NCT NUMBER:
DATE:10.08.25

STUDY PROTOCOL

TITLE/PURPOSE OF THE STUDY: EVALUATE THE EFFICACY OF SUBGINGIVAL APPLICATION OF 1.2% LOVASTATIN GEL AS AN ADJUNCT TO CONVENTIONAL NON-SURGICAL PERIODONTITIS THERAPY IN GENERALLY HEALTHY NON-SMOKING AND SMOKING PATIENTS FROM CENTRAL EUROPE: A RANDOMIZED SPLIT-MOUTH CONTROL TRIAL.

II RESEARCH HYPOTHESIS:

MAIN HYPOTHESIS:

H_1: After 6 months, a significant improvement in all analyzed outcome parameters (IBD, PD, mSBI, PI, CAL) compared to baseline is observed in both the treatment and placebo groups. Treatment efficacy is significantly higher in the treatment group than in the placebo group, and non-smoking patients show a better response to treatment than smokers.

SPECIFIC HYPOTHESES:

$H_1$1: After 6 months, the values of all outcome parameters (IBD, PD, mSBI, PI, CAL) improve significantly compared to baseline in both the treatment and placebo groups.

$H_1$2: The treatment group shows significantly greater improvement in all analyzed parameters (IBD, PD, mSBI, PI, CAL) after 6 months compared to the placebo group.

$H_1$3: Non-smoking patients show significantly better treatment outcomes compared to smokers, regardless of the intervention group.

$H_1$4: The interaction between treatment and smoking status is significant, meaning that the effectiveness of treatment in the treatment group compared to placebo differs between smokers and non-smokers.

III TYPE OF STUDY
A double-blind, randomized, split-mouth control study.
IV STUDY GROUPS:
STUDY GROUP 1: patients generally healthy, no general illnesses, non-smokers, diagnosed with periodontal disease.
Inclusion criteria:
Generally healthy patients of both sexes aged 18-59 years with diagnosed periodontal disease (at least one pair of non-adjacent sites with PD≥5 mm or CAL ≥4 mm and vertical bone loss ≥ 3 mm) with no history of periodontal disease treatment or antibiotic use in the past 6 months. An eligible tooth must be alive, with no history of endodontic treatment.
Exclusion criteria:
Statin allergy, people receiving systemic statin therapy, alcoholics, immunocompromised patients, pregnant or breastfeeding women.
STUDY GROUP 2: Generally healthy patients, no general illnesses, active smokers with diagnosed periodontal disease.
Inclusion criteria:

Generally healthy patients of both sexes aged 18-59 years with diagnosed periodontal disease (at least one pair of non-adjacent sites with PD≥5 mm or CAL ≥4 mm and vertical bone loss ≥ 3 mm) with no history of periodontal disease treatment or antibiotic use in the past 6 months. The patient must be a current tobacco smoker.
A patient will be classified as a current smoker if he or she smokes more than 10 cigarettes a day regularly for at least five years.
STUDY METHODOLOGY:
After qualification and consent to participate in the study, oral hygiene instruction, determination of clinical, radiological and periodontal parameters (IDB, PD, mSBI, CAL, PI) will be performed on each patient. Each patient will be assigned 2 sites that meet the inclusion criteria. After the SRP procedure is applied within the qualified gingival pockets, a gel drug (1.2% concentration of lovastatin) will be applied to the first designated site, and a placebo gel (split-mouth design) will be applied to the second site. One operator will perform the non-surgical treatment step, and then a second operator will independently perform the subgingival application of lowastatin/placebo gel into the pockets to maintain double-blinding of the study. The patient does not know which site is being treated with the drug. Random assignment of seats to a procedure will be provided by randomization. Reassessment of clinical and radiological parameters will be performed after 3 and 6 months. During the observation period, patients will be under the care of a hygienist to maintain optimal levels of oral hygiene throughout the study.
The synthesis of the lovastatin gel will follow the recipe of Thylin et al.
Thylin MR, McConnell JC, Schmid MJ, et al. Effects of simvastatin gels on murine calvarial bone. J Periodontol. 2002;73(10):1141-1148. doi:10.1902/jop.2002.73.10.1141
The substrates will be purchased from Sigma-Aldrich company.

V DEFINITION OF ENDPOINTS
- radiological parameter: IBD (Intrabony Defect Depth) – a parameter measured as the distance from the edge of the alveolar crest to the bottom of the bony defect; assessed on cone beam computed tomography (CBCT) scanning
- clinical parameter; PD (Probing Depth – pocket probing depth) – the distance between the gingival margin and the bottom of the gingival pocket, measured with a calibrated periodontal probe, expressed in mm.
- clinical parameter: mSBI (modified Sulcus Bleeding Index-enhanced gingival pocket bleeding index) – an index used to determine the severity of inflammation in the periodontal tissues; determines the percentage of the study area that is inflamed)
- clinical parameter: PI (Plaque Index – plaque index) – an index that evaluates the patient's level of oral hygiene on a numerical scale of 1-4
- clinical parameter: CAL (Clinical Attachment Level) – the distance between the enamel-cement junction (CEJ) and the bottom of the gingival pocket; expressed in mm.

