~0.5 reported to be genome-wide significant in a CRP GWAS for genotyping using TaqMan Technology.^{24,25} The TaqMan assay contained 1.5 μ l of TaqMan master mix, which is the polymerase; 0.0375 μ l of TaqMan probe (the marker for each gene investigated); and 1.4625 μ l of double distilled water (ddH₂O), comprising 3 μ l of the master mix. At the completion of the PCR process, the plates were read using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied

Biosystems, Foster City, CA) Fast Real-Time PCR System controlled by the SDS software, version 2.4. All the files with samples and markers were imported into Progeny, version 7.6.04 (Progeny Genetics, LLC, Delray Beach, FL) to check for Mendelian errors and discrepancies in each file. The final genotype data was then exported for analysis. A mechanical error in initial blood processing machinery yielded DNA concentrations that did not allow proper analysis in some isolated samples.

Statistical Analysis

Based on a combined sample size of 30, assuming a two-sided test and Type I error of $\alpha = 0.05$, the pilot had expected power of 80% to detect correlations of .468 or greater in magnitude (+/-). For $n = 15$, that is, within either the obese or normocentric group, the same power was anticipated to detect correlations of .616 or greater (in absolute value). Nonparametric statistical approaches were used to assess differences among the proinflammatory cytokines and CRP values in the GCF. The Spearman rank correlation was used to assess whether there was an increasing or decreasing relationship between 2 variables of interest. The exact Wilcoxon Rank Sum test was used to assess whether the distribution of a biomarker or clinical parameter differed between obese and normocentric subjects. The level of Type I error was set at 0.05.

Nonparametric statistical approaches were also used to assess differences among the SNP values. Because the mode of inheritance was not known, the way genotype translates into effect was not known. Assessment required consideration of 3 possible models corresponding to

dominant, recessive, and additive effects. The exact Kruskal-Wallis test was used to assess whether the distribution of CRP in the GCF differed among the 3 genotypes at each of the 6 candidate SNPs. For significant results ($P < 0.05$), pairwise comparisons among genotypes were made using exact Wilcoxon Rank Sum tests. The Spearman rank correlation was used to assess whether there was an increasing or decreasing relationship between mean CRP level and the number of copies of the minor allele (nonparametric analogue to an “additive” genetic model). Exact Wilcoxon Rank Sum tests were used to assess whether the distribution of CRP differed with subgroups defined by assuming that the minor allele acted in accordance with either a dominant or a recessive mode of inheritance. Exact significance probabilities were obtained in conjunction with Hardy-Weinberg testing (Statistical Genetics Utility program HWE2; Jurg Ott, Rockefeller University, NY, NY: <http://www.jurgott.org/linkage/util.htm>). All other analyses were performed using SAS® software, Version 9.3. (SAS Institute Inc, Cary, NC).

Results

A total of 30 subjects were seen, 15 male subjects and 15 female subjects. Of the normocentric subjects, 7 (46.7%) were female; of the obese subjects, 8 (53.3%) were female. Genotype data were attained from 22 to 24 of the 30 subjects. Characteristics of the study population are shown in Table 1, including descriptive statistics for GCF biomarkers, adiposity indices, clinical measures, and age for the obese and normocentric subgroups.

TABLE 1: Study Population Characteristics.

Variable	Mean	Std Dev	Median	Minimum	Maximum
Normocentric Subjects (N): N = 15					
Obese Subjects (O): N = 15					
Age N	40.07	13.04	36	25	64
O	48.33	13.99	49	28	70
BMI N	22.70	2.27	23.01	18.73	25.55
O	37.55	5.28	37.94	31.01	50.79
Waist N	76.4	9.5	77.0	60.0	90.0
Circumference O	114.2	17.9	110.5	96.5	164.5
CRP * N	0.87	1.16	0.39	0.01	3.68
O	3.70	3.55	3.37	0.07	12.27
IL-8 † N	192.61	139.15	121.90	86.87	587.56
O	228.30	147.91	172.17	54.11	606.95
IL-10 † N	0.61	0.29	0.59	0.07	1.28
O	1.07	0.82	0.85	0.09	3.38
IL-1β † N	3.43	3.41	2.69	0.21	12.89
O	9.15	9.05	5.39	1.46	34.36
TNF-α † N	1.39	0.86	1.25	0.35	3.11
O	2.23	2.44	1.49	0.31	8.11
Plaque Index N	0.31	0.34	0.17	0.00	1.00
O	0.84	0.63	1.00	0.00	2.00
Gingival Index N	0.47	0.50	0.33	0.00	1.50
O	0.92	0.60	1.00	0.00	2.00

* in GCF (nanograms per 30 seconds).

