

# Lactoferrin in Gingival Crevicular Fluid and Peripheral Blood during Experimental Gingivitis

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## ABSTRACT

**Objectives:** Lactoferrin (LF) is an iron binding protein and stored in the specific granules of granulocytes. It is released by degranulation following granulocyte activation. A positive correlation was previously reported between periodontitis and LF titers of gingival crevicular fluid (GCF) and blood. The purpose of this study was to examine alterations of GCF and blood levels of LF (LF-GCF and LF-BL, respectively), employing the experimental gingivitis model.

**Methods:** Twelve systemically healthy volunteers, aged 19-21, were selected. Pre-experimental phase of hygiene was followed by a 14-day experimental gingivitis phase in which subjects refrained from all oral hygiene procedures. After that subjects resumed optimal plaque control for 21 days of recovery period. At days 0 (baseline), 14 and 35 gingival crevicular fluid (GCF) and blood samples were collected and plaque index (PI), gingival index (GI), bleeding on probing (BOP) and probing pocket depth scores were recorded. LF levels were measured with commercial enzyme-linked immunosorbent assay (ELISA) kit.

**Results:** PI, GI, BOP and LF-GCF increased significantly after 14 days of experimental gingivitis period and decreased significantly after reinstatement of oral hygiene measures ( $P<.05$ ). LF-BL appeared to follow the same pattern. Significant negative correlation was detected between the level of LF-BL and BOP at day 14 ( $P<.05$ ), whereas significant positive correlation was noticed between LF-BL and clinical scores PI, GI and BOP at day 35 ( $P<.05$ ).

**Conclusions:** LF-BL followed the same pattern with LF-GCF and clinical scores during the experimental gingivitis and recovery periods, although alterations of the LF-BL appeared statistically insignificant. (Eur J Dent 2009;3:16-23)

**Key words:** Lactoferrin; Gingival crevicular fluid; Gingivitis; Peripheral blood; Periodontal index.

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## INTRODUCTION

The periodontal diseases has complex etiology and has been proposed that it is the host response to the long-term bacterial challenge, including increased neutrophil response and the release of proinflammatory mediators, which may be related with initiation and exacerbation of the systemic diseases and conditions. When compared to periodontally healthy subjects, significantly higher plasma white cell counts has been reported for subjects with gingivitis and periodontitis, and suggested that those high white cell counts might be related with systemic diseases such as myocardial infarction.<sup>1</sup> Neutrophilic granulocytes play an important role at the primary defense of the host against pathogens and immunogenic materials.<sup>2</sup> They are reported to be present in increased numbers within the gingival pocket and to be the predominant leukocytes in the pocket epithelium and adjacent connective tissue in periodontal diseases.<sup>3-5</sup>

Lactoferrin (LF), an iron binding protein with some antibacterial properties is stored in the specific (secondary) granule of the granulocytes and mainly released during migration and influences granulocyte functions such as adhesion and chemotaxis.<sup>3,6</sup> LF has been proposed as a marker of the number of granulocytes.<sup>7</sup> Following the activation of neutrophils, degranulation of specific granules with an instantaneous release of LF, has been demonstrated previously.<sup>6,7</sup> LF titers in various body fluids, including sputum and blood, were reported to be correlated with the presence of inflammation, such as periodontitis.<sup>8</sup> Significantly higher levels of LF-GCF have been found at both periodontitis and gingivitis compared to healthy sites and those titers also reported to be decreased to the healthy sites' LF levels after periodontal treatment.<sup>9,10</sup>

The human experimental gingivitis study model, introduced by Löe et al,<sup>4</sup> has shown that qualitative effect of dental plaque created by total withdrawal of oral hygiene procedures leads to gingival inflammation and since this pioneering work of Löe et al, many studies have been undertaken using experimental gingivitis study model.<sup>11-14</sup>

The purpose of our present study was to examine the alterations of LF levels in GCF and peripheral venous blood, resulting from the bacterial plaque

accumulation in a healthy mouth, employing the experimental gingivitis model, in order to examine whether local gingival inflammation has some systemic influence.

