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Pro-inflammatory biomarkers during experimental gingivitis in patients with type 1 diabetes mellitus: a proof-of-concept study

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Abstract

Aim: To compare gingival crevicular fluid (GCF) biomarker levels and microbial distribution in plaque biofilm (SP) samples for subjects with type 1 diabetes (T1DM) *versus* healthy subjects without diabetes during experimental gingivitis (EG).

Materials and Methods: A total of nine T1DM patients and nine healthy controls of age and gender similar to the T1DM patients were monitored for 35 days during EG. Hygiene practices were stopped for 3 weeks, and GCF, SP, plaque index (PI) and gingival index were determined. IL-1β, IL-8, MMP-8 and MMP-9 were quantified by enzyme-linked immunosorbent assay, and SP samples were assessed by DNA–DNA hybridization for a panel of 40 subgingival microbial species.

Results: IL-1β levels in T1DM patients were elevated compared with healthy individuals, and showed differences between groups at 7–21 days while healthy patients showed IL-1β increases from baseline to 14–21 days ($p<0.05$). Differences were observed in MMP-9 levels between patients with and without T1DM at 7–14 days ($p<0.05$). Orange complex species and PI measurements displayed a superior correlation with biomarker levels when compared with other complexes or clinical measurements during EG.

Conclusions: The mean GCF biomarker levels for IL-1β and MMP-8 were most significantly elevated in T1DM subjects compared with healthy individuals during EG, not resulting from differences in the mean PI or microbial composition.

Key words: biomarker; diabetes; gingival crevicular fluid; gingivitis; IL-1β; MMP

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Gingivitis can be described as gingival inflammation maintaining a stable level of alveolar bone, and junctional epithelial attachment (Champagne et al. 2003, American Academy of Periodontology 2005). The reversible process of gingivitis can be resolved with plaque removal from the tooth surface, although individual host responses are influenced by environmental and host-related factors. Specific patient cohorts, such as those with type 1 diabetes (T1DM), are at a higher risk of developing gingivitis, and have a higher prevalence of gingivitis when compared with the overall population of children and adolescents (Ryan et al. 2003). Characterization of chronic gingivitis is traditionally based on the presence of bleeding, oedema, redness, and an increased flow of gingival crevicular fluid (GCF). Although gingivitis occurs over time as a steady-state inflammatory response, gingivitis can be induced in otherwise healthy patients by stopping oral hygiene practices (Löe et al. 1965, Deinzer et al. 2007).

The experimental gingivitis (EG) method was developed to induce gingivitis in healthy patients. In these classical studies, a microbial shift from a Gram-positive- to a Gram-negative-dominated

Conflict of interest and source of funding statement

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species population was observed after 4–7 days before the clinical demonstration of gingivitis. Individual variability in the time required to develop clinical signs of gingivitis during EG trials has also been recognized, although patients had relatively equal levels of oral health at study commencement, and trials have been shown to yield reproducible results with the same study population (Löe et al. 1965, Trombelli et al. 2006, 2008). More recently, EG studies have attempted to identify signature cytokine markers in the early stages of inflammation in both experimental and natural gingivitis models (Trombelli et al. 2006, Deinzer et al. 2007).

Gingival crevicular fluid exists in the gingival sulcus as a serum transudate, and as an inflammatory exudate in the case of tissue inflammation. Because the non-invasive nature of GCF collection techniques, there is growing interest for its use in monitoring oral inflammation, potentially aiding in early recognition of periodontal disease (Christodoulides et al. 2007, Lamster & Ahlo 2007, Rai et al. 2008). The inflammatory host response to an oral bacterial challenge is a critical determinant in patients' health or disease outcome. Patients exhibiting similar levels of plaque accumulation may respond very differently to the plaque biofilm based on their individual susceptibility to inflammation or disease, leading to a distinctive high or low response (Trombelli et al. 2006). The development of techniques to quantify a patient's risk for disease development would be a critical advancement in determining individual susceptibility with the possibility of early disease detection and intervention. A greater understanding of a patient's systemic health could lead to more individualized treatment strategies for inflammatory oral disease, with the opportunity to rely on the patient's current health profile rather than a historical confirmation of disease.

