J Clin Periodontol 2010; 37: 324–333 doi: 10.1111/j.1600-051X.2010.01543.x

Journal of Clinical Periodontology

Changes in gingival crevicular fluid inflammatory mediator levels during the induction and resolution of experimental gingivitis in humans

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Offenbacher S, Barros S, Mendoza L, Mauriello S, Preisser J, Moss K, de Jager M, Aspiras M. Changes in gingival crevicular fluid inflammatory mediator levels during the induction and resolution of experimental gingivitis in humans. J Clin Periodontol 2010; 37: 324–333. doi: 10.1111/j.1600-051X.2010.01543.x

Abstract

Aim: The goal of this study is to characterize the changes in 33 biomarkers within the gingival crevicular fluid during the 3-week induction and 4-week resolution of stent-induced, biofilm overgrowth mediated, experimental gingivitis in humans.

Methods: Experimental gingivitis was induced in 25 subjects for 21 days followed by treatment with a sonic powered toothbrush for 28 days. Clinical indices and gingival crevicular fluids were collected weekly during induction and biweekly during resolution. Samples were analysed using a bead-based multiplexing analysis for the simultaneous measurements of 33 biomarkers within each sample including cytokines, matrix-metalloproteinases (MMPs) and adipokines. Prostaglandin-E_2 was measured by enzyme-linked immunoadsorbant assay. Statistical testing using general linear models with structured covariance matrices were performed to compare stent to contralateral (non-stent) changes in clinical signs and in biomarker levels over time.

Results: Gingivitis induction was associated with a significant 2.6-fold increase in interleukin 1-β (IL-β), a 3.1-fold increase in IL-1α and a significant decrease in multiple chemokines as well as MMPs-1, -3 and 13. All changes in clinical signs and mediators rebounded to baseline in response to treatment in the resolution phase.

Conclusions: Stent-induced gingivitis is associated with marked, but reversible increases in IL-αa and IL-1β with suppression of multiple chemokines as well as selected MMPs.

Key words: biofilm; crevicular fluid; gingivitis; inflammation; periodontal disease

Accepted for publication 20 December 2009

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.

This work supported by a research grant provided by Philips Oral Healthcare Inc. and NIH grants RR00046 and UL1RR025747. Drs. de Jager and Aspiras are employees of Philips Oral Healthcare Inc., Dr. Offenbacher has also received lecture honorarium support from Philips Oral Healthcare.

The gingival crevicular fluid (GCF) is a serum transudate that is enriched with microbial and host products that arise as a result of the current inflammatory dynamics of the host–biofilm interaction. Recently, the application of suspension bead multiplexing methods for the simultaneous analysis of multiple inflammatory mediators has been used to characterize the levels of 16 inflammatory mediators that are associated with naturally occurring gingivitis at the biofilm gingival interface (Offenbacher et al. 2007). These studies have shown, for example, that chronic gingivitis is associated with increases in several mediators of inflammation including interleukin 1β (IL-1β, 1.35-fold), interleukin 6(IL-6, 2.1-fold), regulated on T-cell activation, normal T-cell expressed and secreted (RANTES, 2.5-fold) and monocytic chemotactic

protein-1(MCP-1, 2.2-fold) (Offenbacher et al. 2007). Earlier studies in models of experimental gingivitis demonstrated an increase in GCF levels of IL-1β and leukotriene B4 (LTB_4), a marker of neutrophil activation (Heasman et al. 1993). Indeed there are many studies that have demonstrated in gingivitis a dominance of neutrophil markers of recruitment, such as IL-8 and neutrophil degranulation, including β glucuronidase (Lamster et al. 1994), elastase (Soder et al. 2002) and other matrix-metalloproteinases (MMPS) (Soder et al. 2002). More recently, the experimental gingivitis model has been adapted to include the use of intra-oral stents that cover only selected teeth to be worn only during routine oral hygiene (Burrell & Walters 2008) to permit the study of localized changes in biofilm overgrowth and inflammation. By using localized stents unilaterally the effects of contralateral biofilm maintenance can be compared within individuals to study localized biofilm overgrowth and cross-arch differences in GCF inflammatory response as a way to adjust for site-specific changes that determine clinical and inflammatory mediator status within an individual. In this study we have re-examined the classic experimental gingivitis model using this updated stent-induced biofilm overgrowth model (SIBO) in conjunction with immunoadsorbant bead suspension array multiplexing tools, which permit the simultaneous analyses of 33 inflammatory mediators within each GCF sample, to evaluate the changes in the GCF composition over time. This was a hypothesis-generating experiment with the goal of identifying new candidate biomarkers within the GCF, which co-vary with the changes in clinical status. As a secondary goal, we were interested whether within the gingivitis subjects there was a homogeneous inflammatory phenotypic response or whether there were clusters of individuals that displayed different GCF biomarker signatures.

Methods

Clinical study design and patient enrollment

This study design represents a modification of the ''Experimental gingivitis in healthy volunteers'' study as originally described by Loe et al. (1965). We recruited and enrolled subjects with naturally occurring gingivitis, defined as some bleeding upon probing present, typically in at least 10% of sites, as these subjects were more likely to develop experimental gingivitis in the course of this study (Trombelli et al. 2006). The study design involved a 1-week hygiene phase, a 3-week induction phase using two stents and a 4-week resolution phase. The study design and informed consent were approved by the UNC Human Subjects Review Committee. Twenty-six subjects were consented and enrolled with 24 subjects completing the study between 13 November 2006 and 30 April 2007. For eligibility all subjects had at least 20 teeth (excluding third molars) and $\geqslant$18 sites with bleeding to gentle probing. Exclusion criteria included: (1) treatment with antibiotics for any medical or dental condition within 1 month before the screening examination; (2) chronic treatment (i.e., 2 weeks or more) with any medication known to affect periodontal status (e.g., phenytoin, calcium antagonists, cyclosporin, anticoagulants, non-steroidal anti-inflammatory drugs, aspirin) within 1 month of the screening examination; (3) ongoing medications initiated $<$3 months before enrollment (i.e., medications for chronic medical conditions must be initiated at least 3 months before enrollment); (4) participants with clinically significant organ disease including impaired renal function, heart murmur, history of rheumatic fever or valvular disease, or any bleeding disorder; (5) participants with active infectious diseases such as hepatitis, HIV or tuberculosis; (6) severe unrestored caries, or any condition that was likely to require antibiotic treatment during the study, including the need for prophylactic antibiotic; (7) individuals who self-reported use of any tobacco products or who have used tobacco products within the previous 6 months of the screening examination; (8) individuals who were pregnant, or expect to become pregnant within the next 3 months and individuals nursing. Participants were excluded from the study or analysis or discontinued if any of the following conditions were met: (1) changes in the participant's medical status or medications; (2) use of any antibacterial rinses; (3) use of non-study dentifrices, toothbrush or dental floss during the no-hygiene and resolution phases of the study; (4) use of irrigating devices; (5) participant's inability or noncompliance to wear their stents or shields over one mandibular and one maxillary sextant during daily brushing procedures; (6) use of oral antib iotics and non-steroidal anti-inflammatory drugs during the trial. Acute use of oral acetaminophen was permitted. Participants requiring treatment for an acute medical or dental condition during the study were withdrawn from the trial.

