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# Effect of azithromycin, as an adjunct to nonsurgical periodontal treatment, on microbiological parameters and gingival crevicular fluid biomarkers in generalized aggressive periodontitis

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*Background and Objective:* To study the effectiveness of azithromycin in combination with nonsurgical periodontal therapy on clinical and microbiological parameters, and on the MMP-8 and TIMP-1 levels in gingival crevicular fluid, over a 6-mo time-period in patients with generalized aggressive periodontitis.

*Material and Methods:* Thirty-two patients with generalized aggressive periodontitis were included in this randomized, double-blind, placebo-controlled, parallel-arm study. They were randomly assigned to azithromycin or placebo groups (500 mg once daily for 3 d). Probing depth, clinical attachment levels, presence of bleeding on probing and plaque were recorded. Gingival crevicular fluid samples were obtained from one single-rooted tooth, while microbiological samples were obtained from two single-rooted teeth, all with a probing depth of ≥ 6 mm. Microbiological parameters were analyzed by quantitative real-time PCR for *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Fusobacterium nucleatum*, *Prevotella intermedia* and total bacteria. Gingival crevicular fluid biomarkers were determined by immunofluorometric assay and ELISA.

*Results:* All clinical parameters improved, and microbiological parameters and gingival crevicular fluid MMP-8 levels significantly decreased, over the 6-mo period ($p < 0.05$); both groups demonstrated similar improvements. The azithromycin group presented a higher percentage of deep pockets resolved (probing depth reduction of ≥ 3 mm from baseline) compared with the placebo group at 1 mo ($p < 0.05$).

*Conclusion:* Adjunctive azithromycin therapy provides no additional benefit over nonsurgical periodontal treatment on clinical parameters, microbiological parameters and gingival crevicular fluid biochemical markers investigated in patients with generalized aggressive periodontitis.

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Aggressive periodontitis, characterized by rapid and severe periodontal destruction, comprises a heterogeneous group of periodontal diseases affecting adolescents and young adults (1,2). It is subclassified as localized or generalized in relation to the extent of the periodontal destruction (2). There may be an association between a specific microbial environment and a modified host response during the development of the aggressive forms of periodontitis (3). Previous studies have shown that alteration in host defense-cell functions, the increased expression of a wide variety of immunological and genetic risk factors, as well as the presence of a pathogenic oral biofilm could be attributed to the pathogenesis of the aggressive form of periodontal disease (3–6). Studies have shown evidence of an association of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia, Fusobacterium nucleatum* and *Prevotella intermedia* and generalized aggressive periodontitis, rather than the amount of microbial dental plaque (4,7–9).

Management of aggressive periodontitis has always presented a challenge for clinicians and there are still no established protocols and guidelines for efficiently controlling the disease. Owing to the high susceptibility of the host, conventional nonsurgical therapy of periodontal disease is generally insufficient in the management of aggressive periodontitis (10). Therefore, adjunctive use of systemic antimicrobials has been suggested, and several antibiotics or combination therapies have been used in generalized aggressive periodontitis in this respect (11,12).

Azithromycin is a systemic antibiotic that has drawn attention as an adjunctive antimicrobial in the treatment of periodontal disease owing to the favorable pharmacological properties and low incidence of adverse effects (13,14). It has a wide antimicrobial spectrum of action towards aerobic and anaerobic gram-negative organisms and is effective in the treatment of several systemic, intraoral and facial infections (15). Clinical studies have demonstrated that high concentrations of azithromycin persist in many tissues for 7–10 d following a very simple dosage regime (16–18). Azithromycin has shown promising anti-inflammatory properties besides well-known antimicrobial functions in several systemic diseases (14,19–21). It plays a role in host defense by modulating the functions of inflammatory cells. It exhibits inhibitory effects on oxidant production by stimulated cells and modulate proinflammatory and anti-inflammatory cytokine release by these cells (14,19–21).

The above properties appear to make azithromycin an ideal candidate adjunctive antibiotic for use in association with conventional periodontal therapy. Clinical studies investigating the effectiveness of adjunctive azithromycin in the management of chronic periodontitis are controversial (22–27). To date, only one study has investigated the clinical benefits of azithromycin in the treatment of generalized aggressive periodontitis (28). To the best our knowledge, no reports have examined the effectiveness of adjunctive azithromycin therapy on clinical and microbiological parameters and on gingival crevicular fluid biomarkers concomitantly in patients with generalized aggressive periodontitis. Therefore, the hypothesis tested was that significant differences are present in clinical and microbiological parameters and in gingival crevicular fluid MMP-8 and TIMP-1 levels in patients with generalized aggressive periodontitis receiving nonsurgical periodontal therapy in combination with systemic azithromycin compared with those receiving only nonsurgical periodontal therapy over a 6-mo period. The aim of the present work was to investigate the efficacy of azithromycin on clinical, microbiological and biochemical parameters beyond that obtained by nonsurgical periodontal therapy alone.

## Material and methods

### Study population

Consecutive patients with generalized aggressive periodontitis (17 men and 15 women; 18–38 years of age) were included from the Department of Periodontology, School of Dentistry, Ege University, İzmir. Before participation, the purpose and procedures of the study were fully explained to the participants, and all gave written informed consent in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the Ege University School of Medicine. Subject recruitment started in 2004 and was completed by the end of 2006.

### Inclusion and exclusion criteria

Complete medical and dental histories were taken from all subjects. Patients were excluded from the study if they had severe medical disorders (including diabetes mellitus), immunological disorders, any history of systemic disease, a known hypersensitivity to any type of macrolide, if they had received antibiotics or other medicines or periodontal treatment within the past 6 mo and if they were pregnant. Cigarette smoking status was self-reported by the patients at the screening visit. Subjects smoking $\geq 10$ cigarettes per day were not included in the present study.

