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Comparison of experimental gingivitis with persistent gingivitis: differences in clinical parameters and cytokine concentrations

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Background and Objective: Experimental gingivitis has been studied extensively as a well-controlled laboratory model of gingivitis. It is unclear, however, how experimental gingivitis compares with persistent plaque and gingivitis in more naturalistic settings. The present study compares both conditions in a randomized controlled design.

Material and Methods: Twenty-six students suffering from plaque and gingivitis were randomly assigned to either a persistent gingivitis or an experimental gingivitis condition. Subjects with persistent gingivitis continued their habitual (i.e. insufficient) oral hygiene behaviour, resulting in persistence of plaque and gingivitis. Experimental gingivitis consisted of initial prophylaxis and subsequent total neglect of oral hygiene. Crevicular interleukin-1β and interleukin-8 and clinical data were assessed weekly.

Results: After 4 wk, subjects with experimental gingivitis showed significantly more plaque accumulation ($p = 0.005$), higher interleukin-1β ($p = 0.037$), and lower interleukin-8 ($p = 0.043$) concentrations than subjects with persistent gingivitis. Whereas in experimental gingivitis we observed considerable fluctuations in clinical and immunological parameters over the 4-wk period, persistent gingivitis was characterized by little fluctuation, indicating that we were monitoring an inflammatory steady state.

Conclusion: The data indicate that conditions observed after 4 wk of experimental gingivitis are not comparable with persistent gingival inflammation in a naturalistic setting. Results are discussed with respect to current studies, indicating that chronic inflammation may reflect a stage of down-regulated pro-inflammatory response.

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The inflammatory host response is a core aspect of periodontal disease. It is this response, rather than toxic products from pathogenic bacteria, which is thought to be the immediate cause for periodontal breakdown. Therefore, during the last few years basic and clinical periodontal research have focused on the host immune response to periodontal pathogens.

One model studied extensively in this context is the experimental gingivitis model. In this model, perfect oral cleanliness and gingival health is established first; subsequently, gingivitis is induced by neglect of oral hygiene. Originally developed more than 40 years ago by Löe *et al*. (1), an

increasing number of researchers, including those in our group, decided to employ the experimental gingivitis model to achieve a better understanding of the host's immune response to periodontal bacteria under certain conditions (2–12); experimental gingivitis is thus a well-controlled and often-analysed condition increasing our understanding of the host's response to undisturbed plaque accumulation.

To what degree, however, does the experimental gingivitis model compare to more naturalistic conditions of persistent plaque and gingivitis? Is the immune response observed after 28 d of experimental gingivitis similar to what we observe in persistent gingivitis? As it is persistent gingivitis, rather than experimental gingivitis, which precedes chronic periodontitis, such information might help to integrate findings better from experimental gingivitis studies.

The present study thereby compared experimental gingivitis and persistent gingivitis in a randomized controlled trial. Both clinical signs of gingivitis and pro-inflammatory cytokines (interleukin-1β and interleukin-8) in gingival crevicular fluid were assessed weekly, for a period of 4 wk, in either experimental gingivitis or persistent gingivitis.

Materials and methods

Subjects

Subjects were 13 female and 13 male students (age 25.3 ± 5.5 years). They were recruited by internet-announcement on the homepage of the University and by flyers distributed throughout the campus; for participation they received monetary compensation. Subjects were included in the study when they showed clinical signs of plaque-induced gingivitis (i.e. bleeding on probing and visible plaque at adjacent surfaces) in at least five of six papillae under study.

Exclusion criteria applied as a result of potential impacts on gingival health, or because of ethical reasons, were as follows: any clinical signs of periodontitis at any tooth (pocket depth of more than 3.5 mm at any of six sites per tooth or clinical attachment loss); current orthodontic or dental treatments; untreated caries; defect fillings; diseases of the immune system; infections of any kind; acute allergic responses (e.g. acute hay fever); any history of neurological of psychiatric disease; nicotine consumption of more than five cigarettes per day or drug abuse; pregnancy; regular use of any medication, including calcium-antagonists, anticonvulsives, immunostimulants or immuno-suppressives prior to the study; use of any of these substances from 1 wk prior to the study until the end of the study; and/or use of antibiotics or antipyretics within a period of 6 wk prior to until the end of the study. Furthermore, we also excluded students who reported any type of enduring strain (e.g. preparation for major examinations) within 2 wk prior to the study until its end.