Patient protocol and procedures

At the screening visit (Day 14) informed consent was obtained, medical history, height, weight and vital signs were collected, a full-mouth clinical examination was performed and impressions were made for stent fabrication. Full-mouth examinations included oral cancer screening plus gingival indices [GIs, Loe & Silness (1963)], plaque indices [(PIs, Silness & Loe (1964)], probing depths (PD), attachment level (AL) and bleeding on probing (BOP) scores (yes/no) at each of six sites per tooth. Customized stents were fabricated for each subject within the General and Oral Health Center. Stents were fabricated to resemble an acrylic mouthguard but extended to cover approximately 2 mm over gingival margins. Stents formed a seal and rested on the gingiva, but were relieved on the tooth and tissue side except for the occlusal surfaces to avoid disturbing plaque or gingival tissues. One week later at Visit 2 (Day 7) the exam was repeated and biological samples were collected. GCF was collected at 16 sites: eight from the stent teeth and eight from the contralateral control teeth using methods described previously (1), with no plaque removal before GCF sample collection. Subgingival plaque was collected (1) at two sites on the stent teeth and the contralateral control teeth, selecting those two teeth in the middle of the stent sampling the mesiobuccal subgingival sites. A prophylaxis was performed at the end of that visit (Day 7) consisting of mechanical scaling, tooth polishing and oral hygiene instruction (modified Bass tooth brushing, flossing).

Following a 1-week standard hygiene phase, participants returned for the baseline (Day 0) visit. From that point forward for a 3 week, no-hygiene phase, subjects abstained from brushing and flossing teeth in two right posterior sextants via placement of acrylic stents. At baseline (Day 0) GCF and plaque samples were collected, clinical exams performed, vitals, adverse events

recorded and medical histories updated. At this visit subjects were give two stents and given instructions for use. All participants refrained from all oral hygiene procedures (i.e., tooth brushing, flossing or use of interdental aids) in two sextants (up to six teeth in each arch). This was pre-selected as the upper right (teeth #1–6) and lower right (#27–32). If there were fewer than four teeth in a sextant then the contralateral sextant was assigned. Participants were directed to continue plaque control procedures to the remaining four sextants using the fluoride dentifrice dispensed at Day 7 (Visit 2); however, flossing was to be discontinued entirely to avoid unintentional flossing of non-stent teeth. During daily brushing procedures, participants wore removable customized acrylic stents to cover the two experimental sextants and to encourage participant compliance. Subjects were seen at Day 7, 14 and 21 for GCF collection during this induction phase and clinical exams were performed at each visit to assure patient safety and compliance. At Day 21 (Visit 6) plaque samples were also taken. At Day 21 subjects were given a Sonicare Elite (Philips Oral Health Care Inc., Snoqualmie, WA, USA) toothbrush and were provided with both oral and written instructions for use twice a day for 2 min. Subjects used the Sonicare toothbrush daily at home without flossing for 2 weeks returning on Day 35 for a repeat examination and biological sampling and again at Day 49 (4 weeks after reinitiating oral hygiene). At Day 49 rescue prophylaxis was provided for subjects that needed additional care to restore their oral health. Thus, Day 21 represented the peak of gingivitis induction and Day 49 the resolution.

Examiner training and calibration

Clinical examiners were calibrated before commencement of the study for training of study procedures and for documentation of acceptable intra- and inter-examiner measurement reliability. This examiner calibration and training is under a separate protocol which is performed within the GO Health Center. Examiners were standardized on measurements of the PI, GI, PD, AL, BOP PI, GI, PD, clinical AL and BOP. The two participating examiners were found to have an intra-class κ coefficient of agreement of 0.96 and 0.94 for PD and 0.94 and 0.92 for AL measurements, as compared with the Gold standard examiner. Clinical parameters were measured using a manual University of North Carolina (UNC-15) periodontal probe. For experimental gingivitis measures, these parameters were measured at six periodontal sites per tooth for teeth within the two experimental gingivitis sextants and for teeth in the other four sextants for control purposes. Summary statistics and comparisons were made using measurements at stent teeth and the contralateral non-stent teeth.

Analysis of GCF inflammatory mediators

Sixteen GCF samples were collected per participant on Days 7, 0, 7, 14, 21, 35 and 49. Four samples were collected from each quadrant sampling buccal sites at the distal of the second premolar, mesial and distal of the first molar, and at the mesial of the second molar. If one of these teeth was absent, the rule was to sample from the next available (mesial) tooth. Samples were collected onto filter paper strips (Pro Flow Inc., Amityville, NY, USA), and the volume determined using a Periotron 8000® (Harco Electronics Limited, Winnepeg, MB, Canada). Samples were wrapped in aluminium foil, sealed in a cryovial and placed into liquid nitrogen chair-side. Strips were stored in liquid nitrogen (-180°C) until mediator analysis. Each sample was collected into a pre-labelled sample vial that was bar coded with unique identifiers for the study and specimen identifier without participant identifiers. The average GCF volume collected from each pocket area was 0.20 μl. All laboratory analyses were performed blinded from sample, visit or patient information. Four of the GCF samples were analysed for each mediator, two from the stent area and two from the contralateral non-stent. GCF samples were used to quantify specific mediator levels within these samples by ELISA and/or using the Fluorokine® MAP cytokine multiplex kits (R&D Systems Inc., Minneapolis, MN, USA) and Luminex® analyser system (Luminex Corporation, DeSoto, TX, USA). Four different sets of four GCF samples were eluted differently as four different multiple cytokine plate reagent systems were used: the Cayman PGE2 assay, the Cayman cytokine panel, the R&D MMP panel and the R&D obesity panel. Local levels of inflammatory biomarkers were determined following methods described by Offenbacher et al. (2007). The following list of biomarkers were assayed followed by the mean minimum detection level in picograms per millilitre shown in brackets [ ]: prostaglandin E2 (PGE2) [15.0], IL-1β [0.27] IL-6 [0.36], IL-1α [0.24], nIL-2 [0.89], IL-4 [1.75], IL-5 [0.33], IL-10 [0.13], interferon-γ (IFN-γ) [0.31], granulocyte/monocyte colony stimulating factor (GM-CSF) [1.05], MCP-1 [0.16], macrophage inflammatory protein α (MIP-1α) [1.19], RANTES [1.08], tumour necrosis factor α (TNF-α) [0.60], fibroblast growth factor-basic protein (FGF basic) [1.82], granulocyte colony stimulating factor (G-CSF) [0.57], IL-17 [0.39], IL-1 receptor antagonist (IL-1ra) [2.06], MIP-1β [0.44], thrombopoietin (TPO) [2.81], vascular–endothelial growth factor (VEGF) [0.81], MMP-1 [4.4], MMP-12 [1.2], MMP-13 [15.9], MMP-2 [25.4], MMP-3 [1.3], MMP-7 [16.9], MMP-8 [8.9] and MMP-9 [7.4]. All mediator values were corrected for elution volume, assay dilution and GCF volume and expressed as a GCF concentration. Mean values and standard deviations were computed for stent sites (four values) and non-stent sites (four values) for each patient at each visit. Log mean values of mediator concentrations were computed for comparisons, as the mean values were not normally distributed.

