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ORIGINAL ARTICLE

The effect of metronidazole on the presence of *P. gingivalis* and *T. forsythia* at 3 and 12 months after different periodontal treatment strategies evaluated in a randomized, clinical trial

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Abstract

Objective. The benefit of full-mouth disinfection (FDIS) over traditional scaling and root planing (SRP) in the treatment of chronic, destructive periodontitis remains equivocal and it is not known whether the use of adjunctive antibiotics may enhance the effect of FDIS. Therefore, the aim of this study was to evaluate the effect of conventional SRP completed over 21 days *or* 1-day FDIS, with *or* without systemically delivered adjunctive metronidazole (MET) on the presence of *P. gingivalis* and *T. forsythia* after 3 and 12 months. ***Materials and methods.*** One hundred and eighty-four patients with moderate-to-severe periodontitis were randomly allocated to one of four treatment groups; (1) FDIS+MET; (2) FDIS+placebo; (3) SRP+MET; (4) SRP+placebo. Prior to treatment, pooled subgingival samples were obtained from the five deepest pockets. The same sites were sampled again 3 and 12 months after treatment. All samples were analyzed for *P. gingivalis* and *T. forsythia* by PCR, whereas *A. actinomycetemcomitans* and other bacteria were identified by culture techniques. ***Results.*** At baseline, 47% of the samples were positive for *P. gingivalis,* while almost all samples were positive for *T. forsythia.* The occurrence of *P. gingivalis* and *T. forsythia* was significantly reduced at 3 and 12 months after treatment in the FDIS+MET group, but not in the other treatment groups. ***Conclusion.*** FDIS+MET had a significant effect in patients with *P. gingivalis* and *T. forsythia,* resulting in a significant reduction in number of patients where these micro-organisms could be detected at 3 and 12 months post-therapy.

Key Words: *microbial diagnosis, scaling and root planing, full mouth disinfection, one-day treatment, antibiotics, RCT*

Introduction

Scaling and root planing (SRP) are the main components of any successful periodontal treatment [1]. However, some patients experience little effect of this purely mechanical approach [2], which has been explained by the presence of a specific infection component [3,4] recalcitrant to SRP alone [5]. In such cases adjunctive antibiotic therapy [6] has been used, albeit with varying results [7–10]. Conventional SRP is typically carried out over a period of 2–3 weeks, whereas adjunctive antibiotic regimens are recommended to last 8–10 days depending upon the type of drug administered. However, in any of these scenarios, the possibility for re-inoculation and re-infection may cause recurrence of the periodontal disease. In fact, the possibility of re-infection of treated sites was precisely the rationale underpinning the introduction of the 'Full Mouth Disinfection' (FDIS) concept by Quirynen et al. [11], who suggested that FDIS would reduce the risk of re-infection of a previously disinfected area before the completion of the SRP. Moreover, the FDIS approach was hypothesized to result in an instantaneous reduction of the pathogenic microbiota to levels manageable for the host's immune system, whereby the FDIS could enhance the effect of the mechanical instrumentation. In line with this, Teughels et al. [12] reported more favorable clinical and microbiological results with the FDIS strategy than with SRP alone, whereas later studies have found little or no additional effect of this technique [13,14]. Recently, Preus et al. [15] found no difference in clinical effect between SRP and FDIS when combined with systemically delivered, adjunctive metronidazole (MET) in patients with a metronidazole-sensitive subgingival