† in GCF (picograms per 30 seconds). BMI–Body Mass Index. CRP C-reactive protein. GCF–Gingival crevicular fluid.

We first considered the inflammatory markers in relation to our measures of adiposity. Distribution of CRP levels in GCF differed significantly between obese vs normocentric subjects ($P = 0.0096$). Median CRP level in obese subjects was 3.37 vs 0.39 (ng/mL) in normocentric subjects (Figure 1). Other differences between the obese and normocentric subjects were noted via the exact Wilcoxon Rank Sum procedure and included the following: IL-1 β ($P = 0.021$; median of 5.39 pg/30sec in obese subjects vs 2.69 in normocentric subjects); plaque index ($P = 0.017$; median

of 1.00 in obese subjects vs 0.17 in normocentric subjects); and gingival index ($P = 0.047$; median of 1.00 in obese subjects vs 0.33 in normocentric subjects) (Table 2). Evidence was found of an increasing relationship between the level of CRP in the GCF with both BMI ($r = 0.46$; $P = 0.01$) and waist circumference ($r = 0.49$, $P = 0.0062$) (Table 3, Figure 2). Furthermore, there was evidence of an increasing relationship between BMI and three other measures: IL-10 ($r = 0.378$; $P = 0.0392$), IL-1 β ($r = 0.427$; $P = 0.0186$), and plaque index ($r = 0.361$; $P = 0.0497$) (Table 3).

TABLE 2: Descriptors for Obese and Normocentric Groups.

OUTCOME	Normocentric	Obese	<i>P</i> -value*
Serum CRP reaching threshold	1	5	---
Median CRP in GCF (ng/mL)	0.39	3.37	$P < 0.001$
Median IL-1 β in GCF (picograms per 30 seconds)	2.69	5.39	$P = 0.02$
Median Plaque Index	0.17	1.00	$P = 0.02$
Median Gingival Index	0.33	1.00	$P = 0.047$

* Significance probability associated with the test of the null hypothesis that the distribution of the outcome of interest is the same in the obese and normocentric groups, based upon the exact Wilcoxon Rank Sum test.

CRP – C-reactive protein. GCF – Gingival crevicular fluid.

TABLE 3: Relationship Between Systemic Factors and Local Inflammation.

GCF CRP concentration relationship with:	<i>r</i> -value*	<i>P</i> -value†
BMI	$r = 0.46$	$P = 0.01$
Waist Circumference	$r = 0.49$	$P < 0.01$
Increasing relationship between BMI and:		
IL-1 β		$P = 0.02$
IL-10		$P = 0.04$
Plaque Index		$P < 0.05$

*Spearman's rank correlation coefficient: The relationship associated with the extent one variable increases with the increase of another.

†Significance probability associated with the test of the null hypothesis that the distribution of the outcome of interest is the same in the obese and normocentric groups, based upon the exact Wilcoxon Rank Sum procedure.

BMI – Body mass index. CRP – C-reactive protein. GCF – Gingival crevicular fluid.

FIGURE 1: Box plot describing the distribution of C-reactive protein (CRP) measured in the gingival crevicular fluid (GCF) in 15 normocentric and 15 obese subjects. Median CRP level in obese subjects was 3.37 vs 0.39 in the normocentric (ng/mL). Distribution of CRP levels in GCF differed significantly between obese vs normocentric subjects ($P < 0.001$).

FIGURE 2: A: Plot depicting the relationship between the level of C-Reactive protein (CRP) measured in the gingival crevicular fluid (GCF) and body mass index in 30 subjects.

FIGURE 2: B: Plot depicting the relationship between the level of CRP measured in the GCF and waist circumference in 30 subjects. Evidence was found of an increasing relationship between the level of CRP in the GCF with both BMI ($r = 0.46$; $P = 0.01$) and waist circumference ($r = 0.49$, $P < 0.001$). These plots demonstrate the relationship between measures of adiposity and oral inflammation.

Of the obese subjects, 5 of 15 presented with serum CRP at or above the 0.5 mg/dL threshold, relative to 1 of 15 normocentric subjects presenting at threshold.