Analysis of microbial composition

Subgingival plaque samples collected on Days 7, 0, 21 and 49 were processed to detect and quantify the levels of periodontal pathogens using checkerboard DNA–DNA analysis as described previously. Plaque samples were obtained from the mesial surfaces of the four first molars including the two selected sextants using a sterile curette. Samples from each site were placed into a separate Eppendorf tube containing 150 μl of TE buffer. One hundred millilitres of 0.5 M NaOH was added to each sample and the samples were frozen at -80°C. Each plaque sample was processed independently to identify and quantify 15 periodontal pathogens to examine for changes in biofilm composition over time under the stent. The microbes identified are presented in the results section.

Statistical analyses

Changes from baseline in clinical parameters over time (e.g., PI scores, GI scores, PD and ALs) were compared for the stent side *versus* the non-stent

side with statistical tests based on general linear models with structured covariance matrices. Specifically, an unstructured covariance matrix for stent and non-stent side clinical parameters was combined with an equi-correlation (e.g., compound symmetric) structure over time in Kronecker-product fashion (Galecki 1994). In this manner each patient served as their own control over time comparing stent to non-stent sites and comparisons were computed relative to baseline levels among subjects during induction. In addition, mean values of GCF mediator levels were also analysed by general linear models in a similar manner by comparing the log of the mean GCF concentration on the stent side (four GCFs assayed independently) to the non-stent side (also four sites). An overall *F*-test for the side (stent *versus* non-stent) by time interaction was computed for each clinical or mediator level to test for a difference in trends between stent and non-stent sides over time during the induction phase. Statistical testing assumed an α-value of <0.05 as significant and comparisons were made between baseline (Day 0) and the peak of induction (Day 21) and between resolution and at peak of induction (Day 49 *versus* Day 21). Regardless of the significance of the *F*-test for the interaction, *t*-statistics were used to examine for mediator-specific changes at Day 21 *versus* Day 0 and Day 49 *versus* Day 21. All tests used the Kenward–Roger degrees of freedom small-sample correction (Kenward & Roger 1997). *p*-values were not adjusted for multiple comparisons, so changes in single mediators should be interpreted with caution.

Cluster analyses were performed to examine for subjects who formed groups that exhibited similar changes in inflammatory mediators, irrespective of clinical signs. Cluster analyses were performed using PROC CLUSTER which hierarchically clusters people based upon the change in levels of inflammatory mediators within the gingival crevicular fluid. The number of clusters chosen was based upon the values obtained from the SAS ''Gap statistic'' analysis. Only mediators which demonstrated significant overall changes from baseline to Day 21 were used to develop clusters. Each resulting cluster contained subjects who had similar patterns of inflammatory response mediators as a result of biofilm overgrowth. Clustering resulted in three different inflammatory clusters (IC). Each of these three ICs was characterized by describing the mediator changes observed comparing baseline to Day 21 and the mean changes in clinical signs, testing for statistical significance by GLM methods. An $\alpha<0.05$ was considered statistically significant.

Results

Study implementation

Thirty-eight subjects were screened to reach our target enrollment. Two subjects were withdrawn before study completion: one for non-compliance with appointment times and the other because she missed Visit 2 and would miss another visit due to a travel conflict. There were five protocol violations that did not result in data censure or subject discontinuance or withdrawal: (1) one subject used a non-study toothpaste on one evening and the following morning; (2) one subject took three dosages of 25 mg Meclizine (antihistamine) for vertigo over 3 days; (3) one subject forgot to use stents while brushing on one morning stating she brushed without stents that morning but did not brush stent area. Subject was advised to always put stents in place before brushing; (4) one subject began a 14-day regimen of Prilosec; (5) one subject flossed in non-stent areas during first week of induction, but discontinued thereafter. There were no adverse events reported during the study. The mean age of the 25 subjects was 44.0 (standard error, 12.7), comprising 10 African Americans, 15 Caucasians, 20 females and five males. There were no significant differences among the ICs in the distribution of race, sex, age or mean body mass index.

Changes in clinical signs during induction and resolution of experimental gingivitis

The SIBO during the 3-week induction phase was associated with an overall increase in clinical signs of inflammation and plaque scores that was limited to the stent area. In Table 1 the time trends for the stent side during induction phase (integrating from baseline through Day 21) were significantly different than the time trends for the non-stent side at $p<0.001$ for PI, GI and BOP scores. One practical advantage of this stent model, as compared with the total abstinence of oral hygiene model is that subjects prefer (1) to be able to limit the extent of gingival inflammation to selected posterior regions of the mouth, (2) to be able to use dentifrice and normal brushing habits on the remainder of dentition and the tongue to improve oral freshness. Using general linear models with structured covariance matrices, the increases seen in PI and GI are statistically significant by 1 week and increase in magnitude through Day 21. After re-instating oral hygiene by the initiation of Sonicare usage at Day 21, at Day 35 and again at Day 49 there is a statistically significant decrease in mean PI and GI as compared with Day 21 and a resolution to baseline values. For example, the mean GI increased from 0.92 (0.06) [mean (standard error)] at baseline to 1.45 (0.07) ($p<0.001$) at day 21 which is the peak of the induction phase, dropping to 0.83 (0.06) at resolution. A similar pattern was seen for extent BOP which achieved statistical significance by 14 days. The slight increases in PD during induction were also statistically significant and ALs increased at day 14 but not at Day 21. Thus, there was an induction of experimental gingivitis under the stent that was fully and readily reversed by the use of the Sonicare toothbrush. None of the subjects required a rescue prophylaxis at Day 49.

Changes in GCF inflammatory mediators during induction and resolution of experimental gingivitis

Thirty three mediators were analysed as shown in Table 2 (cytokines) and Table 3 (MMPs, adipokines and prostanoids). All mediators were detectible in $>90\%$ of samples with the exception of IL-5, which is a mediator of allergic responses (acute hypersensitivity) and was not detected in any GCF sample. In Table 2 at baseline the levels of these detectible cytokine mediators fall into three concentration groups of constitutively ''low'' expression levels ($\sim$1–10 ng/ml including IL-17, IFN-γ, IL-6, IL-10 GM-CSF, TNF-α, TPO, RANTES, IL-4 and IL-2). None of these ''low basal secretion'' mediators show any significant increase during the induction phase, but RANTES shows a significant 1.9-fold decrease. There are some ''intermediate'' level mediators at baseline in the range of 10–1000 ng/ml that includes MCP-1, FGF, MIP-1β, G-CSF, VEGF, ENA-78, MIP-1α and

IL-1β. Of these mediators only IL-1β demonstrates a significant increase during SIBO gingivitis. The change in GCF IL-1β at baseline goes from log mean values of 2.84–3.23 at Day 21, which represents an increase from 676–1737 ng/ml, or a 2.6-fold increase.