## MATERIALS AND METHODS

### Patient selection

Twelve dental students attending Gazi University Faculty of Dentistry was recruited voluntarily. This participant group consisted of all systemically healthy males in age ranging from 19 to 21 years. They were selected on the presence of 24 teeth or more, absence of pockets  $\geq 5$  mm and absence of approximal attachment loss.<sup>15,16</sup> No subject wore any orthodontic or prosthetic appliances.<sup>17</sup> The use of any medication that might interfere with the outcome of the study, during the past 6 months, was an exclusion criterion, such as anti-inflammatory agents, antibiotics, and immunosuppressive drugs. All of the participants were non-smokers. The study protocol was approved by Institutional Review Board at Gazi University, School of Medicine and all subjects were asked to give an informed written consent to participate, after a thorough explanation of the procedures and objectives of the study.

### Experimental design

Experimental design of the study consisted of 3 parts as pre-experimental, experimental and recovery periods (Figure 1):

- Pre-experimental period: Two weeks prior to the experimental period participants received a thorough dental prophylaxis and instruction in oral hygiene. During the following week professional tooth cleaning was repeated. Participants were regularly examined and their oral hygiene was monitored to achieve maximum gingival health. When participants had less than 20% approximal bleeding sites, they entered a 14 day experimental gingivitis trial period.<sup>15,16</sup>
- Experimental period: Examiners made sure of the participants gingival health at the baseline of this period via clinical indices, then participants were led to enter this period by cessation of all plaque control measures.<sup>15,16</sup> At day 0 (baseline) and 14 clinical parameters were recorded, and peripheral venous blood and GCF samples were taken.

- Recovery period: After 14 day experimental period without any oral hygiene measures, participants received professional prophylaxis, instruction in oral hygiene and they were encouraged to return their daily oral hygiene measures. The professional tooth cleaning was repeated and periodontal condition regularly examined in order to achieve maximum gingival health during this 21-day recovery period.<sup>5</sup> At the end of the 21 day recovery period clinical indices were repeated and peripheral venous blood and GCF samples were taken.

### Periodontal examination

Following clinical parameters were recorded for monitoring the gingival situation of the participants before and during the experimental period and control period: Plaque index (PI),<sup>18</sup> gingival index (GI),<sup>19</sup> bleeding on probing (BOP)<sup>20</sup> and probing pocket depth (PD). All clinical parameters were measured with a William's probe calibrated in millimeters.

### GCF sampling

For collection of GCF, prefabricated sterile paper strips (Periopaper® GCF Strips, Proflow, Amityville, NY) were used. The sites to be sampled

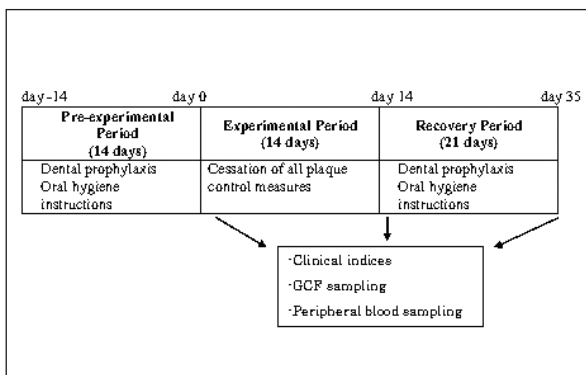


Figure 1. Experimental design of the study.

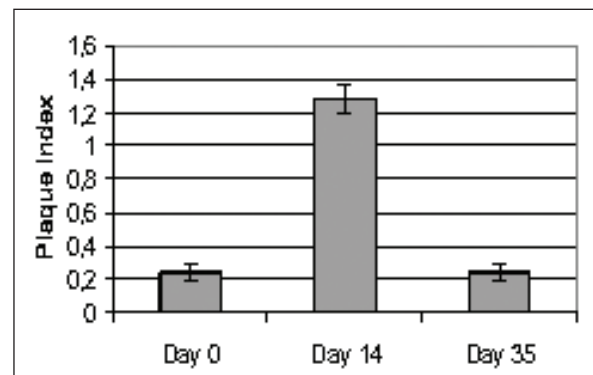


Figure 2. Plaque index (\*: P<.05).