Systemic inflammatory diseases such as diabetes alter the host environment, and are predicted to increase the patient's vulnerability to gingivitis due to changes in the inflammatory response to microbial challenges (Lalla et al. 2000, Noack et al. 2000, Mealey & Rose 2008, Salvi et al. 2008). T1DM is a chronic autoimmune disease in which the immune system selectively destroys the insulin-producing β-cells of the pancreas, resulting in hyperglycaemia due to the lack of insulin secretion (Skyler 2007). Individuals with diabetes mellitus have impaired neutrophil and macrophage functioning, and altered collagen production in addition to exaggerated collagenase activity (Lalla et al. 2000, Noack et al. 2000, Mealey & Rose 2008), perhaps leading to the patient's heightened inflammatory state, as interactions with advanced glycation end-products (AGEs) have been shown to increase macrophage secretion of pro-inflammatory mediators (Mealey & Rose 2008). It has been shown recently that some T1DM patients may have gene polymorphisms accentuating the host response, such as the IL-6 -174 genotype (Raunio et al. 2009), and that it may also predict periodontal disease susceptibility. Research has also shown that the consequences of hyperglycaemia over time, such as hyperlipidaemia and vascular damage, may not only result in greater complications associated with diabetes but may also increase the patient's risk for development of severe periodontal disease (Noack et al. 2000). It was recently discovered that patients with T1DM may be more likely to have a decreased level of osteocalcin, a marker for bone formation, signifying a decreased ability to form bone in T1DM patients with or without periodontitis (Lappin et al. 2009). Coordination of dental and metabolic care could improve the overall health of the patient, directing combinatorial supervision of patients with diabetes at risk for severe periodontitis, and perhaps additional systemic complications.

Inflammation of tissues is associated with modified cytokine and inflammatory mediator expression patterns, such as those seen with matrix metalloproteinases (MMPs). MMPs are proteolytic enzymes involved in normal extracellular matrix remodelling, but have also been implicated in impaired wound healing, tumour progression, and destructive conditions such as Crohn's disease and atherosclerosis (Shiau et al. 2006, Giannobile 2008). MMPs can be considered host-modulatory agents, as they are involved in altering and activating proteins and specific chemokines (Giannobile 2008). Studies have shown significantly increased levels of GCF IL-1β, and activation of plasma MMP-9 and MMP-2 in patients with diabetes as compared with healthy patients, and also in patients diagnosed with periodontal disease (Champagne et al. 2003, Shiau et al. 2006, Christodoulides et al. 2007, Mealey & Rose 2008). It has been proposed that shifts in cytokine or inhibitor equilibrium may give rise to additional therapeutic strategies to reduce inflammation (Yucel et al. 2008).

Although there have been a number of reports (Zhang et al. 2002, Lalla et al. 2006, Soder et al. 2006, Christodoulides et al. 2007, Bildt et al. 2008, Rai et al. 2008, Schierano et al. 2008, Yucel et al. 2008) on biomarkers and oral inflammation, few studies have looked at relationships between gingivitis, microbiota, and oral biomarker levels in patients with T1DM. Therefore, the aim of this study was to investigate the presence of GCF biomarkers and subgingival plaque microbial distribution in patients with and without T1DM during EG as a secondary analysis of prospectively collected data from the EG and T1DM study (Salvi et al. 2005).

Materials and Methods

This was a secondary analysis of a prospective cohort study of EG and T1DM (Salvi et al. 2005).

Study population

The study protocol was approved by the Ethical Committee of the Canton of Berne, Switzerland before the start of the study. A total of 18 Caucasian subjects (nine patients with T1DM and nine without diabetes) were included in the present study, as presented in supporting information, Fig. S1 (Salvi et al. 2005). Study subjects with diabetes were recruited from the Department of Endocrinology and Diabetology of the University Hospital of the University of Bern. Nine patients without Diabetes of age and gender similar to the diabetes patients were recruited from the Department of Periodontology and Fixed Prosthodontics of the University of Berne. All subjects were recruited and age-matched by a single examiner in February to October of 2002. Enrolled patients with a confirmed diagnosis of T1DM had undergone insulin therapy $\geqslant 12$ months, had a mean glycosylated haemoglobin level $\leqslant 9.5\%$ in the previous 12 months, had a minimum dentition of 24 teeth, a probing depth of $\leqslant 4$ mm at six sites/tooth, and no diabetes-related systemic conditions or other significant medical conditions. Enrolled patients with diabetes had an average age of 25.6 ± 5.8 years, ranging from 16 to 35, with a mean glycosylated haemoglobin level of $8.1 \pm 0.7\%$ and an average duration of diabetes of 9.0 ± 5.3 years. The healthy patients without diabetes enrolled in the study had an average age

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of 24.8 ± 5.7 years, ranging from 15 to 36, with a mean glycosylated haemoglobin level of 5.7 ± 0.2%.

