



# The application of antimicrobial photodynamic therapy (aPDT, 670 nm) and diode laser (940 nm) as adjunctive approach in the conventional cause-related treatment of chronic periodontal disease: a randomized controlled split-mouth clinical trial

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## Abstract

**Objectives** The aim of the present randomized controlled split-mouth clinical trial is to evaluate the effectiveness of the adjunctive use of photodynamic low-level laser therapy (670 nm), applying methylene blue as photosensitizer, and the effectiveness of a diode laser (940 nm) compared with conventional non-surgical mechanical treatment in a group of patients with chronic periodontal disease.

**Materials and methods** Twenty-one patients with moderate to severe periodontal disease with presence of 3 or more quadrants, each containing at least three sites with periodontal pocket depth (PPD) of  $\geq 5$  mm, were included in the study. Periodontal treatment comprising scaling and root planing (SRP) was accomplished for the whole mouth. Applying a split-mouth design, each quadrant was randomly treated with SRP alone (control group), SRP with diode laser (diode group), and SRP with photodynamic therapy (photodynamic group).

**Results** All treatment modalities in this study lead to statistically significant improvements in the evaluated clinical parameters at 3 months and 6 months compared with baseline. There was no statistically significant difference regarding PD and BOP between groups. There was only a tendency for greater reduction of PD in the diode group for deep pockets at 3 months, but not statistically significant.

**Conclusions** After 6 months of evaluation, the high intensity diode laser and the antimicrobial photodynamic therapy have not shown any additional benefits to the conventional periodontal treatment.

**Clinical relevance** The diode or photodynamic laser therapy in conjunction with conventional SRP does not seem to be superior in reducing probing depth and bleeding on probing than SRP alone 6 months after treatment. More studies are necessary to prove the actual need of these types of lasers in the periodontal clinical practice.

**Keywords** Periodontitis, chronic · Diode laser · Photodynamic therapy · Photosensitizing agent

## Introduction

Nowadays, there is a heated debate about the effectiveness of laser applications for the treatment of periodontal and

peri-implant disease and their use as adjunctive tools in the maintenance phase of therapy. Scaling and root planing is an example of a minimally invasive procedure since it is a conservative, cause-related therapy aiming to eliminate etiologic factors from the root surface [1]. Conventional periodontal therapy contributes to the resolution of inflammation and includes the control of biofilm, supragingival and subgingival scaling, root planing and the adjunctive use of chemical agents. The reduction in the microbial load and bacterial metabolic products leads to a reduced inflammatory response and improved tissue healing. Moreover, treatment outcomes may not always be successful for moderate and deep periodontal pockets [2] and for complete removal of bacterial endotoxins and calculus

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deposits from the root surface [3]. Furthermore, access to areas such as furcations, cavities, grooves, and molar's distal regions is limited.

Currently, high-power output lasers are used adjunctively to scaling and root planing or as tools in minimally invasive surgery. In addition, therapeutic, low-power output lasers are employed for cellular stimulation and the activation of antimicrobial agents following scaling and root planing. The complexity of the tissues of the periodontal pocket results in different optical properties, a fact which is very important for the absorption of each wavelength's photons. The antimicrobial action of diode laser irradiation is mainly based on thermal effects and absorption of the incident energy at the pigmented cell membrane of most periopathogenic bacteria [4, 5].

A number of studies reporting on the application of diode lasers in non-surgical therapy have focused on a therapy combining mechanical instruments and laser devices to achieve a greater reduction of bacterial load in the periodontal pocket. A group of studies showed significant improvement in clinical and immunological parameters compared with scaling and root planing up to 6 months after treatment [6–9] while other studies failed to demonstrate that combination therapy could provide any additional clinical [10] and microbiological therapeutic benefit [11–13].

Photodynamic therapy is a treatment modality based on the activation of exogenous photosensitizing agents by a light source. Photosensitizers are dyes composed of molecules capable of absorbing light energy and using it to promote chemical reactions in cells and the surrounding tissues. The substance is applied inside the periodontal pocket and is activated by a light source outside the pocket or with a fiber. A photosensitizer bonded to bacteria can be activated by light of the appropriate wavelength in the presence of oxygen to generate singlet oxygen and free radicals that are cytotoxic to microorganisms, mainly as a result of damage to the cytoplasmic membrane and DNA. This process is referred as antimicrobial photodynamic therapy [14, 15].