It is noteworthy that three mediators in this ''intermediate'' basal secretion group demonstrate significant decreases during gingivitis induction: MCP-1 showing a significant 1.4-fold decrease at Day 21, MIP-1β demonstrates a 10-fold decrease and ENA-78 showing a 1.6-fold decrease. It is interesting that all four mediators that show suppression are chemokines. At baseline the highest basal secretion levels of mediators are in the range of >1–69 μg/ml and include IL-1α, IL-8 and IL-1ra. IL-1α demonstrates a significant 3.09-fold increase by Day 21 and IL-1ra levels do not change during induction. The increase in both IL1$\acute{a}$ and IL1β in the absence of increasing IL-1ra is consistent with a marked increased bioavailablity of both IL1á and IL1β during induction. IL-8 levels drop to about half the basal level at Day 21. It is significant that all mediators which changed during the induction phase all reversed to baseline during the resolution phase in concordance with the clinical reversion to pre-stent levels. Only five cytokines showed significant differences in time trends between stent and non-stent sides during induction (as indicated by asterisks in Table 2): TNF-α, MIP-1β, IL-1a, IL-1b and IL-8. During induction TNF-α, MIP-1β and IL-8 decreased, whereas both IL-1α and IL-β increased. These changes in these five mediators over time met this more stringent criterion for consistent suppression or induction during disease expression.

Table 1. Clinical indices during induction and resolution of experimental gingivitis

	Baseline	Stent in place			Resolution	
		day 7	day 14	day 21	day 35	day 49
Mean PI*	0.08 (0.06)	0.82 (0.08)	0.89 (0.07)	1.06 (0.08)	0.13 (0.04)	0.09 (0.03)
Mean GI*	0.92 (0.06)	1.11 (0.06)	1.34 (0.06)	1.45 (0.07)	0.92 (0.06)	0.83 (0.07)
Extent BOP*	26.1 (3.36)	34.7 (3.78)	48.2 (3.92)	52.0 (4.59)	35.1 (3.09)	36.4 (3.00)
Mean PD	1.82 (0.04)	1.81 (0.04)	1.93 (0.04)	1.94 (0.05)	1.91 (0.04)	1.90 (0.04)
Mean AL	1.28 (0.05)	1.31 (0.05)	1.37 (0.04)	1.36 (0.04)	1.40 (0.04)	1.36 (0.04)

Mean clinical values (standard error) for stent side.

*F-test comparing stent and non-stent time trends, p-value <0.0001.

Days 7–21 indicates $p<0.05$ as compared with baseline, Days 35 and 49 highlight indicates $p<0.05$ as compared with Day 21.

BOP, bleeding on probing; PI, plaque indices; GI, gingival indices; PD, probing depths; AL, attachment level

Changes in GCF MMPs, adipokines and arachidonate metabolites during induction and resolution of experimental gingivitis

Changes in GCF levels of MMP-1 (collagenase-1), MMP-3 (stromelysin-1) and MMP-13 (collagenase-3) are significant by 1 week and are consistently suppressed two- to threefold throughout induction, as shown in Table 3. These three MMPs also demonstrated a statistically.

Table 2. The gingival crevicular fluid biomarker level at baseline and during induction and resolution of stent-induced biofilm overgrowth associated experimental gingivitis

Cytokine level	Baseline	Induction (stent in place)			Resolution	
Log mean ng/mL (standard error)		day 7	day 14	day 21	day 35	day 49
IL-5	–	–	–	–	–	–
IL-17	0.08 (0.04)	0.00 (0.03)	0.00 (0.03)	0.04 (0.03)	0.04 (0.03)	0.00 (0.03)
IFN-γ	0.19 (0.08)	0.29 (0.08)	0.26 (0.07)	0.27 (0.07)	0.28 (0.06)	0.18 (0.04)
IL-6	0.20 (0.07)	0.14 (0.07)	0.16 (0.07)	0.21 (0.08)	0.28 (0.07)	0.22 (0.05)
IL10	0.38 (0.08)	0.59 (0.08)	0.37 (0.07)	0.32 (0.06)	0.34 (0.06)	0.37 (0.06)
GM-CSF	0.38 (0.08)	0.21 (0.08)	**0.17 (0.07)**	0.31 (0.07)	0.30 (0.07)	0.26 (0.04)
TNF-α*	0.47 (0.08)	**0.19 (0.09)**[a]	**0.21 (0.08)**[a]	0.28 (0.09)	0.49 (0.11)	**0.54 (0.09)**
TPO	0.65 (0.11)	0.55 (0.11)	0.73 (0.11)	0.62 (0.11)	0.63 (0.11)	0.62 (0.10)
RANTES	0.69 (0.14)	**0.34 (0.14)**	0.47 (0.12)	**0.42 (0.12)**	0.49 (0.10)	0.34 (0.08)
IL-4	0.72 (0.13)	0.64 (0.13)	0.63 (0.13)	0.71 (0.13)	0.66 (0.11)	0.85 (0.11)
IL-2	0.85 (0.13)	1.16 (0.13)	0.95 (0.11)	1.08 (0.12)	**0.46 (0.12)**[a]	0.93 (0.10)
MCP-1	1.12 (0.05)	1.02 (0.06)	1.01 (0.06)	**0.98 (0.07)**	**1.10 (0.06)**	**1.14 (0.06)**
FGF	1.23 (0.13)	1.45 (0.12)	1.36 (0.09)	1.49 (0.08)	**1.27 (0.08)**	**1.19 (0.12)**
MIP-1β*[b]	1.82 (0.08)	**0.85 (0.10)**[b]	**0.86 (0.12)**[b]	**0.82 (0.15)**[b]	**1.47 (0.12)**[b]	**1.54 (0.10)**[b]
G-CSF	1.89 (0.12)	1.70 (0.10)	1.87 (0.07)	1.88 (0.07)	1.84 (0.07)	1.96 (0.06)
VEGF	2.13 (0.06)	2.09 (0.05)	2.15 (0.04)	2.16 (0.04)	2.14 (0.04)	2.19 (0.04)
ENA-78	2.42 (0.08)	2.36 (0.08)	2.26 (0.07)	**2.20 (0.07)**	2.35 (0.06)	**2.41 (0.04)**
MIP-1α	2.44 (0.05)	**2.30 (0.05)**	2.37 (0.04)	2.37 (0.04)	2.36 (0.04)	2.42 (0.03)
IL-1β*[a]	2.83 (0.11)	2.89 (0.10)	3.12 (0.09)	**3.24 (0.08)**[a]	**2.93 (0.06)**[b]	**2.97 (0.07)**
IL-1α*[a]	3.10 (0.13)	3.40 (0.11)	**3.56 (0.09)**[a]	**3.59 (0.07)**[a]	**3.18 (0.06)**[b]	**3.26 (0.07)**[a]
IL-8*[b]	3.39 (0.06)	**2.93 (0.07)**[b]	**3.00 (0.06)**[b]	**3.08 (0.06)**[b]	3.26 (0.07)	**3.29 (0.05)**
IL-1ra	4.84 (0.04)	4.86 (0.05)	4.87 (0.04)	4.89 (0.04)	**4.79 (0.03)**	4.89 (0.03)

Mediator concentrations are shown as the mean log of each mediator with standard error expressed in ng/ml gingival crevicular fluid at each visit. Cytokine abbreviations are described in text. Bold indicates $p<0.05$.