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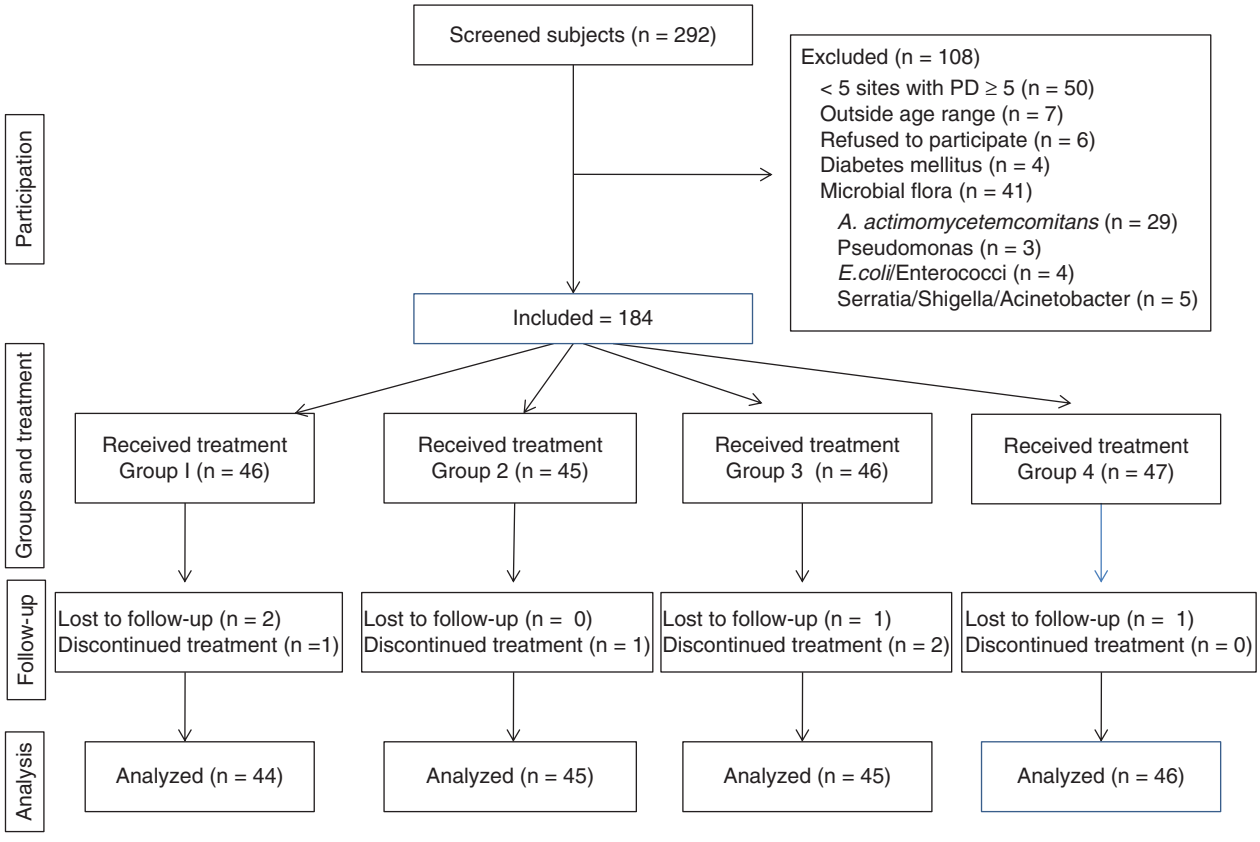
microbiota. Despite the lack of long-term studies, FDIS has been adopted as a more efficient way to treat periodontitis [16].

The purpose of the present study was to describe the detectable presence of the red complex bacteria [17] *Porphyromonas gingivalis* and *Tannerella forsythia* among patients treated with conventional SRP completed over 21 days or 1-day FDIS, with *or* without systemically delivered adjunctive MET.

Materials and methods

The data for the present study originate in a randomized double masked, four-arm, placebo-controlled clinical intervention trial carried out among 184 patients with severe chronic destructive periodontitis and the study rationale, design as well as the 1-year clinical results have previously been described in detail [15]. Briefly, patients were recruited among objects referred to a periodontal specialist clinic for treatment and underwent a 3-month pre-study hygiene phase, subsequent to which eligibility for the trial was assessed using the following criteria: aged between 35–75 years; no prior systematic periodontal treatment experience; no systemic diseases or continuous medication known to affect the severity or progression of periodontitis; and at least five sites remaining with a pocket depth ≥5 mm after completion of the pre-study hygiene phase. Since the antibiotic of choice in this trial was MET, patients harboring species with known resistance to and/or known allergies or adverse reactions from this antibiotic were excluded [15] (Figure 1).

