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Effect of laser-assisted reconstructive surgical therapy of peri-implantitis on protein biomarkers and bacterial load

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Abstract

Objectives: This randomized clinical trial assessed changes in protein biomarker levels and bacterial profiles after surgical reconstructive therapy of peri-implantitis and investigated whether the adjunctive use of Er:YAG laser impacts protein biomarker and microbial outcomes.

Materials and Methods: Twenty-four patients received surgical reconstructive therapy for peri-implantitis with guided bone regeneration following mechanical debridement with (test) or without (control) the adjunctive irradiation of Er:YAG laser. Bacterial and peri-implant crevicular fluid (PICF) samples were collected over 6 months and analyzed with bacterial qPCR and luminex multiplex assays.

Results: Surgical reconstructive treatment significantly affected the concentration of PICF protein biomarkers, including a 50% reduction in IL-1β between 2 and 4 weeks ($p < .0001$). Both MMP-9 ($p < .001$) and VEGF ($p < .05$) levels steadily decreased after treatment. In the laser group, the peak increase in IL-1β was attenuated at 2 weeks, followed by significant reduction in MMP-9 ($p < .01$) and VEGF ($p < .05$) across all follow-up appointments compared with the control nonlaser group. The total bacterial load was reduced 2 weeks after treatment, especially in the laser group, but recolonized to presurgical levels after 4 weeks in both groups ($p < .01$). The composition of selective pathogens varied significantly over the follow-up, but recolonization patterns did not differ between groups.

Conclusions: Reconstructive therapy of peri-implantitis significantly altered PICF protein biomarker and microbial levels during the healing process. The adjunctive use of Er:YAG laser significantly modulated the inflammatory response through reduced levels of MMP-9 and VEGF during the postsurgical period. The bacterial load was reduced immediately after therapy, but recolonization was observed by 4 weeks in both groups.

KEYWORDS
bone implant interactions, bone regeneration, clinical trials, guided tissue regeneration, bone regeneration, lasers, microbiology, periodontology, wound healing

1 | INTRODUCTION

Peri-implantitis is a prevalent chronic inflammatory disease of the peri-implant supporting tissues characterized by an accelerating pattern of disease progression (Derks et al., 2016a, 2016b; Schwarz et al., 2018). Clinical outcomes of peri-implantitis treatment remain unpredictable and reported recurrence rates are as high as 44% (Carcuac et al., 2020) and 60% (Renvert et al., 2018). Nonsurgical treatment of peri-implantitis has limited efficacy (Bassetti et al., 2014; Esposito et al., 2013), due to the limited access to decontaminate the implant surface and an inability to correct bony disharmonies. Surgical interventions generally result in more favorable outcomes for probing depth (PD) reduction (Di Gianfilippo et al., 2020; Froum et al., 2015; Khoury & Buchmann, 2001; Mercado et al., 2018) and radiographic bone gain (Deppe et al., 2007; Mercado et al., 2018; Roccuzzo et al., 2017). Notwithstanding, long-term success is often lower than 60% (Heitz-Mayfield et al., 2018; Isehed et al., 2018; Mercado et al., 2018; Ravida et al., 2022; Roccuzzo et al., 2017). Many treatment modalities have been developed and tested over the years for surgical treatment of peri-implantitis (Di Gianfilippo et al., 2020). However, no clear evidence exists to define the most predictable approach thus further research is warranted for the treatment of peri-implantitis.

Laser therapy has become a promising adjunctive tool for surgical treatment of peri-implantitis (Schwarz et al., 2009). Er:YAG (erbium-doped yttrium aluminum garnet) lasers are primarily absorbed by water, which has well-documented microbial decontamination, anti-inflammatory, and bio-stimulatory properties in various preclinical studies. Its bactericidal effects on titanium surface can reach 99% efficacy irrespective of the implant surface characteristics and without surface damage (Giannelli et al., 2017; Kreisler et al., 2002). In addition, the use of Er:YAG lasers for implant surface decontamination during peri-implantitis therapy can aid in greater new bone-to-implant contact (BIC) in a canine model (linear bone gain: 3.37 vs. 1.83 mm; laser vs. control) (Nevins et al., 2014).

A recent randomized clinical trial from our group investigated the adjunctive use of a Er:YAG laser during surgical reconstructive treatment of peri-implantitis (Wang, Ashnagar, et al., 2021). All patients achieved significant PD reduction up to 6 months follow-up, with the laser-irradiated group showing an additional 0.8 mm of PD reduction compared with the control group. Further research is warranted to define deeper-level outcomes for early detection of subclinical signs of disease and relapse (Larsson et al., 2016; Wang, Hao, et al., 2021). Analysis of protein biomarkers has gained attention as a noninvasive diagnostic tool for detection of subclinical signs of disease through analysis of pro-inflammatory mediators (e.g., Interleukin 1β, IL-1β), tissue remodeling matrix metalloproteinase (MMPs), signals related to angiogenesis (vascular endothelial growth factors, VEGF), or cell proliferation amongst others (Giannobile et al., 1995; Kinney et al., 2014; Li & Wang, 2014; Oringer et al., 1998; Steigmann et al., 2020; Tatarakis et al., 2014). Cross-sectional studies using peri-implant crevicular fluid (PICF) reported high sensitivity and specificity for IL-1β and tissue inhibitor metalloproteinase (TIMP)-2 to differentiate healthy and diseased peri-implant tissues (Wang et al., 2016). Longitudinal investigations reported that IL-1β and VEGF correlated with peri-implant disease severity (Renvert et al., 2015). In addition, IL-1β, VEGF, and IL-6 were found to be reduced in patients with successful disease resolution (Renvert et al., 2017). Therefore, regulators of the upstream immune and inflammatory response such as IL-1 β, of collagen cycle such as MMPs/TIMPs, and revascularization factors as VEGF are among the most investigated and promising biomarkers for detection of subclinical peri-implant disease.