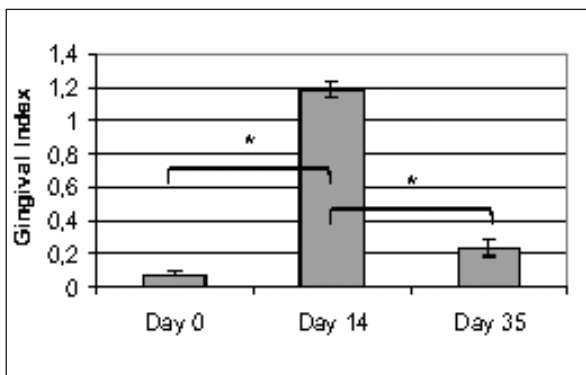


Figure 3. Gingival Index (\*: P<.05).

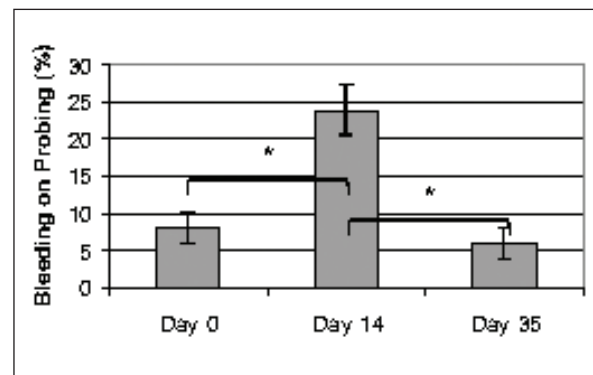


Figure 4. Bleeding on probing (\*: P<.05).

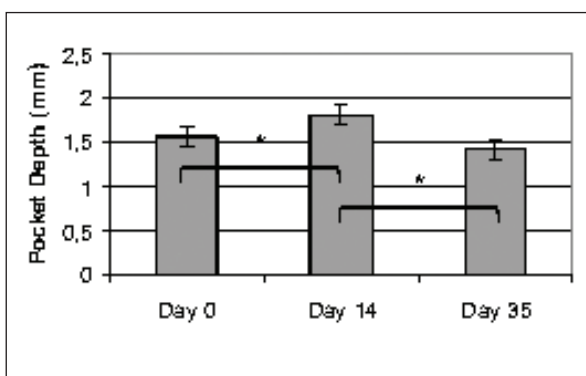


Figure 5. Pocket depth.

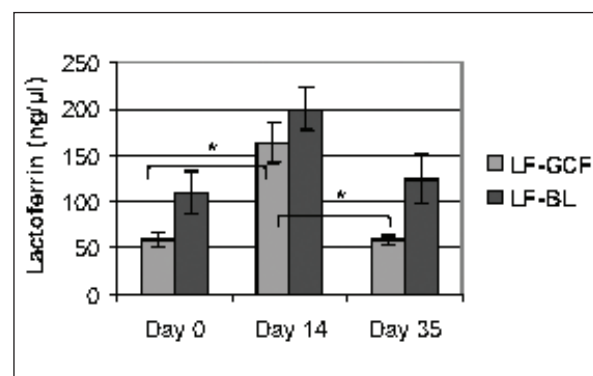


Figure 6. The levels of lactoferrin in GCF (LF-GCF) and blood (LF-BL) (\*: P<.05).

were isolated with cotton rolls and visible supragingival plaque was removed carefully by cotton pellets. The area was gently air-dried in an apico-coronal direction and 30 sec. later paper strips were inserted into the gingival crevice until mild resistance was felt and left there for 30 sec.<sup>21</sup> Strips contaminated by dental plaque, saliva, bleeding or exudates were discarded. Strips were stored in a labeled microcentrifuge tube and frozen at -70°C until further processing. Prior to assaying strips were eluted into 300 µl of 20mM phosphate buffer, pH 6.0, containing 0.15 M NaCl and 0.1% Tween 20.<sup>22</sup>

#### Blood sampling

Six milliliters of peripheral venous blood was drawn into glass test tubes that contain EDTA as an anticoagulant. After the separation of plasma, the samples were stored at -70°C until further processing.