Finally, we evaluated how SNPs were associated with variations in serum CRP levels related to phenotypes of GCF CRP levels. Departure from Hardy-Weinberg proportions were assessed and only noted for SNP rs4129267 ($P = 0.019$). The exact Kruskal-Wallis test was used to assess a significant association between our phenotype (GCF CRP level) and genotype for 2 of the SNPs (rs4129267, $P = 0.034$ and rs4420638, $P = 0.032$), without consideration for confounding factors (Table 4).

Discussion

In this clinical pilot study, we evaluated relationships among pro-inflammatory and anti-inflammatory

markers in GCF, measures of adiposity, and clinical indices; compared the expression of CRP levels in serum with GCF; and assessed the association among CRP in GCF and each of 6 selected SNPs identified as significant to serum CRP levels by GWAS.^{24,25}

One of our key findings was how obesity-related inflammation influences the physiologic processes of the oral cavity. Median CRP levels in GCF in our obese subjects were nearly 10 times greater than in normocentric subjects. These groupings were defined by BMI, though differences in body composition and body fat distribution can limit the usefulness of BMI. As such, waist circumference was also utilized to better assess our subjects' adiposity. An increasing relationship between the level of CRP in the GCF was noted with both BMI and waist circumference, to comparable extents.

TABLE 4: Genotypes of Six Candidate Genes and Testing for Differences in the Distribution of Mean GCF CRP.

SNP	Major/ Minor Alleles (A/B)	Number of Subjects	Genotype Counts			Minor Allele Frequency (Standard Error)	H-W TEST Exact <i>P</i> -value*	<i>P</i> -Value for Kruskal- Wallis Test†
			# of AA	# of AB	# of BB			
rs2794520	C/T	23	10	11	2	0.326 (0.069)	1.00	0.76
rs4129267	C/T	23	13	5	5	0.326 (0.069)	0.02	0.03
rs4420065	C/T	23	10	11	2	0.326 (0.069)	1.00	0.87
rs1260326	C/T	24	12	10	2	0.292 (0.066)	1.00	0.22
rs1183910	C/T	22	12	8	2	0.273 (0.067)	0.62	0.59
rs4420638	A/G	24	13	10	1	0.250 (0.063)	1.00	0.03

*Significance probability associated with the exact test assessing conformance to Hardy-Weinberg proportions.

†Significance probability associated with the exact Kruskal-Wallis test of the null hypothesis that the distribution of mean CRP in the GCF is the same in the 3 groups defined by SNP genotype.

CRP – C-reactive Protein. GCF – Gingival crevicular fluid. SNP – Single nucleotide Polymorphism.

Other noted differences between the obese and normocentric subjects included IL-1 β GCF levels being approximately double the concentration in obese subjects relative to normocentric subjects. An increasing relationship between BMI and IL-10, IL-1 β , and plaque index was also noted. It is interesting to highlight here how pro-inflammatory IL-1 β and anti-inflammatory IL-10 were both increased with BMI, but our other markers were not. Elevation of both pro- and anti-inflammatory cytokines is consistent with the findings of Schmidt et al, which noted a general increase in cytokines in obesity (Brogden). Additionally, plaque index was noted to be 6 times greater in obese subjects, and gingival index was noted to be 3 times greater in obese subjects. It should be considered that local factors such as plaque and gingival indices may contribute to the inflammatory markers assessed

in the GCF and may also contribute to systemic inflammation.

Chronic inflammation in the periodontium reflects the response to plaque containing a polymicrobial biofilm. Periodontal disease can be thought of as an imbalance between bacterial insult and host response in a susceptible host, with degradation of the dental supporting structures mediated by the immune response.³⁰ Intimate knowledge of these processes would facilitate understanding and development of additional novel strategies, such as targeted gene therapy for antagonists that could limit progression of inflammatory infiltrate and recruitment of osteoclasts. Novel strategies can also repurpose old methods to counteract pathogenic processes. For example, Cicek Ari et al note that statin use decreased IL-1 β and MPO while increasing IL-10

in GCF, raising the question of whether statins may attenuate periodontal disease to some degree.³¹

IL-1 β , IL-8, IL-10, TNF- α , and CRP were chosen in this study to provide insight on pro-inflammatory and anti-inflammatory processes of the host response. A larger array of inflammatory markers is recommended with a larger cohort to include such markers as IL-6, which was intentionally not included given its close relationship with CRP and our desire to gather a diverse initial assessment. Determination of these markers longitudinally with incidence and progression of periodontal disease would allow etiological factors to be more thoroughly investigated.