Human research on the effect of Er:YAG lasers on surgical treatment of peri-implantitis is scarce, and to the best of our knowledge, no studies have reported on the longitudinal changes in protein biomarkers and bacterial recolonization after laser-assisted surgical treatment. Therefore, the primary aim of the present study was to investigate the impact of the adjunctive use of an Er:YAG laser in reconstructive therapy regarding bacterial decontamination and inflammatory modulation. Our secondary aim was to explore the dynamic changes of bacterial pathogens and protein biomarkers in the peri-implant crevicular fluid as an assessment of the healing process.

2 | MATERIALS AND METHODS

The study was conducted in fully accordance with the Helsinki Declaration as revised in 2013 and compliant with the Consolidated Standards of Reporting Trials (CONSORT) guidelines. The study was approved by the local ethical committee Health Science Institutional Review Board (HUM00124386) and registered on ClinicalTrials.gov (NCT03127228). This clinical trial has been published in part in (Wang, Ashnagar, et al., 2021; Wang, Hao, et al., 2021). The outcomes from this same patient cohort with regard to oral fluid protein biomarker levels and microbial profiles are reported in the present study.

This study was designed as a randomized patient/examiner double-masked clinical trial on the effect of Er:YAG laser used as adjunct of a reconstructive therapy of peri-implantitis. Twenty-four systemically healthy adults with one dental implant diagnosed with peri-implantitis were recruited to join the study. Implants were considered eligible and included if presented with radiographic evidence of ≥2 mm of bone loss, PD ≥5 mm, positive for bleeding and/or suppuration on probing, while in function for more than 6 months (Sanz & Chapple, 2012). Patients were excluded in cases of implant mobility, current smoking, pregnancy, medications, or diseases known to affect bone or connective tissue metabolism, and in cases antibiotics usage within the last 2 months. Half of the patients served as controls with mechanical debridement and reconstructive treatment; the other half in the test group received the same treatment as the control group plus additional irradiation with Er:YAG laser. Both patients and examiners (including sampling) were masked about the randomization. Additional details on the study design have been reported previously (Wang, Ashnagar, et al., 2021). Each patient had a total of six study visits (Figure 1) that included baseline (V1), surgical

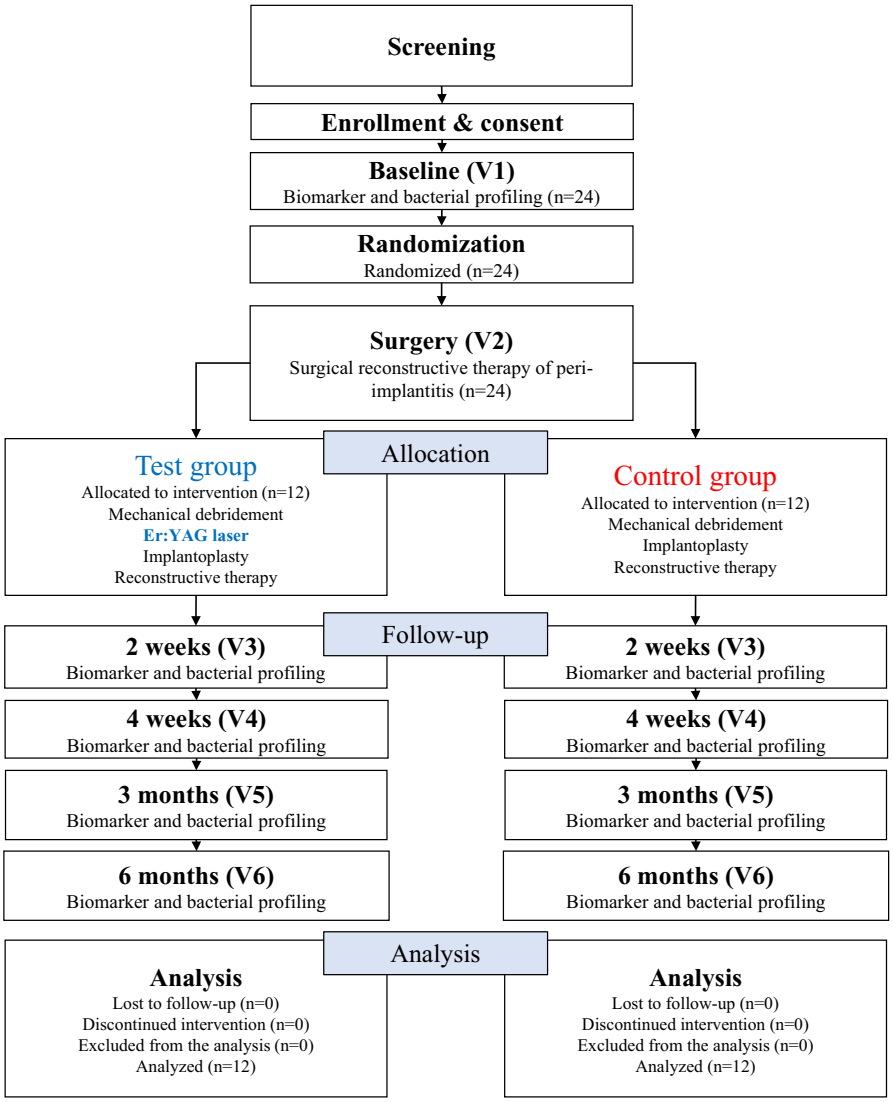

FIGURE 1 Randomized clinical trial study design. Twenty-four patients satisfied the inclusion criteria and attended a total of six study visits and followed up for 6 months. All patients were treated with surgical reconstructive therapy with ($n = 12$) or without ($n = 12$) Er:YAG laser irradiation. Peri-implant crevicular fluid and bacterial plaque samples were collected preoperatively and during four postoperative visits throughout a 6-month period.

