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Clinical and microbiological benefits of metronidazole alone or with amoxicillin as adjuncts in the treatment of chronic periodontitis: a randomized placebo-controlled clinical trial

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Abstract

Aim: To evaluate the effects of the adjunctive use of metronidazole (MTZ) or MTZ+amoxicillin (AMX) in the treatment of generalized chronic periodontitis (ChP).

Materials and methods: Fifty-one subjects ($n = 17$/group) were randomly assigned to receive scaling and root planing (SRP) only or combined with MTZ (400 mg t.i.d.) or MTZ+AMX (500 mg t.i.d.) for 14 days. Clinical and microbiological examinations were performed at baseline and 3 months post-SRP. Nine plaque samples/subject were analysed by checkerboard DNA–DNA hybridization for 40 bacterial species.

Results: Subjects receiving MTZ+AMX exhibited a greater mean gain of clinical attachment, reduction in probing depth (PD) in intermediate and deep sites and a lower percentage of sites with PD $\geqslant$ 5 mm at 3 months, in comparison with those treated with SRP only ($p < 0.05$). The major benefit from the adjunctive use of MTZ was a greater reduction in PD in deep sites. SRP+MTZ+AMX was the only treatment that significantly reduced the levels and proportions of all red complex pathogens and elicited a significantly greater beneficial change in the microbial profile in comparison with SRP only.

Conclusion: The adjunctive use of MTZ+AMX offers short-term clinical and microbiological benefits, over SRP alone, in the treatment of non-smokers subjects with generalized ChP. The added benefits of MTZ were less evident.

Key words: amoxicillin; chronic periodontitis; metronidazole; microbiology; periodontal disease; scaling and root planing

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Conflict of interest and source of funding statement

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Among the various antibiotics used in the treatment of chronic periodontitis (ChP), metronidazole (MTZ) alone or combined with amoxicillin (AMX) seems to be the most favourable. MTZ is very efficient against strict anaerobic bacterial species, characteristic of the main pathogens associated with the onset and progression of ChP. The benefits of the adjunctive use of MTZ in periodontal treatment were initially pointed out by Loesche and colleagues in the 1980s (Loesche et al. 1984, 1987, 1991) and its association with AMX was shown to be effective in treating patients with aggressive or refractory periodontitis infected with *Aggregatibacter actinomycetemcomitans* (van Winkelhoff et al.

1989, 1992). Later on, other studies indicated that the combination of MTZ and AMX was also promising to treat ChP (Berglundh et al. 1998, Winkel et al. 2001, Rooney et al. 2002, Pahkla et al. 2006, Moeintaghavi et al. 2007, Matarazzo et al. 2008, Cionca et al. 2009, 2010).

To date, only two studies have directly compared the clinical effects of MTZ and MTZ+AMX in the treatment of ChP (Rooney et al. 2002, Matarazzo et al. 2008). Both studies indicated better results in terms of reducing full-mouth mean probing depth (PD) and gaining clinical attachment when MTZ and AMX were used as adjuncts to scaling and root planing (SRP), superior to the effects observed with the association of only MTZ. Although the microbiological effects of these antibiotics were not directly studied in non-smokers, Matarazzo et al. (2008) compared these two treatment protocols as regards their ability to change the subgingival microbial profile of smokers with ChP. Subjects who took either adjunctive MTZ or MTZ+AMX showed a better response to treatment than those treated with SRP alone. The greatest benefits in clinical parameters as well as in the composition of the subgingival microbiota were achieved when the two antibiotics were combined. This therapy led to the most striking reductions in the levels and proportions of the three periodontal pathogens from the red complex, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia,* and the greatest increase in the proportions of the beneficial *Actinomyces* and purple complex species.

Therefore, the aim of the present study was to evaluate the clinical effect and the changes occurring in the subgingival microbial profile when non-smoker subjects with generalized ChP are treated with SRP+MTZ or SRP +MTZ+AMX, by directing comparing the outcomes of these two treatment protocols with those obtained with SRP alone.

Material and Methods

Sample size calculation

There is a general consensus in the periodontal literature that a difference of 1 mm between treatments for PD or clinical attachment level (CAL) changes at initially deep sites would be clinically relevant (Greenstein 2003, Guerrero et al. 2005, Matarazzo et al. 2008, Feres et al. 2009, Griffiths et al. 2011). Therefore, the ideal sample size to ensure adequate power in this clinical trial was calculated considering differences of 1.0 mm between each of the test groups and the control group for the mean CAL change in sites with initial PD $\geqslant$ 7 mm. Furthermore, the standard deviation of 1.0 mm for CAL change in initially deep sites was determined based on our earlier study of subjects receiving SRP alone or combined with MTZ or with MTZ+AMX (Matarazzo et al. 2008). Based on these calculations, it was determined that 17 subjects per group would be necessary to provide an 80% power with an α of 0.05.

Subject population

Subjects with previously untreated periodontitis were selected among the population referred to the Periodontal Clinic of Guarulhos University (Guarulhos, SP, Brazil) for treatment. Detailed medical, periodontal and dental histories were obtained. Subjects who fulfilled the inclusion criteria were invited to participate in the study. All eligible subjects were fully informed of the nature, potential risks and benefits of their participation in the study and signed a Term of Informed Consent. This study protocol was approved previously by the Guarulhos University's Ethics Committee in Clinical Research.

Inclusion and exclusion criteria

All subjects were in good general health and presented with at least 15 teeth excluding third molars and teeth indicated for extraction. All subjects were diagnosed with generalized ChP, based on the current classification of the American Academy of Periodontology (Armitage 1999). The inclusion criteria were as follows: $\geqslant$30 years of age and a minimum of six teeth with at least one site each with PD and CAL $\geqslant$ 5 mm, as well as at least 30% of the sites with PD and CAL $\geqslant$ 4 mm and bleeding on probing (BOP). The exclusion criteria were as follows: previous subgingival periodontal therapy, extensive prosthetic rehabilitations, allergy to AMX or MTZ, smoking, pregnancy, systemic diseases that could affect the progression of periodontal disease (e.g. diabetes and immunological disorders), long-term administration of anti-inflammatory medication, need for antibiotic pre-medication for routine dental treatment and antibiotic therapy in the previous 6 months.