Patients were diagnosed as having generalized aggressive periodontitis according to the clinical and radiographic criteria proposed by the 1999 International World Workshop for the Classification of Periodontal Disease and Conditions (2). Patients were included if they presented at least 16 teeth. All patients with generalized aggressive periodontitis demonstrated a generalized pattern of severe destruction, clinical attachment level of $\geq 5$ mm and probing depth of $\geq 6$ mm on eight or more teeth (at least three of which were other than central incisors or first molars) as well as radiographic bone loss of $\geq 30\%$ of the root length on those affected teeth. Additionally, clinical attachment loss was not consistent with the amount of plaque accumulation or local contributing factors.

### Investigator's calibration

The periodontal status of each patient was assessed by a single calibrated

examiner with experience in clinical trials (B.H.). Intra-examiner calibration was achieved by examination of 10 patients with periodontitis. The examiner measured the probing depth at six aspects of each tooth in the upper jaw, three times in each subject, 3 d apart, before beginning the study. The intra-examiner reproducibility for probing depth and clinical attachment loss measurements was assessed, and the interclass correlation coefficient was 0.985 (95% confidence interval: 0.968–0.993) for probing depth and 0.935 (95% confidence interval: 0.863–0.969) for clinical attachment loss.

### Study design and treatment

This clinical trial was designed as a randomized, double-blind, placebo-controlled, parallel-group study of 6 mo duration. It included two groups: a test group who received scaling and root planing (SRP) plus adjunctive azithromycin; and a control group who received SRP plus adjunctive placebo. The control group received placebo capsules. Subjects in the test group received a bottle containing 500-mg azithromycin tablets (Zitromax™; Pfizer İstanbul, Turkey), which were inserted into opaque capsules so that their appearance and packaging were identical to that of the placebo capsules. All capsules were filled with cornstarch. As a result, both the test and the placebo medication looked identical.

The outline of the study protocol is presented in Fig. 1. During the screening, the dichotomous presence of supragingival plaque as well as the presence of bleeding on probing (BOP) for each site was recorded. Thereafter, the full-mouth probing depth and clinical attachment level were measured using a standard manual probe (Williams periodontal probe; Hu-Friedy, Chicago, IL, USA) at six sites around each tooth (mesio-buccal, buccal, disto-buccal, disto-lingual, lingual and mesio-lingual). The cemento–enamel junction, or an anatomical landmark in the event that a restoration margin was present if the cemento–enamel junction was not visible, was used as a reference for clinical attachment loss measurements. Measurements of clinical periodontal parameters were repeated 1, 3 and 6 mo after completion of the medication. All clinical periodontal parameters of each patient were assessed by one examiner (B.H.) who was blinded to treatment assignment.

Subjects were scheduled for baseline sampling of gingival crevicular fluid and subgingival plaque 2 d after screening. All patients received oral hygiene instruction, and full-mouth SRP was performed in each quadrant under local anesthesia during four sequential visits. SRP was performed by the same calibrated, trained and blinded study investigator (B.H.) in a standardized manner. After completion of the SRP, patients were scheduled for sampling of gingival crevicular fluid and subgingival plaque and for medication. Antimicrobial therapy (azithromycin or placebo) was given to all patients at the end of the last treatment visit. Both antibiotic and placebo medications were administered once daily for 3 d. Any adverse effects from antibiotic intake and compliance, as reported by patients, were recorded throughout the study period. Two weeks after medication, participants were scheduled for gingival crevicular fluid and microbiologic sampling. Gingival crevicular fluid sampling was repeated at 2 wk and at 1, 3 and 6 mo after completion of the medication, while microbiologic sampling was repeated at 2 wk and at 1 and 6 mo after completion of the medication. Reminders of how to maintain good oral hygiene and maintenance therapy (i.e. removal of any supragingival plaque and calculus) was administrated at every visit during the study period.

### Randomization and allocation concealment

Patients were assigned consecutive and ascending numbers at the enrolment visit. Each subject was given a code number. Before the start of active therapy, the study coordinator randomly allocated the study subjects to

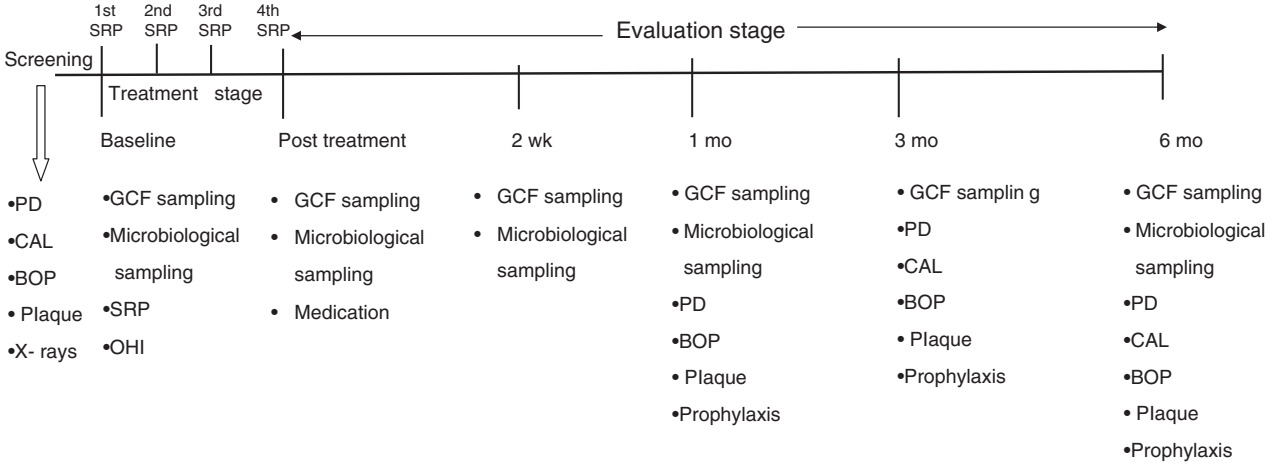


*Fig. 1.* Outline of the study. BOP, bleeding on probing; CAL, clinical attachment loss; GCF, gingival crevicular fluid; OHI, oral hygiene instruction; SRP, scaling and root planing.

