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ORIGINAL ARTICLE

Phytocannabinoids and gingival inflammation: Preclinical findings and a placebo-controlled double-blind randomized clinical trial with cannabidiol

Petr Jirasek[1,2] | Alexandr Jusku[1,2] | Jana Frankova[3] | Marketa Urbankova[4] | Daniel Diabelko[5] | Filip Ruzicka[5] | Barbora Papouskova[6] | Karin Chytilova[7] | Jiri Vrba[3] | Jakub Havlasek[3] | Katerina Langova[8] | Jan Storch[9] | Iva Voborna[1,2] | Vilim Simanek[3,†] | Jan Vacek[3]

[1]Institute of Dentistry and Oral Sciences, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

[2]Institute of Dentistry and Oral Sciences, University Hospital Olomouc, Olomouc, Czech Republic

[3]Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

[4]Department of Clinical and Molecular Pathology, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

[5]Department of Microbiology, Faculty of Medicine of Masaryk University and St. Anne's University Hospital, Brno, Czech Republic

[6]Department of Analytical Chemistry, Faculty of Science, Palacky University, Olomouc, Czech Republic

[7]Department of Oral and Maxillofacial Surgery, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

[8]Department of Medical Biophysics, Faculty of Medicine and Dentistry, Palacky University in Olomouc, Olomouc, Czech Republic

[9]Department of Advanced Materials and Organic Synthesis, Institute of Chemical Process Fundamentals of the Czech Academy of Sciences, Prague, Czech Republic

Correspondence
Jan Vacek, Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Hnevotinska 3, 775 15 Olomouc, Czech Republic.
Email: jan.vacek@upol.cz

Present address
Marketa Urbankova, Department of Radiology, University Hospital Olomouc, Olomouc, Czech Republic

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Abstract

Objective: The aim of this study was to: (1) evaluate the anti-inflammatory effects of cannabidiol (CBD) on primary cultures of human gingival fibroblasts (HGFs) and (2) to clinically monitor the effect of CBD in subjects with periodontitis.

Background: The use of phytocannabinoids is a new approach in the treatment of widely prevalent periodontal disease.

Materials and Methods: Cannabinoid receptors were analyzed by western blot and interleukin production detected using enzyme immunoassay. Activation of the Nrf2 pathway was studied via monitoring the mRNA level of heme oxygenase-1. Antimicrobial effects were determined by standard microdilution and 16S rRNA screening. In the clinical part, a placebo-control double-blind randomized study was conducted (56 days) in three groups ($n = 90$) using dental gel without CBD (group A) and with 1% (*w/w*) CBD (group B) and corresponding toothpaste (group A – no CBD, group B – with CBD) for

[†]Deceased (February 11th 2023)

home use to maintain oral health. Group C used dental gel containing 1% chlorhexidine digluconate (active comparator) and toothpaste without CBD.

Results: Human gingival fibroblasts were confirmed to express the cannabinoid receptor CB2. Lipopolysaccharide-induced cells exhibited increased production of pro-inflammatory IL-6 and IL-8, with deceasing levels upon exposure to CBD. CBD also exhibited antimicrobial activities against *Porphyromonas gingivalis*, with an MIC of 1.5 μg/mL. Activation of the Nrf2 pathway was also demonstrated. In the clinical part, statistically significant improvement was found for the gingival, gingival bleeding, and modified gingival indices between placebo group A and CBD group B after 56 days.

Conclusions: Cannabidiol reduced inflammation and the growth of selected periodontal pathogenic bacteria. The clinical trial demonstrated a statistically significant improvement after CBD application. No adverse effects of CBD were reported by patients or observed upon clinical examination during the study. The results are a promising basis for a more comprehensive investigation of the application of non-psychotropic cannabinoids in dentistry.

KEYWORDS
cannabidiol, inflammation, microbiota, oral hygiene, periodontium, phytocannabinoid

1 | INTRODUCTION

Periodontitis is an inflammatory disease that can cause the irreversible loss of periodontal ligament and alveolar bone.[1] It affects around 20%–50% of the world's population,[2] and is one of the most common causes of tooth loss.[3] The maintenance of oral health or the progression to disease is closely related to the oral microbiota.[4] One of the most serious pathogens in the development of periodontitis are bacteria of the red complex which include *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*.[5] The presence of periodontopathogens and their virulent components activates the innate immune system (*e.g.* macrophages, antigen-presenting dendritic cells, natural killer (NK) cells and neutrophils) and cells of the adaptive immune system (T and B lymphocytes), leading to the release of pro-inflammatory cytokines, interferon-gamma (IFN-γ), interleukin-1 and 6 (IL-1 and IL-6), interleukin-17 (IL-17), tumor necrosis factor-alpha (TNF-α), and enzymes, especially collagenases (matrix metalloproteinase).[6] If regulation of the inflammatory process fails, deleterious changes can occur in the periodontal tissues.[7]

The combination of mechanical plaque removal with anti-inflammatory and/or anti-bacterial drugs or other active substances are the main approaches to inhibiting the development of periodontitis. New low-molecular-weight therapeutics are also being sought that meet three criteria: anti-inflammatory, anti-bacterial, and an acceptable safety profile. One currently studied group of substances that can meet these criteria is the non-psychotropic phytocannabinoids, especially cannabidiol (CBD). Phytocannabinoids are a group of secondary metabolites produced by plants of the *Cannabis* genus, and they can be divided into psychotropic (the main representative is Δ^9-tetrahydrocannabinol, THC) and non-psychotropic (the main representative is CBD).[8–10] In addition to CBD, non-psychotropic phytocannabinoids include cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN), cannabidivarin (CBDV), and others.

