Severe Periodontitis Enhances Macrophage Activation via Increased Serum Lipopolysaccharide

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Objective—In periodontitis, overgrowth of Gram-negative bacteria and access of lipopolysaccharide (LPS) to circulation may activate macrophages leading to foam cell formation. We investigated whether periodontal treatment affects proatherogenic properties of low-density lipoprotein (LDL) and, thus, macrophage activation.

Methods and Results—LDL was isolated and characterized before and after treatment from 30 systemically healthy patients with periodontitis. Production of cytokines and LDL cholesteryl ester (LDL-CE) uptake by macrophages (RAW 264.7) was determined. Baseline periodontal variables correlated positively with serum LPS and C-reactive protein concentrations, as well as macrophage cytokine production and LDL-CE uptake. LPS concentration correlated positively with serum concentration of oxidized LDL and cytokine production. Higher cytokine production and LDL-CE uptake were induced by LDL isolated from patients with elevated number of affected teeth before treatment. Patients with serum LPS concentrations above the median (0.87 ng/mL) at baseline had higher serum high-density lipoprotein (HDL) cholesterol (baseline versus after treatment, 1.30±0.19 versus 1.48±0.28 mmol/L; $P=0.002$) and HDL/LDL ratio (0.31±0.01 versus 0.34±0.10; $P=0.048$), but lower serum LPS concentration (1.70±0.49 versus 0.98±0.50 ng/mL; $P=0.004$) and autoantibodies to β_2-glycoprotein I (0.11±0.06 versus 0.09±0.04 ELISA units; $P=0.022$) after treatment.

Conclusions—Our results suggest that in systemically healthy patients, the infected/inflamed area in periodontitis is associated with macrophage activation via increased serum LPS concentration. **(*Arterioscler Thromb Vasc Biol*. 2004; 24:2174-2180.)**

Key Words: inflammation ■ infection ■ lipoprotein metabolism ■ serum lipopolysaccharide

Chronic bacterial infections, including periodontitis, have been associated with an increased risk of atherosclerosis and coronary heart disease.[1] The mechanisms by which chronic infections increase the likelihood of atherosclerosis are not clearly defined, but the prerequisite is believed to be the host response to a long-term systemic exposure to certain pathogens. In periodontitis, deepened periodontal pockets create increased subgingival space for bacterial deposits, and gingival inflammation leads to microulcerations in periodontal pocket epithelium.[2] The pathological changes provide periodontal bacteria and their components access to systemic circulation, eg, during eating or tooth cleaning. Bacterial translocation from periodontal pockets may cause systemic release of inflammatory mediators and acute-phase proteins leading to monocyte activation and alterations in the lipoproteins toward more atherogenic profile.[3–6]

The central cells in the arterial wall, which mediate development of atherosclerotic lesions, are activated macrophages. They release cytokines, which enhance inflammation and tissue destruction. They also accumulate excess cholesterol that is esterified and stored in the cytoplasm, converting macrophages into foam cells.[7] Lipopolysaccharide (LPS) isolated from Gram-negative bacteria, such as the periodontal pathogen *Porphyromonas gingivalis*, induces cytokine production and macrophage-derived foam cell formation in the presence of exogenous low-density lipoprotein (LDL).[8] In the circulation, LPS associates with all lipoprotein classes, but it may promote atherogenesis when it invades the arterial wall complexed with LDL.[9,10] An LDL–LPS complex is recognized as minimally modified LDL, which readily binds to macrophage LPS receptors.[11] Furthermore, activated macrophages promote oxidation of LDL in vitro by a mechanism that depends on release of superoxide, myeloperoxidase, lipoxygenase, NADPH oxidase, thiol recycling, or transition metal ions.[12] Most importantly, these phenomena feed one another: monocyte/macrophage activation results in cytokine

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production and LDL oxidation, which further activates the cells to accumulate cholesterol, leading to foam cell formation and in later steps to formation of cholesterol crystals. It has been shown in an animal model that host response to infection and inflammation increases the concentration of oxidized lipids in serum and induces LDL oxidation in vivo.[13] Oxidized LDL (oxLDL) itself is also a cytotoxic agent, which contributes to cell injury, smooth muscle cell proliferation, leukocyte chemotaxis, and foam cell formation.[14]