one of the two study groups by means of a computer-generated randomization list. The allocation concealment was preserved as follows. The randomization list was kept by one of the authors (G.A.) until all treatment and follow-up visits were complete and the statistical analysis had been performed. The medications were given to the patients by another author (G.E.) who was blinded to the clinical recordings of the patients. The study was planned as double-blind, and the patient and the examiner who performed the treatment and the sampling (B.H.) were unaware of the medication. Analysis of gingival crevicular fluid and subgingival samples was performed by the authors (G.Ö., C.V., T.T., T.S.) who were not aware of treatment allocation. The biostatistician was also blinded to treatment assignment for the duration of the study.

### Gingival crevicular fluid sampling

At each recall visit, gingival crevicular fluid sampling was performed before recording the clinical parameters and performing microbiological sampling. Gingival crevicular fluid samples were taken from the mesio-buccal aspects of a single-rooted tooth exhibiting a probing depth of ≥ 6 mm. Before gingival crevicular fluid sampling, the supragingival plaque was removed from the interproximal surfaces with a sterile curette; these surfaces were dried gently by an air syringe and were isolated by cotton rolls. Gingival crevicular fluid was collected using filter paper (Periopaper; ProFlow, Inc., Amityville, NY, USA). Paper strips were carefully inserted into the crevice until mild resistance was felt and were left there for 30 s (29). Care was taken to avoid mechanical injury. Strips contaminated with blood were discarded (30). The volume of fluid in each strip was determined using the Periotron 8000 (Proflow, Amityville, NY, USA) and the strip was then placed in a sterile polypropylene tube and kept at −40°C until required for analysis. The readings from the Periotron 8000 were converted to an actual volume (μL) by reference to the standard curve.

### Analysis of collagenase-2 (MMP-8) and TIMP-1

Collagenase-2 (MMP-8) levels in the gingival crevicular fluid samples were determined using a time-resolved immunofluorometric assay, as described by Hanemaaijer *et al*. (27). The MMP-8-specific monoclonal antibodies 8708 and 8706 (Medix Biochemica Oy Ab, Kauniainen, Finland) were used as catching and tracer antibodies, respectively. The tracer antibody was labeled using europium-chelate. The assay buffer contained 20 mM Tris–HCL, pH 7.5, 0.5 M NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/L of dietyhlenetriaminepentaacetic acid. Samples were diluted in assay buffer and incubated for 1 h, then incubated for 1 h with the tracer antibody. Enhancement solution was added, and after 5 min fluorescence was measured using a 1234 Delfia Research Fluoremeter (Wallac, Turku, Finland).

The specificity of the monoclonal antibodies against MMP-8 was the same as that of polyclonal MMP-8 antibodies (31). TIMP-1 levels in the gingival crevicular fluid samples were determined by ELISA (R&D Systems, Amersham, Little Chalfont, Bucks., UK) according to the manufacturer's instructions. Gingival crevicular fluid samples were assayed at a dilution of 1 : 15 for TIMP-1. The ELISA for TIMP-1 detects native, complexed and fragmented species of TIMP-1. The upper limit of detection for total TIMP-1 in the ELISA is 1.25 ng/mL. The results were reported as total MMP-8 (pg/sample) and total TIMP-1 (pg/sample) in the sample. Calculation of the concentration data in each sample was performed by dividing the amount by the volume of the sample.

### Subgingival plaque sampling

Following sampling of gingival crevicular fluid, the subgingival plaque samples were collected from the mesio-buccal surface of two preselected single-rooted teeth, with a probing depth of ≥ 6 mm, using two standardized no. 30 sterile paper points: one was inserted at a 45° angle and the second was inserted parallel to the tooth axis; both were left in place for 30 s. The paper points were transferred to sterile empty polypropylene tubes and stored at −40°C.

### Microbiological procedures

Genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) and stored at −20°C until the quantitative real-time PCR (qRT-PCR) procedure was performed. qRT-PCR was performed using the LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) and the double-stranded DNA-binding dye SYBR Green I or hybridization probes using species-specific primers for five periodontopathic bacteria (*P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *T. forsythia* and *F. nucleatum*) and total bacteria, as described previously (32).

### Sample size calculation

The primary outcome variable was probing depth changes. The sample size required to ensure adequate power of this clinical trial was calculated before starting the study. Considering a mean difference of at least 0.5 mm between whole-mouth probing depth values of azithromycin and placebo groups and assuming the standard deviations to be 0.5 in both groups, it was determined that 13 subjects per group would be necessary to provide 80% power with an alpha of 0.05.

### Statistical analysis

In the present study, per-protocol analysis was applied for statistical comparisons. Chi-square analysis was used to test for gender differences as well as smoking status between both groups, while an age difference was tested using the Student's *t*-test. Clinical variables were calculated for within a patient at each visit and then patient was regarded as the unit of analysis. Gingival crevicular fluid and microbiological data obtained from study site per patient as well

as the percentage of subjects positive for the investigated species were also averaged for azithromycin and placebo groups at all time-points. Before analysis of the total bacteria and all five target bacteria over time, as well as analysis of gingival crevicular fluid and microbiological data the levels of MMP-8 and TIMP-1 were transformed to logarithms (base 10) in order to generate distributions that more closely resembled the normal distribution and to stabilize the variance.

