Study of the Antimicrobial Effect of an Ethanolic Extract of Propolis in Periodontal Disease

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Abstract: Periodontal disease encompasses gingivitis and periodontitis and is one of the most common chronic infections in the adult population. This study aimed to evaluate the influence of Spanish propolis extract (EEP) on the effect of the clinical and microbiological parameters as an adjuvant to scaling and root planning in patients undergoing supportive periodontal therapy (SPT). Forty chronic periodontitis patients were randomly assigned into two groups for the treatment. In the control group (n = 20), the sites were treated by scaling and root planning followed by gingival irrigation with physiological saline and in the test group (n = 20), the sites were treated by scaling and root planing followed by subgingival placement of EEP. At baseline (BL), bleeding on probing positive (BOP+) sites with probing pocket (PPD) ≥ 4 mm were defined as study sites. Plaque index, PPD, BOP, clinical attachment level (CAL), and subgingival plaque were evaluated at BL and 1 month later. The results showed a significant clinical improvement (p < 0.05) in the PPD, CAL and BOP+ comparing them with BL and one month after the periodontal treatment and a significant reduction (p < 0.05) for Tannerella forsythensis, Porphyromonas gingivalis, Prevotella intermedia and Treponema denticola in both groups. In addition, the improvement of clinical parameters was observed with subgingival use of EEP and also statistically significant differences between groups were observed (p < 0.05) such as reductions of BOP+ % and reduced counts of T. forsythensis and P. gingivalis, considered as the “key pathogens” for the periodontal diseases. Our results suggest prophylactic and therapeutic potential for EEP against periodontal diseases, improving clinical parameters, reducing gingival bleeding and decreasing bacterial counts of T. forsythensis and P. gingivalis. The subgingival use of EEP represents a promising modality as an adjuvant in periodontal therapy to avoid microbial resistance and other adverse effects.

Keywords: propolis; oral healing; periodontal disease; antimicrobial effect

1. Introduction

Periodontal disease encompasses gingivitis and periodontitis. Gingivitis, or inflammation of the gums which leads to bleeding gums, is considered an early form of periodontal disease. Periodontal disease is one of the most common chronic infections in the adult population [1]. Periodontitis develops over time with the accumulation of dental plaque, bacterial dysbiosis, periodontal pockets formation, gingival recession, tissue destruction and alveolar bone resorption, which can ultimately lead to tooth loss.
Periodontal disease may contribute to the organism’s overall inflammatory burden, worsening conditions such as diabetes mellitus and atherosclerosis [2] systemic inflammation being one of the main reasons for this association [3]. The immune response from the interaction between inflammatory cells and pathogenic bacteria like P. gingivalis, T. forsythensis, P. Intermedia, B. forsythus and A. actinomycetemcomitans can be crucial for the development of inflammatory processes in the surrounding tissue of the teeth.

All forms of periodontitis are treated by scaling and root planing (SRP) risk factor elimination or minimization, and daily home care and professional prophylaxis during the subsequent follow-up appointment with dentists. After clinical detection of periodontal bone adhesion and/or bone resorption, the goal of treatment is controlling inflammation and preventing disease progression and create the conditions that will help the patient to maintain healthy, functional and comfortable dentition in the long term [4] because when tissue and bone loss has occurred, it is permanent.

Scaling and root planning can be limited by the inability to reach deep areas, making it difficult to eliminate all bacteria. Therefore, the use of antibacterial agents is recommended to reduce the use of surgical approaches to periodontal pockets [5]. This helps to achieve effective drug levels at points where systemic drug intake is minimal and the risk of antibiotic-resistant strains is low [6].

The clinical use of antibiotics and other antimicrobial agents, as adjuvants for the treatment of periodontitis, is really common [7]. Recently, special attention has been paid to natural therapies. There is considerable evidence that herbal products, essential oils, and purified phytochemicals have proved to be an abundant source of biologically active compounds [8]. Approximately 80% of the population of the developing countries still use traditional medicines for their health care [9]. There have been many reports of traditional plants for the treatment of periodontal diseases. They showed the ability to inhibit the growth of oral pathogens and decreased bacterial adhesion responsible for the formation of dental plaque. Traditional plants could be the first step in developing gingivitis reducing risk factors and contributing to the maintenance of oral health to avoid bacterial recolonisation [5,10]. In this sense, Sakagami et al. [11] suggested the efficacy of GTF (glucosyltransferase) inhibitors and ARBs (Angiotensin II receptor blocker) to prevent biofilm formation and periodontitis, respectively. Different Lactobacillus species and the two-peptide bacteriocin PLNC8 αβ on P. gingivalis have been investigated to study possible effects on supporting the host immune system against invading pathogens; the probiotics tested represent valid support for SRP and benefit several clinical indexes [12].