Oxidized forms of phospholipids, cholesterol fatty acyl esters, and apolipoprotein B in oxLDL are highly antigenic molecules that give rise to production of autoantibodies in humans.[15] Major portion of autoantibodies to oxLDL belong to a family of cross-reacting antiphospholipid antibodies, a heterogeneous group of antibodies with regard to their specificity. Besides oxLDL, their antigenic epitopes are recognized in phospholipids, such as phosphatidylcholine and cardiolipin, and in phospholipid-binding proteins, such as β_2-glycoprotein I (ie, apoH) and prothrombin. As a consequence of their binding to their antigens, LDL–immunoglobulin complexes are formed. These immune complexes readily bind to macrophage surface Fcγ receptors, which have no negative feedback regulation, and consequently the macrophages are transformed into foam cells.[16,17] In fact, elevated levels of antiphospholipid antibodies have been found to be markers or predictors of accelerated atherogenesis and thrombosis.[18–20]

The aim of the study was to investigate whether periodontitis is connected with proatherogenic properties of LDL. For this, LDL was isolated and characterized from 30 adult patients with periodontitis before and after periodontal treatment. Isolated individual LDL preparations were applied on macrophage cultures, and subsequent production of cytokines and uptake of LDL by macrophages were determined. Serum analyses included lipid profiles, LPS concentrations, oxLDL concentrations, and serum autoantibody levels against phospholipids, as well as antibody levels to 2 periodontal pathogens, *Actinobacillus actinomycetemcomitans* and *P. gingivalis*.

Methods

Study Subjects

Thirty adult patients with periodontitis (16 males, 14 females; mean age, 49.3 years; SD, 7.08 years) were included in the study. The patients were selected among those seeking periodontal treatment in a specialist clinic in Helsinki, Finland. The odontological inclusion criteria of the study patients were as follows: (1) patients had at least 24 natural teeth; (2) >6 teeth were affected by periodontitis as determined by clinical (distance from the cementoenamel junction to the bottom of periodontal pocket exceeding 1 mm at approximal sites) and radiographic (distance from cementoenamel junction to alveolar bone margin exceeding 3 mm) periodontal attachment loss; (3) periodontal tissues were inflamed as assessed by generalized gingival redness, edema, and gingival bleeding on probing; (4) patients had had no periodontal therapy during the 6 preceding months.

The inclusion criteria for systemic health status of the patients were as follows. Patients had no diagnosed systemic diseases. This was verified by collecting anamnestic information of the systemic health and medications by a questionnaire that was completed by each patient before the dental examination. The questions presented concerned any continuous medical care either currently or previously, allergy, diagnosed cardiovascular diseases, hypertension, diabetes, rheumatoid arthritis, kidney or liver diseases, epilepsy, gastrointestinal problems, ulcer disease, HIV infection, or possible other systemic diseases. Additional questions included the presence of endoprostheses, such as joint prostheses, artificial heart valves, or pacemakers. Whenever required, the dentist elucidated to the patient any unclear details in the questionnaire. Patients had no acute infections within 2 months before entry into the study. None had received antibiotic treatment during the 6 preceding months. All patients volunteered in the study and signed an informed consent. The Ethical Committee of the Institute of Dentistry at the University of Helsinki approved the study design.

The periodontal treatment was conducted according to the odontological needs of the patients. All patients received local anesthesia during mechanical periodontal therapy comprising scaling and root planning and gingivoplasty when indicated. In adjunction, 7 patients received metronidazole 500 mg twice daily for 7 days. Except for enhanced oral hygiene, the patients were not advised to change their dietary or smoking habits during the study. Clinical periodontal examination was performed as described[21] before treatment and ≈3 months after it. All teeth in the dentition were examined at 6 sites for probing pocket depth, gingival bleeding, or appearance of pus on probing. Radiographic examination before treatment included panorama radiographs with peri-apical radiographs when indicated.

Serum and Plasma Analyses

Serum and plasma samples were taken both before (baseline) and after periodontal treatment. The samples were preserved at −70°C until use. LPS concentration in serum samples was determined by kinetic Limulus Amebosyte Lysate test kit with a chromogenic substrate (Diagnostica & Analys Service AB, Gothenburg, Sweden) on diluted (1:5, vol/vol in endotoxin-free water) samples. Total cholesterol and triglyceride concentrations were assayed by enzymatic methods,[22] high-density lipoprotein (HDL) cholesterol was determined by a direct method, and LDL cholesterol concentrations were calculated using Friedewald formula.[23] Concentration of oxLDL was determined by the enzyme-linked immunosorbent assay method (Oxidized LDL ELISA; Mercodia Ltd, Uppsala, Sweden). Serum IgG class antibody levels to *A. actinomycetemcomitans* and *P. gingivalis* were determined by multiserotype enzyme-linked immunosorbent assay as described.[24] Serum antiphospholipid antibody levels were determined by enzyme-linked immunosorbent assay as described previously.[25–27] These included anti–β_2-glycoprotein I (anti–β_2-GPI), anticardiolipin (anti-CL), antiphosphatidylcholine (anti-PC), antibodies to oxLDL (anti-oxLDL), and antiprothrombin. C-reactive protein (CRP) concentrations were determined by a sensitive immunoassay (UC CRP ELISA; Eucardio laboratory) and plasma fibrinogen by the Clauss method.[28]