treatment of peri-implantitis (V2), 2-week and 4-week follow-ups (V3 and V4, respectively), as well as 3-month and 6-month follow-ups (V5 and V6, respectively). Bacterial plaque and peri-implant crevicular fluid (PICF) samples were collected at baseline (V1) and at every follow-up examination (V3, V4, V5, and V6). Clinical measurements and standardized radiographs were obtained at baseline (V1), at 3 and 6 months (V5 and V6). Clinical measurements were recorded by two calibrated masked examiners (MA and JK) as reported in Wang, Ashnagar, et al. (2021). Operator calibration was conducted as described in (Di Gianfilippo et al., 2021). Inter-examiner agreement (Lin's concordance correlation coefficient, CCC) and intra-examiner agreement (intraclass correlation coefficient, ICC) were of 0.79 (CCC; 95% CI 0.74–0.84), 0.93 (ICC for MA; 95% CI 0.89–0.96), and 0.84 (ICC for JK; 95% CI 0.72–0.91), respectively.

2.1 | Patient allocation and treatment procedures

Patients were screened and enrolled between June 2017 and May 2018. On the first examination (V1), all participants signed the informed consent and received sampling of PICF and bacteria, clinical measurements, a standardized radiograph, and a periodontal maintenance. The periodontal maintenance consisted in single session of full-mouth supragingival debridement with hand scalers and piezo instruments. At the day of the surgical intervention (V2), the group allocation was revealed only to the surgeon. Patient randomization allocation was described in the previous publication (Wang, Ashnagar, et al., 2021). Patients and examiners were kept unaware of the group assignment until the end of the study. Allocation criteria and allocated sequence were concealed by the study coordinator (JK) and available only for surgeon operators during surgical intervention (CWW and HLW).

All surgical procedures were performed by two expert board-certified periodontists (CWW and HLW) between 2017 and 2018 in the Department of Periodontics and Oral Medicine of the University of Michigan. All patients received surgical reconstructive treatment of peri-implantitis, with the adjunct of laser for the test group. Details of the surgical intervention have been previously published (Wang, Ashnagar, et al., 2021). Of note, implants allocated in the test group received Er:YAG laser irradiation for removal of granulation

tissue from the infrabony defect, implant surface decontamination, and tissue modulation. Surface decontamination of test patients was performed with the laser using slow 0.5 mm/s vertical and horizontal linear movements (panel setting: 50 mJ/pulse, 25 pps). Low-energy laser was also applied over the bony defect and flap inner tissue (panel setting 30 mJ/pulse, 20 pps). A simulated placebo laser treatment was performed in the mouth of patients allocated to the control group with irradiation of a wet gauze placed over the vestibule close to the implant location.

After degranulation and decontamination, a bone wax was placed to protect the infrabony defect from titanium particles when implantoplasty of the suprabony threads was performed. Then, the infrabony component of the defect was grafted with bone allograft particulate (MinerOss and Grafton, BioHorizons, Alabama, USA) and covered with an absorbable acellular dermal matrix (ADM) membrane (Alloderm GBR, BioHorizons, Alabama, USA). Tension-free primary closure was performed with polytetrafluoroethylene (PTFE) sutures (Cytoplast, BioHorizons, Alabama, USA), and the wound was protected with a surgical dressing (Coe-Pak Periodontal Dressing, Patterson Dental, Minnesota, USA). All patients were provided with antibiotics (Amoxicillin, 500 mg tabs, three times/day for 10 days) and analgesic nonsteroidal anti-inflammatory drugs (Ibuprofen 600 mg tabs, prn). All patients were followed at 2 weeks and at 4 weeks for dressing and suture removal. They also returned for follow-up visits at 3 months and at 6 months after the intervention, when they received clinical and radiographic examination and a nonsurgical maintenance.

2.2 | Peri-implant crevicular fluid sampling and processing

Peri-implant crevicular fluid was collected with sterile paper strips from the deepest pocket of each implant at baseline examination (V1), 2 weeks (V3), 4 weeks (V4), 3 months (V5), and 6 months (V6) after surgical intervention. Crown surfaces were isolated by cotton rolls and gently dried with air. A single sterile paper strip (Oraflow Inc., Smithtown, NY, USA) was inserted in a standardized interproximal site until mild resistance from the pocket was felt and left stable in place for 30 s. After removal, a waiting period of 90 s elapsed before the next sampling. The process was repeated until collection of two samples without evident blood contamination. Finally, samples were placed into a sterile polypropylene tube and stored at −80°C.

Peri-implant crevicular fluid samples were processed all at once after the conclusion of the clinical portion of the study. A 20-μL extraction solution (10 mg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% human serum albumin in phosphate-buffered saline) was pipetted directly onto the cellulose portion of each PICF strip and secured at the top of a 12×75 mm polystyrene culture tube using a cap to hold it in place. The tubes holding the paper strips were centrifuged at 2000 rpm at 4°C for 5 min. Each strip was washed and centrifuged five times to obtain a total elution volume of 100 μL. The samples were then stored at −80°C until array analysis, and scanning was performed according to the manufacturer's protocol (RayBiotech, Norcross, GA).

2.3 | Bacterial plaque sampling and processing

Bacterial plaque and DNA were also collected with sterile paper cones from each implant at V1, V3, V4, V5, and V6 immediately after PICF sampling. Sterile paper cones were inserted deep into the pocket in standardized interproximal sites. Sterile cones were left for 30 s in place, and after removal, a wait period of 90 s elapsed before the second sampling. Samples were placed into a sterile tube containing RNA 500 μL of stabilizing buffer and stored at −20°C.