Clinical procedures

Before the experimental phase of the study, patients underwent prophylaxis, including scaling and polishing beginning in January of 2003. Patients were directed to carry out oral hygiene practices for 3–4 weeks following prophylaxis, during which gingival health was assessed by plaque index (PI) and gingival index (GI) scores. To begin the experimental phase, patients were instructed to refrain from all oral hygiene practices for 21 days, resuming oral hygiene practices following the 21-day exam, continuing for an additional 2 weeks until Day 35, as depicted in Fig. 1. Clinical assessments and patient sampling were performed by an additional examiner, masked to each subject's medical status. All patient visits were completed by July 2003.

GCF collection and isolation

GCF collection sites were selected at the baseline visit from the mesiobuccal sites of teeth from all four quadrants. Four samples were collected from each quadrant, per time point, per patient, totalling 1440 GCF samples for all patients. Before sampling, patients underwent supragingival plaque removal and isolation of the collection area. GCF was collected using methylcellulose paper strips for 30 s. Replicates of each quadrant were separated in an aluminium foil, placed in sterile Eppendorf vials, and stored at −80°C until elution. All patient samples were coded by a randomly assigned number and letter sequence at the time of collection by the clinical centre, before procurement by the laboratory centre. All study personnel analysing patient samples and statisticians evaluating patient results were blinded to patients' medical status for the duration of the study.

The absorbed fluid was eluted from each strip with five wash–centrifugation cycles using a buffer containing 200 mM PMSF in methanol, 1 mg/ml Aprotinin, 30% human serum albumin, and 1 × phosphate-buffered saline, pH 7.4. GCF fluids from multiple strips were pooled for per quadrant analysis, totalling approximately 100 μl per strip and 400 μl per quadrant. GCF fluid elution samples were stored at −80° until analysis by enzyme-linked immunosorbent assay (ELISA).

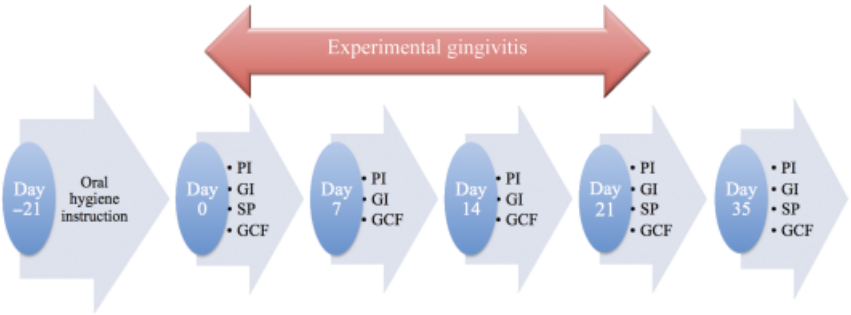

Fig. 1. Experimental gingivitis study timeline.

Biomarker analysis by ELISA

Biomarker selection was based on human cytokine microarray colorimetric assay results (Allied Biotech Inc., Ijamsville, MD, USA), and previously studied inflammatory proteins. The pilot microarray analysis of 14 human cytokines included patient samples from both groups over the complete time course of the study. Biomarkers having protein levels >0 were then selected for further analysis with the entire patient population.

IL-1β, IL-8, MMP-8, and MMP-9 levels were determined by ELISA assays (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. GCF samples were assayed at dilutions (1:5) for IL-8 and (1:10) for IL-1β, MMP-8, and MMP-9, and evaluated in duplicate. The commercial kits detected total (active and pro-) MMP-8 and MMP-9, and active IL-1β. Biomarker quantification was performed using a Multiskan Ascent plate reader. Results for IL-1β and IL-8 levels are reported as pg/ml, and as ng/ml for MMP-8 and MMP-9.

Subgingival bacterial isolation and analysis

The identified test areas were isolated with cotton rolls following the removal of supragingival plaque. Subgingival bacterial samples were collected with Gracey curettes from four mesiobuccal sites as determined at the baseline visit. Samples were collected at baseline, Days 21 and 35. Patient samples were combined for each time point. The subgingival plaque samples were pooled with 600 μl Tris-EDTA (10 mM tris-HCl, 1 mM EDTA, pH 7.6), adding 400 μl 0.5 NaOH after dilution.