Isolation and Characterization of LDL

Very-low-density, LDL, and HDL were isolated by sequential ultracentrifugation[29] and stored at −70°C. LDL preparations (d=1.019 to 1.063 g/mL) were dialyzed against phosphate-buffered saline (PBS) (10 mmol/L phosphate, pH 7.4, containing 150 mmol/L NaCl) overnight, and characterized for particle size, conjugated dienes, and lag time during Cu^{2+} oxidation. The LDL particle size was determined by native gradient gel electrophoresis in 2% to 12% polyacrylamide gradient gels.[30] High molecular weight standard (Amersham Pharmacia) and one LDL reference sample stored at −70°C were run in each gel. After staining, the LDL patterns were captured in a digital format and analyzed with Kodak Digital Science 120 digital camera and 1D™ analysis software. LDL oxidation in vitro was determined by monitoring Cu^{2+}-induced oxidation of LDL. The formation of conjugated dienes at the wavelength of 234 nm was continuously followed for at least 3.5 hours as described previously.[31]

Labeling of LDL

LDL cholesteryl ester (CE) moiety was labeled with [^{3}H]-cholesteryl oleate (Amersham Biosciences, Piscataway, NJ) as described.[32] Briefly, 250 μCi of tracer (in toluene, Amersham Biosciences) and

TABLE 1. Correlation Coefficients (r) Between Clinical Periodontal Variables and Serum Parameters at Baseline

	Suppuration§	Bleeding Pockets¶	Periodontal Pockets‖	CRP	Anti-PC	Anti-Prothrombin	Anti–β_2-GPI	Anti-CL	Anti-oxLDL	LDL Particle Size	LDL Cholesterol	LPS Concentration	OxLDL Concentration	Maximal Rate	Maximal Dienes	Oxidation Lag-Time	TNF-α	IL-1β
CE-uptake†	0.011	**0.364***	**0.469**	−0.288	−0.023	0.080	**0.413**	−0.044	0.172	**−0.370**	0.228	0.056	−0.302	**−0.362**	**0.469**	0.274	**0.645**	**0.602**
IL-1β†	**0.450**	**0.369**	**0.518**	**0.598**	**0.736**	0.133	**0.472**	**0.799**	0.087	−0.299	0.305	−0.002	−0.288	**−0.479**	0.009	0.331	**0.468**	**1**
TNF-α†	0.118	**0.396**	**0.423**	−0.171	−0.176	−0.104	0.232	0.029	0.078	−0.096	**0.410**	**0.338**	0.137	−0.048	**0.539**	**0.393**	**1**	
Oxidation lag-time‡	0.214	0.284	0.235	0.075	0.173	−0.100	0.175	0.123	−0.004	−0.015	0.168	0.227	−0.009	0.025	−0.055	**1**		
Maximal dienes‡	−0.052	−0.034	0.052	**−0.349**	−0.158	0.184	0.040	−0.174	0.065	0.151	**0.344**	−0.092	−0.080	−0.117	**1**			
Maximal rate‡	−0.246	−0.179	−0.274	−0.215	−0.237	−0.238	**−0.341**	−0.290	−0.218	0.287	−0.258	0.080	0.173	**1**				
OxLDL concentration	0.019	−0.109	0.015	0.114	−0.165	−0.113	−0.102	−0.230	−0.048	0.267	**0.474**	**0.369**	**1**					
LPS concentration	**0.417**	**0.391**	0.307	−0.189	−0.151	0.215	0.073	−0.203	0.028	−0.082	0.232	**1**						
LDL cholesterol	**0.412**	0.080	**0.431**	0.226	−0.131	0.156	0.154	−0.173	0.149	0.060	**1**							
LDL particle size	−0.199	**−0.497**	**−0.441**	0.158	−0.129	−0.215	−0.155	−0.067	−0.168	**1**								
Anti-oxLDL	−0.061	−0.195	−0.143	−0.035	0.180	**0.392**	0.173	**0.395**	**1**									
Anti-CL	0.079	−0.159	−0.110	**0.487**	**0.956**	0.103	0.163	**1**										
Anti-β_2-GPI	0.054	−0.214	−0.092	−0.016	0.088	0.218	**1**											
Anti-prothrombin	0.113	−0.128	0.164	0.042	0.096	**1**												
Anti-PC	0.159	−0.087	−0.048	**0.481**	**1**													
CRP	**0.468**	0.054	0.202	**1**														