The trial had four treatment arms: FDIS+MET (Group 1); FDIS+placebo (Group 2); SRP+MET (Group 3); and SRP+placebo (Group 4). Groups 1 and 2 received full mouth SRP completed within a single workday (FDIS) using two sessions of 65 min each, 2-h apart. In Groups 3 and 4 the SRP was completed using two 65-min sessions, 21 days apart. Subsequent to all mechanical treatment sessions, in all groups, the patients rinsed for 1-min with 10 ml 0.2% chlorhexidine (CHX) (Corsodyl 2 mg/ml mouth rinse, Smithcline Beecham Ld, Brentford, UK) and, following mechanical instrumentation, all sulci and pockets were filled with CHX gel (Corsodyl Gel 1%, Smithcline Beecham Ltd). In addition, patients in Groups 1 and 3 received MET (Flagyl 400 mg, Sanofi Aventis, Lysaker, Norway), 400 mg ×3 for 10 days, starting the day before the two mechanical treatment sessions in Group 1 and the



One person who should have been allocated to group II, was mistakenly allocated to group IV, whereby the four groups comprised 46, 45, 46 and 47 patients respectively.

Figure 1. Flow diagram showing the steps in the identification of participants for the trial adapted with permission from Preus et al. (2013). Reproduced with the permission of the American Academy of Periodontology, as copyright holder for [15].

day before the second SRP session (day 20) in Group 3. Patients in Groups 2 and 4 received pharmacy-packed placebo tablets according to the same scheme as for Groups 1 and 3, respectively. Group assignment was performed according to a random allocation table [18]. Four patients left the study during the active treatment phase; one died, two were diagnosed with cancer and one with diabetes mellitus. Data were, therefore, available for 180 patients at the 12-month follow-up (Figure 1).

Additional details regarding patient recruitment, eligibility assessment, in- and exclusion criteria, randomization procedures, patient flow and clinical assessments can be found in the aforementioned publication [15]. The project protocol was approved by the Privacy Ombudsman for the Norwegian Universities (#15768) and Regional Committee for Medical Research Ethics, (Oslo, Norway) (REC South East 2.2006.3573/S-06458b). The US National Institutes of Health Clinical Trials Registry number (http://www.clinicaltrials.gov) is NCT01318928.

Microbiological sampling

Prior to inclusion/exclusion, subgingival bacterial samples were obtained from the five deepest pockets twice, with 3-week interval. The same five sites were sampled at 3 and 12 months after treatment, using the same harvesting method.

The project leader (HRP) performed the sampling as follows: Second to clinical registrations the full surfaces of the five subgingival sampling sites were sampled by a curette (LM syntette. LM Instruments; LM-Instruments Oy, Parainen, Finland) and the harvest transferred to 1 ml PBS in a sterile CryoTube (Nunc Store-it™ Cryo-Tubes, 1.8 ml (working volume), Thermo Fischer Scientific, Roskilde, Denmark). One paper point was inserted for 10-s for the planktonic state in each site and subsequently used to wipe off any visible, remaining substance on the scaler from that site. This procedure was repeated for all five sites and the samples pooled in one tube. After closing the lid and vigorous shaking, one half of the harvest was stored at −20°C for future, retrospective microbiological analyses with alternate techniques. The other half of the harvest was transferred to a transport medium (SSI Transport Medium (Stuarts) (Statens serum Institutt, Copenhagen, Denmark) for immediate processing at UniLabs (UniLabs, Skien, Norway; Microbiological laboratory licensed by Norwegian Health Authorities for Oral Microbiology).

Microbiological diagnosis

All samples reached the laboratory within 24 h of sampling, without experiencing transport temperatures above or below those recommended. The diagnosis was received within 3 weeks of sampling. In the laboratory, samples were vortexed and seeded on blood agar (horse blood) and Tryptic Soy Serum Bacitracin Vancomycin agar (TSBV) (horse serum) [19] medium for identification of *A. actinomycetemcomitans* and other bacteria with known in- or low sensitivity to MET [15]. The seeded samples were cultured at 35°C, aerobically, as well as in 5% CO_2 in air, for 5 days, and bacterial colonies described and identified by physical appearance and biochemical tests [20]. Microbiological diagnoses also included the red complex bacteria [17] *P. gingivalis* and *T. forsythia* as well as those species that had caused subject exclusion, i.e. *A. actinomycetemcomitans*, Pseudomonas, *E. coli*, Enterococci, *Serratia*-, *Shigella*- and *Acinetobacter*- species (Figure 1).