The Shapiro–Wilk test was applied to each variable to assess whether the clinical, microbiological and biochemical variables were distributed normally. As the data achieved normality, the general linear model for repeated-measures analysis of variance was used to detect intragroup and intergroup differences in clinical, microbiological and biochemical data. Where there were significant differences, the Bonferroni test was used for post-hoc analysis, which takes into consideration the multiple comparisons. The significance level was set at $p < 0.05$.

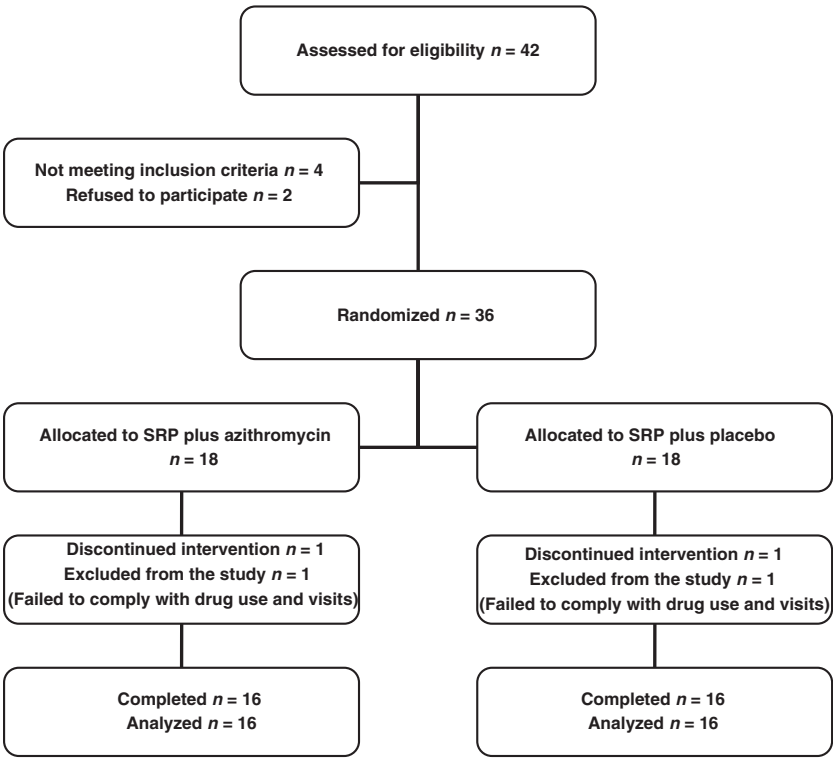


*Fig. 2.* Flow chart of participation in the study. SRP, scaling and root planing.

## Results

### Patient retention and demographics

The flow chart of the experimental study design is presented in Fig. 2. Of the 36 patients who entered into the study, 32 completed all examinations throughout the 6-mo study period. During this 6-mo trial, two patients from the azithromycin group and two from the placebo group dropped out because they were unable to attend regular maintenance appointments. Random assignment resulted in 16 patients in the azithromycin group and 16 patients in the placebo group (Fig. 2). Treatment with azithromycin was well tolerated and none of the patients complained of any adverse effects from the use of either azithromycin or placebo capsules.

Study groups completing the 6-mo follow-up had similar demographic characteristics. The sex distribution (nine men and seven women in the azithromycin group and eight men and eight women in the placebo group) and mean ages (28.75 ± 4.4 years in the azithromycin group and 29.56 ± 5.9 years in the placebo group) were similar. The smoking habits of patients were similar in both groups. Nine out of 16 patients in the azithromycin group and 10 out of 16 patients in the placebo groups were never smokers; and 43.8% of the patients in the azithromycin group and 38.5% of patients in the placebo group smoked < 10 cigarettes per day. Never smokers and smokers smoking < 10 cigarettes per day were evenly distributed in both groups ($p > 0.05$).

### Clinical results

The distribution of sites subgrouped by baseline probing depth as well as number of teeth in azithromycin and placebo groups is outlined in Table 1. Shallow, mild-to-moderate and deep sites were distributed identically between groups at baseline. The number of teeth present was also similar in azithromycin and placebo groups ($p > 0.05$).

The confidence intervals of the whole-mouth clinical parameters for azithromycin and placebo groups are shown in Table 2. At baseline, there were no significant differences in clinical periodontal parameters between the study groups ($p > 0.05$). The periodontal conditions of azithromycin and placebo groups improved markedly between baseline and 6 mo ($p < 0.05$). This was evidenced by a decreased probing depth and a substantial reduction in BOP and plaque scores. The reduction in mean probing depth in moderate (baseline 4–6 mm) and deep (baseline probing depth ≥ 7 mm) sites, and improvements in the whole-mouth clinical attachment loss scores, were similar for both groups at all time-points ($p > 0.05$). Azithromycin and placebo groups showed similar BOP and plaque scores at all time-points ($p > 0.05$) (Table 2).

Analysis of sites with a baseline probing depth of ≥ 7 mm revealed that the percentage of sites that exhibited improved probing depth by ≥ 3 mm were significantly higher in the adjunctive azithromycin group than in the placebo group at 1 mo ($p < 0.05$) (Table 3).