[a]$p<0.01$,

[b]$p<0.001$, induction values vs. baseline, resolution values vs. Day 21.

*Time trend for the stent side through Day 21 is significantly different than the time trend for the non-stent side at $p<0.05$.

Table 3. The gingival crevicular fluid biomarker level at baseline and during Induction and resolution of stent-induced biofilm overgrowth associated experimental gingivitis

Mediator level	Baseline	Induction (stent in place)			Resolution	
Log mean ng/mL (standard error)		day 7	day 14	day 21	day 35	day 49
Matrixmetalloproteinases						
MMP-1*[a]	1.86 (0.12)	1.24 (0.14)[b]	1.29 (0.12)[b]	1.36 (0.12)[b]	1.90 (0.11)	**1.87 (0.11)**[a]
MMP-3*[b]	1.98 (0.10)	1.31 (0.12)[b]	1.54 (0.11)[a]	1.60 (0.11)[a]	2.23 (0.10)[b]	**2.01 (0.10)**
MMP-7	2.40 (0.08)	2.56 (0.08)	2.40 (0.07)	2.36 (0.06)	2.36 (0.05)	2.43 (0.05)
MMP-13*[a]	2.51 (0.14)	1.97 (0.16)[a]	1.85 (0.14)[a]	2.05 (0.14)	2.70 (0.12)[b]	**2.68 (0.11)**[b]
MMP-8	4.93 (0.08)	4.85 (0.09)	5.09 (0.08)	5.16 (0.07)	5.02 (0.07)	5.07 (0.07)
MMP-9	5.50 (0.07)	5.20 (0.08)[a]	5.39 (0.07)	5.54 (0.07)	5.56 (0.06)	5.61 (0.06)
Adipokines						
Serpin-E1*	2.72 (0.07)	2.40 (0.07)[b]	2.36 (0.07)[b]	2.41 (0.08)[a]	2.69 (0.06)[a]	**2.65 (0.06)**
Complement-D	3.31 (0.06)	3.03 (0.07)[a]	3.17 (0.06)	3.31 (0.06)	3.49 (0.05)	3.40 (0.05)
CRP	3.58 (0.10)	3.34 (0.08)	3.55 (0.07)	3.63 (0.10)	3.64 (0.08)	3.51 (0.08)
Adiponectin	3.69 (0.06)	3.50 (0.07)	3.66 (0.07)	3.69 (0.08)	3.90 (0.06)	3.79 (0.07)
Resistin	4.57 (0.06)	4.37 (0.06)	4.48 (0.06)	4.50 (0.06)	4.67 (0.06)	4.61 (0.07)
Arachidonic acid metabolites						
PGE2	2.52 (0.08)	2.19 (0.09)	2.39 (0.09)	2.42 (0.09)	2.47 (0.10)	2.50 (0.09)

Mediator concentrations are shown as the mean log of each mediator with standard error expressed in ng/ml gingival crevicular fluid at each visit. Mediator abbreviations are described in text. Bold indicates $p<0.05$:

[a]$p<0.01$,

[b]$p<0.001$, induction values vs. baseline, resolution values vs. Day 21.

*Time trend for the stent side through Day 21 is significantly different than the time trend for the non-stent side at $p<0.05$.

Significant difference in time trends between stent and non-stent sides during the induction phase. MMP-9 also shows a transient decrease at 1 week that is no longer significant at Day 21. In contrast MMP-8 increased 1.7-fold at Day 21. As with the changes in cytokine levels during resolution, the MMPs that were suppressed during induction returned to baseline levels during resolution. Complement D, CRP, adiponectin and resistin all show a significant transient 1-week suppression in level that quickly rebounds, but Serpin-E1 shows a continued suppression during induction with a 2.0-fold reduction at 21 days. The level of Serpin–E1 also displays dynamic resiliency and rebounds during resolution. Levels of PGE_2 show a 7-day suppression, but did not demonstrate significant changes at induction or resolution.

Clinical and GCF mediator changes seen in IC subgroups

Although the general pattern of overall changes in inflammatory mediator levels shown in Tables 2 and 3 described the mediator perturbations for the entire study group, this general pattern was not evident looking at individual patients. In other words, individual subjects during the induction of gingivitis seemed to have some of the mediator changes present in the overall pattern, but many were missing or different. For this reason we sought to determine how these mediator changes during induction grouped together by using cluster analysis to see if the response of the overall group was due to different groups of subjects with similar patterns of inflammatory responses that might represent different ICs, each group with different patterns of mediator response that comprises a different inflammatory phenotype. This might explain why the overall mediator response of the whole group was not predictive of what any single patient might experience. For that reason we performed clustering analyses based upon changes in GCF mediators that occurred between baseline and Day 21. This resulted in subdividing the group into three different ICs with 13, six and six subjects, respectively. The clinical and mediator responses associated with these three ICs, (labelled IC1, IC2 and IC3) are shown in Table 4, emphasizing the baseline and peak of gingivitis induction (Day 21) data time points. Mean values that are statistically significant are indicated in boldface. However, the sample size is relatively small with only six subjects in each of the two smaller clusters and these are not adjusted for multiple comparisons, so statistical significance is less important than the descriptive nature of these analyses. Overall, there are little differences in the magnitude of the changes in clinical signs comparing these three different ICs. Baseline BOP and GI scores tended to be a little higher in IC2, but overall the magnitude of the plaque accumulation and the inflammatory response seen at Day 21 was quite similar among the three ICs.

During induction IC1 has a significant increase in IL-1α, IL-1β, IL-6, MMP-8 and MMP-9 with a significant decrease in MIP-1β. The mediator pattern shift seen during induction for IC2 is only suppression with a decrease in MIP-1β and MMP-1 and a range of adipokine response suppression that included Complement-D and Serpin-E1. Finally, IC3 demonstrated increases in both IL-1α and IL-1β as seen in IC1, but this is also accompanied by chemokine suppression including IL-8, MCP-1, MIP-1β, as well as depressed expression of MMP-1, MMP-3 and MMP-13. IC3 also shows lowered Serpin-E1 at Day 21. Thus, the segregation of subjects into ICs results in three different inflammatory response phenotypes, each of which produce similar, but not identical clinical responses.