The collected bacterial samples were processed all at once after the end of the clinical phases of the study. Each paper cone was transferred to a Power Bead Lysis Tube with 200 μL of the pellet and supernatant. DNA extraction was performed using DNeasy PowerSoil Kit (Qiagen, Shallowater, TX, USA), and the final DNA elution was carried out in 100 μL of kit-provided C6 solution. DNA quantity and quality were determined using a NanoDrop2000 (Thermo Scientific, Shallowater, TX, USA). 1.0 μL of the template DNA was used to perform the qPCR reaction using Taqman® Master Mix (Applied Biosystems, Shallowater, TX, USA) in StepOnePlus Real-Time PCR System (Applied Biosystems, Shallowater, TX, USA). Bac2F and Bac2R primers and the corresponding Bac2 probe were used for the reaction. DNA from Escherichia coli was used as a standard. The sample was run on quantitative PCR in triplicate. The PCR reaction was carried out with an initial holding stage of 50°C for 2 min followed by 95°C for 10 min. The cycling stage consisted of 40 cycles of 95°C for 15 s, followed by 60°C for 1 min.

2.4 | Statistical analysis

The overall trend of protein biomarker level and the laser treatment effect on protein biomarker levels during the healing process represented the primary outcomes of this study, and they were assessed using the generalized estimating equations (GEE) model through the R package "geepack" (Højsgaard et al., 2006). To overcome the large variance and non-normality of the bacterial profiles, we assessed the statistical significance of the overall trend of bacterial profiles and the effect of the laser on bacterial levels during the healing process using a nonparametric longitudinal data model as implemented in the R package "nparLD" (Noguchi et al., 2012), followed by the Benjamin–Hochberg method for multiple tests correction. To assess the statistical significance of overall protein biomarker levels/bacterial profile differences between different times, ANOVA was conducted. Post hoc paired t-tests were performed to identify significant protein biomarker/bacterial changes between time points. To assess the laser treatment effect at different time points, post hoc two-sample t-tests were conducted. Pearson correlation was used to estimate the relationship among protein biomarkers, bacterial profiles, and clinical measurements. The original power

calculation of the trial was based on clinical parameters provided in (Wang, Ashnagar, et al., 2021).

3 | RESULTS

Twenty-four patients (age: 64.9 ± 11.2 years; 14 males and 10 females) were enrolled and randomly assigned to test ($n = 12$) and control ($n = 12$) groups. Groups were homogeneous regarding baseline demographic and clinical variables. All patients received their predetermined treatment without protocol deviation and completed the 6 months of follow-up without dropout. No implants were lost leading to a survival rate of 100% within the study period. Baseline demographics, clinical characteristics, and outcomes were previously reported in (Wang, Ashnagar, et al., 2021).

3.1 | Protein biomarker analysis

Levels of IL-1β and MMP-9 from the twenty-four patients showed a significant fluctuation over the healing phase and follow-up visits ($p < .0001$ and $p < .001$ respectively; Figure S1). IL-1β was associated with a 25% increase at 2 weeks after surgical intervention and a 50% reduction between 2 and 4 weeks ($p < .0001$). Similarly, MMP-9 increased 10% during the first 2 weeks and decreased 16% to levels lower than baseline at 4 weeks ($p < .05$). IL-1β and MMP-9 remained stable for the following examinations without any statistically significant fluctuation. Although there was no significant peak increase, VEGF level significantly decreased over time ($p < .05$) with a more notable reduction that occurred between 2 and 4 weeks (155.3 ± 27.0 vs. 86.2 ± 14.2 pg/mL; 2 vs. 4 weeks) despite ANOVA measures between individual time points being nonsignificant (Figure S1).

Surface disinfection with Er:YAG laser produced reduced levels of protein biomarkers at all four time points compared with the control intervention, with statistical significance achieved for MMP-9 ($p < .01$) and VEGF ($p < .05$) (Figure 2). Laser disinfection attenuated the postoperative peak increase in IL-1β, but statistical significance was not achieved (2 weeks, test vs. control: $p = .08$). MMP-9 and VEGF levels were successfully reduced by laser treatment ($p < .01$), with statistical significance that was maintained for MMP-9 at the last follow-up examination ($p < .001$).

3.2 | Bacterial load and microbial profile analysis

Reconstructive surgical treatment of peri-implantitis successfully reduced total bacterial load for the full cohort, especially at 2 weeks ($p < .05$, Figure 3). Overall bacterial load decreased nearly twofold during the 2 weeks following the intervention (6.5 ± 9.9 vs. 3.3 ± 5.8 ng/μL; baseline vs. 2 weeks); however, bacteria recolonized to preoperative levels as early as 4 weeks. When individual red-complex pathogens from the twenty-four patients were tested (Figure S2), *Tannerella forsythia* and *Treponema denticola* were