*Table 1.* Number of sites (percentage) subgrouped by baseline probing depth in azithromycin and placebo groups

| | Azithromycin group *n* (%) | Placebo group *n* (%) |
|---|---|---|
| No. of teeth | 26.31 ± 2.4 | 25.75 ± 2.4 |
| Baseline probing depth 0–3 mm | 51.63 ± 25.1 (34.18 ± 17.7) | 62.87 ± 14.8 (45.1 ± 12.3) |
| Baseline probing depth 4–6 mm | 63.31 ± 21.8 (42.19 ± 12.9) | 60.1 ± 18.9 (37.73 ± 10.9) |
| Baseline probing depth ≥ 7 mm | 39.56 ± 20.9 (23.63 ± 18.5) | 36.75 ± 13.6 (17.17 ± 11.8) |

### Total amount and concentration of MMP-8 and TIMP-1 in gingival crevicular fluid

The total amounts of MMP-8 in the gingival crevicular fluid of patients in the study groups are presented in Fig. 3A. Azithromycin and placebo groups had similar total amounts of MMP-8 in the gingival crevicular fluid at baseline ($p > 0.05$). After therapy with azithromycin plus SRP or with placebo plus SRP, the total amount of MMP-8 in the gingival crevicular fluid showed marked improvements at the 2-wk and 3-mo time-points ($p < 0.05$). There were no significant decreases in the total amount of MMP-8 in the gingival crevicular fluid compared with baseline at other study time-points ($p > 0.05$). The total amounts of MMP-8 in the gingival crevicular fluid of azithromycin and placebo groups were similar at all time-points ($p > 0.05$).

When the gingival crevicular fluid MMP-8 data were expressed as concentration, there was no significant difference between azithromycin and placebo groups in the baseline concentration of MMP-8 in gingival crevicular fluid ($p > 0.05$). The concentration of MMP-8 in the gingival crevicular fluid from azithromycin and placebo groups showed marked improvements at the 2-wk time-point ($p < 0.05$), but no significant differences were found at other study time-points compared with baseline ($p > 0.05$). The concentration of MMP-8 in the gingival crevicular fluid from azithromycin and placebo groups was found to be similar at all study time-points ($p > 0.05$) (data not shown).

The total amounts of TIMP-1 in the gingival crevicular fluid from the study groups are given in Fig. 3B. The total amount of TIMP-1 in the gingival crevicular fluid was similar in azithromycin and placebo groups at baseline ($p > 0.05$). In neither group did the total amount of TIMP-1 in the gingival crevicular fluid change during the study period ($p > 0.05$). There was no statistically significant difference in the total amount of TIMP-1 in the gingival crevicular fluid from azithromycin and placebo groups at any time-point ($p > 0.05$).

*Table 2.* 95% Confidence intervals (CI) of clinical variables at study time-points, and intragroup and intergroup comparisons

| Variables | Study time-point | Azithromycin group ($n = 16$) 95% CI | | Placebo group ($n = 16$) 95% CI | | ANOVA Intragroup | Intergroup |
|---|---|---|---|---|---|---|---|
| Probing depth (mm) | Baseline | 4.05 | 4.81 | 3.79 | 4.31 | $p < 0.000$, baseline vs. other time-points | $p = 0.179$, no significant difference between study groups at any time-point |
| | 1 mo | 2.42 | 2.80 | 2.32 | 2.55 | | |
| | 3 mo | 2.22 | 2.53 | 2.25 | 2.46 | | |
| | 6 mo | 2.17 | 2.46 | 2.11 | 2.29 | | |
| Probing depth (4–6 mm) | Baseline | 5.18 | 4.94 | 5.08 | 4.84 | $p = 0.000$, baseline vs. other time-points | $p = 0.632$, no significant difference between study groups at any time-point |
| | 1 mo | 2.99 | 2.59 | 3.04 | 2.66 | | |
| | 3 mo | 2.77 | 2.39 | 2.73 | 2.39 | | |
| | 6 mo | 2.67 | 2.33 | 2.57 | 2.23 | | |
| Probing depth (≥ 7 mm) | Baseline | 8.23 | 7.71 | 8.14 | 7.50 | $p = 0.000$, baseline vs. other time-points | $p = 0.608$, no significant difference between study groups at all-time points |
| | 1 mo | 4.16 | 3.64 | 4.04 | 3.58 | | |
| | 3 mo | 3.64 | 3.13 | 3.74 | 3.04 | | |
| | 6 mo | 3.48 | 2.86 | 3.38 | 2.84 | | |
| Clinical attachment loss (mm) | Baseline | 5.33 | 6.31 | 4.93 | 5.57 | $p = 0.000$, baseline vs. other time-points | $p = 0.217$, no significant difference between study groups at any time-point |
| | 1 mo | 3.79 | 4.57 | 3.54 | 4.02 | | |
| | 3 mo | 3.64 | 4.34 | 3.40 | 3.91 | | |
| | 6 mo | 3.57 | 4.29 | 3.35 | 3.86 | | |
| BOP (%) | Baseline | 65.97 | 83.53 | 65.3 | 79.63 | $p = 0.000$, baseline vs. other time-points | $p = 0.584$, no significant difference between study groups at any time-point |
| | 1 mo | 15.68 | 24.25 | 18.07 | 25.00 | | |
| | 3 mo | 14.44 | 21.99 | 16.57 | 25.80 | | |
| | 6 mo | 14.17 | 21.61 | 15.60 | 22.33 | | |
| Plaque (%) | Baseline | 93.48 | 98.73 | 85.22 | 97.50 | $p = 0.000$, baseline vs. other time-points | $p = 0.793$, no significant difference between study groups at any time-point |
| | 1 mo | 32.20 | 42.73 | 31.21 | 42.36 | | |
| | 3 mo | 29.68 | 36.96 | 29.47 | 37.79 | | |
| | 6 mo | 25.07 | 28.41 | 25.67 | 28.90 | | |

*Table 3.* Mean number (percentage) of deep pockets (≥ 7 mm probing depth at baseline) that exhibited probing depth reduction of ≥ 3 mm from baseline

| | Azithromycin group *n* (%) | Placebo group *n* (%) |
|---|---|---|
| 1 mo | 26.8 ± 5.5 (86.3 ± 10.4)* | 16.6 ± 5.9 (76.8 ± 14.8) |
| 3 mo | 28.5 ± 5.9 (91.3 ± 7.1) | 19.0 ± 7.4 (85.5 ± 15.3) |
| 6 mo | 29.0 ± 5.9 (92.9 ± 10.6) | 19.9 ± 6.9 (88.2 ± 14.3) |

* Significant difference from placebogroup ($p < 0.05$).