Forty subgingival species were counted for each pooled sample, using the checkerboard DNA–DNA hybridization technique (Socransky et al. 1994, 2004, Salvi et al. 2005). Standards were used at concentrations of 10^5 and 10^6 cells of each species. Signal detection was carried out using the Storm Fluorimager 840 (Amersham Biosciences, Piscataway, NJ, USA). In order to obtain a full, detailed account of the identified bacteria, the digitized information was analysed by a software program (ImageQuant, Amersham Biosciences) allowing comparison of signals against standard lanes of known bacterial amounts (10^4 and 10^5 cells) in the appropriate checkerboard slot. Signals were converted to absolute counts by comparisons with these standards, and studied as the proportion of sites defined as having $\geq 1.0 \times 10^4$ and $\geqslant 1.0 \times 10^5$ bacterial cells. Cross-reactivity was routinely tested in the microbiology laboratory between known pure bacterial reference bacterial strains purchased, or obtained from other laboratories. Quality control results were consistent with those reported in previously published research findings (Socransky et al. 2004).

Statistical analysis

Data were analysed using GraphPad Prism™ and R (R Foundation for Statistical Computing, Vienna, Austria). Biomarker data are presented as mean values ± standard error (SE). Two-sample *t*-tests were used to assess between-group differences in the mean biomarker protein levels at each time point. Paired *t*-tests were used to assess the mean within-group changes from baseline to each future time point. For each of the 18 patients, we computed the area under the curve (AUC) of the baseline to Day 21 biomarker protein levels for each of the four biomarkers; we used these AUC

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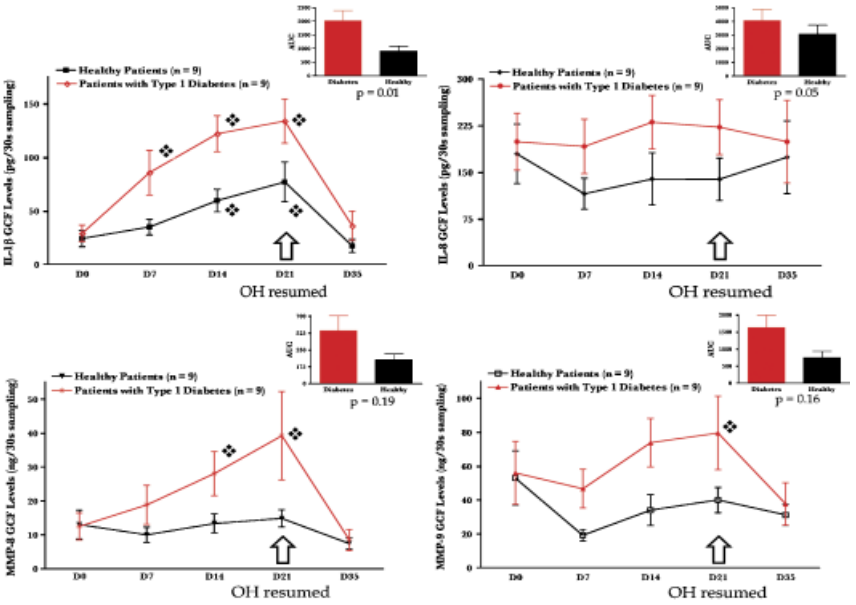

Fig. 2. Longitudinal biomarker profiles (± SEM) during experimental gingivitis utilizing enzyme-linked immunosorbent assay quantification methods. Data points in red indicate patients with diabetes; data points in black indicate healthy patients without diabetes. The area under the curve analysis of the two patient groups for each biomarker is inset into the corresponding panel. Arrows indicate oral hygiene (OH) reinstatement. (a) IL-1β gingival crevicular fluid (GCF) levels. (b) IL-8 GCF levels. (c) MMP-8 GCF levels. (d) MMP-9 GCF levels. *Significant difference within patient group from baseline to the later time point ($p<0.05$).

values as a summary of the overall biomarker levels in each patient. The median AUC levels are displayed in Fig. 2, and between-group differences in the median AUC were assessed using a Wilcoxon rank-sum test. The Wilcoxon rank-sum test was used to establish between-group differences in the median total DNA probe counts and the relative proportions of complex species. Pearson's correlation coefficients were used to summarize the association between biomarker levels and complex species proportions or clinical measurements at baseline and Day 21, demonstrated in Tables 1–3.

Results

Clinical and microbial findings

Thorough presentations of the clinical and microbiological findings have been described previously (Salvi et al., 2005). All patients included in the study remained compliant throughout the protocol, and all 18 patients were therefore included in the data analysis as having measurable outcomes. No adverse events were reported at any point during the study.

No significant differences in the PI scores between the two patient groups were observed over the course of the study; PI scores significantly increased over the 21-day period, followed by a decrease from Days 21 to 35 for both patient groups.