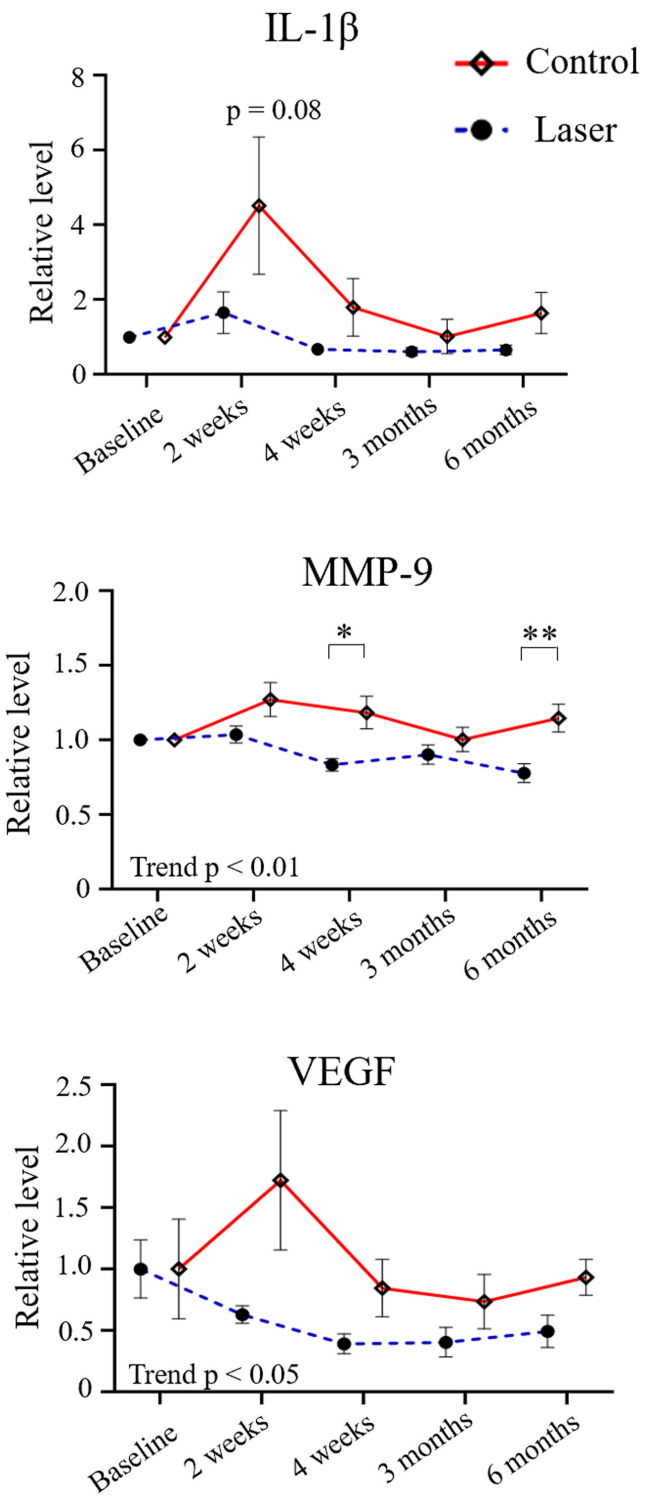

FIGURE 2 Longitudinal changes in interleukin-1β (IL-1β), matrix metalloproteinase (MMP)-9, and vascular endothelial growth factors (VEGF) for the two treatment groups during the healing process after surgical reconstructive therapy of peri-implantitis. Data were presented as mean ± standard error. Statistical significance was reported for between-group comparisons and was indicated as follows: $^{*}p < .05$; $^{**}p < .001$.

significantly decreased by the treatment at 2 weeks (*T. forsythia*: $p < .0001$; *T. denticola*: $p < .01$), while *Porphyromonas gingivalis* level remained unchanged (*P. gingivalis*, $p = .1$). *Tannerella forsythia* and

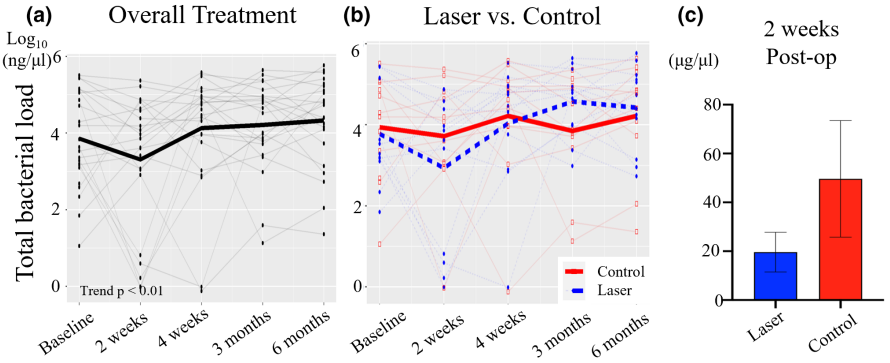

FIGURE 3 Total bacterial load for the whole cohort of patients (a) and the comparison for the two treatment groups (b) at baseline and over a period of 6 months after reconstructive treatment of peri-implantitis. The intergroup difference at the critical 2-week follow-up between control and experimental laser group was shown (c). The trend for panel A ($p < .01$) indicated the overall significance for variation during the follow-up period.

T. denticola responded similarly to the surgical treatment between baseline and 2 weeks (bacterial count decreased by 5.8 times for *T. forsythia* and 13.5 times for *T. denticola*), and remained stable at a low level between 2 and 4 weeks. However, both of these periopathogens recolonized to preoperative relative ratio between 4 weeks and 3 months (Figure S2). No significant time effect was noted for other bacteria, with the exception of *Streptococcus mutans* ($p < .01$) and *Fusobacterium nucleatum* ($p < .05$) which decreased after the intervention to recolonize after 6 months.

The Er:YAG laser did not significantly alter the microbial profile, despite a trend noted with reduced load for the test group. The average total bacteria counts were lower at 2 weeks in the test group compared with the control (19.6 ± 28.2 vs. 49.6 ± 79.1 µg/µL; test vs. control; $p = .2$) but did not reach statistical significance.