*Statistically significant ($P<0.05$) coefficients are in bold face.
†LDL-CE taken up and cytokines produced by macrophages.
‡Parameters of Cu^{2+}-induced LDL oxidation.
§Number of periodontal pockets with suppuration.
¶Number of periodontal sites with bleeding on probing.
‖Number of teeth with deepened periodontal pockets.

175 μg of phosphatidylcholine were dried under nitrogen and suspended with 1 mL PBS. The mixture was sonicated on ice for 10 minutes, after which 1200 μL of LPDS and 6 mg of LDL (as total protein) were added. The mixture was incubated at 37°C for 18 hours and labeled LDL was re-isolated by ultracentrifugation at density 1.063 g/mL. The preparation was dialyzed against PBS and stored in aliquots at −70°C.

Incubation of Isolated LDLs With Macrophages

Permanent mouse RAW264.7 macrophages were cultivated in RPMI 1640 medium containing 10% fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The cells were revived exactly 2 weeks before the experiments and passaged 4 times. On the day before the experiment, the cells were harvested using a rubber policeman, counted under a microscope, and divided at a density of 1.5×10^5 cells/mL on 6-well plates. Before starting the experiment the next day, the medium was changed into the macrophage SFM medium (Gibco), supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 ng/mL Leucomax (1.67 million international units; Novartis, Finland; stock 2.5 μg/mL in sterile water). [^{3}H]-cholesteryl oleate LDL (20 μg/mL, specific activity 2.68 cpm/ng) was added in the medium. LDL isolated from samples taken before and after periodontal treatment were added on the cells in a concentration of 80 μg/mL in PBS. To find out if activation of macrophages involved LDL oxidation, the incubations were performed in the absence and presence of α-tocopherol in a final concentration of 50 μmol/L (stock 50 mol/L in ethanol). Aliquots of medium (50 μL) for determination of cytokine concentrations were taken after 24 hours of incubation and stored at −70°C until analyzed. After 48 hours of incubation, the media were removed, and the cells were washed twice with PBS containing 2% bovine serum albumin and twice with PBS. One milliliter of 0.3 N NaOH was added into each well, and the cells were lysed for 30 minutes at 4°C on a shaker. Protein concentration was determined by the method of Lowry.[33] Homogenous cell lysates (500 μL) were transferred to scintillation vials and counted for radioactivity after storage at 4°C overnight with the scintillation cocktail. Cytokine concentrations (tumor necrosis factor [TNF]-α and IL-1β) were determined from the culture media by commercial methods (Mouse TNF-α and Quantikine® M Murine; Immunodiagnostic and R&D Systems Inc, respectively).

Statistical Analyses

The statistical significance of the differences in the continuous variables between the study groups was tested with the Mann–Whitney U test. The statistical significance of the differences between the samples taken before and after periodontal treatment was tested with the Wilcoxon signed ranks test. The 2-tailed Pearson correlation was used for correlation analyses, in which skewed distributions were corrected using quartiles of the variables in the analysis. All statistical analyses were performed with SPSS 10.0 for Windows program.

Results

A 2-tailed Pearson correlation analysis was performed to examine relationships between clinical periodontal variables and serum parameters before treatment (Table 1). The number of pathologically deepened periodontal pockets, and periodontal pockets bleeding on probing correlated positively ($P<0.05$) with the production of IL-1β ($r=0.518$, $r=0.369$) and TNF-α ($r=0.423$, $r=0.396$) and uptake of LDL cholesterol ($r=0.469$, $r=0.364$) by macrophages. Number of pockets with bleeding on probing and those with suppuration correlated positively with serum LPS concentration ($r=0.391$, $r=0.417$). Number of sites bleeding on probing correlated negatively with LDL particle size ($r=-0.497$) and number of sites with suppuration correlated positively with CRP ($r=0.468$).