Experimental design and treatment protocol

In this double-blinded, randomized, parallel-design and placebo-controlled clinical trial, subjects were randomly assigned (*see* ''Subjects' randomization and allocation to therapies'') to one control and two test groups ($n = 17$ per group) as follows: control: SRP; test 1: SRP+MTZ (400 mg t.i.d. for 14 days); and test 2: SRP+MTZ (400 mg t.i.d. for 14 days)+AMX (500 mg t.i.d., for 14 days). Subjects in the control group received MTZ and AMX placebos and subjects from the MTZ group received an AMX placebo, both t.i.d.., for 14 days. The antibiotic or placebo therapies started immediately after the first session of mechanical instrumentation.

During the initial phase, all subjects received instruction on proper home-care techniques and full-mouth supragingival scaling. They were also given the same dentifrice (Colgate Total®, Anakol Ind. Com. Ltda- Kolynos do Brasil - Colgate Palmolive Co, São Bernardo do Campo, SP, Brazil) to use during the period of the study. SRP was performed by two trained periodontists (M. P. S. and T. A. O. S.) in four to six appointments lasting approximately 1 h each, using manual instruments and under local anaesthesia. The end point for each SRP appointment was ''smoothness of the scaled roots'', which was checked by one of the study coordinators (M. Fe.). The two therapists were randomized according to the different treatment groups so that each one treated approximately the same number of patients in each group. An overall full-mouth SRP was performed during the first treatment visit, and in the following appointments, the scaling of specific groups of teeth began, starting with those presenting the deepest pockets. Treatment of the entire oral cavity was completed in a period lasting between 19 and 21 days, and all sites with PD $\geqslant$ 5 mm were scaled within the first 2 weeks (i.e. during the course of antibiotic administration). All subjects received microbiological and clinical monitoring at baseline and at 3 months post-therapy.

Subjects' randomization and allocation to therapies

Each subject was given a code number during the enrolment visit and the study coordinators (M. Fe. and L. C. F.) used a computer-generated table with 51 numbers randomly distributed into three blocks to allocate them to one of the three therapeutic groups. Guarulhos University Pharmacy prepared the antibiotics and placebo capsules, which were stored in identical opaque plastic bottles. The bottles were sent to the study coordinators, who marked the code number of each subject on a set of four bottles (two for each week), according to the therapy assigned. The coded bottles were given to the examiners (G. M. S. S. and J. A. V. M.), who at no time during the study had any access to information about the contents of the bottles or the assignment of subjects to the three therapies. In addition, all study personnel, including the biostatisticians, the laboratory technicians and participants, were blinded to treatment assignment during the study. Code breaking was conducted by the study coordinators only after the statistical analysis was performed.

Compliance and adverse events

The subjects were asked to bring the bottles containing the medication at the end of each week, when compliance was checked. The bottles contained 21 capsules of each placebo, MTZ or AMX, sufficient for 1 week of medication (21 capsules, 3 times/day for 7 days). During these visits, subjects returned the old bottle containing the placebo or the antibiotic and received a new bottle of medication/placebo. Subjects also answered a questionnaire about any self-perceived side-effects of the medications on the last day of drug/placebo administration. Two study assistants conducted this inquiry, and were also responsible for calling the subjects every 2 days to monitor compliance. These assistants were not examiners or therapists in this study.

Clinical monitoring

Clinical monitoring was performed by the two calibrated examiners (see ''Investigator's calibration'') and the treatment was carried out by another two clinicians (M. P. S. and T. A. O. S.). Thus, the examiners and the clinicians were masked to the nature of the treatment groups. Subjects were clinically monitored at baseline and at 3 months post-therapy. The presence or absence of visible plaque (without using disclosing agents), gingival bleeding, BOP and suppuration, as well as PD (distance in mm from the gingival margin to the bottom of the probeable gingival pocket) and CAL (distance in mm from the cementoenamel junction – (CEJ) - to the bottom of the probable gingival pocket) were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) in all teeth, excluding third molars. If the CEJ was located subgingivally, the examiners determined its exact location visually, by gently displacing the ginigval margin with the periodontal probe, or by tactile sensitivity. If the CEJ was absent, i.e. due to tooth abrasion or a buccal crown, a reference point closest to its original position was considered and recorded on the subject's chart (e.g. margin of a restoration or abrasion). The PD and CAL measurements were recorded to the nearest millimetre using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA).

Investigator's calibration

The two examining researchers (G. M. S. S. and J. A. V. M.) participated in the calibration exercise that was performed in 10 non-study subjects with ChP. Each examiner measured one quadrant per subject. The quadrant chosen should have at least six teeth. If a quadrant presented fewer than six teeth, the following quadrant was chosen. For better standardization, quadrant #1 was the first choice, followed by #2, #3 and #4, respectively. Initially, the first examiner measured PD and CAL in a given quadrant and 15 min. later, the second examiner measured the same quadrant. Sixty minutes later, this same protocol was repeated, but the order of the examiners was changed. Therefore, all 10 subjects were probed twice in the same visit by each of the two examiners. Upon completion of all measurements, the intra- and inter-examiner variability for PD and CAL measurements were assessed. Calibration was carried out according to Araujo et al. (2003) and the standard error (SE) of measurement was calculated. The inter-examiner variability was 0.09 mm for PD and 0.31 mm for CAL. The mean intra-examiner SE variability was 0.11 mm (PD) and 0.21 mm (CAL) for the first examiner (G. M. S. S.), and 0.16 mm (PD) and 0.19 mm (CAL) for the second examiner (J. A. V. M.).