3.3 | Correlations among protein biomarkers, microbial profiles, and clinical variables

Pearson heatmap analysis showed strong correlations between protein biomarker and bacterial pathogens. Positive correlations existed among pro-inflammatory mediators, and positive correlations were noted among bacterial pathogens. Negative correlations existed between anti-inflammatory cytokines and microbial pathogens, or between early bacterial colonizers and pro-inflammatory mediators (Figure 4). A positive correlation linked pro-inflammatory mediators, such that IL-1β, with MMP-9 (*r*:0.52) and VEGF (*r*:0.52); and MMP-9 with VEGF (*r*:0.44). A positive correlation was also revealed among red-complex pathogens; *T. forsythia* correlated with *P. gingivalis* (*r*:0.31) and *T. denticola* (*r*:0.40). Total bacterial load was positively correlated with *T. denticola* (*r*:0.29) and *Prevotella intermedia* (*r*:0.28). Correlations were noted between bacterial colonizers and protein biomarker levels. In particular, a positive correlation was noted between *F. nucleatum* and IL-1β (*r*:0.23), MMP-9 (*r*:0.30), and VEGF (*r*:0.23). Negative correlation existed between early colonizers and pro-inflammatory mediators, as well as between bacterial pathogens and anti-inflammatory cytokines. *Streptococcus mutans* negatively correlated with IL-1β (*r*:−0.34), MMP-9 (*r*:−0.14), and VEGF (*r*:−0.12).

Pearson correlation analysis was used to determine whether protein biomarker and specific bacteria correlated with changes in clinical variables between baseline and 6 months. *Porphyromonas gingivalis* negatively correlated with mean PD reduction ($r = -0.50$, $p = .01$) and deepest PD reduction ($r = -0.41$, $p = .05$) with statistical significance. A negative correlation was noted between the 6-month mean PD reduction and IL-1β (*r*:−0.24), MMP-9 (*r*:−0.21), and *T. denticola* (*r*:−0.15), but such correlations did not reach statistical significance (Figure S3). Similarly, a nonstatistically significant negative correlation was noted between the 6-month reduction in the deepest PD site and IL-1β (*r*:−0.24), and *T. denticola* (*r*:−0.31) (Figure S4). A composite clinical definitions of treatment failure was created as PD ≥6 mm and radiographic bone loss ≥3 mm and positive bleeding on probing at 6 months and tested with the investigated variables. None of the selected protein biomarkers and microbial pathogens were significantly associated with the treatment failure except for *T. denticola*. When the 24-week level of *T. denticola* is lower than baseline, there is higher chance the case will satisfy the definition of treatment failure ($p = .051$).

4 | DISCUSSION

The present randomized clinical trial investigated the longitudinal changes of protein biomarkers and bacterial load from peri-implantitis lesions treated with reconstructive therapy with or without the adjunctive use of Er:YAG laser. In general and within the included cohort, selected protein biomarker levels fluctuated with an immediate postoperative increase followed by a decrease to a level lower than at baseline. Additional laser application for the test group significantly impacted the levels of MMP-9 and VEGF in PICF up to 6 months follow-up. The increase in the IL-1 at 2 weeks post-op was attenuated in the laser group compared with control, but the use of laser did not influence its level after 3 and 6 months. Bacterial

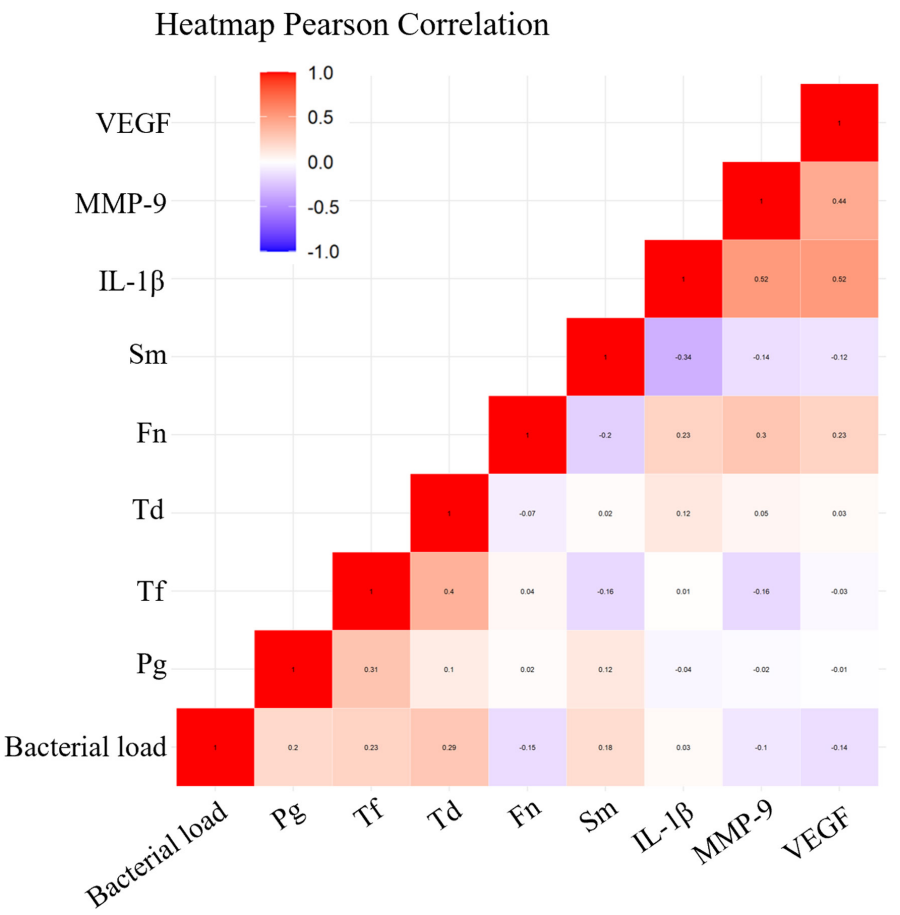

FIGURE 4 Heatmap correlation among total bacterial load, bacterial species, and protein biomarkers. Each box is positioned at the intersection of the respective target, and it is colored with the Pearson correlation spectrum scale. The red color indicates positive correlation, and the blue color negative correlation. The intensity of the color represents the strength of the correlation. Abbreviations. Pg: Porphyromonas gingivalis, Tf: Tannerella forsythia, Td: Treponema denticola, Fn: Fusobacterium nucleatum, Sm: Streptococcus mutans, IL-1β: interleukin-1β, MMP-9: matrix metalloproteinases-9, VEGF: vascular endothelial growth factor.

counts reduced after therapy in both groups without statistically significant differences between treatment modalities.