Patients with diabetes mellitus had more sites with GI scores $\geqslant 2$ on Days 7 and 21. Similar to the PI results, the GI scores significantly increased over the 21-day period, followed by a decrease from Days 21 to 35 for both patient groups.

Biomarker findings

As shown in Fig. 2, the mean levels of biomarkers IL-1β, MMP-8, and MMP-9 were the highest at Day 21 for both healthy subjects and those with diabetes. The mean IL-1β levels were significantly elevated above baseline in patients with diabetes at Days 7, 14, and 21 ($p<0.05$), and in healthy patients at Days 14 and 21 ($p<0.05$). Patients with diabetes also displayed significant differences in the mean MMP-8 protein levels between baseline and Days 14 and 21 and MMP-9 protein levels between baseline and Day 21 ($p<0.05$).

Figure 2 also displays evidence that the mean GCF biomarker protein levels were higher during EG in patients with Diabetes than healthy individuals. Significant differences were observed between the two patient groups for the mean IL-1β protein levels at Days 7, 14, and 21 ($p<0.05$), and the mean MMP-9 levels at Days 7 and 14. Between-group differences in the mean MMP-8 levels were seen at Days 14 and 21, although the differences were not statistically significant ($p = 0.065$ and 0.086, respectively). The inset barplots in Fig. 2 display the mean areas AUCs of each biomarker for patients with diabetes and healthy individuals. Although all four biomarkers had a higher mean AUC in diabetes patients than healthy individuals, only the difference in IL-1β was

Table 1. GCF-derived IL-1β, IL-8, MMP-8, and MMP-9 levels correlated with microbiota and clinical measurements during experimental gingivitis in healthy patients

	Day 0	Day 21	AUC
Red complex			
IL-1β	0.14	0.43	0.27
IL-8	0.20	0.24	0.02
MMP-8	0.15	0.01	−0.06
MMP-9	0.22	−0.15	−0.27
Orange complex			
IL-1β	0.20	0.41	0.29*
IL-8	0.44	0.05	0.09
MMP-8	0.47	0.34	0.03
MMP-9	0.33	0.19	0.42
Blue complex			
IL-1β	−0.02	0.10	−0.27
IL-8	−0.03	0.24	0.07
MMP-8	−0.10	−0.11	−0.31
MMP-9	−0.12	0.40	−0.16
Purple complex			
IL-1β	0.00	−0.13	−0.23
IL-8	0.01	−0.15	−0.15
MMP-8	-0.06	−0.62	−0.62
MMP-9	0.01	0.13	−0.38
Green complex			
IL-1β	0.07	0.47	0.29
IL-8	0.45	0.22	0.05
MMP-8	0.53	−0.10	0.06
MMP-9	0.33	0.43	0.46
Yellow complex			
IL-1β	−0.43	0.20	−0.29
IL-8	−0.23	0.35	0.34
MMP-8	−0.35	−0.06	−0.28
MMP-9	−0.39	0.54	−0.04
Plaque index			
IL-1β	0.51	0.22	0.07
IL-8	0.54	0.13	0.13
MMP-8	0.52	0.29	0.15
MMP-9	0.76**	0.32	0.49
Gingival index			
IL-1β	−0.45	0.20	−0.17
IL-8	−0.12	0.60*	0.55
MMP-8	0.01	0.43	0.12
MMP-9	−0.12	0.43	0.44

AUC, area under the curve; GCF, gingival crevicular fluid.
*$0.05<p<0.10$, **$p<0.05$.

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Table 2. GCF-derived IL-1β, IL-8, MMP-8, and MMP-9 levels correlated with microbiota and clinical measurements during experimental gingivitis in patients with diabetes

	Day 0	Day 21	AUC
Red complex			
IL-1β	0.13	0.20	0.22
IL-8	0.28	0.01	0.16
MMP-8	0.15	0.22	0.41
MMP-9	0.29	− 0.06	0.11
Orange complex			
IL-1β	0.57	0.08	0.10
IL-8	0.76**	0.12	0.13
MMP-8	0.56	0.08	0.34
MMP-9	0.62*	− 0.22	0.05
Blue complex			
IL-1β	0.26	0.40	0.08
IL-8	0.46	− 0.32	− 0.27
MMP-8	0.37	− 0.29	− 0.35
MMP-9	0.69*	− 0.30	0.32
Purple complex			
IL-1β	0.04	− 0.01	− 0.24
IL-8	0.15	− 0.09	− 0.22
MMP-8	0.13	− 0.17	− 0.06
MMP-9	0.34	0.09	0.28
Green complex			
IL-1β	− 0.25	− 0.15	0.09
IL-8	− 0.06	− 0.30	− 0.18
MMP-8	− 0.18	− 0.18	0.14
MMP-9	− 0.12	− 0.42	− 0.31
Yellow complex			
IL-1β	− 0.07	− 0.15	− 0.40
IL-8	0.01	− 0.19	− 0.26
MMP-8	− 0.03	− 0.25	− 0.23
MMP-9	0.18	− 0.04	0.26
Plaque index			
IL-1β	0.18	0.86**	0.03
IL-8	0.60*	0.37	− 0.12
MMP-8	0.42	0.41	0.09
MMP-9	0.77**	0.07	− 0.18
Gingival index			
IL-1β	0.02	0.44	0.17
IL-8	0.39	0.26	0.01
MMP-8	0.21	0.25	− 0.14
MMP-9	0.58*	0.21	0.29