CBD is a substance with proven anti-inflammatory, cytoprotective, immunomodulatory, and anxiolytic effects.[11,12] According to recent studies, it can suppress the production of pro-inflammatory cytokines by reducing the cellular immune response. A reduction in the production of some inflammatory mediators such as IFN-γ, TNF-α, IL-1β, and IL-10 appears to be important for the anti-inflammatory function of CBD.[13] CBD affects the endocannabinoid system via the CB1 and CB2 receptors, and it acts biphasically and pleiotropically, *e.g.* see interactions with PPAR-γ[14] and other regulatory pathways. In terms of cytoprotective mechanisms, the interaction of CBD with the Nrf2 pathway plays an important role.[15] The CB2 receptor is expressed in the periodontal cells at the gene and protein level. The CB1 receptor has not been detected at the protein level.[16] Our knowledge of the antimicrobial action of CBD on G-negative/anaerobic, facultatively anaerobic bacteria, or directly on selected periodontopathogens is very limited.[17,18]

The aim of this study was to evaluate the anti-inflammatory and cytoprotective effect of CBD, CBN, CBG, and CBC on normal human gingival fibroblasts (HGFs), an accepted in vitro model.[19] The second goal was to clinically monitor the effect of CBD on periodontal tissues in subjects with periodontitis and this included microbiological evaluation.

2 | MATERIALS AND METHODS

2.1 | Phytocannabinoids, dental gels, and toothpastes

CBD and CBG were purchased from CBDepot Ltd. at 99% purity. CBC and CBN were synthesized as previously described.[20] Corsodyl 1% chlorhexidine digluconate dental gel from GlaxoSmithKline was used. Intervention: group A (placebo): dental gel placebo (no CBD) and placebo toothpaste (no CBD); group B (CBD group): dental gel and toothpaste both with CBD (1%, *w/w*); group C: Corsodyl 1% chlorhexidine digluconate dental gel (active comparator) and placebo toothpaste (no CBD). All gels and toothpastes were prepared by CB21 Pharma Ltd. (Brno, CZ). The detailed composition of gels and toothpastes is described in Section 1 of the Data S1.

2.2 | Preclinical findings

Human gingival fibroblasts were obtained during the surgical removal of impacted wisdom teeth from a healthy volunteer according to the standard protocol. Cell viability was assessed using an MTT assay.[21] After 24 h of simultaneous treatment with lipopolysaccharide (LPS) from *P. gingivalis*[22] (1 μg/mL, SMB00610, Sigma-Aldrich), the cells were incubated for 24 and 48 h with two concentrations of CBD or other phytocannabinoids (0.5 μM or 0.25 μM) in serum-free medium. An ELISA Human IL-6 Kit, ELISA Human IL-8 Kit (Peprotech), and Human Total Human MMP-2 Duo Set ELISA (Bio-Techne R&D Systems Ltd.) were used for analyses of IL-6, IL-8, and MMP-2, respectively, in the cell culture medium after the treatment as per the manufacturer's protocol. Western blot analysis was used for the detection of CB1 and CB2 receptors in cell lysate. Quantitative real-time PCR was used for Nrf2 pathway investigations. An ultra-performance liquid chromatograph (ACQUITY I-Class) system was interfaced with a Waters Synapt G2-S Mass Spectrometer with ESI operating in positive ionization mode (Waters); for details on LC–MS analysis of phytocannabinoids, see ref. 23 For microbiology testing, a standard microdilution method according to the Clinical and Laboratory Standards Institute (Annapolis Junction, USA) was performed.

The preclinical experimental details can be found in Section 2 in Data S1.

2.3 | Clinical trial

2.3.1 | Study design

The study was designed as a placebo-control double-blind randomized clinical trial after obtaining the approval of the Ethics Committee of Olomouc University Hospital (Approval Number: 36/20) and registered at ClinicalTrials.gov (NCT05498012).[24] The randomized controlled trial was conducted in compliance with the Consolidated Standards of Reporting Trials (CONSORT) guidelines[25] and Helsinki Declaration of 1975, as revised in 2013.[26]

Patients were recruited monocentrically in three groups. The CONSORT diagram of the study is shown in Scheme 1. Patients were treated three times with dental gel without (group A) and with 1% *w/w* CBD (group B). Dental gel was applied to the periodontal pockets. Additionally, participants in group A ($n=30$) used placebo toothpaste without CBD daily without restrictions for 56 days to maintain oral health, while participants in group B ($n=30$) used toothpaste containing 1% *w/w* CBD under the same conditions. Meanwhile, the third group, group C ($n=30$), used Corsodyl dental gel containing 1% *w/w* chlorhexidine digluconate (active comparator) and placebo toothpaste daily.

2.3.2 | Population

The recruitment of patients from November 2020 to February 2022 was from those referred to the Institute of Dentistry and Oral Sciences, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic, and those who fulfilled the inclusion criteria were invited to participate in the study. For baseline characteristics, see Table 1.

The inclusion criteria were a stage I-IV periodontitis diagnosis according to the 2017 Classification of Periodontal and Peri-Implant Diseases and Conditions by the American Academy of Periodontology and the European Federation of Periodontology,[1] age 35–65 years, number of natural teeth ≥16, no evident physical or mental impairment and signed the informed consent form. Those excluded were those with chronic diseases (*e.g.* diabetes mellitus, oncological diseases), increased bleeding (medications: anticoagulants, antiplatelet agents, bleeding diathesis); pregnant and lactating women; immunosuppressed patients and patients with an autoimmune disease; tobacco smokers; users of cannabis or cannabis products; systemic ATB therapy during the last 3 months; patients with removable prosthesis; patients with braces; parallel participation in another clinical trial.

The sample size calculation (power analysis, with required sample size $n=21$) was based on the three arms of the study. To provide a power of 80%, a significance level of 0.05, and differences in MGI (modified gingival index) between 0 and 7 days (group A 0.08; group B 0.2; group C 0.08) and a standard deviation of 0.14, 21 patients per group would be necessary. Due to the anticipated use of non-parametric methods, the sample size was increased by 10%. We needed at least 21 patients for each group (calculated by the program TIBCO STATISTICA version 13.4.0.14., see Table S1). To compensate for anticipated dropouts, 90 patients were recruited and allocated to three groups (30 each).

2.3.3 | Randomization and allocation concealment

All participants gave their written informed consent after verbal and written information was provided. After confirming entry criteria,

SCHEME 1 CONSORT flow diagram on subject enrollment, allocation, follow-up and analysis.