Intersite variability was noted in some early assessment of our data by tooth number, which we subsequently assessed by patient. This may be relevant in cases of acute periodontitis, where particular sites in the mouth are affected. However, in terms of study design, it was felt that pooling the sites allowed for cost efficiency while still providing an appropriate resolution of data. Blood glucose, blood pressure, and periodontal examination measurements were not included in our statistical analysis. Their use in our study was to aid in identifying pathology that may necessitate exclusion from the study population. These measures did not identify any subjects with previously-undiagnosed diabetes, severe hypertension, or aggressive periodontitis.

Our assessment of a correlation between CRP serum and GCF levels was limited by the use of a clinically relevant threshold. Consequently, much of the data set registered as below threshold, precluding any quantitative analysis of the relationship. Though inconclusive, the data is encouraging for future investigation, with 5 of 15 obese subjects presenting with serum CRP at or above the 0.5 mg/dL threshold compared to only 1 at the threshold from the normocentric group. Correlation between CRP levels in the GCF and serum warrants further investigation. Future investigations ought to have a more sensitive

serum CRP laboratory test to allow analysis of quantitative serum CRP measures. The question we hope to address is whether GCF concentration accurately reflects serum concentration (a straightforward filtration constant) or whether inflammation, gingival biotype, and other host factors alter the presence of CRP in the GCF predictably.

CRP is present in the GCF as serum exudate, so the nature of its dispersal into the GCF may provide a basis for measurement of GCF CRP as an accurate reflection of serum CRP concentration. Although our GCF and serum correlation was unable to be quantitatively evaluated, our findings are consistent with the current body of literature. Pradeep et al conducted a similar study with 4 groups of 10 subjects ranging from healthy periodontium and nonobese to chronic periodontitis and obese. They found a significant difference between serum and GCF levels of hs-CRP between the 4 groups.³² Further assessment of the contents of GCF and the way they reflect systemic considerations may provide novel non-invasive assessment tools. Additionally, it has been noted previously that GCF may be a reliable medium for screening disease activity in periodontal disease.^{23,33}

To the best of our knowledge, this is the first study to investigate SNPs in CRP genes and their association with CRP levels in the GCF. Our pilot results indicate there is potential for genetic variation to impact the expression of oral inflammatory markers, but a larger sample size is required. The range of 22–24 results reported for a given SNP is reflective of borderline concentrations in a couple of samples yielding PCR results for some SNPs but not others. The issue with processing was purely mechanical, and we assess that the methods described are feasible to apply to a large cohort. The selected SNPs were genome-wide significant with minor allele frequency (MAF) of ~ 0.5 . This MAF will help maximize the presence of variants observed in our study. Larger cohorts should investigate a

comprehensive selection of SNPs associated with altered serum CRP levels. To assess possible epigenetic regulation, it would be worthwhile to investigate whether levels change over time, within the same individual, as a function of environmental pressures. Genetic variations and their impact on chronic inflammatory conditions are important topics as precision medicine techniques advance.

Caution regarding multiple testing applies here, and none of these results would be significant after any reasonable adjustment for multiple testing. The impact of sex, age, and ethnicity on the amount and distribution of body fat and the possible effects on pro-inflammatory cytokine levels should be considered. Future investigations ought to weigh whether to have a range of ages or a select age group to minimize such differences. We must also consider how our groups are divided and be aware of obesity/normocentric group associations with confounders such as diet, activity levels, dental/medical access, socioeconomic status, and oral hygiene. Additional processes may impact results and should be controlled through exclusion. Recent infections or wounds, antibiotic use, diabetes, autoimmune conditions, and connective tissue disorders are among those that should be considered when designing future studies.

Conclusion

Understanding the dynamics of inflammatory markers in the oral cavity will provide insight for better understanding of the pathogenesis of chronic inflammatory conditions and facilitate development of diagnostic markers and novel treatment strategies for such conditions. This pilot study provides the foundation for future investigations by characterizing factors that influence GCF inflammatory biomarkers, including measures of adiposity, serum inflammatory biomarkers, and genetic variation. Our methods are feasible on a larger scale and our initial results are promising.

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Conflict of Interest: There are no conflicts of interest to report.

Informed consent: Informed consent was obtained and we adhered to the IRB approved protocol (IRB ID# 201307772) in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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