There should be still many unknown substances that are useful for treating oral diseases in the natural kingdom. Spanish propolis can be one of the natural substances used in periodontal disease because as previously reported [13].

Propolis is a non-toxic resinous substance produced by bees that has anti-inflammatory, antibacterial, antifungal, and antioxidant properties, among others [14,15], which has attracted the attention of researchers, in medical and dental care. Its composition is quite complex based on vegetable resins (50%), waxes (30%), essential and aromatic oils (10%) and pollen and other organic substances (10%). Its mains components are flavonoids and phenolic esters such as artepiline C, gallic acid, catechins, ursolic acid and bacarin showing bacteriostatic activity producing blisters in the bacterial membrane [13]. Its components have anti-inflammatory, antibacterial and immunomodulating properties too, which are very useful to treat candidiasis, aphthous ulcers, gingivitis and periodontitis [16–18].

The purpose of this study was to evaluate the effect of subgingivally delivered ethanolic extract of Spanish propolis as an adjuvant to scaling and root planning in the treatment and maintenance of periodontitis.

2. Materials and Methods

2.1. Ethanolic Extract of Spanish Propolis

Propolis samples were supplied by Verbiotech I+D+i S.L., (Granada, Spain). A broad analysis reveals approximately 50% resin and vegetable balsam, 30% wax, 10% essential
and aromatic oils, and 5% pollen and 5% other compounds. Raw propolis chunks scraped directly from the frames and boxes of beehives were provided by Verbiotech I+d+i S.L. (Granada, Spain). The propolis formulation was prepared under aseptic conditions. 20 g of unrefined propolis was crushed and dissolved in 100 mL of 66% ethanol (Sigma-Aldrich, St. Louis, MO, USA). The mixture was kept at room temperature (25 °C) for 28 days and subsequently filtered by gravity using a Whatman 1004125 Grade 4 Qualitative Filter Paper Standard (Whatman Maidstone, UK) and Protran R nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Total Phenolic Assay

A lyophilized sample of 0.5 g of raw propolis chunks was weighed and phenolic and flavonoid products were extracted with 50 mL 80% aqueous methanol on an ultrasonic bath Model 2510 EMS (Hatfield, MA, USA) for 20 min. A portion (1 mL) of the extracts was centrifuged at 14,000 rpm for 5 min. The total phenolic content of propolis and vegetable product was determined by the Folin–Ciocalteau assay [19]. The extract was oxidized with the Folin–Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the obtained blue colour was measured at 760 nm after 60 min. Using gallic acid as standard total phenolic content (the calibration curve was generated using a concentration of 2.5–50 mg/L) was expressed as mg gallic acid equivalents (GAE)/100 g of fresh weight. Data reported were from three replications.

2.3. Total Flavonoid Assay

Total flavonoid content was measured by the aluminium chloride colorimetric assay [20]. A fraction (1 mL) of the extract (0.5 g propolis) extracted with 50 mL 80% aqueous methanol solution or a standard solution of catechins (20, 40, 60, 80, 100 mg/L) was added to 10 mL vial containing 4 mL bidistilled H2O. Subsequently, 0.3 mL of 5% NaNO2 was added to the flask. After 5 min, 0.3 mL of 10% AlCl3 was added. After 6 min, 2 mL of 1M NaOH solution was added to bring the total volume to 10 mL with bidistilled H2O. The solution was mixed and the absorbance at 510 nm was measured with a blank. Total flavonoid content was expressed in mg of catechin equivalent (CE) per 100 g of fresh weight. Samples were analysed in triplicates.