P. gingivalis and *T. forsythia* were identified by real-time TaqMan PCR with PCR primers targeting the 16S rRNA gene. Semi-quantitative results were generated by reference to standard curves of a 10-fold dilution of DNA sample *of P.gingivalis* and *T. forsythia* DNA with known concentration (Table I). PCR primers and probes targeting species-specific regions of the 16s rRNA genes of *P. gingivalis* (PginF: GGACTAAAACCGCATACACTTGTATTA, PginR: CGCATGCCTATCTTACAGCTATAAAT, PginT: FAM-TGCATGATATTACAAGGAAA –MGB) and *T. forsythia* (BfoF: GGGTGAGTAACGCGTATGTAACCT, BfoR: TGCGGAACCCCTGTTTTATG, BfoT: FAM-CGCAACAGAGGGATAA –MGB) were designed using Primer Express (Applied Biosystems, Foster City, CA) and tested *in silico* for inclusivity (binding to all known variants of target sequences within the bacterial species) and specificity (absence of binding to other bacterial species) was performed by Sequence targeting and 'Basic Local Alignment Search Tool' (BLAST) [21]. *A. actinomycetemcomitans* was quantified as high, moderate and low yield by culture. The semi-quantitative

Table I. Detection level and range for semi-quantification of *T. forsythia* (T.f), *P. gingivalis* (P.g) number of cells/ml of sample and *A. actinomycetemcomitans* (A.a) in CFUs. UniLabs, Skien, Norway.

	Low	Moderate	High
T.f	$<4 \times 10^7$ cells	$4 \times 10^7 \leq$ cells $<1 \times 10^8$	$\geq 1 \times 10^8$ cells
P.g	$<1 \times {}^{105}$ cells	$1 \times 50^5 \leq$ cells $<1 \times 10^7$	$\geq 1 \times 10^7$ cells
A.a*	<10 CFU	10< CFU <50	≥50 CFU

*By culture; CFU (Colony Forming Units) were counted on the plate based on a seeded volume of 40–50 µl.

Table II. Results of semi-quantitative analyses for *P. gingivalis* at baseline (BL) and at 3 and 12 months following treatment in the four treatment arms.

		P. gingivalis			
Treatment group	Time	Not detected *n* (%)	Low *n* (%)	Medium *n* (%)	High *n* (%)
FDIS + Metronidazole	BL	26 (57.8)	1 (2.2)	1 (2.2)	17 (37.8)
	3 months	41 (91.1)	2 (4.4)	2 (4.4)	0
	12 months	39 (86.7)	3 (6.7)	2 (4.4)	1 (2.2)
FDIS + Placebo	BL	21 (46.7)	0	2 (4.4)	22 (48.9)
	3 months	22 (49.9)	8 (17.8)	9 (20.0)	6 (13.3)
	12 months	23 (51.1)	10 (22.2)	9 (20.0)	3 (6.7)
SPR + Metronidazole	BL	20 (45.5)	1 (2.3)	4 (9.1)	19 (43.2)
	3 months	28 (63.6)	8 (18.2)	6 (13.6)	2 (4.6)
	12 months	25 (58.1)	6 (14.0)	8 (18.6)	4 (9.3)
SPR + Placebo	BL	28 (60.9)	1 (2.2)	1 (2.2)	16 (34.8)
	3 months	32 (69.6)	7 (15.2)	5 (10.9)	2 (4.4)
	12 months	33 (71.7)	4 (8.7)	6 (13.0)	3 (6.5)
Overall	BL	95 (53.1)	3 (1.7)	8 (4.5)	74 (41.3)
	3 months	123 (68.7)	25 (14.0)	22 (12.3)	10 (5.6)
	12 months	120 (67.4)	23 (12.9)	25 (14.0)	11 (6.2)

ranges for *P. gingivalis* and *T. forsythia*, as well as the direct quantifications of *A. actinomycetemcomitans*, are shown in Table I.

Statistics

Differences between treatment groups in the detection, elimination or resurgence frequencies of *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans* were tested using the two-sample proportion test.

Results

At baseline, almost all subjects were positive for *T. forsythia*, while *P. gingivalis* was detected in 47% of the patients (Tables II and III). Overall, the

Table III. Results of semi-quantitative analyses for *T. forsythia* at baseline (BL) and at 3 and 12 months following treatment in the four treatment arms.