Azithromycin and placebo groups had a similar concentration of TIMP-1 in gingival crevicular fluid at baseline and at all study time-points ($p > 0.05$). In neither group did the gingival crevicular fluid TIMP-1 concentration change during the study period ($p > 0.05$) (data not shown).

### Microbiological findings

The detection frequency of the selected periodontopathogens was calculated as the percentage of patients positive for the selected pathogen given for the sampling sites (Fig. 4A,B). *A. actinomycetemcomitans* was only detected in a small number of patients at each observation time-point. Only two out of the 16 patients in the azithromycin group and five out of the 16 in the placebo group were positive for *A. actinomycetemcomitans*. After adjunctive azithromycin therapy, one of two *A. actinomycetemcomitans*-positive patients was still positive at the end of the study period, while in the placebo group one out of five *A. actinomycetemcomitans* patients was positive at the end of the study period. Low prevalence prevented any within- or between-group statistical comparison.

At baseline there were no significant differences between azithromycin and placebo groups regarding the *P. gingivalis*, *P. intermedia*, *T. forsythia* and *F. nucleatum* levels and total bacteria at sampling sites ($p > 0.05$). In both azithromycin and placebo groups, *P. gingivalis*, *P. intermedia* and *T. forsythia* levels and total bacteria showed a significant decrease from baseline to the end of the study period ($p < 0.05$) (Fig. 4C,D). The *F. nucleatum* levels decreased to the 2-wk time-point ($p < 0.05$), after which no decrease was observed in either group ($p > 0.05$). Although the levels of the pathogens declined, more than half of the sites initially positive for these species were also positive after treatment (Fig. 4C,D). Azithromycin and placebo groups exhibited similar levels of *P. gingivalis*, *P. intermedia*, *T. forsythia*, *F. nucleatum* and total bacteria at all study time-points ($p > 0.05$).

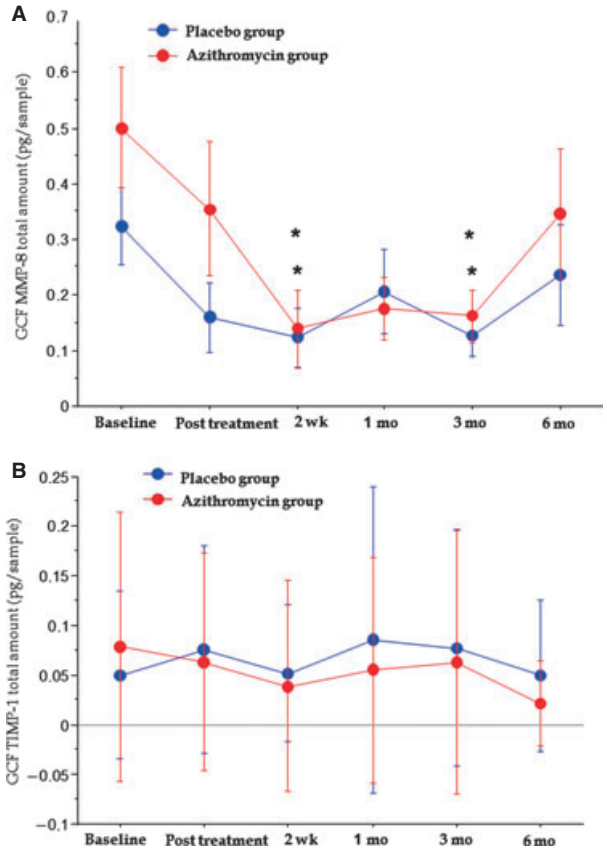


*Fig. 3.* (A) Total amount of MMP-8 (pg/sample) in the gingival crevicular fluid of both azithromycin and placebo groups from baseline to 6 mo. *Significant difference from baseline in azithromycin and placebo groups (repeated-measures analysis of variance, $p < 0.05$).

## Discussion

The present study evaluated the effectiveness of azithromycin as an adjunct to nonsurgical periodontal therapy on clinical and microbiological parameters and on gingival crevicular fluid biomarkers in patients with generalized aggressive periodontitis. On the basis of the present findings, it can be concluded that adjunctive azithromycin provides no additional benefit over nonsurgical periodontal treatment on clinical periodontal parameters or on the microbial species and gingival crevicular fluid biomarkers investigated in patients with generalized aggressive periodontitis. Adjunctive azithromycin seems to be effective in deep pockets by ≥ 3 mm reduction compared to baseline in a short term but this benefit was not observed the rest of the study period. To the best of our knowledge this is the first study to investigate the adjunctive effect of azithromycin on microbiological and gingival crevicular fluid biomarkers, in addition to clinical parameters, in patients with generalized aggressive periodontitis.