Serum LPS concentration had a positive correlation with serum oxLDL concentration ($r=0.369$) and the production of TNF-α by macrophages ($r=0.338$) (Table 1). LDL cholesterol uptake by macrophages had a positive correlation with anti–β_2-GPI levels ($r=0.413$) and a negative correlation with LDL particle size ($r=-0.370$). IL-1β production by macrophages correlated positively with CRP ($r=0.598$), anti-PC

TABLE 2. Comparisons of Patient Groups Divided by Medians of 3 Periodontal Parameters at Baseline

	Suppuration*			Periodontal Pockets†			Bleeding on Probing‡		
	Mean (SD)			Mean (SD)			Mean (SD)		
Parameter	≤2 sites n=15	>2 sites n=12	P§	<13 teeth n=14	≥13 teeth n=15	P§	<55 sites n=13	≥55 sites n=14	P§
Serum and plasma									
Total cholesterol (mmol/L)	6.08 (0.87)	6.29 (1.23)	0.922	6.02 (0.64)	6.35 (1.24)	0.541	5.93 (0.65)	6.40 (1.27)	0.452
HDL cholesterol (mmol/L)	**1.56 (0.48)**	**1.19 (0.19)**	**0.010**	**1.63 (0.48)**	**1.19 (0.15)**	**0.000**	1.39 (0.31)	1.40 (0.51)	0.610
HDL/LDL ratio	**0.42 (0.16)**	**0.29 (0.08)**	**0.028**	**0.44 (0.15)**	**0.29 (0.08)**	**0.001**	0.38 (0.14)	0.35 (0.15)	0.583
Triglycerides (mmol/L)	**1.30 (0.58)**	**1.86 (0.69)**	**0.037**	1.37 (0.58)	1.69 (0.70)	0.206	1.44 (0.63)	1.65 (0.73)	0.382
LPS concentration (ng/mL)	1.06 (0.59)	1.13 (0.81)	0.124	0.91 (0.61)	1.26 (0.70)	0.169	**0.83 (0.54)**	**1.33 (0.73)**	**0.043**
OxLDL concentration (mU/L)	7.58 (2.26)	7.10 (2.14)	0.625	7.26 (2.01)	7.45 (2.25)	0.930	7.40 (2.44)	7.34 (2.00)	0.884
Anti–β_2-GPI (EU)	0.08 (0.04)	0.10 (0.04)	0.241	0.08 (0.06)	0.10 (0.04)	0.326	0.09 (0.04)	0.09 (0.04)	0.865
Anti-PC (EU)	**0.13 (0.08)**	**0.54 (0.86)**	**0.025**	0.39 (0.79)	0.24 (0.30)	0.844	0.36 (0.82)	0.26 (0.31)	0.207
Anti-prothrombin (EU)	0.15 (0.18)	0.15 (0.06)	0.942	**0.11 (0.04)**	**0.18 (0.17)**	**0.035**	0.17 (0.19)	0.13 (0.05)	0.846
CRP (mg/mL)	**0.92 (0.68)**	**2.40 (1.79)**	**0.011**	1.40 (1.40)	1.74 (1.49)	0.475	1.64 (1.51)	1.57 (1.53)	0.738
Fibrinogen (g/L)	3.40 (0.47)	3.73 (0.53)	0.092	0.62 (0.51)	3.58 (0.57)	0.062	3.41 (0.47)	3.68 (0.54)	0.174
LDL									
LDL particle size (nm)	**21.9 (0.25)**	**21.6 (0.41)**	**0.021**	**21.9 (0.21)**	**21.6 (0.38)**	**0.007**	**21.9 (0.26)**	**21.7 (0.39)**	**0.049**
Oxidation lag-time (min)	55.6 (12.4)	64.4 (19.2)	0.231	58.9 (10.4)	61.9 (20.0)	0.896	53.2 (9.7)	65.4 (18.7)	0.120
Maximal dienes (μmol/mg)	44.6 (6.5)	41.4 (4.4)	0.150	42.3 (5.5)	44.2 (6.4)	0.471	43.8 (4.1)	42.6 (7.1)	0.332
Macrophage experiments									
CE-uptake (μg/mg)	2.87 (2.16)	2.70 (1.01)	0.380	**2.37 (1.76)**	**3.61 (1.82)**	**0.014**	2.56 (1.69)	3.02 (1.77)	0.357
IL-1β (ng/mL)	**0.93 (1.03)**	**2.92 (1.93)**	**0.004**	**1.29 (1.90)**	**2.60 (1.68)**	**0.014**	1.46 (1.45)	2.14 (2.03)	0.440
TNF-α (ng/mL)	45.0 (21.9)	39.9 (12.4)	0.590	**37.0 (12.8)**	**50.4 (20.2)**	**0.012**	36.5 (11.8)	48.5 (21.4)	0.061

*Number of purulent periodontal pockets.
†Number of teeth with deepened periodontal pockets.
‡Number of periodontal sites with bleeding on probing.
§Mann–Whitney *U* test.