Photodynamic therapy has demonstrated significant antimicrobial activity at periodontal pathogenic bacteria in combination with conventional periodontal treatment. Application of photodynamic therapy in experimental periodontal disease conditions showed promising results. The results in prospective clinical studies are presented controversial. Several studies on the effects of photodynamic therapy, adjunctive to scaling and root planning, have reported short-term clinical benefits compared with control groups [16–18].

The aim of the present randomized controlled split-mouth clinical trial is to evaluate the effectiveness of the adjunctive use of photodynamic low-level laser therapy (670 nm), applying methylene blue as photosensitizer, and the effectiveness of a diode laser (940 nm) compared with conventional non-surgical mechanical treatment in a group of patients with chronic periodontal disease.

## Materials and methods

### Subject selection

Twenty-one systemically healthy patients were recruited in the Postgraduate Clinic of Preventive Dentistry, Periodontology and Implant Biology at the Dental School of Aristotle University of Thessaloniki between March 2016 and April 2017. The sample size of our study was calculated after conducting a statistical analysis. That was determined to provide 90% power to recognize a significant difference of 1 mm at measuring sites between groups with a 95% confidence interval ( $\alpha = 0.05$ ) and assuming a standard deviation of 1.5 mm, considering the changes in mean probing depth. This study was approved by the Ethics Committee of the Dental School of Aristotle University. The mean age of the population was  $48.2 \pm 8.2$  years, and almost two-thirds of the subject were female (13 women). Informed consent was obtained from all individual participants included in the study.

The inclusion criteria were:

1. Moderate to severe periodontal disease with presence of 3 or more quadrants in the oral cavity, each containing at least three sites with periodontal pocket depth (PPD) of  $\geq 5$  mm
2. Age  $\geq 20$
3. Light to moderate smokers ( $< 10$  cigarettes/day). We excluded the heavy smokers and just included the light to moderate smokers ( $< 10$  cigarettes/day) corresponding to a significant percentage of patients at our daily practice.

The exclusion criteria were:

1. Medication (i.e., antibiotics) affecting healing of periodontal and peri-implant tissues in the last 6 months
2. Systemic diseases that affect the appearance and progress of periodontal disease
3. Pregnancy or lactating
4. Non-surgical and/or surgical periodontal treatment for pocket reduction received over the last 1 year

### Experimental design

The present study was designed as a split-mouth randomized controlled clinical trial, and conducted for a follow-up period of 6 months. Using a randomization chart, the quadrants of the oral cavity were assigned to the 3 investigated treatment groups. Laser groups were a diode laser group and a photodynamic therapy group and the third group was the control group. The sites in the SRP group (control group) received only mechanical therapy. The diode laser group received mechanical therapy and additional application of a 940-nm diode

laser. In the photodynamic group, antimicrobial photodynamic therapy was performed by using a transgingival low-level GaAlAs 670 nm diode laser device after mechanical periodontal therapy.

The laser groups were always diametrically opposite chosen (to avoid “carry-cross” effect between laser quadrants). In the first session, oral hygiene instruction was given. All patients practiced the oral hygiene procedure over a week to ensure adequate plaque control prior to baseline examination. In the next session, full-mouth SRP was carried out for all patients by applying ultrasonic (EMS®, Switzerland, type A and P tips) and hand instruments (Gracey curettes 3–4, 11–12, and 13–14, Hu-Friedy, Chicago, IL, USA). The procedure was performed under local anesthesia, within one or two appointments. Different treatment approaches were assigned randomly to the three dental quadrants. Laser irradiation of test groups was conducted in three sessions, the first 48 h after SRP, and the second and third with intervals of 7 days.

In the diode laser group, the additional therapy was performed using Ezlase (Biolase Ezlase 940, Biolase Technology, Irvine, CA) 940 nm wavelength, 2 W power average output, operated in continuous mode. A 300- $\mu$ m-diameter tip (E3–4) was used for each patient. The tip was inserted into the pocket and was operated with a sweeping movement, apically to coronally direction for 30 s. The operator was the same during the whole process and checked fast the fiber tip for any possible debris. The fiber was used with the proper angulation, and by tactile effect, it was assured that the irradiation aimed at the inner portion of the sulcus and not at the cementum surface. The power density was calculated at 2.831 KW/cm<sup>2</sup>. The entire volumes of periodontal pockets were irradiated. During the first irradiation, there was an amount of granulation tissue removed, but at the second and third irradiation, we used the laser device to reduce subgingival bacterial loads and promote healing.