Microbiologic assessment

Sample collection

Nine subgingival plaque samples were collected at baseline and at 3 months post-SRP from nine non-contiguous interproximal sites per subject. The selected sites were randomized in different quadrants and subsets according to baseline PD, three samples in each of the following categories: shallow ($PD \leqslant 3$ mm), intermediate (PD 4–6 mm) and deep ($PD \geqslant 7$ mm). After the clinical parameters had been recorded, the supragingival plaque was removed and the subgingival samples were taken using individual sterile mini-Gracey curettes (#11–12) and immediately placed in separate Eppendorf tubes containing 0.15 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). One hundred microlitres of 0.5 M NaOH was added to each tube and the samples were dispersed using a vortex mixer.

Checkerboard DNA–DNA hybridization

Counts of 40 bacterial species were determined in each sample, using the checkerboard DNA–DNA hybridization technique (Socransky et al. 1994). The microbiological analysis was entirely performed at the Laboratory of Microbiology of Guarulhos University. The samples were boiled for 10 min. and neutralized using 0.8 ml of 5 M ammonium acetate. The DNA released was then placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA), concentrated on a 15×15 cm positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN, USA) and fixed to the membrane by baking it at 120°C for 20 min. The membrane was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labelled whole genomic DNA probes for 40 bacterial species were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using the antibody to digoxigenin conjugated with alkaline phosphatase and chemiluminescence

detection. The last two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. Signals were evaluated visually by comparison with the standards at 10^5 and 10^6 bacterial cells for the test species on the same membrane by a calibrated examiner (k-test = 93%). They were recorded as: 0 = not detected; $1 < 10^5$ cells; 2 = $\sim 10^5$ cells; 3 = 10^5–10^6 cells; 4 = $\sim 10^6$ cells; or 5 = $> 10^6$ cells. The sensitivity of this assay was adjusted to allow the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. This procedure was carried out in order to provide the same sensitivity of detection for each species. Failure to detect a signal was recorded as 0, although conceivably, counts in the 1–1000 ranges could have been present.

Primary and secondary outcome variables

This study compared the clinical and microbiological effects of two antibiotic protocols with one control treatment. The primary outcome variable was the mean CAL changes at 3 months post-SRP in sites with baseline PD $\geqslant$ 7 mm. The secondary outcome variables were the mean PD change in sites with baseline PD $\geqslant$ 7 mm, the mean CAL and PD changes in the full-mouth as well as in sites with baseline PD between 4 and 6 mm, the mean changes in individual full-mouth mean CAL, difference in the number of sites with PD $\geqslant$ 5 mm or PD < 5 mm, as well as the mean changes in the levels and proportions of the 40 bacterial species analysed (individually or as complexes).

Statistical analysis

The mean percentage of sites with visible plaque, gingival bleeding, BOP and suppuration, as well as the mean PD and CAL were computed for each subject and then averaged across subjects in all groups. Similarly, the changes in PD, CAL and BOP over time were examined in subsets of sites according to the initial PD of 4–6 and $\geqslant$ 7 mm. Values for each clinical parameter were averaged separately within both PD categories in each subject and then averaged across subjects in the treatment groups. The mean counts ($\times 10^5$) of individual bacterial species were averaged within each subject and then across subjects in the three groups. The percentage of the total DNA probe counts was determined initially in each site, then per subject and averaged across subjects in the three groups.

The significance of differences among the three groups for the clinical and microbiological parameters was sought using the Kruskal–Wallis and Dunn's multiple comparison test. The Wilcoxon test was used to test differences between the two time-points. The level of significance was set at 5%. Adjustments were made for multiple comparisons when the 40 bacterial species were evaluated (Socransky et al. 1991). In brief, an overall p of $0.05 = 1\text{-}(1-k)^{40}$ was computed, where k is the desired individual p value. Therefore, p values of < 0.00127 was considered statistically significant at $p < 0.05$.

Results

Subject retention

Subject recruitment started in June 2008 and was completed by the end of August 2009. Figure 1 presents the flow chart of the study design. All subjects returned