		T. forsythia			
Treatment group	Time	Not detected *n* (%)	Low *n* (%)	Medium *n* (%)	High *n* (%)
FDIS + Metronidazole	BL	0	37 (82.2)	4 (8.9)	4 (8.9)
	3 months	24 (53.3)	21 (46.7)	0	0
	12 months	25 (55.6)	20 (44.4)	0	0
FDIS + Placebo	BL	1 (2.2)	34 (75.6)	7 (15.6)	3 (6.7)
	3 months	4 (8.9)	40 (88.9)	1 (2.2)	0
	12 months	7 (15.6)	38 (84.4)	0	0
SPR + Metronidazole	BL	1 (2.3)	31 (70.5)	10 (22.7)	2 (4.6)
	3 months	19 (43.2)	25 (56.8)	0	0
	12 months	7 (16.3)	35 (81.4)	0	1 (2.3)
SPR + Placebo	BL	0	35 (76.1)	8 (17.4)	3 (6.5)
	3 months	6 (13.0)	40 (87.0)	0	0
	12 months	9 (19.6)	37 (80.4)	0	0
Overall	BL	2 (1.1)	137 (98.9)	29 (16.2)	12 (6.7)
	3 months	53 (29.6)	126 (70.4)	1 (0.6)	0
	12 months	48 (27.0)	130 (73.0)	0	0

prevalence for these two species was reduced at 3 and 12 months following treatment, to 32% and 33%, respectively, for *P. gingivalis* (Table II) and 70% and 73% for *T. forsythia* (Table III). *A. actinomycetemcomitans* was infrequently detected at 3 and 12 months after treatment, while *Enterobacteria*, *Pseudomonas spp* and the other micro-organisms remained only sporadically diagnosed. These species were, therefore, not subject to further analyses.

Table II shows that, at baseline, *P. gingivalis* was present in 'high' quantities when detected. The detection frequency was clearly reduced after treatment in all treatment groups, although prevalence at patient level was most clearly reduced in the FDIS+MET group (Group 1).

While almost invariably detected at baseline, *T. forsythia* was most commonly present in no more than low quantities (Table III). There was clear evidence of reduced detection frequency of *T. forsythia* in all treatment arms, although prevalence was more clearly reduced in the two treatment groups receiving MET (Table III).

Table IV outlines the pattern of detection frequency of *P. gingivalis* at the three sampling time points in each of the four treatment groups. In a few cases, *P. gingivalis* could be detected at 3 or at 12 months, even though the baseline sample had had no detectable *P. gingivalis*. Reduction below detection level of *P. gingivalis* from the sampled sites following treatment was most pronounced in the FDIS+MET group, where 73.7% (95% CI = [53.9%; 93.5%]) of the baseline positive patients remained with no detected *P. gingivalis* at both 3 and 12 months following treatment. Statistically significant reduction below detection level of *P. gingivalis* was also observed in the SRP+MET group (17.4%, 95% CI = [1.9%; 32.9%]) and in the SRP group (22.2%, 95% CI = [3.0%; 41.4%]), but not in the FDIS+Placebo group (8.3%, 95% CI = [−2.7%; 19.4]) (Table IV). The number of patients with undetectable levels of *P. gingivalis* was statistically significantly higher in the FDIS+MET group than in any of the other groups, among which the frequency of non-detectable marker bacteria did not differ significantly.

Table V outlines the pattern of detection of *T. forsythia* in each treatment group at the three sampling time points. Apparent reduction of *T. forsythia* to non-detectable levels from the sampled sites after treatment was most pronounced in the FDIS+MET group, where 42.4% (95% CI = [27.8%; 56.7%]) of the baseline positive patients remained with no detectable marker bacteria at both 3 and 12 months. The corresponding figures for the FDIS+Placebo group, the SRP+MET group and the SRP+Placebo group were 2.3% (95% CI = [−2.1%; 6.7%]), 14.3% (95% CI = [3.7%; 24.9%]) and 4.4% (95% CI = [−1.5%; 10.2%]), respectively (Table V). The no-detection frequency of *T. forsythia* was statistically significantly higher ($p < 0.004$) in the FDIS+MET group (Group 1) than in any of the other groups, but a statistically significant ($p < 0.05$) difference was also found between the SRP+MET and FDIS+Placebo groups. No other statistically significant differences in the no-detection frequency of the marker bacteria were observed.