Independent variable: experimental vs. persistent gingivitis

When included in the study, all students suffered from gingivitis in at least five of six papillae under study (see above). Students were then randomly assigned to either an experimental gingivitis group or a persistent gingivitis group.

Experimental gingivitis —In this group, gingival health had to be established first. Therefore, this group received a prophylactic intervention at least 1 wk prior to study onset: plaque was removed and participants were instructed on how to maintain perfect oral hygiene. Compliance in this instruction was controlled twice a week when bleeding on probing was assessed at all 16 sites under study. Hygiene instruction was repeated and visible plaque removed when necessary. Students were not allowed to participate further when more than one positive bleeding response was observed at the beginning of the study. At the beginning of the experimental gingivitis period, students were instructed to refrain from any oral hygiene procedures for a period of 4 wk. Compliance in this instruction was controlled twice a week. The front teeth were cleaned at these appointments by the dentist to make the experimental condition indiscernible to any naïve person.

Persistent gingivitis —This group represented the common clinical condition of naturally occurring untreated plaque-associated persistent gingivitis. Thus, no alteration of gingival health was intended. Therefore, participants were instructed just to proceed with their regular oral health behavior. All other treatments (including control appointments) were similar to those of the experimental gingivitis group, beside the fact that within this group no tooth cleaning was provided by the dentist at any time.

Dependent variables

Sixteen sites (i.e. the mesial and distal aspects of teeth 5 and 6 of the two upper quadrants) were studied. At these sites, plaque and bleeding and crevicular interleukin-1β and interleukin-8 were assessed weekly. In all cases, gingival crevicular fluid sampling took place prior to assessment of clinical parameters.

Plaque and bleeding

In order to control the clinical condition of persistent gingivitis, we assessed clinical signs of gingivitis [by using the papillary bleeding index of Saxer & Mühlemann, modified by Rateitschak *et al*. (13)] and visible plaque [by using the plaque index of Sillness & Löe, modified by Rateitschak *et al*. (13)] at all 16 sites where gingival crevicular fluid samples were taken; plaque index scores of 0 and 1 were put into one category, as degrees of 1 are only visible after gentle scaling resulting in plaque displacement, which was not intended in this study. All assessments were performed by a trained examiner previously calibrated to another person, with inter-rater accordances of $> 90\%$.

Crevicular interleukin-1β and interleukin-8

Gingival crevicular fluid collection was performed as described in detail previously (3,11). Prior to gingival

crevicular fluid collection, the respective teeth were dried to prevent salivary contamination of the samples. Small paperstrips (Periopaper; Harco, New York, NY, USA) were then placed in the gingival crevice at four sites per tooth (distovestibular, mesiovestibular, distopalatinal, and mesiopalatinal) for 30 s. The volume sampled with each paper was then determined by a previously calibrated Periotron 8000 (Harco). Immediately afterwards, papers were put into a micro test tube kept on ice and containing 800 μL of phosphate-buffered saline plus 1% bovine albumin. When all 16 papers were in the tube it was centrifuged for 5 min. After centrifugation, aliquots of the supernatants were shock-frozen in CO_2 and stored at −80°C until assayed. Analyses for human interleukin-1β and interleukin-8 were performed by commercially available sandwich enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions, with inter- and intra-assay variations coefficients below 10% and a sensitivity of < 1 pg/mL (standard curve: 0–400 pg/ml; Endogen, Cambridge, MA, USA). Cytokine data were reported as standard curve units (ng/mL).

Procedure

At least 1 wk prior to starting the study, patients were randomized to the experimental gingivitis group or the persistent gingivitis group. Randomization was performed by a person not involved in data assessment by drawing a sealed opaque envelope out of a bundle of identical envelopes which contained the respective experimental conditions.

Pretreatments were then accomplished according to the respective condition (see above). On day 0, gingival crevicular fluid samples were taken for the first time. Subjects from the experimental gingivitis group were then instructed to refrain from any oral hygiene procedures, whereas subjects in the persistent gingivitis group were instructed to maintain their regular oral hygiene procedures. From that day on, weekly gingival crevicular fluid samples and clinical assessments took place (appointments were arranged in a way that all assessments were performed within 6–8 d after the last assessment). Within one subject, time of the day of assessments was kept constant (± 30 min). All assessments were performed between 08:00 h and 18:00 h. Treatment groups (experimental gingivitis vs. persistent gingivitis) and gender groups (male vs. female) were stratified with respect to morning and afternoon appointments.