#### Lactoferrin levels

LF levels were determined by a commercial ELISA kit (Calbiochem, Darmstad, Germany) as described in the manufacturer's instructions. Different concentrations of LF (100, 50, 25, 12.5, 6.2, 3.1, 1.6 ng/µl) were prepared by the dilution of lyophilized LF standard, supplied by the manufacturer. The absorbance values (Optical Densities=OD) were measured spectrophotometrically at a wavelength of 450 nm, and the results were evaluated quantitatively according to the ODs of standard LF concentrations. If the measured concentrations of LF in a sample were greater than 100 ng/ml, the sample was re-tested by using further dilutions (1/100).

#### Statistical analysis

The clinical parameters, LF-GCF and LF-BL were expressed as mean±standard error. Repeated measurement variance analysis (ANOVA) was used for assessment of the data obtained at day 0, 14 and 35.<sup>23</sup> The arc-sinus transformation was used for ANOVA assessments of BOP data. Bonferroni test was used for determination of different groups. The Pearson test was used for correlation assessments.<sup>23</sup>

## RESULTS

### Clinical parameters

The mean clinical parameters and their standard errors are summarized in Figures 2-5. PI, GI and BOP scores showed statistically significant increase during the experimental period of 14 days and significant decrease thereafter, as expected ( $P<.01$ ). Pearson correlation and P values are summarized at Tables 1 and 2.

### Lactoferrin levels

The mean data of LF levels (±standard errors) in GCF (LF-GCF) and blood (LF-BL) and their standard deviations are summarized at Figure 6.

The differences between mean LF-GCF levels for time intervals found to be statistically significant ( $P<.01$ ). From day 0 to day 14, mean LF-GCF levels significantly increased ( $58.8\pm7.2$  and  $163.2\pm21.9$  ng/µl, respectively) (Min: 29 ng/µl, Max: 98 ng/µl and min: 72 ng/µl, max: 297 ng/µl, respectively) ( $P<.05$ ), and significantly decreased at day 35 ( $58.7\pm5.4$  ng/µl) (Min: 26 ng/µl, Max: 191 ng/µl) ( $P<.05$ ). The pattern of LF-GCF levels followed during all of the periods of the study appeared to be similar and compatible with pattern of PI, GI and BOP scores, although no statistically significant correlation was detected (Tables 1 and 2).

The mean level of LF-BL at day 0 which was detected as  $112.0\pm23.4$  ng/µl (Min: 1 ng/µl, Max: 255 ng/µl), was increased to  $200.0\pm22.9$  ng/µl (Min: 7 ng/µl, Max: 289 ng/µl) at day 14 and decreased to  $125.0\pm27.5$  ng/µl (Min: 9 ng/µl, Max: 256 ng/µl) at day 35. No statistically significant differences were found between the time intervals. Levels of LF-BL demonstrated almost the same pattern with LF-GCF, PI, GI and BOP at days 0, 14 and 35. However, a significant negative correlation existed between the level of LF-BL and BOP at day 14 ( $P<.05$ ), whereas significant positive correlation existed between the level of LF-BL and scores of PI, GI and BOP at day 35 ( $P<.05$ ) (Tables 1 and 2).

## DISCUSSION

In the present study it was aimed to examine previously hypothesized systemic inflammatory response triggered by dental plaque accumulation via assessment of neutrophil protease LF levels within GCF and peripheral venous blood, employing the experimental gingivitis model. Kowolik et al<sup>5</sup> demonstrated that circulating neutrophils were

gradually increased during the experimental gingivitis period of 21 days and they stated that results of their study support the hypothesis that the accumulation of dental plaque can result in a measurable systemic inflammatory response.