In the photodynamic group, laser irradiation was performed by using a transgingival low-level GaAlAs diode laser device (MED-701, Lasotronic, Switzerland) with a wavelength of 670 nm, 350 mW output power; 1% phenothiazine chloride solution (methylene blue) was used as photosensitizer. Each tooth was irradiated for 2 min (60 s buccally, 60 s palatally/lingually). The tip diameter was 1 cm and the power density was calculated at 0.445 W/cm<sup>2</sup>. The 670 nm diode laser was used at the level of the sulcus entrance and the entire volume of the periodontal pocket was irradiated. Photodynamic laser treatment was also applied in 3 sessions with the same intervals described above in the diode laser group.

### Clinical recordings

The periodontal status of each subject was assessed at baseline and at 3 and 6 months after periodontal treatment. All measurements were performed by one blinded therapist, thereby allowing intra-experimental comparisons of the values. The

examiner underwent calibration training at the beginning of the study. Intraexaminer variability test was performed with  $r = 0.920$  (Pearson’s correlation test).

The following clinical measurements were recorded:

**Probing pocket depth (PD):** By applying a calibrated periodontal probe (Hu-Friedy®, PQW7, USA) with a diameter of 0.5 mm, pocket depth was measured at 6 sites at each tooth (mesio-labial, mid-labial, distal-labial and mesio-palatal/lingual, mid-palatal/lingual and distal-palatal/lingual sites) as the distance from the gingival margin to the bottom of the sulcus.

**Clinical attachment level (CAL):** this was recorded at 6 sites in a manner similar to PD in relation to the cemento-enamel junction.

**Bleeding on probing (BOP):** using the same probing pressure, sulcus bleeding was determined 30 s after probing and was assessed as either presence or absence at 6 sites per tooth.

**PI (Plaque Index):** (O’ Leary et al.1972) presence-absence (6 sites per tooth).

The primary outcome of the study was changes in probing pocket depth (PD) between three groups and the secondary outcomes were changes in CAL, BOP, and PI.

### Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences 24.0 (SPSS Inc. v.24.0; IBM, Chicago, IL, USA). Test of normality was conducted using the Shapiro-Wilk test in conjunction with histograms, P-P and Q-Q plots. Paired *t* test was applied to determine the differences in the means of clinical parameters (PD, probing depth; and CAL, clinical attachment level) between baseline and 3 months and baseline and 6 months within each group. One-Way ANOVA with repeated measures was used to find differences in the means of the clinical parameters (PD, probing depth; and CAL, clinical attachment level) between groups in the same period. The non-parametric McNemar’s  $X^2$  test was applied to determine the differences in the proportions of clinical parameters (PI, plaque index; and BOP, bleeding on probing) between baseline and 3 months and baseline and 6 months within each group. Cochran’s Q test was used to find differences in the proportions of clinical parameters (PI, plaque index; and BOP, bleeding on probing) between groups in the same period. Relationships with a *p* value (*p*)  $\leq 0.05$  were considered as statistically significant. All reported *p* values are two-sided. In the presentation of results, continuous variables are shown as means with standard deviation (SD), while frequencies with percentages are used for categorical variables *N* (%).

### Results

A total of 448 sites in the diode laser group, 417 sites in the photodynamic group, and 389 sites in the control group in 21

patients were included in the current study. Two groups were formed for the evaluation of each therapeutic approach: sites with baseline probing depth between 4 and 6 mm and sites with baseline probing depth  $\geq 7$  mm. The sites with baseline probing depth between 4 and 6 mm totaled 1081 and 173 for  $\geq 7$  mm. All patients completed the 6-month period of the study. Healing occurred in all cases with no adverse effects such as pain, burning sensation, or any other feeling of discomfort.

The three groups showed similar baseline characteristics for the PD, CAL, and BOP with no significant differences. The only significant difference was for PI and for control group compared with test groups.