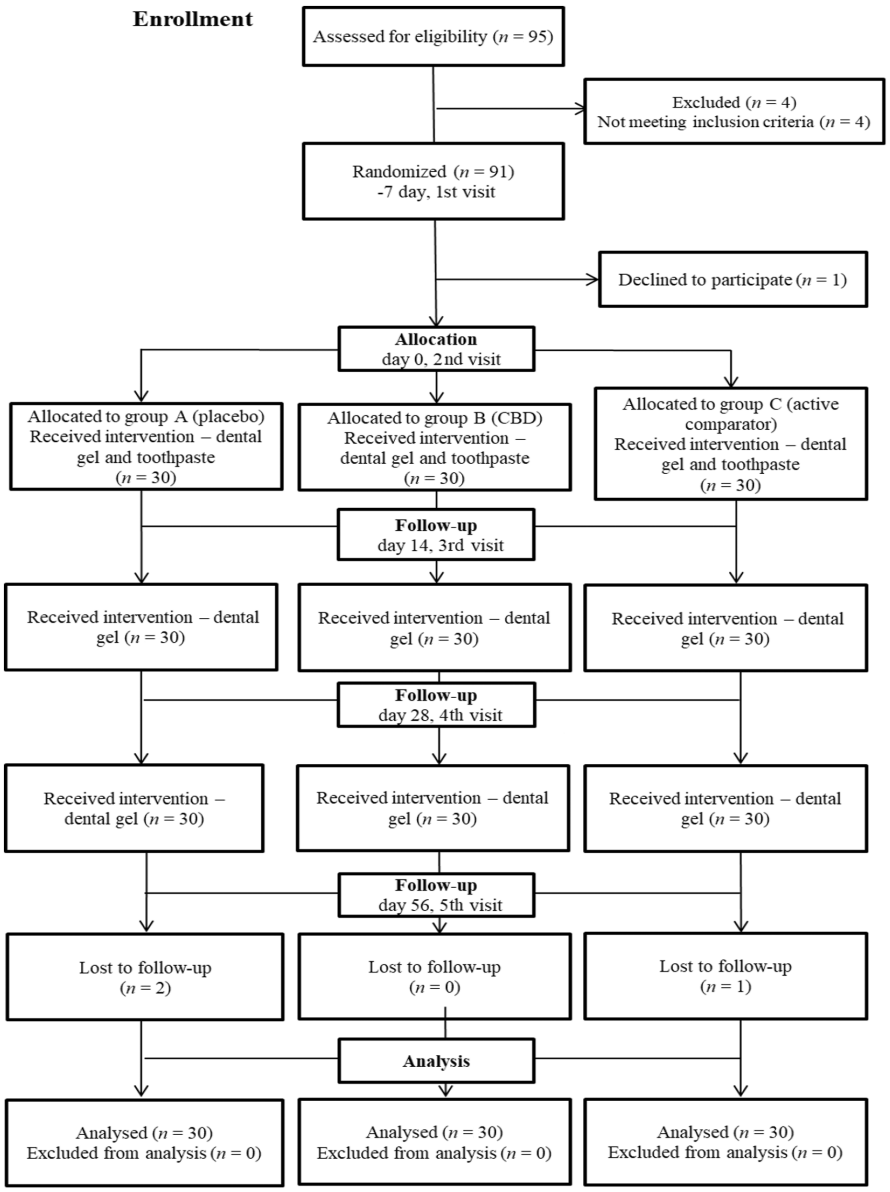

TABLE 1 Baseline characteristics of subjects involved in the study.

Variable[a]	A – placebo (*n* = 30)	B – CBD (*n* = 30)	C – Corsodyl (*n* = 30)	*p*-value
Age (years)	52 ± 8 (36–65)	55 ± 8 (36–65)	51 ± 10 (36–65)	.833[b]
Number of teeth	25 ± 2.5	25 ± 2.6	25 ± 2.7	1.000[b]
Sex (Female %)	53	60	57	.963[c]
Pocket depth (mm)	4.40 ± 0.84 (3.22–6.52)	4.94 ± 1.21 (2.90–7.98)	4.69 ± 1.23 (3.39–8.94)	.175[b]

[a]Mean ± SD (range).
[b]ANOVA.
[c]Fisher's exact test.

they were entered into the study and assigned a patient number. Assignment to group A, B, or C was done using randomization, with a block size of six patients. A staff member not involved in the examination or treatment of the patients provided the patients with sequentially numbered containers with the study products, which were provided by the manufacturer. The composition of the placebo dental gel/toothpaste was formulated to closely resemble that of both the CBD gel/toothpaste and the control Corsodyl. All toothpastes and gels were packed in identical bottles. Both the participants and examiner were blinded to the group assignment. The code for the toothpastes was broken once the study was completed and the dataset was locked.

2.3.4 | Treatment protocol

1. Enrollment (day−7), patients who met the inclusion criteria were documented via performing a panoramic radiograph, Russell's periodontal index, microbiological sampling, removal of supra/sub-gingival plaque, and calculus was performed with ultrasonic tips. The probing pocket depth was also evaluated together with clinical attachment level measure to confirm stage I–IV periodontitis diagnosis. The attachment level was exclusively considered for the diagnostic procedure. The patients were provided with Curaprox CS 3960 Super Soft and CPS 06-011 Prime Start Mix toothbrushes and oral hygiene instructions ('Brush your teeth twice a day with the provided toothpaste for at least 2 min each time you brush'). The bass technique was recommended with a pea-sized amount of toothpaste. The use of other oral hygiene products was strictly prohibited during the intervention.
2. Baseline (day 0), the allocation of patients to 3 groups (groups A, B, C), periodontal health was assessed via a set of periodontal, gingival, and oral hygiene indices. According to the allocation group A, B, or C, dental gels (1–1.5 mL via syringe with stainless-steel cannula) were applied to all periodontal pockets (5 min of exposure) and corresponding toothpastes were handed over for home use to maintain oral health. The patients used the toothpastes to replace their regular dental hygiene for the duration of the study and were continuously checked for proper oral hygiene. From the third visit, each session commenced with an update on adverse effects.
3. On the third visit (day 14), periodontal health was assessed via the set of periodontal, gingival, and oral hygiene indices. Group A, B, or C dental gels were applied to all periodontal pockets (5 min exposure).
4. On the fourth visit (day 28), periodontal health was assessed via the set of periodontal, gingival, and oral hygiene indices. Sulcular fluid samples were also collected for microbiological analysis. Group A, B, or C dental gels were applied to all periodontal pockets (5 min exposure).
5. During the final visit (day 56), the patient's periodontal health was assessed via the set of periodontal, gingival, and oral hygiene indices. Sulcular fluid for microbiology screening was sampled. The total duration of the intervention was set at 56 days.