Although patients had been selected into the trial on the basis of no detectable *Aggregatibacter*

Table IV. Patterns of detection of *P. gingivalis* at baseline (BL) and at 3 and 12 months following treatment in the four treatment arms.

Pattern of presence of *P.gingivalis*			Treatment group			
BL	3 months	12 months	FDIS+MET ($n = 45$)	FDIS ($n = 45$)	SRP+MET ($n = 43$)*	SRP ($n = 46$)
–	–	–	25	18	19	25
–	–	+	1	2	—	1
–	+	–	—	1	1	1
–	+	+	—	—	—	1
+	–	–	14	2	4	4
+	–	+	1	—	5	2
+	+	–	—	2	1	3
+	+	+	4	20	13	9
Elimination %§ (95% CI)			14/19 = 73.7% (53.9, 93.5)	2/24 = 8.3% (−2.7, 19.4)	4/23 = 17.4% (1.9, 32.9)	4/18 = 22.2% (3.0, 41.4)
Resurgence % (95% CI)			1/26 = 3.9% (−3.5, 11.2)	3/21 = 14.3% (−0.1, 29.3)	1/20 = 5.0% (−4.6, 14,6)	3/28 = 10.7% (−0.7, 22.2)

–sample negative; + sample positive.

*One patient who had no sample analyzed at 12 months was positive for *P. gingivalis* at baseline and at 3 months.

§Two-sample proportion test of difference between groups: FDIS+MET vs FDIS, $p = 0.000$; FDIS+MET vs SPR+MET, $p = 0.000$; FDIS+MET vs SRP, $p = 0.002$; FDIS vs SRP+MET, $p = 0.352$; FDIS vs SRP, $p = 0.203$; SRP+MET vs SRP, $p = 0.699$.

Table V. Patterns of detection of *T. forsythia* at baseline (BL) and at 3 and 12 months following treatment in the four treatment arms.

Pattern of presence of *T. forsythia*			Treatment group			
BL	3 months	12 months	FDIS+MET (n = 45)	FDIS (n = 45)	SRP+MET (n = 43)*	SRP (n = 46)
–	–	–	—	1	1	—
–	–	+	—	—	—	—
–	+	–	—	—	—	—
–	+	+	—	—	—	—
+	–	–	19	1	6	2
+	–	+	5	2	12	4
+	+	–	6	5	—	7
+	+	+	15	36	24	33
Elimination %[§] (95% CI)			19/45 = 42.4% (27.8, 56.7)	1/44 = 2.3% (−2.1, 6.7)	6/42 = 14.3% (3.7, 24.9)	2/46 = 4.4% (−1.5,10.2)
Resurgence % (95% CI)			—	0/1 = 0%	0/1 = 0%	—

–sample negative; + sample positive.
*One patient who had no sample analyzed at 12 months was positive for *T. forsythia* at baseline and at 3 months.
[§]Two-sample proportion test of difference between groups: FDIS+MET vs FDIS, p = 0.000; FDIS+MET vs SPR+MET, p = 0.004; FDIS +MET vs SRP, p = 0.000; FDIS vs SRP+MET, p = 0.042; FDIS vs SRP, p = 0.584; SRP+MET vs SRP, p = 0.105.

actinomycetemcomitans, some of the 3 and 12 months samples nevertheless appeared positive for this micro-organism (Table VI). In the two groups receiving MET, 4.6% (95% CI = −1.6%; 10.75%]) of the samples harbored detectable *A. actinomycetemcomitans* at 3 or 12 months, whereas the corresponding figures for the FDIS and the SRP groups were 11.1% (95% CI = [1.9%; 20.3%]) and 10.9% (95% CI = [1.9%; 19.9%]), respectively. The 'resurgence' frequency of *A. actinomycetemcomitans* was not significantly different among the groups.