All treatment modalities in this study caused statistically significant improvements in the evaluated clinical parameters at 3-month and 6-month courses compared with baseline (Tables 1, 2, 3, and 4). There was no statistically significant difference regarding PD and BOP between groups at 3 and 6 months. There was only a tendency for greater reduction of PD in test groups (especially for diode group) for deep pockets  $\geq 7$  mm at 3 months, which did not reach statistically significant levels ( $p = 0.096$ ) (Table 1). Concerning CAL (Table 2), there was only a statistical significant clinical attachment gain found in the diode group in comparison with the control group for shallow pockets 4–6 mm at 3 months ( $p = 0.035$ ).

## Discussion

The aim of this clinical trial was to evaluate and compare the long-term clinical effects of diode laser and photodynamic therapy as adjunctive therapies in the treatment of chronic

periodontitis in combination with scaling and root planing. The findings of the present study did not reveal any additional clinical benefit following the utilization of the diode or photodynamic laser therapy in conjunction with conventional SRP in comparison with mechanical therapy solely in terms of probing depth, CAL, and bleeding on probing 6 months after treatment. There was only a tendency for greater reduction of PD in the diode group for deep pockets at 3 months, which did not reach statistically significant values.

One recent RCT [19] investigating the efficacy of PDT, diode laser, and scaling in the treatment of chronic periodontitis also reported no statistically significant differences between three groups regarding clinical parameters at 3 months after treatment. In the study [15], the researchers compared the clinical effects of laser therapy with a low-power diode laser (810 nm) and photodynamic therapy (using a new photoactivator Emundo®) as adjunctive therapies in the treatment of 20 chronic periodontal patients; a statistical significant greater reduction (0.7 mm) in probing depth at 6 weeks and 3 months for the diode test group was reported. Similarly, a tendency ( $p = 0.096$ ) for greater PD reduction was recorded in our trial in test groups for deep pockets  $\geq 7$  mm at 3 months (0.85 mm for diode group and 0.6 mm for photodynamic group).

Concerning the clinical attachment gain, there were no statistically significant differences between groups at 3 and 6 months except for the significant clinical attachment gain in the diode group in comparison with the control group for shallow pockets (4–6 mm) at 3 months. A recent randomized clinical trial [19] reported similar findings after laser irradiation at 3 months in agreement with other studies concerning diode lasers [13] and photodynamic therapy [20].

**Table 1** Differences in PD parameter (mean (SD)) for moderately deep pockets ( $4 \text{ mm} \leq \text{PD} \leq 6 \text{ mm}$ ) and deep pockets ( $7 \text{ mm} \leq \text{PD} \leq 9 \text{ mm}$ )

	Baseline	3 months	$\Delta$ (0–3 months)	$p^*$ value	6 months	$\Delta$ (0–6 months)	$p^{***}$ value
Moderately deep pockets ( $4 \text{ mm} \leq \text{PD} \leq 6 \text{ mm}$ )							
PD (mm)							
Control	4.80 (0.76)	3.26 (1.05)	1.54 (1.10)	<0.001	3.14 (1.06)	1.66 (1.14)	<0.001
Diode	4.82 (0.78)	3.12 (1.00)	1.70 (1.04)	<0.001	3.19 (1.02)	1.63 (1.07)	<0.001
Photodynamic	4.76 (0.79)	3.18 (1.02)	1.58 (1.08)	<0.001	3.10 (1.01)	1.66 (1.02)	<0.001
$p^{***}$ value	0.613	0.200	0.171		0.453	0.915	
Deep pockets ( $7 \text{ mm} \leq \text{PD} \leq 9 \text{ mm}$ )							
PD (mm)							
Control	7.54 (0.88)	4.25 (1.57)	3.29 (1.88)	<0.001	4.13 (1.57)	3.41 (1.72)	<0.001
Diode	7.67 (0.87)	3.42 (0.97)	4.25 (1.54)	<0.001	3.54 (0.83)	4.13 (1.33)	<0.001
Photodynamic	7.58 (0.83)	3.96 (1.43)	3.62 (1.31)	<0.001	3.83 (1.49)	3.75 (1.42)	<0.001
$p$ value	0.869	0.096	0.100		0.284	0.213	

PD, probing depth

$p^*$  value refers to the statistically significant difference for each group between baseline and 3 months ( $p < 0.05$ ); paired  $t$  test