Ethics

The study design was approved by the local ethical committee and conforms with the guidelines of the World Health Organization (Declaration of Helsinki).

Statistical analyses

To answer the research question (To what degree do the two models compare after 4 wk of experimental gingivitis?) groups were compared with respect to clinical and immunological parameters at this point in time; while differences at earlier time points would not be surprising, we expected the two conditions to bear more resemblance at the end of the experimental gingivitis period. We report two-tailed *t*-tests for independent groups to test the hypothesis that groups would differ. The intended level of significance was $\alpha = 5\%$. When Levene's test indicated variance differences ($p > 0.10$), the *t*-test for unequal variances are reported along with corrected degrees of freedom. Effect sizes *d* for univariate group comparisons are given along with hypothesis tests; *d* is computed as the mean difference between groups divided by the common standard deviation of the groups. According to Cohen (14), effects sizes of $d = 0.2$, $d = 0.5$, and $d = 0.8$ are considered to be small, medium, and large, respectively.

To control for possible gender differences, further *t*-tests were computed to compare male and female participants within one treatment group.

In some cases, measures had no variance within one group under analysis. In these cases, nonparametric group comparisons with exact *p*-values were computed.

To assess temporal stability of clinical and immune parameters during experimental gingivitis and persistent gingivitis we ran repeated-measures analysis of variance within each study group; results of these analyses are reported as greenhouse-geisser corrected values along with original degrees of freedom, greenhouse-geisser ϵ and parital η^2 as indicator of effect size.

Results

The inclusion criterion (gingivitis in at least five of the six papillae under study) and the randomization procedure resulted in complete comparability of both groups with respect to number of sites and severity of plaque and bleeding (all $p > 0.80$) prior to randomization. All but one person of each group were complete non-smokers. The two smokers reported smoking fewer than three cigarettes a day. Exclusion of these two participants did not change any of the statistics. Therefore, the data reported in the following include the two smokers.

The course of gingivitis during the 4-wk measurement period is shown in Figs 1, 2, and 3 for clinical parameters, crevicular interleukin-1β and crevicular interleukin-8, respectively.

Group comparisons after 4 wk

No gender differences were found in any of the measures analysed (all $p > 0.28$), besides severe bleeding responses in the persistent gingivitis group (see below).

Groups did not differ with respect to number of sites with bleeding responses [$d = 0.38$ t(24) $= 0.959$; $p = 0.347$). Papillary bleeding index values of 3 and 4 (i.e. interdental triangle filled with blood or profuse bleeding) were found in one male and two female subjects of the experimental gingivitis group and in no male and five female subjects of the persistent gingivitis group (gender difference within the persistent gingivitis group: exact $p = 0.005$); this resulted in a nearly significant difference between gingivitis groups within women (exact $p = 0.076$), but not within men (exact $p = 0.462$).