2.4. Participants and Enrolment

This is a randomized, controlled, parallel, double-blind study conducted at the Faculty of Dentistry, University of Granada. The study was approved by the Ethics Committee of the University of Granada (reference 819). Forty patients were included in the study (Figure 1). Written consent was obtained from all the subjects and the treatment procedure was explained to the patient before treatment was given. The inclusion criteria were: Patients have explicit consent, need tooth extraction with advanced periodontal disease, and have not received active periodontal treatment in the last 3 months, bleeding of two or more teeth (bleeding on probing positive, BOP+), depth of the periodontal pocket (probing pocket depth, PPD ≥ 4 mm), significant tooth extraction, highly mobile teeth, and age between 50–60 years. Exclusion criteria were: lack of informed consent, systemic illness, allergy to one of the products tested, pregnancy or lactation, use of antibiotics or anti-inflammatory drugs. After enrolment, patients are randomly assigned to test or control groups using a computer-generated sequence. All clinical measurements were performed by the same researcher. This study included two groups. In the control group (n = 20), the site was treated scaling and root planning only, and in the experimental group (n = 20), the site was treated by scaling and root planing followed by subgingival placement of ethanolic extract of Spanish propolis (EEP) (Figure 2). At the first visit, all patients had clinical measurements, bleeding on probe positive (BOP+), Periodontal pocket Deep (PPD) and Clinical attachment level (CAL). We selected the 5 deepest locations, removed the plaque on the surface with a cotton swab, and dried the area. Subsequently, a glass cylinder was inserted into the gum line for 1 min to avoid contact with the remaining oral tissue.
The cylinder was then removed and placed in a test tube, air-dried for a few minutes with the cap open, and the sample was used for qualitative and quantitative microbiological studies by PCR of the following microorganisms: *A. actinomycetemcomitans, T. forsythensis, P. gingivalis, P. intermedia* and *T. denticola*. Periodontal ultrasound treatment was performed on each surface for 30 s. The device used was a Piezon® Master 400 from EMS Swiss Instrument Perio Slim, the power was adjusted to 75%. Polishing was done with Perio-Set® and rubber cups with low abrasive paste (Kerr Hawe®). The EEP gingival sulcus was then washed using a 3 mL UEETEK® graduated pipette in the Vitulia 0.9% physiological test and serum as a placebo in the control group. There was no anaesthesia or antibiotic treatment during the surgery.

**Figure 1.** Flowchart diagram showing the process of the study.

**Figure 2.** Study design. The registered participants were 40 chronic periodontitis patients who had signed the informed consent. They needed periodontal maintenance therapy and had at least two teeth with bleeding on probing positive (BOP+), probing depths equal or greater than 4 mm (PPD ≥ 4 mm) at baseline (BL). Subgingival plaque samples were obtained with sterile vials from test and control sites at baseline and 1-month follow-up.
2.5. Microbial Sampling

Gentle removal of surface plaque was performed with a sterile cotton swab, isolating the sample site with a cotton roll and air-dry prior to sampling. Baseline plaque samples were obtained from deeper pockets using sterile curettage at the test and control sites at baseline (before scaling and planning) and one month after treatment. Subgingival samples were collected in one injection after being gently inserted into the bottom of the sampling site. Samples were collected in sterile vials containing thioglycolate broth and transported to the laboratory for microbiological analysis.

2.6. DNA Extraction and Analysis

DNA extraction was performed according to Avila-Campos [21]. Samples were homogenized and washed twice at 12,000 × g for 10 min. The pellet was resuspended in 300 µL of ultra-pure water and boiled for 10 min. After centrifugation (14,000 × g, 10 min), the supernatant (DNA) was stored and transferred to a new tube to be used as a slide. The quality of the DNA samples was confirmed by agarose gel electrophoresis and all were stored at −20 °C.