(r=0.736), anti–β_2-GPI (r=0.472), and anti-CL (r=0.799) levels, and negatively with maximal rate of LDL oxidation in vitro (r=−0.479).

To evaluate the relationship between clinical and other study variables before treatment, we divided the patients into 2 groups according to the medians of 3 clinical periodontal variables, suppuration, number of deepened periodontal pockets, and gingival bleeding on probing (Table 2). When the median of sites with suppuration (≤2 versus >2) served as a cutoff value, the patient group with higher number of purulent periodontal pockets had a significantly higher CRP concentration (P=0.011), triglyceride concentration (P=0.037), and level of anti-PC antibodies (P=0.025), but lower HDL cholesterol concentration (P=0.010), HDL/LDL ratio (P=0.028), and smaller LDL particle size (P=0.021) than the group with fewer purulent sites. LDL isolated from these patients also induced higher production of IL-1β from macrophages (P=0.004) than LDL isolated from patients with fewer purulent sites.

When the patients were divided into 2 groups according to the median number of teeth with pathologically deepened periodontal pockets (<13 versus ≥13), LDL cholesterol was more readily taken up by the macrophages (P=0.014) in the group having ≥13 teeth with periodontal pockets. Also, LDL isolated from these patients induced significantly higher production of IL-1β (P=0.014) and TNF-α (P=0.012) by the macrophages than LDL isolated from patients with fewer periodontal pockets. The patients with a greater number of teeth with periodontal pockets had significantly lower HDL cholesterol concentrations (P<0.001) and HDL/LDL ratios (P=0.001) but higher levels of antiprothrombin antibodies (P=0.035) than patients with fewer teeth with periodontal pockets.

Finally, when the median number of sites with bleeding on probing was used as a cutoff value, the LDL mean particle size was smaller (P=0.049) and mean serum LPS concentration higher (P=0.043) in the patients with number of sites above (>55) than below (≤55) the median (Table 2).

The patients were additionally divided into 2 groups by the median value of serum LPS concentration (≤87 versus >87 ng/mL) before periodontal treatment (Table 3). Patients with LPS concentration above the median had higher concentrations of oxLDL (P=0.050) and antiprothrombin antibodies (P=0.028) at baseline than patients with LPS concentration below the median. The mean lag time of LDL oxidation was longer in the former than in the latter group (P=0.043). In addition, LDLs isolated from the former group induced higher TNF-α production by the macrophages than LDLs from the latter group (P=0.047).

The mean number of deepened (>5 mm) periodontal pockets and the mean proportion of bleeding sites on probing decreased from 24.1±14.9 to 6.0±6.3 and 62.9±27.7 to 17.5±17.9 after periodontal treatment, respectively. Both changes were statistically significant (P<0.001). Metronidazole treatment in adjunction to mechanical periodontal treatment did not produce clear differences in the study parame-

TABLE 3. Comparison of Samples Before and After Periodontal Treatment in Patients (n=30) With a Low or High Serum LPS Concentration at Baseline