Discussion

The results of the present study have shown that non-surgical treatment, with or without adjunctive MET, leads to a reduction in the detection frequency and the quantities of both *P. gingivalis* and *T. forsythia*. However, the combination FDIS+MET appeared statistically more effective in reducing both *P. gingivalis* and *T. forsythia* below detection levels for up to 12 months following treatment than did any of the other three treatment regimens. This result might suggest that the FDIS does indeed achieve the purported reduction of the risk of re-infection of a previously disinfected area before the completion of the SRP treatment [11]. However, judged from the clinical outcomes of therapy [15], this reduction did not manifest itself in a clinical benefit of FDIS over traditional SRP treatment, but rather as a way to enhance the effect of an antibiotic. Accordingly, MET had a significant, adjunctive clinical effect in patients with a MET-sensitive subgingival micro-biota, but the same-day FDIS approach did not

Table VI. Patterns of detection of *A. actinomycetemcomitans* at 3 and 12 months following treatment in the four treatment arms. Note that patients were selected as being free from detectable *A. actinomycetemcomitans* at baseline (BL).

Pattern of presence of *A.actinomycetemcomitans*			Treatment group			
BL	3 months	12 months	FDIS+MET (n = 44)*	FDIS (n = 45)	SRP+MET (n = 44)	SRP (n = 46)
–	–	–	42	40	42	41
–	–	+	1	2	—	2
–	+	–	—	3	—	1
–	+	+	1	—	2	2
Resurgence % (95% CI)			2/44 = 4.6% (−1.6, 10.7)	5/45 = 11.1% (1.9, 20.3)	2/44 = 4.6% (−1.6, 10.7)	5/46 = 10.9% (1.9, 19.9)

–sample negative; + sample positive.
One patient who had no sample analyzed at 12 months was negative for *A. actinomycetemcomitans* at baseline and at 3 months.

perform any better than the 3-week SRP approach, whether MET was used or not.

It is tempting to statistically correlate the clinical findings with the microbiological diagnoses at baseline and 1 year following treatment. However, it is premature to expect lasting clinical changes based on microbiology as early as 12 months post-therapy. An imminent reduction below detection levels of target micro-organisms is normally found following any therapy, as are also positive clinical effects [7–9]. However, as the microbiota restores itself later in the post-treatment period, the clinical recovery becomes less evident and often is reversed to recurrence. The observed effect of FDIS/SRP + placebo (Groups 2 and 4) for both *T. forsytha* and *P. gingivalis*, with a tendency to lower frequency of detectable levels for *P. gingivalis* and a transition from high to medium or low levels for *T. forsythia*, could be ascribed to altered ecology caused by mechanical treatment or to a combination of the treatment and the CHX gel. Thus, there will be ecological consequences of both the use of an antibiotic and the mechanical treatment alone or in combination. However, as far as we know, the partial impact of any of these treatment ingrediences on the total result is to date unknown.

P. gingivalis and *T. forsythia* are two of the three red complex bacteria [17] that for decades have been implicated as putative periodontal pathogens or rather specific marker bacteria for some periodontal diseases. These species are sensitive to MET. In Norway, oral microbiological diagnosis is publicly subsidized in order to enhance the use of such diagnosis and reduce the use of antibiotics to concur with WHO and EU recommendations [22,23]. However, a reliable, easily accessible and convenient identification of *Treponema denticola*, which is the third member of the red complex, was not available in Norway at baseline and this species was, therefore, not included. Moreover, since the antibiotic of choice in this study was MET, patients with microbiological diagnosis containing *A. actinomycetemcomitans* or other MET-resistant bacterial species were rejected from participation in this study. However, for surveillance reasons, microbiological diagnoses also included these bacteria, although they were infrequently detected.