2.3.5 | Clinical assessment

The overall score of the periodontal, gingival, and oral hygiene indices (Russell's periodontal index,[27] plaque index (PLI), gingival index (GI), and modified gingival index (MGI)[28] measured on four (buccal, mesial, distal, lingual) surfaces,[29–31] and gingival bleeding index (GBI)[32]) after the treatment was the primary outcome. Russell's periodontal index evaluates overall periodontal health, including gingival inflammation and pocket depth. The GI and MGI primarily focus on evaluating gingival inflammation with varying degrees of detail and scoring. While the GI is based on visual and tactile (probing) examinations of the gingiva, the MGI is purely based on visual examination. Additionally, the MGI enhances the sensitivity of the index for lower values. On the other hand, the GBI specifically focuses on evaluating gingival bleeding, providing information about the bleeding tendency of the gingiva, which can be indicative of gingival health. The GBI records the presence or absence of gingival inflammation as determined by bleeding from interproximal gingival sulci.[33] The PLI is used to assess dental plaque accumulation, which is a key factor in the development of gingivitis and other oral health issues. PLI served as an indicator of patient's oral hygiene compliance.

The secondary outcome measured was changes in periodontal pathogen levels from day−7 to day 28 and day 56 (for more details, see Section Clinical microbiology below). A single calibrated examiner (A.J.) performed all treatment interventions and collected all clinical, radiographic, and microbiological samples during all visits, thus can be regarded as standardized in terms of pressure and visual assessment. Previously, the examiner performed a calibration process in 10 patients with periodontitis, measuring one quadrant in each subject. The probing pocket depth and the above-mentioned indices were evaluated twice with a minimum interval of 60 min, and an intra-examiner reproducibility of 97% was achieved. A periodontal examination of the entire mouth was performed by the calibrated examiner at −7, 0, 14, 28, and 56 days after the treatment started, using a North Carolina Probe 54B (LM-Dental, Parainen, Finland). The periodontal probe was inserted with a force of 0.2–0.3 N (ref. 34).

As for the evaluation of adverse effects, adverse reactions could be spontaneously reported by a participant to an attending physician, elicited by the investigator during the trial, or laboratory related.[35]

2.3.5.1 | *Clinical microbiology*

Subgingival sampling (sulcular fluid) was performed. Bacterial DNA detection was based on the methodology of VariOr® Dento (GEN-TREND). The sampling site was isolated with cotton swabs. Using sterile tweezers, a sterile paper pin was then inserted into the bottom of each of the five deepest periodontal pockets and left there for 15 s. After that, the pins with the collected samples were allowed to dry on sterile gauze for 15 min and then placed in a test tube and sent to the laboratory. Dried samples were stable prior to transport within 14 days. If samples were stored for more than 14 days, they were stored at −20°C prior to transport. Bacterial screening was performed using an ExiPrep Plus Bacteria Genomic DNA Kit (Bioneer) using the Exiprep 16 Plus automatic device (Bioneer) using eluates (50 µL for each) of the paper pins. Bacterial DNA detection was done with VariOr® Dento (GEN-TREND). This method is based on the amplification of specific 16S rRNA sequences of periodontal pathogenic bacteria[36] using polymerase chain reaction (PCR) with DIG-UTP labeled oligonucleotides (Roche) followed by hybridization (AutoBlot 3000, MEDTEC) and chemiluminescence detection. The sensitivity of the method

was 1×10^3 bacterial count per 1 μL of sulcular fluid. The semi-quantitative microbiology approach was based on the following scale (bacterial count per 1 μL of paper pin eluate): (−): undetected, corresponds to a bacterial count $<10^3$; (+): weakly positive, bacterial count 10^3–10^4; (++): moderately positive, bacterial count 10^4–10^5; (+++): strongly positive, bacterial count $>10^5$.

2.4 | Statistical data analysis

Data were expressed as means, standard deviation (SD) or median, minimum and maximum values. In the clinical part, normality was tested using the Shapiro–Wilk test. One-way analysis of variance (ANOVA) with Bonferroni tests or Dunnett's post hoc tests were used for independent samples. A repeated-measures ANOVA with Bonferroni correction post hoc tests were used to analyze dependent samples. Non-parametric versions of these tests (*i.e.*, Kruskal-Wallis ANOVA with Dunn's post hoc tests and Friedman's test with Bonferroni correction post hoc tests) were used if the normality assumption was not met. Qualitative data are described using absolute and relative frequencies. The differences between independent samples were tested using Fisher's exact test, McNemar's test of symmetry was used for dependent samples. $p < .05$ was adopted as the level of statistical significance for all analyses, and IBM SPSS Statistics for Windows, Version 23.0. IBM Corp. was the software used. In the preclinical part, the statistical analysis was performed by one-way ANOVA; Dunnett's multiple comparison test was performed using GraphPad Prism.

3 | RESULTS

The preclinical part of the study was carried out on HGFs with CBD, CBN, CBG, and CBC. The inflammatory response was induced using lipopolysaccharide (LPS) produced by *P.gingivalis*. We also evaluated in vitro antimicrobial activity on suspensions of *P.gingivalis* and *Streptococcus mutans*. These preclinical data were then used as the basis for conducting the clinical part aimed at evaluating the effect of CBD in dental gel and toothpaste on periodontal inflammation. The clinical evaluation focused on both the health of the oral cavity soft tissue and the main periodontal pathogenic bacteria count.

3.1 | Preclinical findings

3.1.1 | CB receptors expression

The presence of cannabinoid receptors CB1 and CB2 in HGF cell cultures ($n = 3$) was evaluated. Using monoclonal antibodies, no CB1 receptor was confirmed in the HGFs (data not shown). In contrast, the presence of CB2 receptors was clearly demonstrated. The expression of CB2 receptors was monitored both in native HGFs and after 6 and 24 h incubation with the phytocannabinoids (Figure 1A-C). The expression level of the CB2 receptor was not affected in the presence of the investigated substances.