$p^{**}$  value refers to the statistically significant difference for each group between baseline and 6 months ( $p < 0.05$ ); paired  $t$  test

$p^{***}$  value refers to the statistically significant difference between groups in the same period ( $p < 0.05$ ); one-way ANOVA with repeated measures

**Table 2** Differences in CAL parameter (mean (SD)) for moderately deep pockets (4 mm ≤ PD ≤ 6 mm) and deep pockets (PD ≥ 7 mm)

	Baseline	3 months	Δ (0–3 months)	<i>p</i> * value	6 months	Δ (0–6 months)	<i>p</i> ** value
Moderately deep pockets (4 mm ≤ PD ≤ 6 mm)							
CAL (mm)							
Control	5.29 (1.17)	4.14 (1.59)	1.15 (1.28)	< 0.001	4.05 (1.67)	1.24 (1.35)	< 0.001
Diode	5.30 (1.08)	4.32 (1.56)	0.98 (1.29)	< 0.001	4.32 (1.52)	0.98 (1.25)	< 0.001
Photodynamic	5.49 (1.52)	4.49 (1.77)	1.00 (1.23)	< 0.001	4.45 (1.92)	1.04 (1.19)	< 0.001
<i>p</i> *** value	0.098	0.180	0.137		0.130	0.140	
Deep pockets (PD ≥ 7 mm)							
CAL (mm)							
Control	8.48 (1.31)	6.61 (2.35)	1.87 (1.77)	< 0.001	6.43 (2.39)	2.05 (1.94)	< 0.001
Diode	8.04 (1.26)	5.22 (1.17)	2.82 (1.50)	< 0.001	5.26 (1.10)	2.78 (1.28)	< 0.001
Photodynamic	8.00 (1.21)	5.87 (1.39)	2.13 (1.74)	< 0.001	6.00 (1.62)	2.00 (1.91)	< 0.001
<i>p</i> *** value	0.355	0.035 <sup>A</sup>	0.171		0.050	0.259	

CAL, clinical attachment level

*p*\* value refers to the statistically significant difference for each group between baseline and 3 months (*p* < 0.05); paired *t* test

*p*\*\* value refers to the statistically significant difference for each group between baseline and 6 months (*p* < 0.05); paired *t* test

*p*\*\*\* value refers to the statistically significant difference between groups in the same period (*p* < 0.05); one-way ANOVA with repeated measures

<sup>A</sup> Difference between control and diode

The bleeding on probing index showed a similar reduction in all three treatment groups at 3 and 6 months after treatment. The findings of the present trial concerning BOP are consistent with those of a number of systematic reviews dealing with the effectiveness of diode lasers, showing that their combined application does not produce significant differences at 6 months compared with the control groups. On the other hand, there is an inconsistency with the results of prospective studies concerning applications of photodynamic therapy, which showed a tendency for a 15% reduction in the bleeding on probing at 6 months in comparison with control groups [5].

No differences at 3 months between groups were observed regarding plaque index, while at 6 months, the index decrease was greater in the control group. All patients were given the same oral hygiene instructions during the initiation period of the study, as well as at the scheduled re-examinations. However, there is no documentation in the literature indicating that the application of a laser device or photodynamic therapy

may prevent plaque formation on the irradiated radical surface of the tooth.

We used a 940-nm diode laser because of its high absorption in pigments and hemoglobin and its minimal absorption in water (compared with 980 nm diode lasers) and hydroxyapatite. There are few studies which assess the long-term effectiveness of the adjunctive application of the 940-nm diode laser therapy in the literature.

There are two further studies using a similar methodology (diode laser, Biolase Ezlase 940 nm, Biolase Technology, Irvine, CA) as adjunctive approach in periodontal therapy. The first study [9] included 30 periodontal patients (divided into 2 groups of 15 subjects) with 1-, 3-, and 6-month observation periods. The control group was treated with hand instruments only, while in the test group, scaling and root planing in conjunction with laser radiation was performed. Clinical parameters

**Table 3** BOP (%) at baseline and 3 and 6 months

	Baseline	3 months	6 months
BOP			
Control	81.9%	21.9%	13.3%
Diode	80%	18.4%	14.6%
Photodynamic	79%	18.1%	15.9%
<i>p</i> *** value	0.638	0.379	0.651