Treatment approaches, including the adjunctive use of systemic

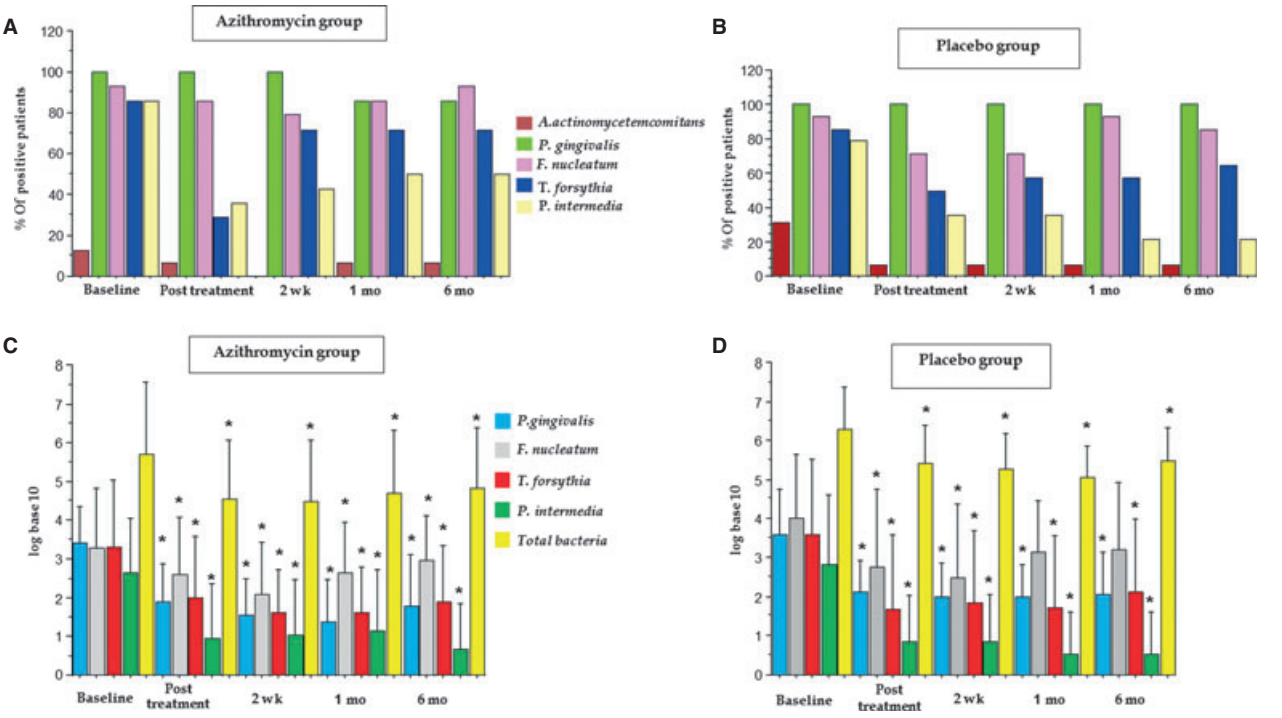


*Fig. 4.* (A) Frequency of detection of different periodontal pathogens in subgingival samples of the azithromycin group at the study visits. (B) Frequency of detection of different periodontal pathogens in subgingival samples of the placebo group at the study visits. (C) Total amount of periodontal pathogens investigated in subgingival samples of the azithromycin group at the study visits. *Significant difference from baseline (repeated-measures analysis of variance, $p < 0.05$). (D) Total amount of periodontal pathogens investigated in subgingival samples of the placebo group at the study visits. *Significant difference from baseline (repeated-measures analysis of variance, $p < 0.05$).

antimicrobials or combination therapies, has been advocated for the management of generalized aggressive periodontitis. Several randomized-controlled clinical trials have shown greater benefit of systemic antimicrobials in the management of patients with generalized aggressive periodontitis when used combination with nonsurgical therapy (11). In the present study, significant improvements in clinical parameters, as indicated by reduced clinical inflammation, probing depth and clinical attachment loss, were observed in patients with generalized aggressive periodontitis after SRP plus azithromycin and SRP plus placebo therapy, and the improvements were maintained over a period of 6 mo. This could be attributable to the high quality of nonsurgical periodontal therapy for which a considerable time was spent in both groups. As expected, SRP leads to the resolution of the inflammatory response and cessation of the progression of periodontal disease, and thereby results in a relative gain of clinical attachment and reduction of probing depth (33,34). Sufficient periodontal maintenance therapy, including supragingival scaling and oral hygiene instruction given to the study patients at recall visits, might clearly have improved the oral hygiene conditions throughout the study period in both groups. The results of the present study are in agreement with reports indicating that patients with generalized aggressive periodontitis respond well to mechanical instrumentation (35–37). It is well known that the efficacy of nonsurgical therapy at an individual site is related to the baseline probing depth and that deeper pockets have greater potential for clinical improvement (33,34). In the present study, the use of azithromycin in combination with SRP in patients with generalized aggressive periodontitis resulted in similar clinical improvements to those obtained by SRP plus placebo therapy in both deep and moderate pockets. On the other hand, when the percentage of severely diseased sites (i.e. probing depth ≥ 7 mm) exhibiting a probing depth reduction of at least 3 mm from baseline were taken into consideration, a significantly greater percentage of sites receiving adjunctive azithromycin was observed to attain this threshold level of probing depth reduction at 1 mo than sites receiving adjunctive placebo, and, although not significant, this improvement could still be seen at 6 mo. Our findings are comparable with those of Haas *et al.* (28), who previously showed that adjunctive azithromycin resulted in significantly higher percentages of teeth with a probing depth reduction of ≥ 1 mm from baseline to 12 mo in patients with generalized aggressive periodontitis. It is known that probing depth reductions of ≥ 3 mm might represent marked improvements of individual sites, which is a very stringent criterion for assessing the success of treatment in deep pockets (33,34). From this point, we might suggest that adjunctive azithromycin has a greater therapeutic

impact than placebo plus nonsurgical therapy in the deepest sites that were not thoroughly cleaned by instrumentation.