3.1.2 | Cytotoxicity of phytocannabinoids

For testing the phytocannabinoids on the HGFs, subtoxic concentrations were used (tested in the range 0.78–25 μM). The growth curves of the HGFs treated with phytocannabinoids are shown in Figure S1. The IC_{50} value was in the range 1–8 μM. The IC_{50} for CBD was 1.3 μM. The data show that concentrations of phytocannabinoids higher than 1.56 μM reduced cell viability. For this reason, we chose subtoxic concentrations in the range of 0.25–0.5 μM CBD for further experiments.

3.1.3 | Inflammatory response

In the HGF model, the inflammatory reaction was induced after 24 h application of 1 μg/mL LPS, also a subtoxic concentration, and this is shown in Figure S2. After the application of LPS, we monitored the levels of pro-inflammatory factors, interleukins IL-6, IL-8 and the production of matrix metalloproteinase, MMP-2[37–39] after 6 and 24 h. LPS-treated HGFs produced two to three times higher amounts of IL-6 and IL-8 vs. LPS non-treated cells for both time intervals (Figure S3A,B). No changes in MMP-2 production were observed, irrespective of LPS application (Figure S3C). The anti-inflammatory agent indomethacin (IND) was used as a positive control. IND is known to inhibit/inactivate the COX-2 enzyme and subsequently the transcription factor, NF-κB, which leads to the suppression of pro-inflammatory cytokine production.[40] The presence of IND did not affect the production of IL-6 or IL-8 in non-LPS-treated cells. In contrast, in LPS-treated HGFs, a ca. 25% decrease in the production of both cytokines was observed after the application of IND compared to the control (Figure S3A,B). A similar (statistically non-significant) decrease in the production of IL-6 and -8 was also found after phytocannabinoid application. This effect was observed exclusively after 24 h of incubation (see red dashed lines, Figure S3).

3.1.4 | Nrf2 pathway activation

In addition to the anti-inflammatory effect in vitro, we also evaluated the ability of CBD to activate the Nrf2 pathway.[41] The effect of the tested compounds on the Nrf2 pathway was evaluated in HGFs by determining relative changes in the expression of the *HMOX1* gene encoding cytoprotective heme oxygenase-1. After the exposure of gingival fibroblasts for 6 h to 5 μM sulforaphane, a potent Nrf2 activator,[42] the levels of HMOX1 mRNA increased 4.3-fold and 4.6-fold (statistically significant at $p < .05$) when normalized to GAPDH mRNA and 18S rRNA levels, respectively (Figure 1D, left). An increase in the HMOX1 mRNA levels was also found after the cell treatment with CBD. In cells not exposed to LPS, a 6-h treatment

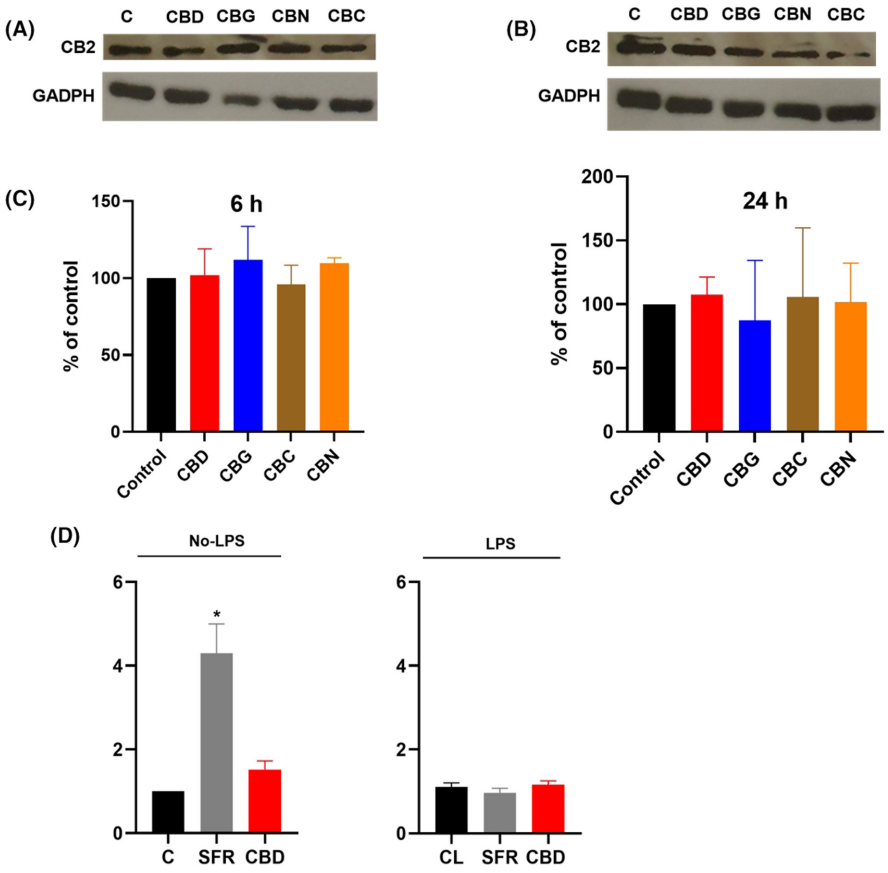

FIGURE 1 The effect of phytocannabinoids (0.5 μM) on the expression of CB2 receptor in HGFs determined by western blot. Representative western blot after (A) 6 h and (B) 24 h. (C) The quantification of CB2 receptor after 6 and 24 h expressed as percentage of control as mean ± SD ($n = 3$). (D) HGFs were incubated in serum-free medium for 24 h in the presence or absence of 1 μg/mL lipopolysaccharide (LPS), and then treated in serum-free medium for 6 h with 5 μM sulforaphane (SFR; positive control), 0.5 μM cannabidiol (CBD), or left untreated 'C' or 'CL'. After treatment, the levels of HMOX1 mRNA were determined using quantitative real-time PCR with results normalized to GAPDH mRNA. Data are means ± SD of three experiments. * $p < .05$, significantly increased versus untreated control without LPS.