AUC, area under the curve; GCF, gingival crevicular fluid.
*$0.05 < p < 0.10$, **$p < 0.05$.

Table 3. GCF-derived IL-1β, IL-8, MMP-8, and MMP-9 levels correlated with microbiota and clinical measurements during experimental gingivitis for all patients

	Day 0	Day 21	AUC
Red complex			
IL-1β	0.13	0.33	0.21
IL-8	0.23	0.13	0.12
MMP-8	0.15	0.22	0.26
MMP-9	0.25	0.01	0.03
Orange complex			
IL-1β	0.29	0.12	0.10
IL-8	0.51**	0.02	0.10
MMP-8	0.47	0.01	0.22
MMP-9	0.41*	− 0.17	0.12
Blue complex			
IL-1β	0.07	0.33	0.13
IL-8	0.24	0.05	0.04
MMP-8	0.14	− 0.03	− 0.06
MMP-9	0.35*	0.08	0.24
Purple complex			
IL-1β	0.00	− 0.09	− 0.23
IL-8	0.06	− 0.12	− 0.21
MMP-8	0.02	− 0.18	− 0.20
MMP-9	0.16	0.04	− 0.01
Green complex			
IL-1β	− 0.02	0.05	0.16
IL-8	0.21	− 0.15	− 0.06
MMP-8	0.19	− 0.15	0.15
MMP-9	0.11	− 0.25	− 0.07
Yellow complex			
IL-1β	− 0.16	0.24	0.01
IL-8	− 0.06	0.20	0.15
MMP-8	− 0.15	0.05	0.05
MMP-9	0.00	0.29	0.34
Plaque index			
IL-1β	0.32	0.31**	0.12
IL-8	0.56*	0.17	0.08
MMP-8	0.43	0.18	0.14
MMP-9	0.74**	0.16	0.16
Gingival index			
IL-1β	− 0.01	0.51	0.36
IL-8	0.24	0.48	0.36
MMP-8	0.12	0.42	0.18
MMP-9	0.37	0.41	0.48**

AUC, area under the curve; GCF, gingival crevicular fluid.
*$0.05 < p < 0.10$, **$p < 0.05$.

statistically significant ($p = 0.01$). Our data showed that in healthy patients, the greatest correlations were observed between orange complex species and biomarker IL-1β, IL-8, and MMP-8 levels, although a trend towards significance was observed between the AUC for the longitudinal curve of IL-1β and orange complex bacteria in Table 1. Orange complex species also correlated at Day 0 with all biomarkers in patients with diabetes, and significance was observed with correlation to the MMP-9 level as shown in Table 2. Interestingly, patients with diabetes displayed a correlation between MMP-9 levels and the blue complex at Day 0, and correlations were also observed between the blue complex and IL-8 at Day 0 and IL-1β levels at Day 21. When both patient datasets were combined, as presented in Table 3, the orange complex again correlated with biomarkers IL-8, MMP-8, and MMP-9 at Day 0, and a significant correlation was found with IL-8, and with MMP-9 levels. Similar to the results with patients with Diabetes, a correlation was found between MMP-9 levels and the blue complex at Day 0, and significance was observed between biomarkers and PI measurements. Similar to healthy patients, the combined data set yielded correlations between the GI measurements and biomarkers IL-8, MMP-8, and MMP-9 at Day 21, although resulting in no statistical significance. GI measurements correlated with IL-8, MMP-8, and MMP-9 levels at Day 21, although patients with diabetes differed in that only IL-1β correlated with GI measurements at Day 21, and a trend towards significance was only observed with MMP-9 at Day 0. PI measurements of healthy patients did show a correlation with all biomarkers at Day 0, while significance was seen with MMP-9 in both patient groups. PI measurements of patients with diabetes did correlate with IL-8, MMP-8, and MMP-9 at Day 0, and with IL-1β and MMP-9 at Day 21; however, significance was only observed with MMP-9 at Day 0 and IL-1β at Day 21.