The present study, intentionally, consisted of solely male, systemically healthy and non-smoker dental student volunteers of the same age group. It has been reported that hormonal influences caused by gender differences and age may alter the response to the bacterial challenge.<sup>5,24,25</sup> Smoking has previously been associated with etiology and pathogenesis of the periodontal diseases and also with the outcomes of the periodontal treatments.<sup>26-28</sup> Smoking has also been reported to have adverse effects on PMN viability and function within GCF of periodontally healthy population.<sup>29</sup>

Human experimental gingivitis, which was first introduced almost half century ago, is still one of the most preferred model for researchers to investigate the nature of periodontal diseases.<sup>24,30</sup> The clinical scores of the present study was consistent with the previous studies recruiting the experimental study model.<sup>13,16</sup>

In GCF, significantly higher levels of LF have been found at both periodontitis and gingivitis sites compared to healthy sites.<sup>9</sup> On the other hand, Gustafsson et al<sup>3</sup> reported that the LF-GCF did not differ significantly between gingivitis, periodontitis with/without destruction and authors claimed that LF-GCF levels were not related to the local severity of the periodontal disease.

In our present study, LF-GCF increased significantly during the course of the plaque

**Table 1.** Pearson correlation between PI, PPD, GI, BOP, LF-BL AND LF-GCF at day-14.

	PI	PPD	GI	BOP	LF-BL
PPD	0.241*				
	0.451†				
GI	0.334	0.290			
	0.289	0.361			
BOP	0.361	0.189	0.790		
	0.250	0.557	0.002		
LF-BL	-0.243	-0.169	-0.502	-0.730	
	0.447	0.599	0.097	0.007	
LF-GCF	-0.032	0.318	0.146	-0.064	0.390
	0.922	0.314	0.651	0.843	0.210

\* : Pearson correlation (r).

† : P value.

**Table 2.** Pearson correlation between PI, PPD, GI, BOP, LF-BL AND LF-GCF at day-35.

	PI	PPD	GI	BOP	LF-BL
PPD	0.377*				
	0.227†				
GI	0.593	0.492			
	0.042	0.104			
BOP	0.353	0.562	0.610		
	0.260	0.057	0.035		
LF-BL	0.630	0.543	0.608	0.650	
	0.028	0.068	0.036	0.022	
LF-GCF	-0.152	-0.217	-0.272	-0.541	-0.517
	0.638	0.498	0.392	0.069	0.086

\* : Pearson correlation (r).

† : P value.

accumulation period which is in agreement with the findings of Fransson et al.<sup>24</sup> In that study of Fransson et al,<sup>24</sup> a very similar protocol, with a similar age group (aged 20-25 years) of participants, was followed as in our present study; however the experimental period was longer (21 days). A strong agreement was found between the two studies by the means of LF-GCF increase from day 0 to 14<sup>th</sup> day of the experimental gingivitis period. Fransson et al<sup>24</sup> reported that LF-GCF increased 2,2 times after 14 days of plaque accumulation (125 ng/ $\mu$ l to 277 ng/ $\mu$ l), which is quite close to our findings that indicate 2.8 times increase of LF-GCF from day 0 to 14 (58.8 $\pm$ 7.2 ng/ $\mu$ l to 163.2 $\pm$ 21.9 ng/ $\mu$ l).<sup>24</sup>

The results of this study also showed that GCF-LF levels decreased significantly after the reinstatement of oral hygiene measures and approached the levels that observed at baseline day 0. This probably reflects the decreasing of neutrophil chemotactic factors in the crevice following plaque elimination and resolution of inflammation. This is in agreement with the results of an earlier study by Adonogianaki et al.<sup>31</sup> Authors examined four acute-phase proteins, including LF, in GCF during the course of a 21 day experimental gingivitis study and reported that the concentrations of LF were significantly elevated during the course of the trial and when plaque control was reestablished these values declined. It has also been suggested previously that LF-GCF might be a suitable marker for monitoring of treatment effects in periodontal disease, particularly periodontitis.<sup>32,33</sup>