Increased levels of MMP-8 in the gingival crevicular fluid are related to the progression of the disease and these levels decrease after SRP in patients with chronic periodontitis (38–40). It has been previously demonstrated that MMP-8 was also present at elevated levels in patients with aggressive periodontitis and mainly derived from fibroblasts, epithelial cells and macrophages (41–43). The persistence of MMP-8 at physiologic levels after treatment has been suggested to be involved in the down-regulation of the inflammatory process and the onset of the reparative phase (44). In the present study, the total amount of MMP-8 in the gingival crevicular fluid decreased nonsignificantly in both groups after completion of nonsurgical periodontal therapy (post-treatment). These levels continued to decrease, to significant levels, 2 wk after completion of SRP with or without azithromycin, and showed a slight increase at 1 mo but a more dramatic reduction once the tissues had time to heal (i.e. following a 3-mo period of maintenance), and the reductions became less obvious at 6 mo in both groups. Both groups had similar levels of MMP-8 in the gingival crevicular fluid during the study period. The levels of TIMP-1 in the gingival crevicular fluid remained unchanged over the study period, with no significant differences between treatment groups observed at any study time-point. The present study is also interesting in terms of showing the levels of gingival crevicular fluid MMP-8 and TIMP-1 in patients with generalized aggressive periodontitis before and after therapy because almost all of the data on gingival crevicular fluid MMP-8 levels in periodontal diseases have been derived from patients with chronic periodontitis. In the present study, reduction in the level of MMP-8 in the gingival crevicular fluid is concomitant with clinical healing as well as a reduction in the microorganisms investigated.

The present study showed a significant decrease in the numbers of *P. gingivalis*, *P. intermedia* and *T. forsythia* in both groups, but none was eradicated. It has been emphasized that the mere presence of putative pathogens does not indicate the absence or presence of disease, but high numbers are required (45). Owing to the low frequency of *A. actinomycetemcomitans* in our patients with generalized aggressive periodontitis, it was impossible to determine the effects of azithromycin on *A. actinomycetemcomitans*. The microbiological goal of periodontal therapy (46,47) has been achieved in the present study, although systemic azithromycin did not result in any additional effect on these levels except for lower *T. forsythia* levels after adjunctive azithromycin therapy. Through the improvement in clinical periodontal parameters after SRP therapy, the local environment becomes less favorable for periodontal pathogens and results in a reduced pathogenic microflora (48). Given the antimicrobial activity of azithromycin *in vitro* (15,18,49), one might expect an additional microbiological effect of systemic azithromycin in the present study. On the other hand, azithromycin, given systemically after completion of nonsurgical therapy, had no additional effect on the periodontal pathogens investigated, but did have a further effect on the percentage of deep pockets that had a reduction in probing depth of at least 3 mm. This might be because when azithromycin was administered after termination of nonsurgical periodontal therapy, the pathogenic flora was already depressed by SRP, gingival inflammation was resolved and perhaps the azithromycin concentration was not sufficiently high, as in inflamed tissues in patients with periodontitis (18) when this drug was given after SRP was completed in 3 wk. Recently, the azithromycin concentrations in gingival crevicular fluid have been shown to be similar in gingivitis and healthy sites (50). Moreover, completion of nonsurgical therapy in 3 wk might have allowed biofilm to recolonize in some sites that had previously been root planed, undermining the effectiveness of the adjunctive antibiotic.

A few studies have reported the adjunctive effect of azithromycin on the oral microbiota in chronic periodontitis, but none in aggressive periodontitis. The first report, by Sefton *et al.* (51), found greater reduction in the counts of black-pigmented anaerobes and spirochetes in patients with chronic periodontitis receiving adjunctive azithromycin compared with controls. In a recent study, the adjunctive use of azithromycin resulted in a significant decrease in the levels of *A. actinomycetemcomitans, P. gingivalis* and *T. forsythia* in deep pockets of patients with chronic periodontitis (25). It has been demonstrated that when SRP was performed in a week (partial-mouth SRP, which was completed in a week) similar microbiological improvement to full-mouth SRP was observed in patients with chronic periodontitis and both are superior to conventional SRP in the depression of pathogenic flora (24,52). Different results could be explained by the above-mentioned factors. Studies are necessary to clarify the adjunctive microbiological effectiveness of azithromycin after partial or full-mouth SRP in patients with aggressive periodontitis.

It has been recommended that systemic antibiotics have to be given immediately after completion of SRP to facilitate the efficacy of antimicrobials after removal of the subgingival biofilm (53,54). On the other hand, there is no agreement about the period of nonsurgical periodontal therapy and the optimal time for the administration of an adjunctive antimicrobial regimen. Recently, it has been recommended for nonsurgical therapy to be complete within a short time-period and for the antibiotic intake to be started on the day of treatment completion (12,55). Data from randomized clinical studies of adjunctive antibiotic treatments of patients with aggressive periodontitis have shown that immediate administration of the drug resulted in a better clinical outcome, especially in initially deep sites (23,56–58). Regarding the adjunctive effect of azithromycin in initially deep sites, the late application of the drug may not be

as effective as the application of antibiotics immediately after SRP (20,47). In the present study, azithromycin was prescribed after nonsurgical therapy was completed in 3 wk and the benefit of the antibiotic is particularly evident in deeper pockets where mechanical debridement is less effective.

Overall, the present study indicates that adjunctive azithromycin, in combination with SRP therapy, had similar effects on clinical periodontal parameters, on the periodontal pathogens investigated and on the gingival crevicular fluid MMP-8 and TIMP-1 levels in patients with generalized aggressive periodontitis. Adjunctive azithromycin seems to be effective in deep pockets, as demonstrated by a reduction in probing depth of ≥ 3 mm compared with baseline at 1 mo, but this benefit was not observed subsequently in the study period. Owing to the known immune-modulatory roles of azithromycin on host defense, further studies are needed to investigate the effectiveness of azithromycin as an adjunct to nonsurgical periodontal therapy on proinflammatory and anti-inflammatory cytokine synthesis in the management of aggressive forms of periodontal disease.

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