Discussion

The EG model has given way to critical research findings since its validation, and it is the aim of the present study to utilize this method to aid in the development of a potential biomarker profile for gingivitis, and subsequently periodontitis. This may be enlisted as a clinical patient-monitoring strategy and early intervention tool, most importantly for those patients at a greater risk for inflammation and disease, such as patients with diabetes. The present clinical experiment was to investigate changes in GCF biomarker protein levels and SP microbial distribution in patients with T1DM *versus* patients without diabetes during a 35-day EG trial. Significant differences of the mean IL-1β, IL-8, and MMP-9 levels between individuals with and without diabetes demonstrated a modified host immune response to a similar bacterial challenge.

The study design involved patients stopping oral hygiene practices over a period of 21 days to induce reversible gingival inflammation. Different cytokine patterns have been observed in patients undergoing EG *versus* patients having persistent gingivitis, where patients in the EG group displayed increased GCF IL-1β and decreased IL-8, in comparison with untreated patients having persistent gingivitis (Deinzer et al. 2007). The EG model allows researchers to use a controlled, reversible model of oral inflammation, although the conditions of EG are not necessarily comparable to cases of per-

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sistent gingivitis (Deinzer et al. 2007). Gingival status is currently assessed using gingival indices, which allows practitioners to evaluate gingival tissue quality, without consideration of pocket depth or extent of bone loss (Löe & Silness 1963). In the present study, significant differences were observed in the percentage of GI scores $\geqslant 2$ in patients with diabetes, as compared with those without diabetes after 7 and 21 days ($p < 0.05$). These results indicate that alterations in biomarker levels during oral inflammation may be simultaneous to or may even precede the clinical observations of inflammation. The undeniable subjectivity of clinical oral inflammation measurements may be problematic in determining slight changes in tissue that could indicate alterations in oral health. Alternatively, GCF analysis allows for a more sensitive identification of the immunological environment of the oral cavity and the systemic environment of the host, potentially before clinical signs present in some patients (Zhang et al. 2002).

It was expected that during the plaque accumulation phase of our study, the presence of biofilm would induce an inflammatory response, leading to a subsequent increase of GCF cytokines such as IL-1β (Zhang et al. 2002, Trombelli et al. 2006). In the present study, IL-1β protein levels in patients with diabetes displayed significant differences during periods without oral hygiene, specifically between baseline and Days 7, 14, and 21. Significant changes in IL-1β levels over time and between the two patient groups indicated a distinctly different response to the bacterial challenge beginning early on, and lasting throughout the period of EG. IL-8 protein levels were the highest for patients with diabetes after Day 14, but in healthy patients, the highest levels were observed at baseline, and increased after oral hygiene restoration. In previous studies, a short-term decrease in IL-8 has been observed during EG. In our study, no significant differences in IL-8 levels were observed either between the two patient groups or within each group over time. Based on previous research, it has been proposed that the biofilm, or its associated organisms, may cause down-regulation of IL-8 production, causing a predisposition to enhanced pro-inflammatory responses once the level of inflammatory challenge is surpassed. This theory may be supported by the episodic inflammatory nature of periodontal disease (Deinzer et al. 2007).

In the current study, the mean GCF biomarker levels were elevated in patients with diabetes as compared with healthy individuals during EG, with the highest MMP-8 and MMP-9 protein levels at Day 21, for both patient groups. Differences in the MMP-8 levels between patient groups were also observed at Days 14 and 21, and although significance was not achieved, more significant results would most likely have been obtained with a larger cohort. It should be noted that the use of ELISA methods in the present study for protein quantification may not allow direct comparison with studies using zymography methods to distinguish between the latent and the activated MMPs. Recent biomarker studies have presented MMP-8 as a candidate for diagnostic testing and patient monitoring due to its significant presence in the saliva of periodontally diseased patients (Giannobile 2008). Significant changes were observed in MMP protein levels for patients with Diabetes between baseline and Day 21, although these changes during the EG period were not observed with healthy patients, implying that the patients' diabetes status lead to the early and significant increase in MMP expression.