TABLE 2 MIC values of phytocannabinoids for strains *Streptococcus mutans* CCM 7409 and *Porphyromonas gingivalis* CCM 3985. CHX: chlorhexidine digluconate.

	P. gingivalis		*S. mutans*	
Compound	MIC median [μg/mL]	MIC range [μg/mL]	MIC median [μg/mL]	MIC range [μg/mL]
CBC	16	16	32	32
CBD	1.5	1–2	16	16
CBN	8	8	32	32
CBG	4	4	8	8
CHX	1	1	1	1–2

with CBD increased the level of HMOX1 to 1.5-fold compared to the untreated control when normalized to GAPDH mRNA.

In contrast, no changes in the expression of the *HMOX1* gene were induced by the tested compounds in HGFs pretreated with LPS (Figure 1D, right). CBD uptake in HGF cells ($n = 3$) was confirmed by LC–MS because the Nrf2 pathway is localized intracellularly. CBD levels in the culture medium and HGF cell homogenates after 6 and 24 h incubation with 0.5 μM CBD were monitored as well. A concentration of 2.2 μg/mL (after 6 h of incubation) and 4.4 μg/mL (after 24 h of incubation) was determined in the cell lysate. No free CBD was detected in the culture media after either 6 or 24 h of incubation.

3.1.5 | Antimicrobial efficacy

We also evaluated the antimicrobial action of the phytocannabinoids. MIC was determined for *P. gingivalis* and *S. mutans* using the standard microdilution method. Even though phytocannabinoids primarily exhibit antimicrobial activity against G-positive bacteria,[17,18] a clear inhibition of growth was observed in the anaerobic G-negative *P. gingivalis*, primarily after CBD application. MIC values for CBD were comparable to the chlorhexidine (CHX) positive control. With *S. mutans*, which we classify as a G-positive facultatively anaerobic pathogen, CBG exhibited the lowest MIC values (Table 2).

3.2 | Clinical trial

Patients were treated with dental gel without (group A) and with CBD (group B). Dental gel was applied to the periodontal pockets. Additionally, participants in group A used toothpaste without CBD daily without restrictions, while participants in group B used toothpaste containing CBD. Meanwhile, the third group, group C, used Corsodyl dental gel and toothpaste without CBD daily. The

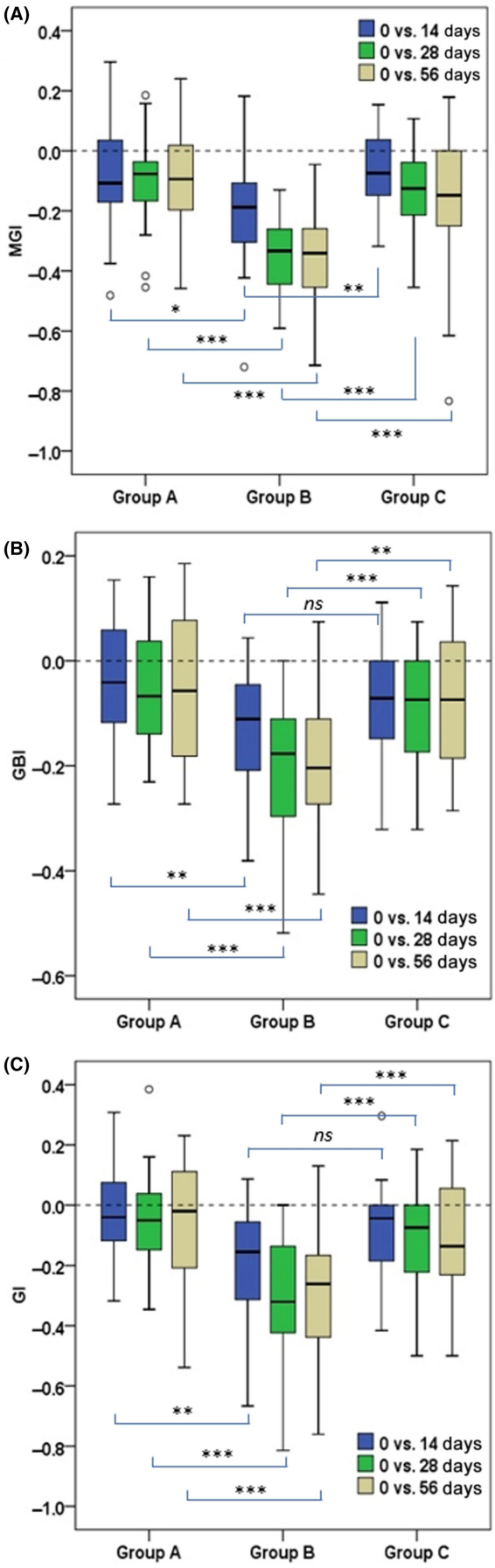

FIGURE 2 Box plots of (A) modified gingival index – MGI, (B) gingival bleeding index – GBI and (C) gingival index – GI for groups A (placebo), B (CBD), C (active comparator). The horizontal line is the median value, the lower edge of the box indicates the value of the 1st quartile (25. percentile), the upper edge the 3rd quartile (75. percentile). The clamps show the maximum and minimum measured values. The observation whose distance from the edge of the box (*i.e.* quartile) is more than 1.5 times the length of the box (*i.e.* interquartile range) represents an outlier. The differences for individual indices are plotted in different colors. Bonferroni post hoc text, * $p < .05$, ** $p < .01$, *** $p < .001$, *ns* = no significance. No statistically significant changes between group A and C were found for all indices.

CONSORT diagram of the study is shown in Scheme 1. No side effects[35] were perceived either by the patient or by the investigator.

3.2.1 | Primary outcomes

Periodontal and hygiene indices were evaluated (Table S2). The Russell periodontal index showed no changes during the study. In contrast, changes were observed for the remaining indices. Graphically, the results are depicted as box plots (Figure 2). In addition to the indices shown in Figure 2, we found a decrease in PLI in all groups. The Friedman test showed statistically significant differences on days 0, 7, 28, and 56 in all groups: group A, $p = .022$, group B: $p < .0001$, and group C: $p = .001$.