Reported literature exploring protein biomarkers for peri-implant disease diagnostics is mostly composed of cross-sectional studies (Darabi et al., 2013; Faot et al., 2015; Ghassib et al., 2019; Gurlek et al., 2017; Rakic et al., 2014, 2020; Severino et al., 2011, 2016; Wang et al., 2016; Zani et al., 2016), while prospective studies with intervention and longitudinal observation are limited. In an experimental peri-implant mucositis model, IL-1β level and clinical signs of inflammation changed to reflect the phases of the study with an increase during the plaque-accumulation period, followed by a decrease during the resolution phases (Chan et al., 2019). In a prospective trial on nonsurgical treatment of peri-implantitis, lower levels of IL-1β and VEGF were documented from implants with favorable clinical outcomes when compared to poor responders (6 months after treatment, good vs. bad responders: IL-1β: 88.4 ± 98.5 vs. 496.7 ± 684.3; VEGF: 242.3 ± 169.7 vs. 568.4 ± 428.6) (Renvert et al., 2017). Interestingly, stable outcomes were achieved in only 22% of the treated patients and the authors speculated that the nonsurgical modality of intervention was insufficient in terms of access to allow for a proper debridement of the contaminated implant surface. As compared to Renvert et al.'s cohort, the patients included in the present study presented with more advanced peri-implantitis lesion, and received surgical treatment for peri-implantitis that allowed adequate access for removal of bacterial biofilm and debridement of inflamed peri-implant tissue, resulting in improved outcomes after therapy. The levels of IL-1β, MMP-9, and VEGF correspond to the healing after reconstructive surgery with a postoperative peak at 2 weeks, followed by a gradual decrease during the longer-term follow-up. Measurements for these biomarkers at 6 months postoperative were lower than those at baseline. In line with the existing evidence, the present study validated that the levels of protein biomarkers can reliably track phases of wound healing and correlate with the level of clinical inflammation. Despite the significant variation of protein biomarker levels, statistically significant differences observed between laser-treated and nonlaser-treated patients for MMP-9 and VEGF were sustained even after 6 months follow-up.

Microbiological research of peri-implantitis pathogenic flora has traditionally targeted the well-studied red complex pathogens and a subset of species from the orange complex. Many authors have previously reported that levels of *T. denticola*, *T. forsythia*, and *P. gingivalis* were increased in peri-implantitis samples compared with those in healthy mucosa (da Silva et al., 2014; Koyanagi et al., 2013; Monje et al., 2020; Polymeri et al., 2021; Sanz-Martin et al., 2017; Shi et al., 2022; Shibli et al., 2008). Many authors also agreed on the role that orange complex pathogens play in bridging together different species and contributing towards the maturation of the peri-implant microbial community (Ghensi et al., 2020; Kroger et al., 2018; Lafaurie et al., 2017; Monje et al., 2020; Sanz-Martin et al., 2017). In the present cohort, surgical reconstructive therapy for the treatment of peri-implantitis successfully reduced PD, as well as the total bacterial load and the relative percentage of pathogens

within the residual biofilm. While overall reconstitution of the baseline biofilm took 4 weeks, red and orange complex pathogens recolonized over the following 3–6 months. Other human trials with clinical and microbiological evaluation supported the results of the present study as the overall bacterial count was reduced only in the short term (Hallstrom et al., 2017), while the count of specific pathogens remained at lower levels over the course of 3–6 months (Bassetti et al., 2014). Our results also suggest that early biofilms are less pathogenic as reflected by lower levels of red and orange complex bacteria, and open the field to future directions related to personalized maintenance care (Nieri et al., 2020). In a recently published parallel study (Wang, Hao, et al., 2021), machine-learning clustering of peri-implant immune infiltrate predicted the outcome of surgical therapy and correlated with the alpha Faith's phylogenetic diversity. Patients with favorable immunolandscape signature (increased M1/M2 macrophage ratio) had reduced recolonization of pathogenic bacterial species and improved treatment outcome after 6 months.