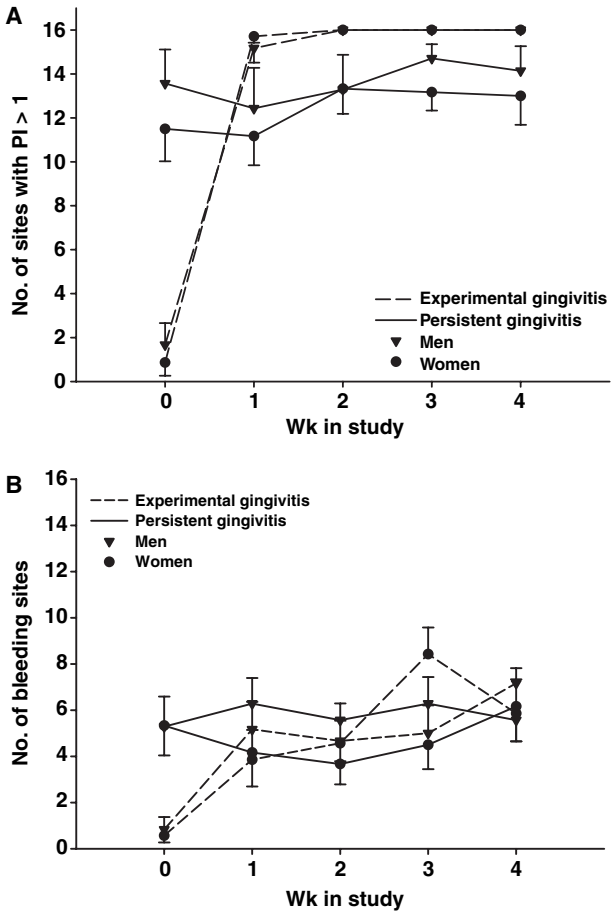

Fig. 1. Plaque index and bleeding response in experimental and persistent gingivitis. Mean and standard error of the mean of the number of sites showing plaque indices above 1 (A), and a positive bleeding response (B), are shown for the 16 sites where gingival crevicular fluid samples were taken. Subjects in the experimental gingivitis group (six men, seven women) refrained from any oral hygiene for 4 wk, whereas subjects in the persistent gingivitis group (seven men, six women) proceeded with their habitual oral hygiene behaviour. PI, plaque index.

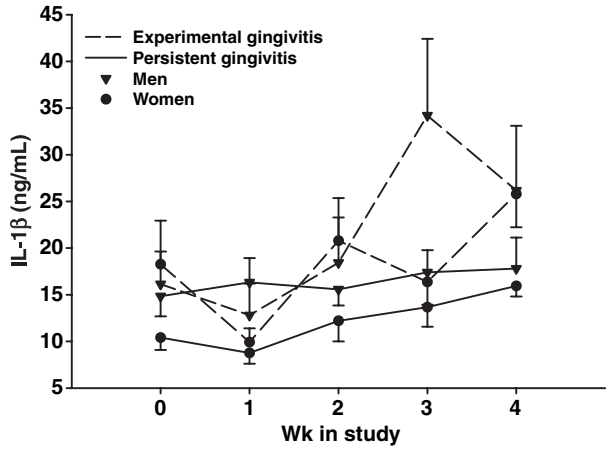

Fig. 2. Crevicular interleukin-1β in experimental and persistent gingivitis. Mean and standard error of the mean of standard curve units (ng/mL) are shown for the 16 sites under study. Subjects in the experimental gingivitis group (six men, seven women) refrained from any oral hygiene for 4 wk, whereas subjects in the persistent gingivitis group (seven men, six women) proceeded with their habitual oral hygiene behaviour. IL-1β, interleukin-1β.

Plaque accumulation, of degree 2 and 3, was observed at all 16 sites of all participants of the experimental gingivitis condition. Within the persistent gingivitis group, a mean of 13.62 sites (standard deviation ± 3.01) was affected. This resulted in a significant difference between gingivitis groups (exact $p = 0.005$).

With respect to interleukin-1β, significant group differences were observed [$d = 0.89$; $t(17.8) = 2.258$; $p = 0.037$]; mean concentrations in the experimental gingivitis group (25.96 ± 12.83 ng/mL) were higher than those in the persistent gingivitis group (16.94 ± 6.55 ng/mL).

A significant group difference was also observed for interleukin-8 [$d = 0.84$; $t(24) = 2.137$; $p = 0.043$], where the mean concentration in the experimental gingivitis group (54.32 ± 27.30 ng/mL) was lower than that of the persistent gingivitis group (84.61 ± 43.20 ng/mL).

Effects of time on clinical and immune parameters under experimental gingivitis and persistent gingivitis conditions

To assess whether parameter means varied over time, repeated-measures analysis of variance over the 4-wk study period were computed for the experimental gingivitis and persistent gingivitis groups, respectively. Gender-by-time analyses revealed neither significant main effects of gender nor significant gender-by-time interactions (all $p > 0.07$). Thus, data of both genders were pooled for further analyses of time effects.

Within experimental gingivitis, significant effects of time were observed for number of sites with bleeding [$\epsilon = 0.30$; $F(4/48) = 12.843$; $p < 0.001$; $\eta^2 = 0{,}51$], interleukin-1β [$\epsilon = 0.65$; $F(4/48) = 4{,}635$; $p = 0.011$; $\eta^2 = 0{,}28$], and interleukin-8 [$\epsilon = 0.36$; $F(4/48) = 15{,}095$; $p < 0.001$; $\eta^2 = 0.56$]. For experimental gingivitis subjects, no analysis of variance was computed for number of sites with plaque from week 3 onwards because this parameter showed no variance (see Fig. 1).