Significant changes were observed for GI, GBI, and MGI. Post hoc multiple comparison tests between groups showed that CBD patients had the highest MGI values on day 0. The greatest reduction in MGI occurred in the CBD group (Figure 2A). The changes in all observed periods in patients using CBD were statistically significantly higher than in groups A and C. The Friedman test showed statistically significant differences on days 0, 14, 28, and 56 for group A: $p = .003$, group B: $p < .0001$ and group C: $p = .0003$. For all groups, statistically significantly lower values were found on day 56 than on day 0.

For GBI, the Kruskal-Wallis test showed a statistically significant difference between groups on days 28 ($p = .023$) and 56 ($p = .006$). The CBD patients had significantly lower GBI values than the placebos (Figure 2B). The ANOVA showed a statistically significant difference between groups in GBI reduction between day 0 and days 28 or 56. Post hoc tests showed that the changes in group B were greater than those in the other groups. The repeated-measures ANOVA showed statistically significant differences between days 0, 14, 28, and 56 for group A: $p = .047$, group B: $p < .0001$, group C: $p < .0001$. No significant reduction in GBI values between 0 vs. 56 days was confirmed for the placebo group, but for groups B ($p < .0001$) and C ($p = .009$), it was highly significant.

For GI, a statistically significant difference between groups was demonstrated on day 28 ($p = .016$) and 56 ($p = .011$), see Figure 2C.

Group B had significantly lower values than group A. The ANOVA showed a statistically significant difference between groups in GI reduction between day 0 and days 28 or 56. Bonferroni post hoc tests showed that the changes in group B were greater than the changes in the other groups. The repeated-measures ANOVA only showed statistically significant differences between days 0, 14, 28, and 56 in groups B and C. There were no changes in the GI index over time in group A. In group B, the GI index decreased over time, $p < .0001$, as well as in group C ($p = .001$).

3.2.2 | Secondary outcomes

Microbiological samples were taken on days −7, 28, and 56. Statistically significant differences were only found for the bacterium *Eubacterium nodatum* in all groups (group A: $p = .036$; group B: $p = .040$; group C: $p = .024$). In groups A and C, there was no significant difference between the number of bacteria on days −7 and 56. The reduction only occurred between day −7 and day 28. In the CBD group, the highest number of tested bacteria was on day −7; it decreased and then remained at the same level. Decreasing trends in bacterial count were observed for *P. gingivalis* in group B in comparison with groups A and C (Figure 3). Figures S4 and S5 show the numbers of patients with each severity of infection with the respective bacteria during the study (see Data S1).

4 | DISCUSSION

In the preclinical part, the presence of cannabinoid receptors (Figure 1A-C), which are an integral part of the endocannabinoid system,[43,44] was evaluated in HGFs. The CB1 receptor is predominantly expressed in nervous tissue, while CB2 is mainly located in cells of the immune system and associated with the inflammatory response. No CB1 receptor was confirmed in the HGFs. In agreement with our findings, CB1 receptor expression was previously demonstrated on cell gingival models exclusively at the transcriptional but not at the translational level.[16] The presence of the CB2 receptor was clearly demonstrated, a fact which is known from other studies on various peripheral tissue types, see ref. 45 and citations therein.

For experiments on HGFs, subtoxic concentrations of phytocannabinoids up to 0.5 μM were used (Figure S1). The results are consistent with the data using dental cell models and other tissue types, summarized in ref. 11 The inflammatory reaction in HGFs was induced by LPS and monitored by increased levels of IL-6, IL-8, and MMP-2 monitoring (Figures S2 and S3). The decreases in interleukin levels after treatment with phytocannabinoids were not statistically significant for LPS-treated HGFs, most likely because of the quite high interindividual variance in the characteristics of HGF donors. In contrast, the levels of IL-6 and IL-8 were increased in LPS-nontreated fibroblasts, which is probably related to the immunomodulatory effect of phytocannabinoids,[11] which was primarily observed for CBD and CBG after 24 h of incubation. We also evaluated the ability of CBD to activate the Nrf2 pathway. This is one of the main cytoprotective pathways and has recently been demonstrated in CBD-treated (mainly dermal) cells.[15,41] CBD was less effective than sulforaphane in the expression of the *HMOX1* gene for LPS-nontreated cells (Figure 1D). Interestingly, no changes in the expression of the *HMOX1* gene were induced by the tested compounds in HGFs pretreated with LPS. This could be connected to the interplay between the Nrf2 and NF-κB pathways,[15] and should be a subject for further research. The LC–MS method confirmed that CBD is accumulated intracellularly in our experiments. The absence of CBD in culture medium after 24 h of incubation was associated with its uptake kinetics and degradation profile in aqueous solutions as previously reported.[20]

In the context of the preclinical experiments, CBD reduces alveolar bone loss and decreases expression of the receptor activator NF-κB RANKL/RANK in rats with experimentally induced periodontitis. A decrease in neutrophil migration associated with a reduced production of IL-1β and TNF-α was also observed.[46] The effect of