2.7. PCR Amplification

All the clinical samples were included in the PCR analysis. Species-specific primer pairs based on the sequence of the gene 16S rDNA were used according to Ashimoto et al. [22]. The amplification reaction was performed in 25 µL containing 2.5 µL of 10X PCR buffer, 1.25 µL of MgCl₂ (50 mM), 1.0 µL of dNTP (0.2 mM; Invitrogen do Brasil, Sao Paulo, SP, Brazil), 1.0 µL of each primer (0.4 M), 0.25 µL of Platinum Taq DNA polymerase (0.5 U; Invitrogen), 8 µL of sterilized ultra-pure water and 10 ng of DNA. The amplifications were performed in a DNA thermal cycler (Perkin Elmer, GeneAmp PCR System 9700, Norwalk, CT, USA) programmed for 94 °C (5 min), followed by 30 cycles at 94 °C for 30 s; annealing temperature adequate for each primer pairs for 30 s; 72 °C for 30 s, then 72 °C (5 min) to allow the completion of the DNA extension. In each respective PCR amplification DNA from *T. forsythensis* ATCC 43037, *P. gingivalis* ATCC 33277, *P. intermedia* ATCC 25611, *A. actinomycetemcomitans* ATCC 29523, were used as positive controls. A negative control without template DNA was included in each PCR run. Amplified products were compared by electrophoresis in 1% of agarose gel in 1X TBE buffer (1 M Tris, 0.9 M boric acid, 0.01 M EDTA, pH 8.4) (Invitrogen, Waltham, MA, USA), at 60 V, for 2.5 h, stained with ethidium bromide (0.5 mg/mL) and photographed on a UV light transilluminator (Kodak Digital Science System 120 TM). A 1-kb plus DNA ladder (Invitrogen, Waltham, MA, USA) was used as a molecular marker.

2.8. Statistical Analysis

Data are represented as mean ± standard error of the mean (SEM). A descriptive summary of the variables intra-treatment and between-treatments is carried out both before and after the intervention, using centralization and dispersion coefficients. The normality of the variables was checked by the Shapiro–Wilk test. The comparison within each treatment for the quantitative variables was performed using the Wilcoxon test. In the case of the qualitative variable, the comparison within each treatment was made using the McNemar test. The comparison between treatments for the quantitative variables was performed using the Mann–Whitney test. In the case of the qualitative variable, the comparison between treatments was made using the binomial proportions test. A level of p < 0.05 was considered to indicate statistical significance. Statistical analyses were performed using SPSS Version 24.0 (SPSS Inc., Chicago, IL, USA).

3. Results

The pH of propolis formulations (mean of three replications ± SEM) was 5.9 ± 0.48. Chemical analysis revealed that propolis used in the current study contained 388.2 ± 8.2 mg GAE/100 g as total phenolics and 37.0 ± 1.3 mg CE/100 g as total flavonoids.
The results showed a significant clinical improvement ($p < 0.05$) in the PPD and CAL comparing them at the beginning and one month after the periodontal treatment. Although the reduction was more apparent in the test group than in the control group, the differences were not statistically significant when comparing PPD and CAL between the two groups at the follow-up appointment (Table 1).

### Table 1. Effect of the intervention on periodontal status.

| Main Periodontal Parameters at Baseline (BL) and the Month (1 M) | Control | Test |
|---------------------------------------------------------------|---------|------|
|                                                               | Mean    | SEM  | Median | Mean    | SEM  | Median |
| BL-PPD (mm)                                                   | 4.75    | 0.17 | 5.00   | 4.75    | 0.18 | 5.00   |
| BL-CAL (mm)                                                   | 5.75    | 0.29 | 5.50   | 5.80    | 0.33 | 5.50   |
| 1M-PPD (mm)                                                   | 3.60    | 0.17 | 4.00   | 3.30    | 0.15 | 3.00   |
| 1M-CAL (mm)                                                   | 4.55    | 0.28 | 5.00   | 4.45    | 0.26 | 4.00   |
| VAR. PPD (mm)                                                 | 1.20 *  | 0.14 | 1.00   | 1.45 *  | 0.15 | 1.00   |
| VAR. CAL (mm)                                                 | 1.20 *  | 0.17 | 1.00   | 1.35 *  | 0.18 | 1.00   |
| VAR. PPD (%)                                                  | 36.42 * | 5.14 | 33.33  | 46.83 * | 6.02 | 33.33  |
| VAR. CAL (%)                                                  | 30.63 * | 5.72 | 25.00  | 31.46 * | 4.24 | 25.00  |

SD. Standard deviation, SEM standard error of the mean. BL (Baseline) 1 M (one month later), PPD (probing pocket deep), CAL (clinical attachment level). Intra-treatment (Wilcoxon test) * $p < 0.05$ differences were significant when comparing PPD and CAL intra-treatments. Between-groups (Mann-Whitney test) $p \geq 0.05$, differences were not significant when comparing PPD and CAL between groups.