Microbial changes seen in IC subgroups

The changes in inflammatory responses during induction were accompanied by changes in the biofilm composition as reflected by the limited analysis of 15 microbes. The changes in the biofilm load and microbial composition during the induction phase are shown in Table 5. The changes in plaque scores (Table

Table 4. Changes in log mean biomarker level expressed in ng/ml gingival crevicular fluid at baseline and during Induction of stent-induced biofilm overgrowth associated experimental gingivitis for the three distinct inflammatory clusters (ICs)

Clinical sign	IC1 ($n = 13$)		IC2 ($n = 6$)		IC3 ($n = 6$)	
	baseline	Day 21	baseline	Day 21	baseline	day 21
Mean PI	0.17 (0.09)	**1.08 (0.08)**	0.07 (0.10)	**1.04 (0.15)**	0.07 (0.16)	**1.04 (0.15)**
Mean GI	0.90 (0.08)	**1.50 (0.11)**	0.97 (0.16)	1.36 (0.11)	0.87 (0.12)	**1.45 (0.12)**
Extent BOP	26.4 (5.21)	**55.4 (7.80)**	35.3 (7.32)	54.4 (8.12)	16.0 (5.32)	**46.8 (8.88)**
Mean PD	1.79 (0.05)	**1.96 (0.06)**	1.97 (0.07)	2.06 (0.08)	1.73 (0.09)	1.78 (0.09)
Mean AL	1.35 (0.08)	1.52 (0.05)	1.47 (0.15)	1.45 (0.10)	0.97 (0.07)	1.01 (0.08)
Mediator level mean log ng/ml (standard error)						
IL-5	–	–	–	–	–	–
IL-17	0.14 (0.10)	0.09 (0.08)	–	–	–	–
IFN-g	0.24 (0.14)	0.34 (0.11)	0.02 (0.20)	0.32 (0.17)	0.27 (0.12)	0.11 (0.11)
IL-6	0.06 (0.15)	**0.43 (0.12)**	0.16 (0.07)	0.00 (0.06)	0.30 (0.08)	0.05 (0.08)
IL10	0.47 (0.14)	0.42 (0.11)	0.40 (0.15)	0.43 (0.12)	0.25 (0.11)	0.07 (0.11)
GM-CSF	0.37 (0.14)	0.41 (0.11)	0.28 (0.26)	0.37 (0.22)	0.35 (0.10)	0.16 (0.06)
TNF-α	0.35 (0.17)	0.42 (0.13)	0.40 (0.15)	0.07 (0.12)	0.65 (0.14)	**0.27 (0.19)**
TPO	0.71 (0.18)	0.71 (0.14)	0.52 (0.25)	0.46 (0.20)	0.66 (0.25)	0.68 (0.27)
RANTES	0.78 (0.26)	0.41 (0.20)	0.93 (0.36)	0.44 (0.27)	0.54 (0.22)	0.30 (0.21)
IL-4	0.59 (0.16)	0.78 (0.18)	0.58 (0.19)	0.85 (0.16)	0.69 (0.33)	0.51 (0.33)
IL-2	1.04 (0.20)	1.22 (0.15)	0.30 (0.32)	1.00 (0.30)	1.04 (0.09)	0.97 (0.27)
MCP-1	1.12 (0.12)	1.00 (0.09)	1.10 (0.08)	1.09 (0.07)	1.18 (0.09)	0.84 (0.09)
FGF	1.22 (0.20)	1.60 (0.08)	1.12 (0.28)	1.30 (0.21)	1.48 (0.10)	1.54 (0.09)
MIP-1β	1.85 (0.27)	0.99(0.20)	1.62 (0.25)	0.73 (0.19)	1.94 (0.12)	0.62 (0.24)
G-CSF	1.65 (0.17)	1.93 (0.10)	2.04 (0.23)	1.80 (0.16)	1.88 (0.18)	1.84 (0.14)
VEGF	2.00 (0.10)	2.19 (0.06)	2.12 (0.13)	2.15 (0.09)	2.17 (0.09)	2.13 (0.09)
ENA-78	2.39 (0.11)	2.25 (0.09)	2.34 (0.20)	1.92 (0.16)	2.54 (0.10)	2.40 (0.12)
MIP-1α	2.49 (0.08)	2.47 (0.05)	2.33 (0.15)	2.34 (0.16)	2.40 (0.07)	2.24 (0.07)
IL-1β	2.69 (0.21)	3.27 (0.15)	3.17 (0.18)	3.10 (0.14)	2.75 (0.15)	3.33 (0.15)
IL-1β	2.86 (0.19)	3.61 (0.09)	3.63 (0.20)	3.42 (0.16)	3.03 (0.18)	3.66 (0.18)
IL-8	3.26 (0.12)	3.17 (0.11)	3.44 (0.14)	3.10 (0.11)	3.44 (0.09)	2.89 (0.09)
IL-1ra	4.81 (0.07)	4.92 (0.06)	4.82 (0.09)	4.83 (0.08)	4.86 (0.07)	4.92 (0.07)
MMP-1	1.79 (0.15)	1.61 (0.12)	2.17 (0.21)	**1.12 (0.22)**	1.86 (0.23)	**1.08 (0.23)**
MMP-3	1.79 (0.17)	1.76 (0.18)	2.14 (0.30)	1.58 (0.21)	2.07 (0.18)	**1.40 (0.18)**
MMP-7	2.45 (0.11)	2.52 (0.10)	2.69 (0.10)	2.43 (0.10)	2.04 (0.14)	1.98 (0.14)
MMP-8	4.66 (0.11)	**5.36 (0.10)**	5.19 (0.12)	5.01 (0.12)	4.99 (0.16)	5.07 (0.16)
MMP-9	5.26 (0.10)	**5.62 (0.09)**	5.66 (0.13)	5.44 (0.13)	5.59 (0.15)	5.51 (0.15)
MMP-13	2.46 (0.18)	2.25 (0.17)	2.39 (0.23)	2.00 (0.23)	2.68 (0.30)	**1.64 (0.31)**
Adiponectin	3.69 (0.12)	3.73 (0.11)	3.73 (0.10)	3.55 (0.11)	3.70 (0.17)	3.67 (0.18)
Complement-D	3.33 (0.11)	3.38 (0.10)	3.40 (0.08)	**3.16 (0.08)**	3.30 (0.11)	3.30 (0.13)
CRP	3.52 (0.15)	3.53 (0.12)	4.10 (0.16)	3.88 (0.24)	3.16 (0.16)	3.25 (0.30)
Resistin	4.39 (0.09)	4.57 (0.07)	4.81 (0.08)	4.50 (0.14)	4.50 (0.11)	4.38 (0.13)
Serpin-E1	2.60 (0.13)	2.53 (0.13)	2.70 (0.11)	**2.16 (0.11)**	2.78 (0.09)	**2.43 (0.11)**
PGE2	2.40 (0.11)	2.53 (0.14)	2.78 (0.18)	2.35 (0.15)	2.49 (0.15)	2.25 (0.15)

Mean log of each biomarker with standard error is shown at each visit. Cytokine abbreviations are described in text. Bold indicates $p < 0.05$ as compared with baseline.