The ultimate goal of an antibiotic treatment is to eradicate the culprit bacteria from an infection. However, specifically pertaining to periodontitis, more modest goals apply, as reducing the number of target bacteria to levels manageable for the host and their resident subgingival microflora [12,24] is what we hope for. A lasting eradication of marker pathogens from the subgingival microbiota has been considered impossible, since the subgingival periodontal microflora is organized in biofilms [25], which may be impenetrable to antibiotics, and biofilm remnants may escape even the most meticulous SRP in difficult-to-scale areas [26–28]. However, substituting SRP with FDIS may represent an improvement for adjunct antibiotic therapy in periodontics, as FDIS may theoretically enhance the mechanical and chemical destruction of the subgingival biofilm to an extent whereby the bacteria become manageable for the antibiotic. Two important issues control the success potential of the FDIS+MET technique: The quality of the mechanical instrumentation (SRP) and the timing and concentration of the antibiotic as it enters the subgingival pocket via the gingival crevicular fluid (GCF). The antibiotic is not effective unless the biofilm is mechanically removed [29]. Therefore, the SRP/FDIS quality is of the essence and an antibiotic must not be made to substitute for insufficient mechanical cleaning. Moreover, it may take up to 24 h before the Minimum Bactericidal Concentration (MBC) is reached in the GCF [30,31] and the antibiotic must reach this level prior to the mechanical biofilm removal to be effective. This was precisely the rationale underpinning our decision to commence the MET therapy 24 h prior to mechanical treatment in this study, although this administration model seems infrequent for adjunct antibiotics in periodontics [10]. The reason for not initiating the antibiotic earlier than 24-h prior to treatment was the desire to extend the duration of drug action as much as possible after the mechanical treatment *within* the prescribed antibiotic regimen period for a maximum effect on the recolonization process.

Our findings suggest that it is possible to reduce *P. gingivalis* to undetectable levels from periodontal sites for up to 12 months in a substantial number of patients using this specific combination of FDIS +MET. The reason some patients kept their *P. gingivalis* was probably due to the aforementioned remaining biofilm in hard-to-reach areas [26–28]. Moreover, it must be emphasized that the presence of *P. gingivalis* at baseline and through 3 and 12 months was only investigated in the sites representing the five deepest pockets at *pre-treatment* in each patient and it is clearly possible that *P. gingivalis* may have resided undiagnosed in other niches in the oral cavity.

While *T. forsythia* were reduced significantly with FDIS+MET and SRP+MET at both 3 and 12 months, reduction was numerically not as effective for this micro-organism as for *P. gingivalis*. An explanation could be that *T. forsythia* is not as sensitive to MET as anticipated and research is in progress to explore this possibility. Thereby, this finding might indicate that adjunct antibiotic (MET) therapy is less effective, perhaps even unnecessary, in patients who suffer from *T. forsythia*-associated periodontitis, provided there are no other periodontal pathogens in the microbiological diagnosis that warrants antibiotic treatment. Our observation in the SRP+MET group of a substantial number of patients in whom *T. forsythia* seemed eliminated at 3 months, but appeared to have resurged at 12 months, suggests that recording

microbiological results before 12 months in a clinical study may be premature [10].

As a consequence of the inclusion criteria, *A.actinomycetemcomitans* was not diagnosed in any of the participants' pre-study samples. The reason for finding *A.actinomycetemcomitans* after treatment in a few samples was probably related to the method of diagnosis, which was culture, where the detection limit for *A.actinomycetemcomitans* is 10^2–10^3cells/ml in the seeded sample [32,33]. Moreover, it is possible that all four treatment strategies reduced the presence of other biofilm bacteria to such an extent that undetected *A.actinomycetemcomitans* had the opportunity to grow into detectable levels.

When diagnosing bacteria by culture, as with *A. actinomycetemcomitans*, Pseudomonas, *E.coli*, Enterococci, *Serratia*-, *Shigella*- and *Acinetobacter* spp. in this study, it is not possible to set a specific limit for detection. The pre-requisite would be to know the number of Colony Forming Units (CFU) in the sample and, thereby, produce dilution series based on this, but such information does not exist. The findings that *P. gingivalis*, when present, was invariably found in high quantities supports previous observations [34,35] and shows that the periodontitis environment would seem conductive to high quantities of this bacterium. Our observation that *T. forsythia* was almost unequivocally found in low quantities contradicts previous observations [35,36] and indicate that, if at all etiologically associated with destructive periodontal disease, this bacterium may be pathogenic in low numbers. This observation may also rest on the selected frames for low, medium and high quantity detection and research is in progress to investigate these findings and implications.

In conclusion, the results of the present study showed that FDIS+MET resulted in a significant, although different reduction of the number of patients with detectable *P. gingivalis* and *T. forsythia* at both 3 and 12 months post-therapy. The findings corroborate the common observation that *P. gingivalis* and *T. forsythia* may be treated with MET [17], while *A. actinomycetemcomitans* should be treated with other antibiotics [9,10,37].

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