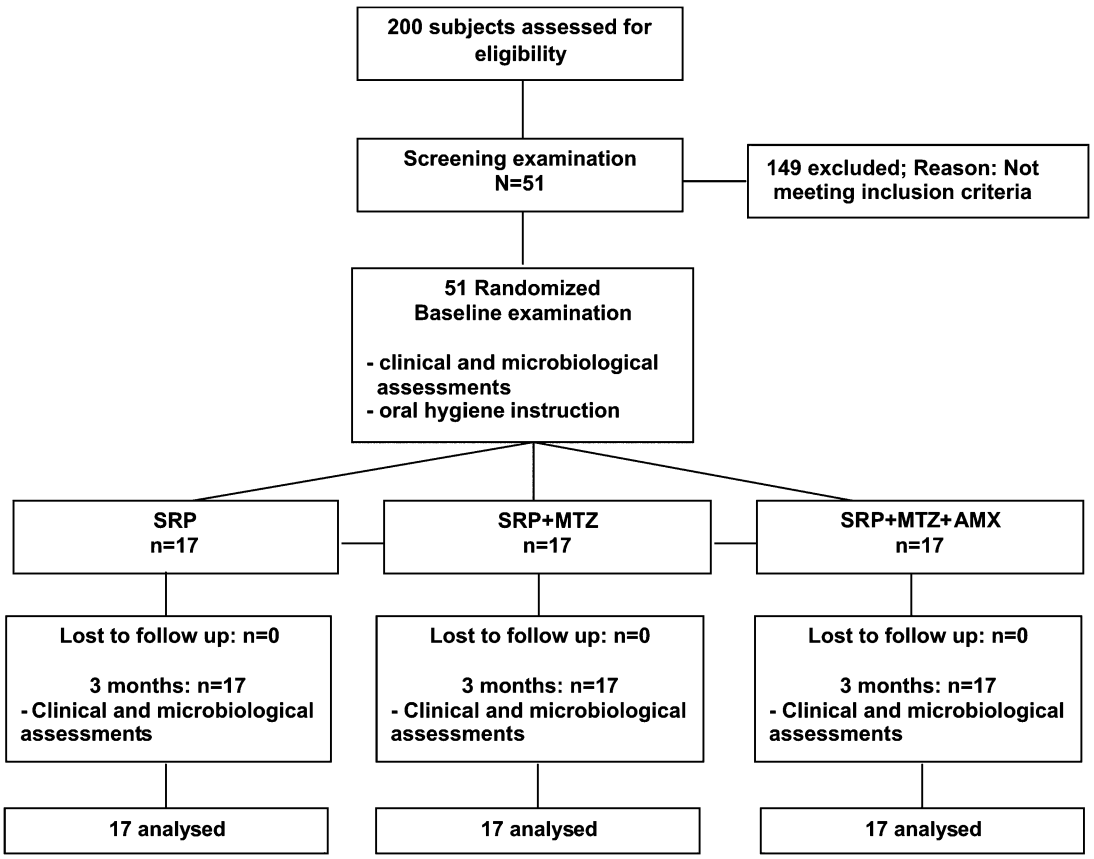

Fig. 1. Flow chart of the study design. SRP, scaling and root planing; MTZ, metronidazole; AMX, amoxicillin.

for the 3-month follow-up visit. Thus, a total of 51 subjects completed the study, 17 subjects per group.

Adverse effects and compliance

Adverse events were reported by four subjects from the MTZ+AMX group, five from the MTZ group and two from the control group, including diarrhoea (MTZ group, $n = 1$); headache (MTZ group, $n = 1$; MTZ+AMX group, $n = 2$); metallic taste (control group, $n = 1$ and $n = 3$ from each test group); vomiting (MTZ group, $n = 1$; MTZ +AMX group, $n = 3$); weakness (control group, $n = 2$); and irritability ($n = 1$ from each test group). Only one subject from the MTZ group reported that the schedules for taking the drugs disturbed his day-to-day activities and, if needed, he would not like to take the drugs again. All the other subjects reported that they would start the treatment again if necessary.

Clinical findings

Table 1 presents the demographic and full-mouth clinical data for all treatment groups, at baseline and at 3 months. No statistically significant differences were observed among groups for any of the parameters evaluated at both time points ($p > 0.05$). In addition, the three treatment protocols led to a significant improvement ($p < 0.05$) in all the clinical parameters. Reductions in the mean PD and gain in the mean clinical attachment between baseline and 3 months are presented in Table 2. Both antibiotic treatments elicited a statistically significant greater reduction in the mean full-mouth PD than SRP alone. In addition, subjects taking adjunctive MTZ+AMX also showed statistically significant improvements in full-mouth CAL as well as in PD and CAL in

Table 1. Demographic characteristics and mean (± SD) full-mouth clinical parameters at baseline and at 3 months post-therapy

Variable	Time-point	Treatment groups		
		SRP ($n = 17$)	SRP+MTZ ($n = 17$)	SRP+MTZ+AMX ($n = 17$)
Gender (male/female)	Baseline^{NS}	7/10	8/9	8/9
Age (years)	Baseline^{NS}	48.9 ± 12.4	41.2 ± 6.1	45.5 ± 9.6
% of sites with:				
PD ⩽3 mm	Baseline^{NS}	54.1 ± 15.2	55.1 ± 15.1	52.2 ± 12.2
PD 4–6 mm	Baseline^{NS}	39.4 ± 13.9	39.5 ± 12.1	38.7 ± 11.4
PD⩾7 mm	Baseline^{NS}	6.5 ± 5.8	6.4 ± 5.7	9.1 ± 7.3
PD (mm)	Baseline^{NS}	3.6 ± 0.5	3.6 ± 0.6	3.8 ± 0.5
	3 months^{NS}	3.0 ± 0.6 *	2.9 ± 0.5 *	2.7 ± 0.4 *
CAL (mm)	Baseline^{NS}	4.2 ± 0.7	4.0 ± 0.7	4.4 ± 0.6
	3 months^{NS}	3.7 ± 0.7 *	3.4 ± 0.6 *	3.6 ± 0.7 *
% of sites with:				
Visible plaque	Baseline^{NS}	82.4 ± 13.0	71.7 ± 17.9	79.0 ± 15.9
	3 months^{NS}	30.1 ± 22.9 *	26.1 ± 11.9 *	30.2 ± 23.7 *
Gingival blending	Baseline^{NS}	34.5 ± 27.1	36.6 ± 25.6	36.8 ± 26.6
	3 months^{NS}	16.9 ± 16.3 *	11.9 ± 13,4 *	16.8 ± 15.5 *
Bleeding on probing	Baseline^{NS}	70.9 ± 22.4	73.3 ± 22.4	72.9 ± 17.2
	3 months^{NS}	32.8 ± 25.1 *	15.7 ± 19.3 *	31.5 ± 25.4 *
Suppuration	Baseline^{NS}	1.0 ± 3.4	2.9 ± 8.7	4.8 ± 7.8
	3 months^{NS}	0.1 ± 0.4 *	0.07 ± 0.2 *	0 ± 0 *

The significance of differences between baseline and 3 months was assessed using the Wilcoxon test (*$p < 0.05$). The significance of differences among groups at each time-point was assessed using the Kruskal–Wallis test ($p > 0.05$; NS, non-significant).
SRP, scaling and root planing; MTZ, metronidazole; AMX, amoxicillin; PD, probing depth; CAL, clinical attachment level.

Table 2. Mean PD reduction and clinical attachment gain between baseline and 3 months post-therapy.