One month later, the levels of bleeding on probe (BOP+) had been significantly reduced ($p < 0.05$) by 90% in the test group vs. control 75% (McNemar test) (Figure 3).

![Figure 3. Levels of bleeding on probing positive (BOP+) both groups after 1 month. No bleeding (BOP) was statistically significant in the test group. * $p < 0.05$.](image)

The presence of *A. actinomycetemcomitans* was not detected in any patients included in the study, so the antimicrobial effect of propolis extract for this bacterium could not be observed. Regarding the bacterial count of the other bacteria, after a month we observed a significant reduction ($p < 0.05$) for *T. forsythensis*, *P. gingivalis*, *P. intermedia*, *T. denticola*, in both groups. When comparing the percentage of decrease in bacteria between the groups, to evaluate the effectiveness of the irrigated substances, we found significant differences, decreasing the number of colonies for *T. forsythensis* and *P. gingivalis* in the test group ($p < 0.05$) (Figure 4).
4. Discussion

The oral cavity is rich in microbiota, and the overgrowth of bacteria can cause many pathologies. Several studies have shown that propolis can limit the growth of bacteria and pathogens responsible for periodontitis due to its antibacterial properties [23]. Propolis solution has a selectively weaker cytotoxic effect on human gingival fibroblasts than chlorhexidine. Additionally, mouthwashes containing propolis have been shown to be effective in healing surgical wounds, which facilitates the use of propolis in mouthwash solutions [24]. Propolis solution can also be used to disinfect toothbrushes [25]. The 3% ethanolic extract of propolis toothpaste was shown to be more effective than commercial toothpaste against periodontitis in a group of patients [26]. Propolis extracts also help treat halitosis, a condition in which patients experience unpleasant breath predominantly due to poor oral hygiene. Propolis toothpaste or mouthwash is used to control the growth of bacterial plaques and pathogenic microbiota that cause gingivitis and periodontitis as a natural remedy [23].

The results of the current study show significant bactericidal action of Spanish propolis extract on \( P. \) gingivalis and \( T. \) forsythensis in bacterial counts one month after periodontal therapy. Regarding the clinical results, in the monthly review, we found a reduction in the average depth of the bags at the 46.83% probing of the test compared to 36.42% in the control group. A significant decrease in bleeding in 90% of the test compared to 75% in the control was also shown. The absence of bleeding is an indicator of periodontal stability [27].

These results agree with Coutinho [28] and Sanghani et al. [29] reporting an improvement in biological and clinical parameters in periodontal tissues and corroborate those of Yoshimasu and Ikeda [13], reporting that \( P. \) gingivalis bacteria at different concentrations were eliminated with the use of propolis in a dose-dependent manner, they suggested that propolis administration in the oral cavity, is an agent with selectivity action against \( P. \) gingivalis in the periodontal pockets while maintaining the homeostatic benefit produced by oral commensal bacteria. Nakao et al. [30] in a double-blind controlled clinical trial in 24 patients with chronic periodontitis investigated topical administration of propolis, curry leaf and minocycline in periodontal pockets, they concluded that propolis treatment significantly improved both PPD and CAL and reduced \( P. \) gingivalis burden in the gingival crevicular fluid. We agree with them although our results were better in the propolis group, although they were not significant with respect to PPD and CAL, fact that can be because the application time was longer than the used in the current study (three times a day for 3 months). Giammarinaro et al. [31] evaluated the efficacy of an antioxidant based formula containing propolis and herbs as an adjuvant therapy to standard non-surgical periodontal treatment when compared to the domiciliary use of chlorhexidine-based formula. Propolis showed better results, although there was no significant difference between the groups.
Additionally, Propolis has been shown to exhibit good antimicrobial activity against a wide range of oral bacteria and inhibit the adherence to glass [32]. It was also shown to be a potent inhibitor of water-soluble glucan synthesis. Uzel et al. [33] also investigated the activity of propolis against several microorganisms. Ethanol extracts of four samples of propolis collected from different geographical regions prevented the appearance of visible growth of microorganisms, due to the presence of many flavonoids. Propolis showed an antibacterial activity similar to chlorhexidine in a study that evaluated the ability of these chemicals to inhibit microbial growth obtained from the saliva of healthy and those with chronic periodontitis [34], in agreement with the results of the current study.