	Low LPS Concentration, ≤0.87 ng/mL n=16			High LPS Concentration, >0.87 ng/mL n=14		
Parameter	Before Mean (SD)	After Mean (SD)	*P**	Before Mean (SD)	After Mean (SD)	*P**
Serum and plasma						
Total cholesterol (mmol/L)	6.07 (0.86)	6.38 (0.97)	0.071	**6.51 (1.29)**	**7.04 (1.32)**	**0.008**
HDL cholesterol (mmol/L)	1.50 (0.50)	1.61 (0.50)	0.072	**1.30 (0.19)**	**1.48 (0.28)**	**0.001**
HDL/LDL ratio	0.40 (0.16)	0.45 (0.20)	0.363	**0.31 (0.01)**	**0.34 (0.10)**	**0.048**
Triglycerides (mmol/L)	1.44 (0.63)	1.55 (0.80)	0.541	1.74 (0.63)	1.94 (0.76)	0.409
LPS concentration (ng/mL)	0.60 (0.20)†	0.78 (0.52)	0.189	**1.70 (0.49)†**	**0.98 (0.50)**	**0.004**
OxLDL concentration (mU/L)	6.73 (1.70)†	6.61 (1.44)†	0.780	8.14 (2.25)†	8.13 (2.36)†	0.975
Anti–β_2-GPI (EU)	0.08 (0.04)	0.08 (0.04)	0.880	**0.11 (0.06)**	**0.09 (0.04)**	**0.022**
Anti-prothrombin (EU)	0.11 (0.04)†	0.11 (0.04)	0.104	0.19 (0.18)†	0.16 (0.14)	0.087
Anti–*A. actinomycetemcomitans* (EU)	21.5 (9.2)	22.1 (9.6)	0.351	19.7 (8.6)	19.4 (8.9)	0.622
Anti–*P. gingivalis* (EU)	26.4 (9.4)	24.4 (8.9)	0.056	**30.6 (12.4)**	**27.8 (11.8)**	**0.005**
CRP (mg/mL)	1.74 (1.58)	1.73 (3.44)	0.992	1.32 (1.21)	1.67 (1.62)	0.899
Fibrinogen (g/L)	3.66 (0.58)	3.52 (0.65)	0.173	3.53 (0.48)	3.50 (0.41)	0.925
LDL						
LDL particle size (nm)	21.8 (0.30)	21.8 (0.38)	0.845	**21.7 (0.37)**	**21.9 (0.37)**	**0.008**
Oxidation lag-time (min)	55.6 (12.4)†	54.9 (8.2)	0.798	66.6 (17.3)†	63.2 (16.1)	0.527
Maximal dienes (μmol/mg)	44.5 (7.0)	44.8 (6.2)	0.623	42.3 (4.3)	42.3 (4.7)	0.826
Macrophage experiments						
CE-uptake (μg/mL)	2.99 (2.00)	3.22 (2.25)	0.255	3.22 (1.76)	3.36 (2.22)	0.331
IL-1β (ng/mL)	1.56 (1.41)	1.81 (1.86)	0.496	**2.43 (2.20)**	**2.14 (2.74)**	**0.048**
TNF-α (ng/mL)	**42.3 (22.6)†**	**52.3 (38.6)†**	**0.041**	45.9 (10.4)†	46.9 (13.6)†	0.507

*Wilcoxon signed ranks test, significance of the differences between the samples before and after treatment.
†Mann–Whitney *U* test, significantly different results between low and high LPS groups.

ters when compared with the patients without metronidazole medication (data not shown). No teeth were extracted during the treatment or the follow-up period.

Supplementation of macrophage growth medium with α-tocopherol decreased LDL cholesterol uptake on average by 8.4% ($P=0.042$) but increased the production of TNF-α by 29% ($P=0.007$). When the statistical analyses were performed for the whole study population and comparisons performed on the results before and after periodontal treatment, serum total cholesterol (6.28±1.08 mmol/L versus 6.70±1.18 mmol/L; $P=0.001$), LDL cholesterol (4.16±1.03 mmol/L versus 4.33±1.15 mmol/L; $P=0.046$), HDL cholesterol (1.40±0.40 mmol/L versus 1.55±0.41 mmol/L; $P<0.001$) concentrations, as well as HDL/LDL ratio (0.35±0.14 versus 0.40±0.17, $P=0.018$) were all higher after periodontal treatment. Furthermore, the mean serum IgG level against *P. gingivalis* was lower after treatment (28.4±10.9 ELISA units versus 26.0±10.3 ELISA units; $P<0.001$). When analyzed for the whole study population, there were no statistically significant differences in other results obtained before and after treatment.

To enable evaluation of the significance of the baseline serum LPS concentration for the influence of treatment regarding lipoprotein metabolism, the patients with serum LPS concentrations below and above the median at baseline (≤87 versus >87 ng/mL) were compared (Table 3). In patients with LPS concentration above the median, significant decreases were observed in serum LPS concentration, level of anti–β_2-GPI, and IgG class antibodies to *P. gingivalis*, as well as increases in serum total cholesterol and HDL cholesterol concentrations and HDL/LDL ratios. In addition, the LDL particle size tended to increase after treatment in this group. Contrarily, in patients with a baseline LPS concentration below the median, no significant changes were observed (Table 3).

Discussion

Both atherosclerosis and periodontitis are inflammatory conditions that commonly are chronic and asymptomatic in nature. Local macrophage activation in periodontium and in arterial intima plays a role in the inflammatory process of both diseases. Formation of macrophage-derived foam cells in the arterial intima is the pivotal step in early atherosclerosis. The prerequisite for foam cell formation is the excess cargo of cholesterol preferentially transported in LDL. Therefore, LDL may be an important mediator between oral infection and arterial inflammation. The aim of the present study was to investigate whether LDL isolated from patients with inflamed periodontitis bear inflammation-induced proatherogenic properties, which can lead to macrophage activation and foam cell formation.