In the present study, PICF was used to monitor the inflammatory/immune processes because of its noninvasive and fast collection, as well as because the possibility to obtain multiple samples from the same implant over different time points. In general, positive correlations were noted between IL-1β and bacterial pathogens. IL-1β positively correlated with MMP-9 and VEGF; this is not surprising as inflammation is a well-orchestrated event coordinated by multiple inflammatory mediators that act synchronously (Hajishengallis et al., 2020). IL-1 is a known inducer for VEGF and MMPs (Inoue et al., 2005) and actively contributes to tissue metabolism through matrix degradation and neoangiogenesis. As for the bacterial–host interaction, *F. nucleatum* positively correlated with IL-1β, MMP-9, and VEGF. This is not surprising either as *F. nucleatum* has potential for activation of Triggering Receptor Expressed on Myeloid Cells (TREM-1), a macrophage-surface receptor involved in the propagation of the inflammation in response to bacterial challenge (Bouchon et al., 2001; Lagha & Grenier, 2016). Of notice, IL-1β, MMP-9, and VEGF positively correlated with *F. nucleatum* but negatively correlated with *S. mutans*, an early colonizer classically associated with symbiotic biofilm. This stresses the existence of a close interrelationship between pathogenic microflora and evoked inflammatory response. However, among red complex pathogens, limited correlations were found with the levels of IL-1β, MMP-9, and VEGF; only *T. denticola* showed slight positive correlation with IL-1β. This conflicts with previous literature supporting increased expression of pro-inflammatory mediators in response to *P. gingivalis*, *T. forsythia*, and *T. denticola* (Holt & Ebersole, 2005). Of note, this study includes samples following surgical therapy of peri-implantitis, and the treatment has significantly reduced the levels of those pathogens. Future mechanistic investigations are needed to clarify the interactions between the host and microbiome. Pro-inflammatory mediators and pathogenic bacteria negatively correlated with reduction in mean PD and deepest PD over the observation period. Furthermore, levels of *T. denticola* associated with treatment failure, defined as PD ≥6 mm and radiographic bone loss ≥3 mm and positive bleeding on probing at 6 months after therapy. These findings reinforce and furtherly expand the literature previously published by Renvert et al., when bacteria and protein biomarkers successfully discriminated patients with favorable vs. unfavorable clinical outcomes 6 months after peri-implant nonsurgical therapy (Renvert et al., 2017).

The present clinical trial is the first human longitudinal investigation on the changes of protein biomarkers and bacterial profile after reconstructive treatment of peri-implantitis based on the existing literature. It is also the first human longitudinal investigation on the effect of adjunctive Er:YAG laser treatment on protein biomarker and bacterial profiles after surgical treatment of peri-implantitis.

A limitation of the study is the reduced sample size that did not allow adjustment of the analysis based on local factors that may have impacted the recolonization of the local microflora, like implant surface characteristics, implant neck macrodesign or restoration contours. In the light of the obtained results, post hoc analyses were used based on the 6-month outcomes of MMP-9, IL-1β, and VEGF, with 12 patients each group and $\alpha = 0.05$. Post hoc analysis for MMP-9 resulted in a power of 0.8761, proving that the included sample size is adequate for analysis of MMP-9. On the contrary, IL-1β and VEGF resulted in a power lower than 0.8. A priori sample size was then calculated with the 6-month levels of IL-1β and VEGF, with a preset power of 0.8 and $\alpha = 0.05$, which resulted to be of 31 patients in each group for IL-1β, and 20 patients each group for VEGF. As being the first study on biomarker evaluation after reconstructive therapy of peri-implantitis with laser vs. mechanical therapy, it was not possible to conduct a priori sample size calculation based on the selected biomarkers, looking at the existing literature. However, results of our pilot trial will help upcoming research by guiding towards a more accurate determination of the sample size for MMP-9, IL-1β, and VEGF from peri-implant crevicular fluid after reconstructive therapy of peri-implantitis.

Another limitation of the study is the use of the definition of peri-implantitis as proposed during the VIII European Workshop in Periodontology (PD ≥5 mm, bone loss ≥2 mm, positive for bleeding/suppuration on probing; Sanz & Chapple, 2012), that is more permissive than the case definition reported during the 2017 World Workshop on Periodontal and Peri-implant Diseases and Conditions (PD ≥6 mm, bone loss ≥3 mm, positive for bleeding/suppuration on probing; Berglundh et al., 2018).

Future study projects, designed after the 2017 World Workshop, will enroll larger sample sizes and adopt the most recent case definition of peri-implantitis. Future studies will further investigate the clinical implications of protein biomarkers and bacteria as adjunctive informative diagnostics to peri-implantitis, and will provide more in-depth assessment of the treatment outcome pertinent to the stability and progression of the disease. Future studies will also propose absolute cutoff levels for protein biomarkers and bacteria able to discriminate peri-implant pathology.

In conclusion and within the discussed limitations, levels of protein biomarker from the peri-implant crevicular fluid and bacterial load from the peri-implant sulcus change after surgical reconstructive treatment of peri-implantitis reflecting the phases of inflammation and healing. Additional irradiation with Er:YAG laser can modulate the inflammatory response through reduced levels of

MMP-9 and VEGF during the postsurgical period. Although surgical reconstructive treatment of peri-implantitis successfully reduced the overall bacterial load, recolonization occurred after 4 weeks.

AUTHOR CONTRIBUTIONS

Riccardo Di Gianfilippo: Data curation (lead); formal analysis (lead); methodology (equal); project administration (supporting); validation (equal); visualization (equal); writing – original draft (lead); writing – review and editing (equal). **Chin-Wei Wang:** Conceptualization (equal); formal analysis (equal); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Yuying Xie:** Formal analysis (lead); investigation (supporting); methodology (supporting); software (equal); validation (supporting); writing – review and editing (supporting). **Janet Kinney:** Data curation (equal); investigation (supporting); project administration (equal); writing – review and editing (supporting). **James Sugai** Data curation (supporting); methodology (equal); investigation (supporting); project administration (supporting); writing – review and editing (supporting). **William V. Giannobile:** Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); investigation (equal); methodology (equal); resources (lead); supervision (equal); validation (equal); writing – review and editing (equal). **Hom-Lay Wang:** Conceptualization (equal); formal analysis (equal); funding acquisition (lead); investigation (equal); methodology (supporting); project administration (supporting); resources (lead); supervision (equal); validation (supporting); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

Dr. H-L Wang is a speaker on behalf of J. MORITA MFG. CORP. and has received honoraria. The other authors do not have any financial interests, either directly or indirectly, in the products or information listed in the article.

DATA AVAILABILITY STATEMENT

Data generated and analyzed during this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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