BOP, bleeding on probing

*p*\*\*\* value refers to the statistically significant difference between groups in the same period (*p* < 0.05); Cochran's Q test

**Table 4** PI (%) at baseline and 3 and 6 months

	Baseline	3 months	6 months
PI			
Control	77.6%	26.6%	21%
Diode	85.5%	26.6%	30.6%
Photodynamic	82.9%	31.6%	27.6%
<i>p</i> *** value	0.038 <sup>A,B</sup>	0.302	0.020 <sup>A</sup>

PI, plaque index

*p*\*\*\* value refers to the statistically significant difference between groups in the same period (*p* < 0.05); Cochran's Q test

<sup>A</sup> Difference between control and diode group

<sup>B</sup> Difference between control and photodynamic group



showed statistically significant improvement in both groups in follow-up periods. Among the groups, a greater improvement in clinical parameters (PD, CAL, BOP) was observed in the diode laser group, which was statistically significant. Furthermore, biochemical parameters were assessed, which indicated a similar post-treatment improvement for both groups. Additionally, a randomized, clinical study comprising 22 patients, who were followed during the maintenance phase of periodontal therapy, compared a combined laser approach to SRP [21]. The test group was treated with mechanical instruments and an adjunctive application of a diode laser at a power of 0.8 W in the same session, while the control group received scaling and root planing only. There were no statistically significant differences found between the groups concerning the clinical parameters (PD, CAL, BOP) at 3 months. The results of the study were in agreement with our findings; however, in all three studies, there was a difference in design regarding the settings of the laser device. Furthermore, there were differences in the frequency of laser applications, as neither of the above 2 studies reported a second repeated session. Finally, irradiation took place in the same session with scaling and root planing in both studies, while in our design, we chose to have a 7-day interval between irradiations.

We chose 3 separate intervals of irradiation to reinforce the antimicrobial action of the laser devices and we chose to wait 48 h after SRP because the 940-nm diode laser shows high absorption in hemoglobin. Scaling and root planing therapy is a process that causes bleeding from the entrance of the sulcus and the point was not to use immediately after scaling the 940-nm diode laser in this situation. Our irradiation target was the inner part of the pockets, and the blood presence would probably affect our results.

There is only a single prospective study using a transgingival GaAlAs low-power device in the literature (MED-701, Lasotronic, Switzerland) according to the principles of photodynamic therapy [22]. The study was published by a German group assessing 1 experimental site and 1 control site selected for each of 19 included participants. The irradiation was applied externally to the sulcus with a 1-cm-diameter tip. The activator substance used was 1% methylene blue solution. The experimental group received a laser application immediately after scaling and root planing, 2 and 6 months after baseline. The results of the study showed a greater improvement in clinical parameters (PD, CAL) in the photodynamic therapy group. We also noted the different design of the study, especially as regards the intervals between applications of photodynamic therapy. The irradiation with the 670-nm diode laser was performed at the level of the pocket entrance (1 mm from gingival margin) with the proper angulation where the width of the free gingiva is maximum 1 mm to 1.5 mm regardless of thick or thin biotype. Accordingly, we did not consider the gingival biotype as a critical factor that would affect the results of the study.

Furthermore, there is an abundance of scientific evidence that heavy smoking has an additive effect on the progression

of periodontal disease and is detrimental to healing after periodontal therapy [23]. Accordingly, we excluded heavy smokers and just included the light to moderate smokers (< 10 cigarettes/day) corresponding to a significant percentage of patients at our daily practice. Accordingly, 7 light to moderate smokers were included in the present study. No comparison between light smokers and non-smoking participants was performed.

However, at present, there is no established protocol for the use of diode lasers as an adjunct to SRP. Further studies are required to assess the long-term effectiveness of the adjunctive application of diode laser therapy and photodynamic therapy with low-power output lasers as adjuncts in the non-surgical treatment of periodontitis.

## Conclusion

- All three treatment modalities in this study lead to statistically significant improvements in the evaluated clinical parameters at 3 months and 6 months compared with baseline. There was no statistically significant difference regarding PD and BOP between groups.
- There was only a tendency for greater reduction of PD in the test groups (especially for the diode group) for deep pockets at 3 months, but it was not statistically significant

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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