The central finding of the present study was that the extent of affected tissue in periodontitis was directly associated with the production of cytokines and enhanced uptake of cho-

lesteryl esters by macrophages, when they were activated by LDL preparations isolated from the patients. The results further indicated that the main mediators between periodontal infection and macrophage activation in serum were LDL cholesterol, LPS, β_2-GPI, and modified phospholipids. All these factors can modify LDL into proatherogenic direction, thereby increasing uncontrolled uptake of LDL by macrophages.[17,34,35] Macrophage activation was significantly and directly associated with several LDL parameters and parameters connected to LDL: LDL cholesterol, serum LPS concentration, small LDL particle size, CRP, and antiphospholipid antibodies.

The intestine is the main and most likely origin of LPS in circulation. However, several earlier articles suggest that serum LPS concentration may also increase in periodontitis,[36–38] although to our knowledge this has not been shown by a direct quantification of serum LPS. However, concentrations of soluble CD14 (sCD14), LPS-binding protein, and antibodies to LPS of periodontal pathogens are elevated in periodontal patients compared with periodontally healthy subjects.[36–38] LPS from dental plaque has also been shown to penetrate the gingiva,[39] and occurrence of bacteremia (number of species and positive cultures) increases with increasing severity of gingival inflammation.[40] Therefore, our results, for the first time to our knowledge, suggest that high serum concentrations of LPS are actually associated with the area of affected tissue in periodontitis. In circulation, LPS associates with all lipoprotein classes and it may initiate atherogenesis, when it is transported into the arterial wall with LDL. In cell cultures, LPS isolated from *A. actinomycetemcomitans* and *P. gingivalis* upregulates the release of inflammatory mediators[41,42] and enhances macrophage foam cell formation.[43,8] These observations support our finding that high serum LPS concentration was associated with increased ability of LDL to activate macrophages in systemically healthy patients with periodontitis.

Anti–β_2-GPI antibodies bind β_2-GPI in oxLDL, where oxidized forms of cholesteryl linoleate serve as ligands specific for β_2-GPI.[17] The positive correlation between cholesteryl ester uptake by macrophages and serum anti–β_2-GPI antibody levels in the present study is therefore supported by an earlier observation that the LDL-immune complexes formed are readily taken up by macrophages transforming them into foam cells.[16,44] No correlation was found between serum anti-oxLDL antibodies and cholesteryl ester uptake by macrophages. This may be due to the oxLDL epitopes used as antigens in the determinations. The epitopes comprised malondialdehyde-LDL, the final oxidized forms of LDL,[45] which may represent epitopes with a too strong modification to be observed among patients with a chronic infection.

Our study holds some limitations. The size of the study population was limited and the results are based on subgroup analyses. Correlating variables with each other produces several probability values, and some of them may represent chance findings. Furthermore, because we collected no dietary data, it could be argued that the participants changed their dietary habits during the periodontal treatment. However, we consider it unlikely that patients with more severe periodontitis would have been more willing to start an antiatherogenic diet, particularly because no dietary counseling was given. Information of possible changes in the smoking habit during the study period was not recorded (number of smokers was 5), but the patients were recommended to not change their habits during the study period. The 3-month follow-up period was selected to ensure detection of periodontal tissue healing caused by periodontal treatment and to avoid interference of periodontal reinfection/re-inflammation or possible acute infections.

We assumed that the wider the extent of periodontitis, the greater the systemic spread of bacteria and their components are, and therefore the bigger the antiatherogenic effects of the treatment become. On the whole, the results support this view. In the analyses, serum LPS concentration was directly associated with number of pockets with bleeding on probing and number of periodontal pockets with suppuration. Increasing number of inflamed deepened periodontal pockets results in increasing subgingival space for bacteria, especially Gram-negative anaerobic species typical of periodontitis. Regarding lipoprotein metabolism, the beneficial effects of periodontal treatment were observed particularly in the group of patients with a high LPS concentration at baseline. Accordingly, the outcome of their treatment was seen as a significant increase in serum HDL cholesterol concentration and decrease in serum LPS and anti–β_2-GPI concentrations. In this group, LDL particle size also increased significantly, although this small mean increase probably does not bear a great physiological significance. However, all these changes are antiatherogenic, preventing macrophage-derived foam cell formation.

In conclusion, our results show that the extent of affected tissue in periodontitis is directly associated with the ability of isolated LDL to activate macrophages in vitro. The main mediators of macrophage activation in LDL were LPS, β_2-GPI, and modified phospholipids. Our results suggest that the infected/inflamed area in periodontitis is associated with macrophage activation via increased serum concentration of LPS.

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