Variable	Treatment groups			
	SRP ($n = 17$)	SRP+MTZ ($n = 17$)	SRP+MTZ+AMX ($n = 17$)	p-value†
PD reduction (Full-mouth)	0.63 ± 0.26^{a}	0.76 ± 0.48^{b}	1.08 ± 0.40^{b}	0.010
Attachment gain (Full-mouth)	0.53 ± 0.27^{a}	0.60 ± 0.45^{ab}	0.76 ± 0.42^{b}	0.013
PD reduction (sites bPD 4-6 mm)	1.06 ± 0.40^{a}	1.36 ± 0.49^{a}	1.77 ± 0.45^{b}	0.001
Attachment gain (sites bPD 4-6 mm)	0.54 ± 0.71^{a}	1.19 ± 0.48^{ab}	1.37 ± 0.39^{b}	0.001
PD reduction (sites bPD⩾7 mm)	2.28 ± 1.11^{a}	3.35 ± 1.04^{b}	3.61 ± 0.63^{b}	0.001
Attachment gain (sites bPD⩾7 mm)	2.09 ± 1.03^{a}	2.67 ± 1.30^{ab}	2.97 ± 0.67^{b}	0.032

The significance of differences among groups was assessed using the Kruskall–Wallis test ($^{\dagger}p$-value) and Dunn's multiple comparison test (different letters indicate significant differences between pairs of groups).
SRP; scaling and root planing; MTZ, metronidazole; AMX, amoxicillin; bPD, baseline probing depth.

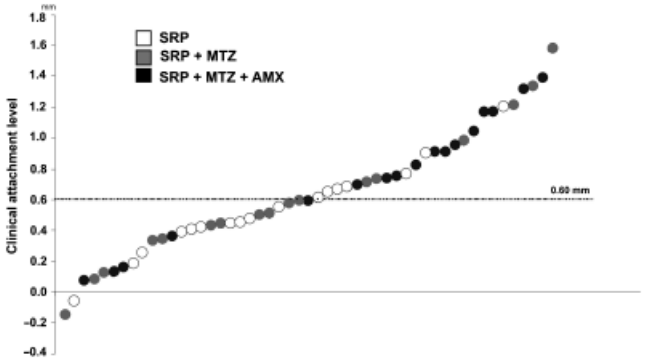

Fig. 2. Plots of the mean changes in individual full-mouth mean clinical attachment level (CAL) between baseline and 3 months post-therapy of subjects in the three treatment groups. The circles represent the mean value of each subject. The dashed line represents the median of change of CAL in all 51 subjects. Positive values represent a gain in CAL, while negative values represent a loss in CAL at 3 months post-therapy. SRP, scaling and root planing; MTZ, metronidazole; AMX, amoxicillin.

Table 3. Mean number and percentage of sites with PD < 5 mm or PD ⩾ 5 mm at baseline and at 3 months post-therapy

Variable	Time-point	Treatment groups			†*p*-value
		SRP	SRP+MTZ	SRP+MTZ+AMX	
PD < 5 mm	Baseline	99.9 ± 24.8 (73.8%) *	108.7 ± 20.4 (73.5%) *	83.4 ± 19.9 (68.0%) *	0.230
	3 months	127.1 ± 25.3 (90.2%)	130.1 ± 18.5 (92.6%)	116.4 ± 17.7 (94.4%)	0.786
PD ⩾ 5 mm	Baseline	39.8 ± 114.9 (27.2%) *	35.1 ± 16.5 (26.5%) *	39.3 ± 15.9 (32.0%) *	0.245
	3 months	13.3 ± 14.9[a] (9.8%)	9.4 ± 8.8[ab] (7.4%)	5.3 ± 4.4[b] (5.6%)	0.020

The significance of differences between baseline and 3 months was assessed using the Wilcoxon test (*$p < 0.05$). The significance of differences among groups at each time point was assessed using the Kruskal–Wallis test (†*p*-value) and Dunn's multiple comparison test (different letters indicate significant differences between pairs of groups).
SRP, scaling and root planing; MTZ, metronidazole; AMX, amoxicillin; PD, probing depth.

initially intermediate (PD 4-6mm) and deep sites (PD ⩾7mm) in comparison with the control group. Adjunctive MTZ also led to a significantly greater reduction in the mean PD in initially deep sites, in comparison with SRP only. The only statistically significant difference observed between the two test groups was a greater mean reduction in PD at initially intermediate sites in subjects who took MTZ+AMX.

The individual changes in the mean CAL from baseline and 3 months post-treatments are presented in Fig. 2. The median of CAL change for all 51 subjects participating in the study was 0.60 mm and is represented by the dashed line in the graph. Most of the subjects who took both antibiotics (13, out of 17) gained attachment equal to or greater than the median of all participants, as opposed to only seven subjects in each of the other two groups.

Table 3 presents the mean number of sites with PD < 5 mm or PD ⩾ 5 mm at baseline and at 3 months post-therapy. All treatments caused a significant increase in the shallow sites as well as a reduction in deep sites. However, at 3 months post-treatments, subjects taking MTZ+AMX had a significantly lower number of remaining deep sites in comparison with those from the control group.

Microbiological findings

At baseline, all subjects were colonized by *P. gingivalis*, *T. denticola*, and *T. forsythia* (red complex) and no significant differences were observed among groups for the counts or the proportions of the individual species. Changes in the microbial profiles between baseline and 3 months post-therapies are represented in Fig. 3 (mean bacterial counts $\times 10^5$) and Fig. 4 (proportions of DNA probe counts). However, the three treatments varied in their abilities to change the subgingival microbial composition. SRP+MTZ+AMX was able to significantly reduce the levels and proportions of the three red complex species, *P. gingivalis*, *T. denticola*, and *T. forsythia*, while only two of them were significantly affected by SRP+MTZ (*P. gingivalis* and *T. denticola*). SRP alone also reduced the levels of these two pathogens, but in terms of proportions, this treatment was able to reduce only *P. gingivalis*. The levels and proportions of three putative pathogens from the orange complex, *Campylobacter rectus*, *Eubacterium nodatum* and *Fusobacterium nucleatum* ssp. *nucleatum*, were reduced by the combination of the two drugs, whereas *E. nodatum* was the only orange complex species significantly reduced in the MTZ group. SRP reduced the levels of *E. nodatum* and *Campylobacter showae*, as well as the proportions of *Streptococcus constellatus* (Figs 3 and 4).