No time effects were observed regarding persistent gingivitis, for pla-

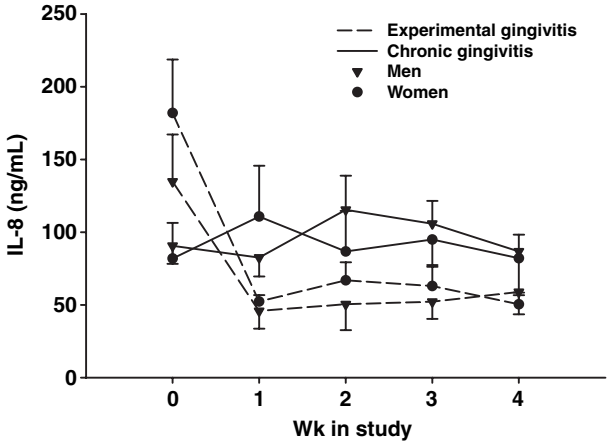

Fig. 3. Crevicular interleukin-8 in experimental and persistent gingivitis. Mean and standard error of the mean of standard curve units (ng/mL) are shown for the 16 sites under study. Subjects in the experimental gingivitis group (six men, seven women) refrained from any oral hygiene for 4 wk, whereas subjects in the persistent gingivitis group (seven men, six women) proceeded with their habitual oral hygiene behaviour. IL-8, interleukin-8.

que [$\epsilon = 0.63$; $F(4/48) = 1{,}295$; $p = 0.292$; $\eta^2 = 0{,}10$), bleeding [$\epsilon = 0.63$; $F(4/48) = 0{,}407$; $p = 0.714$; $\eta^2 = 0{,}03$], interleukin-1β [$\epsilon = 0.65$; $F(4/48) = 2{,}345$; $p = 0.100$; $\eta^2 = 0{,}16$), or interleukin-8 [$\epsilon = 0.70$; $F(4/48) = 0{,}848$; $p = 0.471$; $\eta^2 = 0{,}07$].

Discussion

The present study compared experimental gingivitis with persistent gingivitis in a randomized controlled design. Although both conditions reflect the host's response to plaque bacteria, they differ in at least three major aspects. First, experimental gingivitis is preceded by a prophylactic intervention, which might affect the host's response to subsequently accumulating plaque bacteria. Second, during experimental gingivitis, all oral hygiene procedures are abolished at the sites under study. Patients suffering from persistent gingivitis, in contrast, do show at least some oral hygiene behavior; even though this behavior is insufficient with respect to plaque removal, it still differs from complete neglect of oral hygiene. Third, under experimental gingivitis, plaque is not allowed to accumulate for more than 28 d. To avoid irreversible damage, oral hygiene has to be re-established after this time. In clinically observed persistent gingivitis, insufficient oral hygiene of the patients corresponds with much longer periods of plaque persistence and gingivitis.

These experimental conditions resulted in considerable clinical and immunological group differences. After 4 wk of experimental gingivitis, we observed more plaque accumulation and, at least in women, tentatively less severe bleeding responses than in untreated persistent gingivitis. Furthermore, when compared with persistent gingivitis, subjects of the experimental gingivitis group showed increased crevicular interleukin-1β and reduced interleukin-8 concentrations. While one would not have expected the two conditions to be similar at the beginning of the experimental gingivitis period, it is important to realize that after 4 wk of plaque persistence the two conditions are still not comparable. The commonly used experimental gingivitis design, employed here, as previously (5–9), thereby obviously does not reflect conditions of persistent gingivitis as observed in clinical practice. As the direction of group differences varied between measures, our findings further indicate that the two conditions differ qualitatively, rather than just quantitatively.

It is not plausible to assume that factors other than the differing experimental conditions result in the group differences reported here: application of inclusion and exclusion criteria led to a homogenous group of patients, with a number of potential confounders remaining constant (e.g. number of sites with gingivitis, no periodontal disease, age, education, smoking status, systemic disease, psychosocial stress); potential further confounders were controlled by random assignment to the study groups; additionally, group comparisons prove that no *a priori* differences existed with respect to plaque and gingivitis.