Akca et al. [35] reported that an ethanolic extract of propolis (EEP) similar to the used in the current study inhibited the growth of all planktonic species as much as chlorhexidine. These authors discussed the antibacterial effects of propolis against many microorganisms. This effect is multifactorial, and as a result, the cytoplasm disintegrates cell membranes and cell walls collapse, bacteria are partially degraded and protein synthesis is inhibited. A previous study has shown that solvents and acidic solutions of propolis are more effective against bacteria and can change the pH and concentration of propolis [36]. In addition, bacterial cell wall and their biofilm properties were concluded as adjunct factors, which determine the bactericidal effect of propolis [37]. Furthermore, it was concluded that the cell walls of bacteria and the properties of their biofilms are supporting factors that determine the bactericidal effect of propolis.

Chlorhexidine is a widely used disinfectant and is included in various preparations to prevent infections, such as preoperative skin irrigation, incision preparation, and intraoperative irrigation, and hand antisepsis. However, there are several studies on the safety of chlorhexidine as a wound cleanser and as a local antiseptic around the incision. Previous studies have shown cytotoxicity against naturally proliferating cells [38] and also can be cytotoxic to human fibroblasts, osteoblasts, and lymphocytes in a time and dose-dependent manner [38], which may delay wound healing or lead to increased rates of wound dehiscence. Multiple in vitro studies with Chlorhexidine had demonstrated its cytotoxicity to fibroblast cells [39]. While fibroblasts are a critical cell type in wound healing, myoblasts, osteoblasts are crucial for skeletal muscle repair and bone healing, respectively [40]. Taking into account that the safety of propolis is assured not only by its long history of use as a traditional medicine but also by in vitro and in vivo assessment studies, it is a candidate as an adjunct in periodontal therapies. The use of standardized propolis preparations a safe and less toxic than many other synthetic preparations [41].

Due to the evidence of the link between poor oral hygiene, ongoing chronic inflammation, gingivitis, periodontitis, and systemic diseases such as diabetes, cardiovascular diseases, rheumatoid arthritis and osteoporosis [42], it is essential to achieve periodontal health and stability to avoid bacterial recolonization and bleeding in the oral cavity, using adjuvant therapies that can achieve this objective. It is noteworthy the involvement of the patient in their periodontal maintenance, performing correct oral hygiene at home. In this sense, it has been studied that sonic action heads (SAHs) powered toothbrushes appear to be more effective than brushes with oscillating heads [43], the use of products that do not stain the teeth as propolis and periodic check-ups to control plaque accumulation and gingival inflammation. Future clinical trials in which we test the different administrations of propolis, mouthwashes, toothpastes, chewing gums, ointments, gels to use at home and in the dental office are necessary to better understand their effects and applications.

5. Conclusions

Based on the results obtained, this study shows that the propolis extract has a bactericidal action against periodontopathogenic bacteria especially against *P. gingivalis*, a pathogen that has a key role in the development of periodontal disease. In addition, an improvement of clinical parameters and a reduction of microbiological counts was observed when subgingivally propolis extract was administered in patients with periodontal pockets, reducing specific risk factors for disease progression such as bleeding on probing. The
topical use of propolis may be a promising complement to periodontal therapy to avoid microbial resistance and other adverse effects.

**Author Contributions:** J.D.-C. and J.M.-F. designed the study. M.J.L.-G., E.M.-S., C.R.-B. and M.V.O.-G. performed the experiments. M.J.L.-G., E.M.-S. and J.M.-F. analysed the data. M.J.L.-G., J.D.-C., M.V.O.-G. and J.M.-F. Writing—Original Draft Preparation. E.M.-S., C.R.-B., M.V.O.-G. and J.M.-F. revised and adapted the manuscript. M.J.L.-G., J.D.-C. and J.M.-F. Supervision and Project Administration. All the authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data presented in this study are available on request from the corresponding authors.

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