The levels of the majority of the host-compatible species from the green, yellow, purple complexes and *Actinomyces* species were not affected by treatments, with the exception of *Actinomyces naeslundii*, which was reduced at 3 months post-treatment in the SRP group (Fig. 3). An overall increase in the proportions of several of these beneficial species was observed after treatments. These changes were more noticeable in the SRP+MTZ+AMX group, followed by SRP+MTZ and SRP alone. The proportions of *Actinomyces oris* were significantly increased in subjects who took antibiotics (Fig. 4).

Figure 5 presents the mean proportions of the microbial complexes at baseline and 3 months. The proportion of the red complex was significantly reduced by all treatments. The orange complex was also affected by treatments; however, only the combination of SRP+MTZ+AMX significantly reduced this complex. Overall, after treatments, there was an increase in the proportions of all the complexes that harbour most of the host-compatible bacterial species. The beneficial *Actinomyces* species as well as *Veillonella parvula* plus *Actinomyces odontolyticus* (purple complex) were significantly

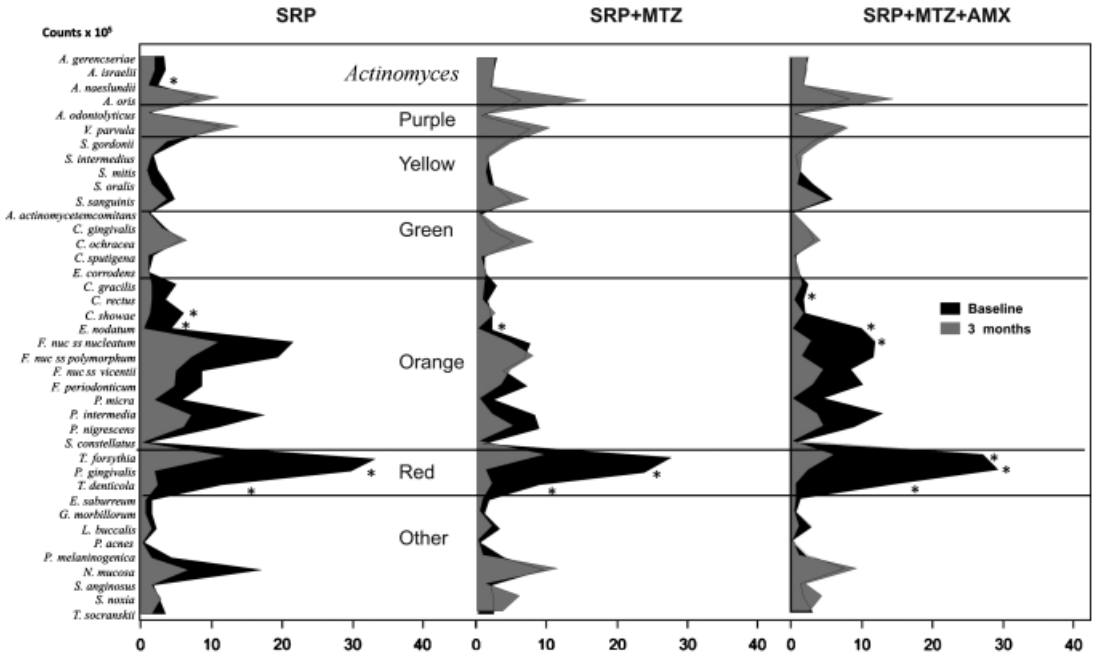

Fig. 3. Mean counts ($\times 10^5$) of the 40 test species at baseline and 3 months post-therapy in the three treatment groups. The species were ordered according to the microbial complexes described by Socransky et al. (1998). The significance of differences within each treatment group between the two time points was assessed using the Wilcoxon test ($^*p < 0.00127$). SRP; scaling and root planing; MTZ, metronidazole; AMX, amoxicillin.

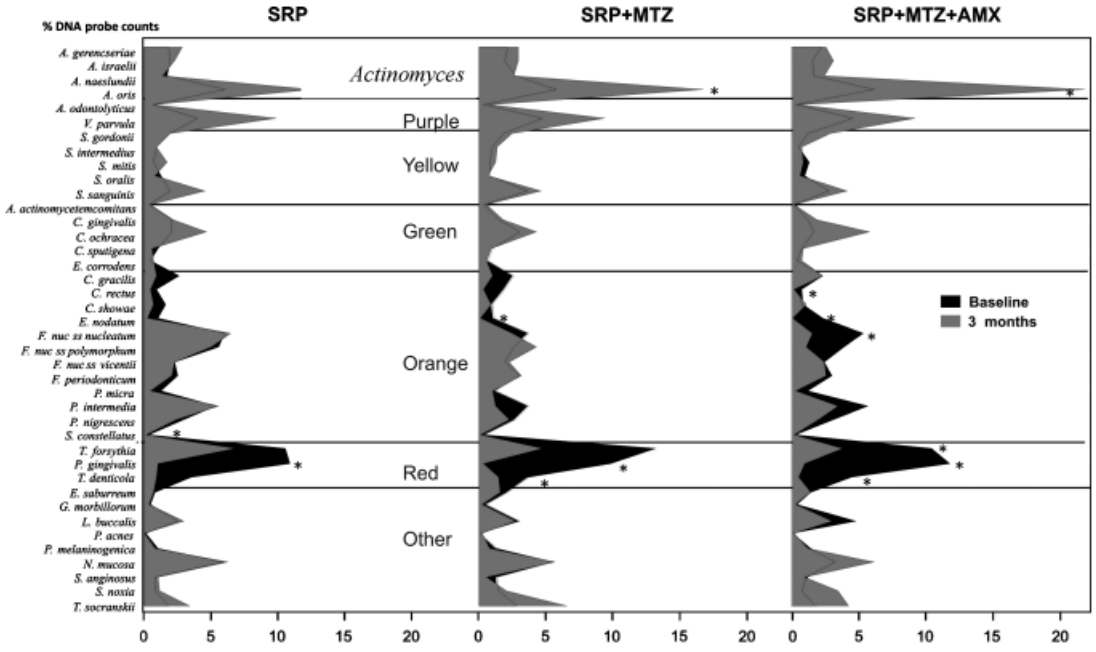

Fig. 4. Mean percentage of DNA probe counts of the 40 test species at baseline and 3 months post-therapy in the three treatment groups. The species were ordered according to the microbial complexes described by Socransky et al. (1998). The significance of differences within each treatment group between the two time points was assessed using the Wilcoxon test ($^*p < 0.00127$). SRP, scaling and root planing; MTZ, metronidazole; AMX, amoxicillin.