As expected from results of earlier studies, considerable fluctuations with respect to plaque, bleeding, interleukin-1β, and interleukin-8 were observed under the experimental gingivitis condition (2–4,6–9,11,12). Interestingly, however, interleukin-8 seems to stabilize rapidly at a low level after markedly increased concentrations at the first measurement. This replicates results from the only other study we know on experimental gingivitis effects on crevicular interleukin-8 concentrations. In this study, which analyzed a 3-d experimental gingivitis period, a 60% decline of interleukin-8 was found from day 0 to day 3 (12). These results indicate that plaque *per se* may induce an interleukin-8 down-regulation. One recent *in vitro* study supports this notion. In that study, human gingival fibroblasts produced less interleukin-8 when cultured together with capsula polysaccharide of *Actinobacillus actinomycetemcomitans* (15). Interestingly, down-regulation of interleukin-8 is currently discussed to be one important pathogenic factor in chronic inflammatory diseases, such as helicobacter-associated duodenal ulcer (16) and Crohn's disease (17). These authors speculate that the down-regulation predisposes to an enhanced pro-inflammatory response in the following when a certain level of inflammatory challenge is exceeded. Such an exaggerated interleukin-8 release has indeed been observed in periodontitis patients compared with periodontally healthy controls (18). This might be considered to be some support to the hypothesis that periodontal breakdown occurs in the context of a sudden boost of a formerly down-regulated pro-inflammatory response. While these notions

are still speculative, they underline how important it would be to analyse the course of plaque-associated immune responses to reach a better understanding of the progression of periodontal disease. Clearly, our study indicates that a 4-wk experimental gingivitis period is far too short to understand what is happening under more chronic conditions.

Within the persistent gingivitis condition, no significant fluctuations were observed over time. Thereby, persistent plaque and gingivitis can be described as an immunological and clinical steady state of the inflammatory response. As discussed above, this state of inflammation seems to be characterized by a comparable moderate and stable pro-inflammatory activity. From clinical observations we also know that this steady state sometimes, and often unforeseeably, becomes disturbed, resulting in an exaggerated inflammatory response and perhaps even periodontal breakdown. One of the major challenges of periodontal research is to find out which factors promote such fluctuations. One way to find an answer to this question are by prospective studies, lasting much longer than the present study and assessing not only the inflammatory condition but also factors discussed to be promoters of an exaggeration. Such studies would enable us to see which alterations precede an exacerbation of disease. In a series of studies, our group found that psychological stress might be such a factor as it induces an increase of pro-inflammatory cytokines in gingival crevicular fluid (2–4).

To our knowledge, this is the first study to compare cytokine concentrations in experimental gingivitis with persistent gingivitis conditions in a randomized controlled design. Furthermore, we do not know any study performing such a controlled comparison with respect to clinical parameters. Early papers (19,20), though analysing gingivitis in humans over a long period of time, provide no information about the comparability of persistent gingivitis and experimental gingivitis because they study only one of the two conditions.

Even though our study has some important merits in that it is the first to compare the course of experimental and more persistent gingivitis in a randomized controlled trial, it also has some restrictions, which should be discussed. First, we have no precise information on the duration of plaque persistence in our patients. All patients suffered from plaque-associated gingivitis when they were included in the study. The last gingival crevicular fluid sampling was taken 5 wk after inclusion in the study. Therefore, we know that plaque and gingivitis in subjects with persistent gingivitis persisted for at least 5 wk. It is, however, more than probable that we were analysing a condition that persisted for a much longer period of time as it would be unrealistic to assume that plaque and gingivitis just happened to occur a few days prior to inclusion in the study. Yet, we have no clear data on that. Another restriction is the representativeness of our patient sample. Our patients were students, suffering from only moderate plaque accumulation and gingivitis. It is a subject of further studies to analyse whether comparable effects would be seen in older patients with plaque and gingivitis of greater severity. Finally, a period of 7 d after prophylaxis might have been too short to re-establish immunological conditions representing full and persistent gingival health in experimental gingivitis subjects, even though clinical health had been re-established. Indeed, future studies should analyse immunological conditions under persistent oral hygiene. This would help us to improve further our understanding of the experimental gingivitis paradigm and its representativeness for other clinical conditions.

In conclusion, the present study clearly shows that subjects in different gingivitis conditions (i.e. experimental gingivitis and persistent gingivitis) do not compare with respect to clinical parameters, interleukin-1β, and interleukin-8. It further shows that persistent gingivitis seems to be a type of immunological and clinical steady state. Further analyses of the characteristics of this steady state, and of the conditions preceding its disturbance, may help us to understand better the pathogenesis of periodontal disease and of other diseases showing similar immunological characteristics (e.g. Crohn's disease and duodenal ulcer).

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