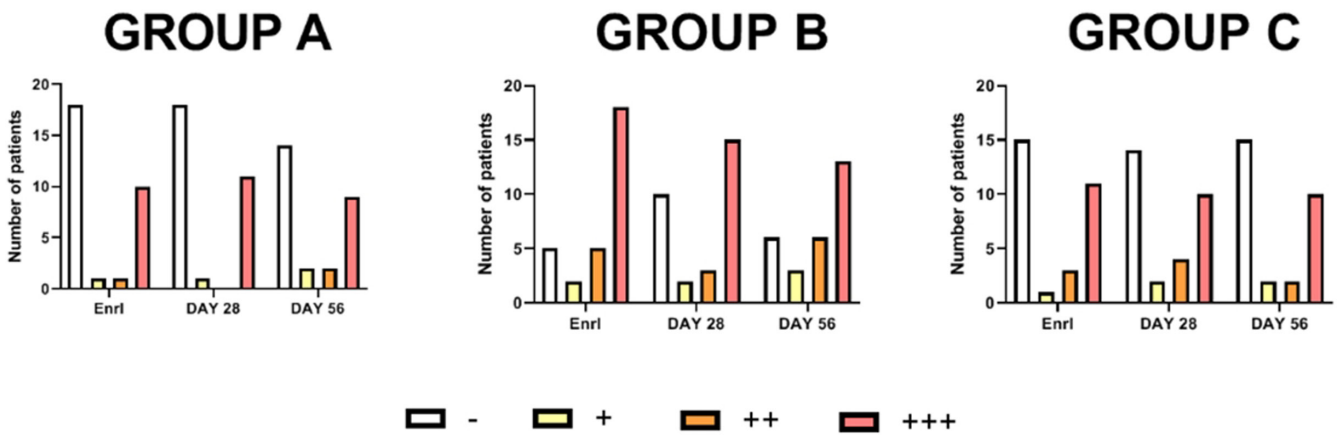

FIGURE 3 Clinical evaluation of *Porphyromonas gingivalis* count. Enrl: enrollment (day −7). For more details, see Figures S4 and S5. (–): undetected, corresponds to a bacterial count $<10^3$; (+): weakly positive, bacterial count 10^3–10^4; (++): moderately positive, bacterial count 10^4–10^5; (+++): strongly positive, bacterial count $>10^5$.

CBD, CBG, and CBDV on the modulation of IL-1β-induced inflammation in HGFs was recently studied. The production of INF-γ, TNF-α, and IL-2 was reduced after the application of the investigated phytocannabinoids. It was also recently demonstrated that CBD treatment promotes HGF proliferation and migration.[47] The in vitro results suggest that each phytocannabinoid could have a unique pharmacological profile and interfere with the immune and endocannabinoid system of HGFs in different ways.[11,48] In contrast to the application of pure cannabinoids, the pro-inflammatory effects, tissue destruction, and periodontal complications in marijuana smokers should be taken in account.[49]

In the clinical part, based on the placebo-controlled double-blind randomized clinical trial, we demonstrate that treatment with CBD dental gel and toothpaste significantly improves GI, GBI, and MGI after 56 days of CBD application in patients with periodontitis (Figure 2), without adverse effects. In addition to the cytoprotective or anti-inflammatory effects of CBD, microbiological evaluations are crucial for both the preclinical and clinical interpretation framework. Our microbiological observations (Table 2) are consistent with those reported[50] and with microbiology studies[51,52] as previously described. Our knowledge of the antimicrobial action of CBD on G-negative/anaerobic, facultatively anaerobic bacteria, or directly on selected periodontopathogens is limited.[17,18] CBD is known to suppress the growth of *P. gingivalis* and *F. alocis*.[50] The aim of one recently published study was to compare the in vitro antimicrobial effects of CBD, CBC, CBN, CBG, and cannabigerolic acid with Oral B and Colgate toothpastes.[51] Colonies of bacteria originating in dental plaque, collected from subjects (aged 18–45 years, $n = 60$), were cultured with a 12.5% cannabinoid solution and directly with the toothpaste samples. The antimicrobial effects of the phytocannabinoids were proven to be greater than those for the established oral hygiene products.[51] Furthermore, in another study, the antimicrobial effects of mouth rinses containing 1% CBD or CBG were tested on subjects aged 18–83 ($n = 72$). Counting of the colony-forming units was used. CBD and CBG were confirmed to have an effect comparable to 0.2% chlorhexidine digluconate.[52] CBD also inhibited the release of membrane vesicles involved in bacterial communication and interaction with the environment.[53] In line with in vitro studies, we (semi-quantitatively) confirmed the effect of CBD on *P. gingivalis* count in patients with periodontitis (Figure 3). However, it should not be overlooked that phytocannabinoids can also trigger the CB2/PI3K axis,[50] which can lead to modulation of the oral tissue response to oral bacteria. In future studies, more comprehensive observations on the effects of CBD on oral bacteria and oral biofilm formation could be considered.

5 | CONCLUSIONS

At the level of both the preclinical data and the placebo-control double-blind intervention trial, we have demonstrated the anti-inflammatory effect of CBD and its ability to inhibit the growth of pathogenic bacteria found in the oral cavity. The most unambiguous result of the study is a statistically significant improvement in GI, GBI, and MGI after 56 days of CBD application in patients with diagnosed chronic periodontitis. No adverse effects of CBD were reported by patients or observed upon clinical examination during the study. The results are a promising basis for a more comprehensive investigation of the application of non-psychotropic cannabinoids in dentistry.

AUTHOR CONTRIBUTIONS

Petr Jirasek DMD, PhD – clinical part coordinator and manuscript writing, Alexandr Jusku DMD, PhD – involved in clinical part, Jana Frankova PhD – HGF isolation and preclinical part, Marketa Urbankova MD, PhD – clinical part assistance, Daniel Diabelko and Filip Ruzicka MD, PhD – microbiology in vitro, Barbora Papouskova PhD – LC–MS analysis of phytocannabinoids, Karin Chytilova MD, DMD, PhD – collection of gingival tissue for HGF isolation, Jiri Vrba PhD and Jakub Havlasek MSc – analysis of Nrf2 pathway activation, Katerina Langova PhD – data statistical analysis, Jan Storch PhD – synthesis of tested phytocannabinoids, Iva Voborna DMD, PhD – conceptualization and infrastructure support, Vilim Simanek MD, PhD, DSc – conceptualization and clinical part design, Jan Vacek PhD – conceptualization, preclinical and clinical part design, manuscript writing.

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CONFLICT OF INTEREST STATEMENT

J.S. is CEO and has financial interests in CB21 Pharma Ltd. (Brno, Czech Republic), CBDepot Ltd. (Teplice, Czech Republic), PharmaCan Ltd. (Prague, Czech Republic), and CB21 R&D Ltd. (Prague, Czech Republic). J.V. and V.S. are involved in the scientific board of CB21 Pharma Ltd. The authors have scientific collaboration and financial support from CB21 Pharma Ltd. and CBDepot Ltd.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Petr Jirasek https://orcid.org/0000-0001-6180-1627
Jan Vacek https://orcid.org/0000-0001-5490-082X

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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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