4) did not differ among the ICs. However there was a significant increase in total counts in IC2 ($p = 0.003$) principally due to increases in *S noxia* ($p = 0.003$) and two Orange complex organisms, *Prevotella intermedia* and *Prevotella nigrescens* (both $p = 0.01$). IC3 demonstrated an emergence of *Actinobacillus actinomycetemcomitans* ($p = 0.003$), *Eikenella corrodens* ($p = 0.03$) and smaller increases in other Red and Orange cluster organisms with borderline significance. In contrast the IC1 group demonstrated a significant increase in Red and Orange complex organisms (except *Campylobacter rectus*, $p = 0.06$), but no increase in total counts. Thus, the IC1 cluster demonstrated significant differences in biofilm composition with a strong emergence of Red and Orange organisms, as compared with the other two clusters. As with the inflammatory mediator data, these *p*-values are not adjusted for multiple comparisons.

Discussion

During the induction of experimental gingivitis there is histological evidence of leucocyte recruitment, vasodilation and epithelial rete peg elongation. The observed increases in IL-1α are consistent with epithelial activation and higher GCF levels of IL-1β are consistent with leucocyte margination, diapedesis and vasodilation. Increases in both IL-1α and IL-1β have previously been described in gingivitis (Heasman et al. 1993) relative to gingival health. However, one unexpected finding was the rather remarkable decrease in multiple chemokines during gingivitis induction. IL-8 is normally present in health to maintain the cleansing efflux of neutrophils into the gingival sulcus to maintain the low levels of bacteria, restricting them to the sulcular environment. The reasons for lower GCF chemokine levels during induction are unclear; it might represent an increased degradation of chemokines within the sulcus or a difference or shift in chemokine compartmentalization in tissue *versus* crevicular fluid. Darveau et al. (1998), demonstrated in culture systems that *Porphyromonas gingivalis* endotoxin was a potent suppressor of chemokine synthesis as compared with the stimulatory activity of enteric LPS species. This finding lead these investigators to suggest that *P. gingivalis* may be capable of disrupting the normal leucocyte efflux from the vascular bed into the gingival pocket where it engages in antimicrobial defenses causing neutrophilic retention and activation within the tissues, a process they referred to as ''chemokine paralysis''. In this study we demonstrate a significant decrease in multiple chemokines within the GCF during induction of gingivitis, including IL-8 (CXCL8), MIP-1β (CCL4), ENA-78 (CXCL5), MCP-1 (CCL2) and RANTES (CCL5), all of which are chemotactic for leucocytes. This suggests that the SIBO induces a transient, but reversible suppression of chemokines. Paradoxically, the timing of this observation of chemokine suppression is coincident with the anticipated histological evidence of leucocyte accumulation occurring within the tissues (Payne et al. 1975, Liu et al. 2001). In addition, in established, naturally-occurring gingivitis there are reports of higher levels of chemokine expression within the tissues and within the GCF, in synchrony with the increased leucocyte infiltration (Tsai et al. 1995). One possible explanation for these conflicting findings are that the chemokines are small peptides cleaved from larger precursor molecules by the action of specific proteases and, in turn, the

Table 5. Unadjusted change in mean log microbial counts (Day 21–Day 0) by inflammatory cluster

	IC1 ($n = 13$)	IC2 ($n = 6$)	IC3 ($n = 6$)	IC1 *p*-value	IC2 *p*-value	IC3 *p*-value
P. gingivalis	**1.78 (0.64)**	0.89 (0.60)	1.59 (0.76)	0.01	0.16	0.051
T. forsynthia	**1.85 (0.80)**	**1.73 (0.75)**	**2.06 (0.95)**	0.03	0.03	0.04
T. denticola	**2.91 (1.14)**	0.47 (1.06)	0.11 (1.34)	0.02	0.67	0.93
Red complex	**1.90 (0.56)**	0.76 (0.53)	0.43 (0.67)	0.004	0.17	0.53
P. intermedia	**2.45 (0.73)**	**1.93 (0.69)**	1.20 (0.87)	0.004	0.01	0.19
P. nigrescens	**3.01 (0.85)**	**2.29 (0.79)**	2.05 (1.00)	0.003	0.01	0.06
F. nucleatum	**2.26 (0.80)**	0.88 (0.75)	**2.10 (0.95)**	0.01	0.26	0.04
C. rectus	1.80 (0.91)	1.31 (0.85)	2.19 (1.07)	0.06	0.14	0.06
Orange complex	**2.41 (0.72)**	1.24 (0.67)	1.21 (0.85)	0.004	0.08	0.17
A. actinomycetemcomitans	**1.52 (0.67)**	0.55 (0.63)	**2.71 (0.79)**	0.04	0.39	0.003
A. viscosus	−0.63 (1.22)	1.20 (0.74)	0.50 (1.05)	0.62	0.13	0.65
C. ocracea	−0.50 (1.36)	−0.69 (1.27)	−0.03 (1.61)	0.72	0.60	0.98
E. corrodens	0.95 (1.13)	0.84 (1.06)	**3.13 (1.34)**	0.41	0.44	0.03
S. intermedius	1.94 (0.96)	0.03 (0.89)	0.98 (1.13)	0.06	0.97	0.40
S. noxia	0.20 (0.79)	**2.06 (0.56)**	0.46 (0.79)	0.80	0.003	0.57
S. oralis	**1.92 (0.88)**	0.41 (0.83)	1.47 (1.04)	0.04	0.63	0.18
V. parvula	0.84 (1.07)	0.34 (1.00)	1.92 (1.27)	0.44	0.74	0.15
Total counts	0.09 (0.34)	**0.87 (0.24)**	0.18 (0.34)	0.80	0.003	0.62

The mean log count change in microbial counts and standard error in parenthesis are shown for each organism and microbial complexes for the 15 organisms measured by DNA–DNA checkerboard using subgingival plaque sample collected at stent sites. Values reflect the change in organism level comparing the peak of gingivitis induction (Day 21) to baseline (Day 0) for each of the three inflammatory clusters. *p*-values reflect paired comparisons of change in specific microbial level at Day 21 vs. Day 0 for each inflammatory cluster. Bold font indicates *p*-values < 0.05, unadjusted for multiple comparisons.

chemokines themselves are degraded by specific proteases [e.g. CD26 (dipeptidyl-peptidase IV) cleaves RANTES and MIP-β (Proost et al. 2006). Thus, the dynamic transient shift in GCF levels and differential compartmentalization of chemokine levels could easily be a consequence of modulation of these proteases that regulate the level within the tissues and/or the GCF. The mechanism for the suppression of MMP-1, -3 and -13 within the GCF is also unclear, but increases in certain cytokines like TGF-β and insulin-like growth factor-1 are known to suppress these MMPs in a coordinated manner (Hui et al. 2001). Alternatively, it is possible that the inhibition in expression may be mediated by the levels of microRNA species which modulate the expression of MMPs by degrading mRNA transcripts. Serpin-E1, also known as the plasminogen activator inhibitor, was also suppressed during induction. This protease inhibitor produced principally by macrophages controls many proteolytic cascades including those involving thrombin and plasminogen and therefore has multiple biological activities including enhancing angiogenesis (Law et al. 2006). Interestingly, many of the chemokines that are transiently suppressed also promote angiogenesis, including IL-8, MIP-1b and ENA-78 (Keeley et al. 2008). Thus, the pattern suggests some transient inhibition of clotting and angiogenesis during this time of increased vasodilation and leucocyte diapedesis.