Significant differences in biomarker levels between time points and patient groups were observed before the endpoint of the EG trial, although no significant difference in plaque levels was observed between patient groups. Differences between healthy patients and those with diabetes, as reflected in biomarker protein levels, are thought to be due to systemic and local effects of the disease such as impaired neutrophil activity and increased macrophage secretion of pro-inflammatory mediators as a result of interactions with AGEs (Mealey & Rose 2008). IL-1β protein levels showed the most promising and distinct biomarker results, as the levels increased very rapidly, creating a clear separation between healthy patients and those with diabetes as early as Day 7, when compared with baseline levels. Therefore, a controlled gingivitis model may be carried out in less than 21 days while potentially decreasing the challenges associated with recruitment and helping to increase patient compliance during the study. The results of the present study are in agreement with previous findings showing that gingival inflammation responses during EG can be consistently detected after 7 and/or 14 days (Trombelli et al. 2006). In comparable studies, subgingival infection patterns were similar between patients with T1DM and those without diabetes, while controlling for periodontal status, indicating that specific factors may have notable effects on the host response to the biofilm, but bacterial colonization may be affected by an alternate set of factors (Lalla et al. 2006). Statistical analysis allowed us to identify discriminators between patient types using IL-1β at Day 14, IL-8 at Day 14, and MMP-9 at Day 7, which, when used together, may aid in predicting oral inflammatory status. Correlations between biomarkers and subgingival plaque species or clinical measurements, as seen in Tables 1–3, yielded mixed results before and following a 21-day absence from oral hygiene. Historically, studies qualitatively analysing microbial populations have yielded mixed results (American Academy of Periodontology 2005). Therefore, it may be of greater necessity to look at a combination of characteristics such as groups of pathogens or individual pathogens along with clinical or oral fluid biomarker components in relation to oral inflammatory status (Ramseier et al. 2009). Ideally, these and other potential biomarkers could help determine a possible relationship between short-term plaque accumulation and inflammatory mediator levels, differentiating not only between health and disease, but could potentially be used to specify patients' level of oral disease, and to measure patients' response to therapeutic measures. Further, the consideration of the measurement of GCF biomarkers by total amount per time of collection (in this study, 30 s) *versus* the measurement of volume has been a topic of discussion in the literature as both being acceptable approaches for the evaluation of GCF biomarkers (Page 1992). Although it is acknowledged that GCF flow increases with inflammation, analysis of the data from a variety of clinical studies of GCF allows a number of conclusions to be drawn on the potential diagnostic significance of GCF. The total amount of the mediator and not the concentration of the mediator in the GCF sample can be reported when timed samples are collected (Lamster 1997). More recently, Orozco et al. (2006) evaluated a variety of interleukins including IL-1β, comparing gingivitis and periodontitis subjects using a similar approach.

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The present findings indicate the need to further examine the potential of utilizing biomarker analysis methods for discrimination between patient groups to observe the implications of systemic diseases on oral health. Because of the limitations of this exploratory study, it would be beneficial to carry out a large, longitudinal, EG study involving patients with diabetes so that one can observe oral inflammation by clinical indication, as it relates to glucose control, and biomarker profiles in multiple tissues. Ideally, the study should involve glycosylated haemoglobin measurements throughout the study, and at its conclusion, in addition to GCF, serum, and saliva biomarker evaluation, potentially with a more ethnically diverse study population. Our findings support research indicating that T1DM patients show a distorted response to inflammation, as observed in GCF (Salvi et al. 2008). The early response seen in patients with T1DM, as a result of destructive autoimmune processes, can help determine molecules or characteristics of interest with a larger sample population, yielding results that can be applied to the general population for identifying inflammation.

Quantifiable biomarker assessment could lead to more sensitive, accurate, and rapid measures of oral health status in varying patient populations, in addition to those of the more subjective clinical indices. It would be of great interest to study a large cohort of patients with diabetes, both Type 1 and Type 2, as well as other patient groups perhaps with other systemic diseases to determine whether these results are applicable to different patient groups. Information on IL-1β and/or specific MMP genotype polymorphisms as compared with protein levels during oral inflammation may provide a further insight into the host risk determinants involved in disease development.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Consort figure depicting patient selection process.

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Clinical Relevance

Scientific rationale for study: Greater knowledge of the relationship between oral inflammation and local biomarker levels in patients with T1DM may lead to improved patient monitoring, early intervention, and subsequent prevention of periodontal disease activity.

Principal findings: Protein profiles for IL-1β, MMP-8, and MMP-9 are higher in patients with diabetes during oral inflammation, indicating distinct differences in the host immune response.

Practical implications: Quantifiable biomarker assessment could lead to more accurate and rapid measures of oral inflammatory disease in addition to more subjective clinical indices. The EG model may achieve desired results in <21 days, further increasing patient compliance.

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