increased in all groups at 3 months. In addition, the SRP+MTZ+AMX treatment increased the proportion of the green complex. Subjects in this group showed significantly lower proportions of pathogens from the orange complex as well as higher proportions of *Actinomyces species* in comparison with those in the SRP group, at 3 months after treatments ($p < 0.05$).

Discussion

This study evaluated the clinical and microbiological effects of two antibiotic protocols in subjects with generalized

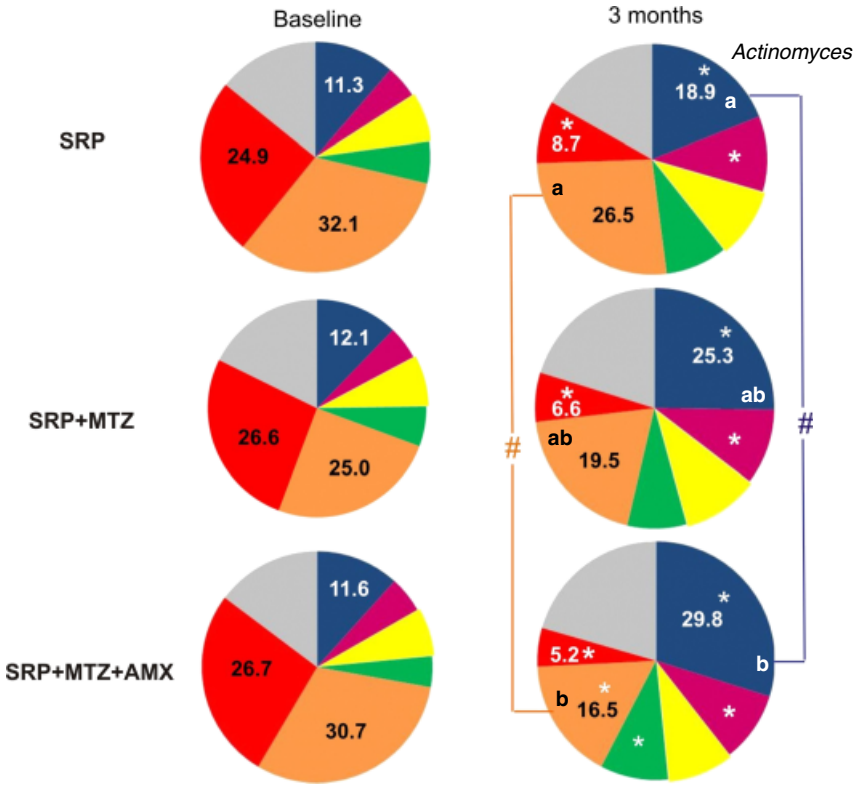

Fig. 5. Pie charts of the mean proportion of each microbial complex at baseline and 3 months post-therapy in the three treatment groups. The colours represent different microbial complexes (Socransky et al. 1998) as well as *Actinomyces* (blue) and ''other'' bacterial species (grey). The significance of differences for each microbial complex was assessed as follows: (1) within each treatment, between baseline and 3 months: Wilcoxon test ($^{*}p<0.05$); (2) among treatment groups at each time point: Kruskall–Wallis ($p>0.05$ at baseline; # indicates $p<0.05$ for *Actinomyces* species and orange complex among the three therapies at 3 months) and Dunn's multiple comparison tests (different letters indicate significant differences between pairs of groups for the *Actinomyces* species and orange complex). SRP, scaling and root planing; MTZ, metronidazole; AMX, amoxicillin.

ChP and compared the outcomes of these treatments with those obtained with mechanical debridement alone. Subjects receiving adjunctive MTZ+AMX exhibited a greater mean clinical attachment gain and reduction in PD in the full-mouth and in initially intermediate and deep sites, in comparison with SRP alone. Although subjects taking adjunctive MTZ also showed a tendency towards greater improvements in these two clinical parameters, the only significant differences in comparison with those obtained with mechanical treatment alone was a greater reduction in the mean PD in the full-mouth and in initially deep sites (Table 2). An additional clinical benefit of the combination of the two antibiotics was the reduction in the number of sites in need of further treatment (PD$\geqslant$5 mm). The MTZ+AMX group had significantly fewer remaining deep sites after treatment (5.3 ± 4.4) in comparison with the control group (13.3 ± 14.9), in agreement with a recent study on the effect of this antibiotic protocol in the treatment of ChP (Cionca et al. 2009). Moreover, the mean number of residual sites in subjects who took MTZ ranged between the values of the other two groups (9.4 ± 8.8, $p>0.05$). This is in agreement with the study of Rooney et al. (2002), who also showed a trend towards a lower percentage of remaining deep sites (PD$\geqslant$6 mm) at 6 months post-therapy, in subjects with ChP treated with SRP+MTZ+AMX, followed by those receiving adjunctive MTZ and by those treated with SRP alone. The number of residual deep sites is considered an important treatment outcome, as a recent comprehensive study by Matuliene et al. (2008) suggested that the persistence of several residual deep sites after treatment may increase the risk of further attachment loss. Another valuable clinical observation in the present study was that the majority of subjects receiving MTZ+AMX had a mean clinical attachment gain within or above the median of gain of all studied individuals, as opposed to the other two groups (Fig. 2). This denotes the constant adjunctive benefit of MTZ+AMX at the subject level, in agreement with two previous investigations (Matarazzo et al. 2008, Mestnik et al. 2010).