One strength of this study design is in the analytical method that enabled us to simultaneously measure multiple cytokines, MMPs and adipokines within each GCF sample to determine changes in mediator concentration at each site relative to each other. The assay system permitted the detection of 33 of the 34 mediators assayed even among healthy gingival sites at baseline. By collecting four GCF strips (two under the stent and two without stent) we could adjust for baseline and cross-arch differences over time such that we can access only those mediator changes which relate to the local changes in inflammatory signs. Most mediators (22/34 or 65%) did not change in concentration and only a third changed significantly (11/34 or 32%) during induction. Because fewer than two mediators would have been expected to change by random chance, assuming an α level of 0.05, and the likelihood of those same mediators also significantly decreasing during resolution is very small (i.e. 0.25% or one chance in 400), these observed changes do not appear to be random observations due to multiple comparison statistical testing issues. It was significant that the use of the Sonicare brush lead to a rapid and complete reversal of both the clinical signs and the biochemical mediators of inflammation from Days 21–49. Furthermore, all mediators that changed during induction also reversed to baseline following therapy coincident with the reversal of clinical gingivitis. This would suggest that these mediators are closely coupled to the changes observed in clinical signs and that these mediators are likely involved in maintaining the subtle homoeostatic tissue changes during reversible fluctuations in biofilm load.

One interesting finding of the study is that the underlying biological phenotype that results in gingivitis appears to differ among individuals. By biological or inflammatory phenotype we are referring to the patient-based cellular and molecular response to the biofilm overgrowth that ultimately results in changes in the clinical phenotype (gingivitis signs). To create an inflammatory phenotype we performed cluster analysis based upon changes in GCF mediators during induction, which resulted in creating three differing inflammatory phenotypes that each resulted in very similar, but not identical, clinical outcomes. Despite the small sample size in these ICs (IC1 = 13, IC2 = 6 and IC3 = 6) there are a few descriptive observations that are noteworthy. First, in the IC1 group, induction was characterized by significant increases in IL-1α, IL-1β, IL-6, MMP-8 and MMP-9 with a significant decrease in MIP-1β. Interestingly, among the six mediators that are significantly increased in this IC only IL-1α and Il-1β are significant for the entire group of 25 subjects. It is intriguing to note that IL-6 is increased and that this response is restricted to only IC1, because IL-6 is a key mediator that orchestrates the transition from acute to chronic inflammation and is elevated in periodontitis (1). Furthermore, in addition to IL-6, increases in MMP-8 and MMP-9 (as well as the non-significant trend for an increase in PGE_2 that is seen in IC1) have all been associated with periodontitis (Okada & Murakami 1998, Soder et al. 2006). Thus, the inflammatory process among IC1 subjects not only differs from the gingivitis clusters IC2 and IC3, but also

has an inflammatory response that appears to be more similar to periodontitis. This raises an interesting possibility that this gingivitis IC may be more susceptible to developing periodontitis than IC2 or IC3. The IC2 group has a suppressive mediator response with decreases in MIP-1β, MMP-1, MMP-3 and a range of adipokine suppression that included Complement-D, resistin and serpin-E1. It is interesting that this group also had a trend for having a higher body mass index (BMI) than the other groups (IC1 = 29.3, IC2 = 34.8, IC3 = 26.1 (No significant differences)). Previous investigations have shown that obesity is significantly associated with increased gingival inflammation (Offenbacher et al. 2007, Andriankaja et al. 2009). The IC3 group has both increases in IL-1α and IL-1β, like IC1, but has a wider range of chemokine suppression including IL-8, MCP-1, MIP-1β, as well as a different MMP suppression profile. Caution must be exercised in interpreting these data, as the sample size within each cluster is too small to enable generalization. Similarly, the sample size is too small to suggest that the overgrowth of total Red and Orange complex bacteria as seen in IC1 is associated specifically with increases in IL-1α, IL-1 β or IL-6, or that the emergence of *A. actinomycetemcomitans* and *E. corrodens* observed in IC3 is associated with the specific changes in cytokines seen in this group, as cited above. It does, however, point to the potential problems in interpreting these complex datasets with multiple mediators and complex microbial changes. Pooling all subjects, as if their inflammatory responses were a homogeneous trait, can lead to incorrect conclusions, as one seeks to understand the underlying biological response structure that determines disease for different individuals. Cellular and molecular pathways are tightly integrated and coordinated with feedback that serves to reinforce specific responses while dampening others. When all patients are pooled, many of these different pathways have the potential to cancel out or regress to the mean. For example, some mediator responses occur only in one cluster (like IL-6 or MMP-13), whereas some mediators were significant overall (like ENA-78), but not within any of the clusters. Admittedly, the small sample size limits the generalizability of the findings, but the data support the concept that clustering may be a helpful tool when applied to larger populations to gain insight into the types of inflammatory phenotypes that result in disease. As a corollary, considering all subjects as having similar inflammatory responses would appear to oversimplify the nature of the host response to biofilm overgrowth. Furthermore, considering the dynamics and the biology of the biofilm-gingival interface, these clustering methods suggest subcategories that reflect patient-specific profiles of emergent bacterial overgrowth which is coupled to a specific cytokine response pattern.

In summary, these data support the concept that dynamic fluctuations in IL-1α and IL-1β represent a significant transient and reversible mediator response that co-varies with changes in clinical signs during the induction and resolution of gingivitis in most subjects. The induction of gingivitis is associated with a suppression of chemokines within the GCF, especially IL-8 and MIP-1β. During the induction of gingivitis in the SIBO model, subsets of discrete inflammatory phenotypes appear to be present within the subject population that are each capable of resulting in similar clinical presentations of disease via differing molecular pathways that are accompanied by differential shifts in microbial composition. Further studies of candidate biomarkers and of ICs will be necessary to confirm these observations.

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Clinical Relevance

Scientific rationale for the study: The underlying molecular response associated with the induction and reversal of experimental gingivitis is not fully understood.

Principal findings: A new stent-induced gingivitis model that creates unilateral experimental gingivitis induces changes in levels of GCF biomarkers and clinical signs that are both fully reversible by power toothbrush treatment.

Practical implications: The resolution of clinical gingivitis was associated with a reversal in specific molecular mediators of inflammation that was altered during the induction phase. However, the pattern of biomarker expression during induction and resolution varied considerably among subjects with similar clinical responses.