Previous studies have pointed out a positive correlation between periodontitis and LF titers in GCF and blood.<sup>8,10</sup> LF is known as a prominent component of PMNs and is released in infected tissues and blood during the inflammatory process. Suomalainen et al<sup>10</sup> found increased amounts of LF in GCF and peripheral blood PMNs, in localized juvenile periodontitis. Furthermore, lower levels of LF was found in periodontitis sites during periodontal therapy, and this was followed by a rapid resumption to normal levels observed in healthy controls. In another study conducted by Martins et al,<sup>8</sup> the results showed that LF titers in sputum, gingival swabs, and blood correlate with the presence of inflammation. Although there are some data about plasma levels of LF at periodontitis, our present study appears to be the

first one to examine plasma levels of LF during the experimental gingivitis.

In the present study, our data showed that LF-BL increased from day 0 to 14, decreased from day 14 to 35, and finally reached almost the baseline values at day 35, the end of the recovery period. Although statistical analysis failed to detect significant positive correlation between clinical scores or LF-GCF and LF-BL at days 0 and 14, still the patterns of LF-BL seemed to be compatible with LF-GCF, PI, GI and BOP scores at baseline, end of the experimental gingivitis and recovery periods. Significant negative correlation ( $P < .05$ ) was detected at day 14 between LF-BL and BOP. On the other hand, significant positive correlation existed between LF-BL and PI, GI and BOP at day 35 ( $P < .05$ ) which suggested that recovery from gingivitis has significant influences on LF-BL.

The pattern of neutrophil protease LF within plasma might simply be associated with momentary release of LF from the specific granules of activated neutrophils by local bacterial insult, such as the pattern of LF-GCF levels.<sup>6,7</sup> On the other hand, insignificance of plasma LF level alteration during the experimental and recovery period might be related with common description of gingivitis as "a local phenomenon" in which migration of neutrophils into the oral cavity, via the gingival crevicular fluid increases, as part of the inflammatory response. This process provides the first line of cellular host defence to localize the oral flora and possibly reduce the likelihood of systemic spread. Moreover, it can be claimed that both statistically insignificant plasma and significant LF-GCF alterations during the same time courses, considered with likeness of their increasing and decreasing patterns, might be attributed to the effectiveness of the first line of host defence for avoiding systemic spread in systemically healthy individuals.

In addition, it is known that serum levels of neutrophil proteases, such as LF and PMN-elastase, have been associated with several mild to moderate inflammatory conditions, such as male genital tract inflammation and *Helicobacter pylori* infection.<sup>34-36</sup> It was demonstrated that LF serum levels boost up to higher levels within 30-120 min of intravenous infusion of lipopolysaccharide or alive *Escherichia coli*, in vivo.<sup>37</sup> Furthermore, not only bacterial contamination, also intense

physical exercise and stress shown to be effective on induction of neutrophil activation, as manifested by release of protease content of neutrophil granules.<sup>38,39</sup> Even if systemic health was one of the inclusion criteria of our study, still LF-BL might be affected from existence of minor undiagnosed inflammations or even from intense physical exercise.

## CONCLUSIONS

In conclusion, the data of the present study demonstrated that the local inflammation created by plaque accumulation during the experimental gingivitis model caused elevated LF levels of GCF and blood. Furthermore, the treatment of local inflammation and returning to daily oral hygiene procedures caused reduction of LF levels in both GCF and BL. However, only LF-GCF alterations were appeared to be statistically significant, in this short term study. Within the limits of this study, our present data contributes to the hypothesis that not only periodontitis, also local microbial dental plaque accumulation and so caused local gingival inflammation might have systemic influences by means of LF release from activated neutrophils. Moreover, further investigations will help better understanding of the possible extents of the potential systemic effects of gingivitis.

## ACKNOWLEDGEMENTS

Authors gratefully acknowledge Fikret Gurbuz, Professor, Ankara University, Faculty of Agriculture, Animal Science Department, Biometry and Genetic Discipline, for his contribution in statistical analyses of the data of the present study.

This study was funded by Gazi University Research Grant 03/2002-30.

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