The clinical outcomes of the SRP in this study were within or even beyond the expected changes for this treatment according to a meta-analysis (Hung & Douglass 2002) and a comprehensive review (Cobb 2002) on the clinical effects of non-surgical periodontal treatment. The authors reported that sites with PD$\geqslant$7 mm would show a mean attachment gain of approximately 1.0 mm (Hung & Douglass 2002) and 1.19 mm (Cobb 2002) after SRP, in comparison with 2.09 mm observed in the present study. This leads us to reflect about a critical point recently raised by Cionca et al. (2009) on the importance of obtaining excellent results with the control therapy in order to allow a clear evaluation of the additional benefits of the test treatments.

The microbiological data of this study demonstrated that the three treatments differed in their ability to alter the subgingival microbial profile associated with periodontal disease to one compatible with a healthy periodontium. Subjects who took MTZ+AMX showed the most beneficial microbiological changes from baseline to 3 months as well as in comparison with those treated with SRP alone. Only in the MTZ+AMX group were the counts and proportions of all three periodontal pathogens from the red complex, *T. forsythia, P. gingivalis* and *T. denticola*, significantly reduced individually (Figs 3 and 4) and as a complex (Fig. 5). This therapy was also more effective against the putative pathogens from the orange complex, as subjects treated with both antibiotics showed significantly lower proportions of this complex in comparison with SRP, at 3 months post-therapy (Fig. 5). In addition, there were higher proportions of the host-compatible *Actinomyces* species in the MTZ+AMX group at 3 months, when compared with SRP only ($p<0.05$) (Fig. 5).

The only previous study that thoroughly compared the microbial effects of MTZ and MTZ+AMX on periodontal treatment was conducted in smokers (Matarazzo et al. 2008). In agreement with our data, the study

detected an advantage for the adjunctive use of MTZ+AMX with SRP, in comparison with only MTZ. However, neither treatment was able to significantly reduce the orange complex pathogens in smokers. In fact, there was an increase in the proportion of these microorganisms at 3 months post-treatment in all groups, and this was particularly noticeable for the *Fusobacterium* species (Matarazzo et al. 2008). The present investigation evaluated a non-smoker population from the same geographic region with a similar degree of disease and observed a significant reduction in the proportion of the orange complex in all groups, especially with the use of MTZ+AMX. Therefore, it could be speculated that the overall poorer response of smokers to periodontal therapy may be related to persisting pathogens from the orange complex. Thus, future studies could investigate possible interactions between substances found in tobacco and these microbial species with the purpose of establishing enhanced therapies for this risk group. This situation illustrates the importance of defining the short-term microbial effect of different treatments in order to guide the next steps of research and, consequently, to optimize the different therapeutic protocols.

In accordance with our data, previous randomized clinical trials also reported that subjects with chronic (Cionca et al. 2010) or aggressive periodontitis (Xajigeorgiou et al. 2006, Mestnik et al. 2010) treated with MTZ+AMX showed a more beneficial change in the subgingival microbial composition than those treated with SRP alone. On the other hand, although in the present study the adjunctive use of MTZ led to a slight advantage over SRP alone in changing the microbial composition, two previous studies detected superior changes in the subgingival microbial profile with the use of this antibiotic, even with a lower dose. Feres et al. (2001) observed that 2 weeks of MTZ (250 mg t.i.d. for 14 days), in combination with SRP, elicited a profound and rapid decrease in the levels and proportions of the red complex, which was virtually eliminated right after treatment. Although a slight recolonization was observed during the monitoring phase, these species remained in very low proportions even at 1 year post-therapy. Haffajee et al. (2008) also described good short-term microbial effects of SRP+MTZ in the treatment of ChP. These differences might be related to the extent of periodontal disease destruction, which was more advanced in the present study in comparison with the study of Feres et al. (2001) and Haffajee et al. (2007, 2008).

It is important to emphasize that this study was designed to test differences (i.e. 1 mm in CAL change in initially deep sites) between the control and each of the test groups, and therefore, the comparisons between the two antibiotic groups should be interpreted with caution. Nevertheless, it should be noted that the effect of the two antibiotic treatments did not differ significantly in most of the clinical outcomes evaluated (Tables 2 and 3), except for a greater reduction in PD at initially intermediate sites in subjects who took MTZ+AMX. The same trend was observed for the changes occurring in the subgingival microbial profiles (Fig. 5). Therefore, the overall interpretation of the clinical and microbiological data suggests that the adjunctive use of MTZ offers some advantages over SRP alone for the treatment of non-smokers with advanced periodontitis, but apparently, in the short term, does not reach the benefits observed with the use of MTZ+AMX. Hence, the data presented here may guide the design of future larger and long-term clinical trials specifically designed to compare the benefits of these two antibiotic protocols in populations with different degrees of periodontal destruction.

The adjunctive use of MTZ+AMX offers short-term clinical and microbiological benefits, over SRP alone, in the treatment of non-smokers subjects with generalized ChP. MTZ also led to additional benefits, although less evident.

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Clinical Relevance

Scientific rationale for the study: Previous investigations have indicated benefits from the adjunctive use of MTZ or MTZ+AMX in the treatment of ChP. However, the clinical and microbiological effects of these two treatments have not yet been directly studied in non-smokers with advanced ChP.

Principal findings: SRP+MTZ+AMX elicited greater improvements in PD and CAL in initially deep and intermediate pockets, and less residual sites (PD $\geqslant$ 5 mm) at 3 months, in comparison with SRP only. This therapy also yielded the most beneficial changes in the subgingival microbial profile.

Practical implications: Subjects with advanced ChP significantly benefit from the adjunctive use of MTZ+AMX.