STATISTICAL ANALYSIS PLAN

A statistical significance level of $\alpha=0.05$ was associated with accepting a 5% risk of making a type I error. The power of the statistical test was assumed to be 90%, considering it sufficient to detect the postulated effects in the studied population.

The effect values (Cohen's *d*) for the baseline variables analyzed (IBD, PD, mSBI, PI, and CAL) were estimated based on available studies that evaluated the effectiveness of periodontal procedures using preparations additionally supporting SRP therapy. The adoption of Cohen's specific intervals *d* was the result of a review of the results of studies that measured clinical effects after six months of follow-up (FU 6 months). This allowed the determination of effect size ranges for a specific parameter and the identification of a target value that was considered most representative in the context of the planned research project.

The ***IBD*** analyzed results published by Pankaj (2017), Kanoriya (2019), Rao (2013) and Kumari (2016), among others. Cohen's *d* values ranged from very high (up to 8.35), which was due to a marked decrease in medial bone loss after pharmacological intervention (Treatment), to values close to zero in the control groups (Placebo). In doing so, a significant scatter was observed due to the variety of adjuvant preparations used, as well as different procedures for including patients in the study. The target value for IBD in this project is ***d* = 1.20**, considering it to be conservative with respect to the highest indications (1.38-8.35) and also representative of moderately high efficacy in the area of periodontal tissue regeneration.

For ***PD*** selected studies (including Pankaj, Kanoriya, Rao, Kumari) presented *d* Cohen values of 2.12-4.60. Such high effect rates are related to the fact that most interventions resulted in a significant reduction in the depth of gingival pockets, especially when antibacterial or regenerative preparations were used. In addition, only a moderate reduction in PD was often observed in the control (placebo) groups. Ultimately, the study design adopted ***d* = 2.00**, treating this value as a realistic yet clinically relevant effect, remaining within the range observed in literature studies (2.12–4.60).

In the context of mSBI, we mainly used Pankaj's (2017) reports, in which d Cohen's values ranging from 2.64 to 2.71 were obtained for different treatment groups. The gingival pocket bleeding index shows significant changes in response to the reduction of inflammation after periodontal treatment, consistent with a large effect size. Thus, the project decided to adopt the value ***d* = 2.50**, considered to be well representative of the large decrease in bleeding with effective assisted treatment.

Very high effect sizes were observed for ***PI***, with the most widely cited data (Gaekwad, 2015; Pankaj, 2017) indicating that Cohen's *d* could exceed 4.5 and even approach 5.5 in some comparisons (placebo vs. therapy). Such values are due to the fact that plaque reduction is one of the most pronounced effects of hygiene procedures and bacterial biofilm control. In order to maintain a certain margin of caution, and at the same time take into account the largest effects documented to date, this project sets a target value of ***d* = 4.00**.

Finally, in the ***CAL*** area, d values ranging from 1.34 to 4.82 and even above have been identified (Gaekwad, 2015; Pankaj, 2017; Kanoriya, 2019; Rao, 2013; Kumari, 2016). Accelerated reconstruction of the connective tissue attachment (re-attachment) or improvement in the stability of the tissues surrounding the tooth usually translates into high *d*, especially when the therapy includes the use of additional regenerative preparations. Taking into account the aforementioned scatter, the project assumed ***d* = 2.50** as a value within the high range of effects noted in the literature, yet achievable under the conditions of pharmacologically assisted procedures.

Thus, the established set of final effects, i.e. $d = 1.20$ for IBD, 2.00 for PD, 2.50 for mSBI, 4.00 for PI, and 2.50 for CAL, was based directly on the analysis of existing data on the therapeutic methods used and the measured differences in periodontal parameters after 6 months. The review and synthesis of the results showed that although there are some methodological differences in the various publications (e.g., type of intervention, type of patients, statistical selection), adopting mean or slightly lower values from the highest ranges allows a reasonable yet high estimate of the expected efficacy in the designed study – in this way, the assumed effect in the designed study is still large and realistic to achieve, while not exceeding the natural spreads observed in the literature.

The statistical test used

The present experiment used **a split-mouth scheme** in which each patient was his own control. Consequently, statistical analysis was carried out using paired t-test of a two-sided nature. Before starting the analysis, the key assumptions of the test will be checked, in particular the normality of the distribution of differences between the values measured in the test and control areas (for each participant). Since the study protocol splits the population into non-smokers and smokers, the test will be conducted independently for each of these subgroups, which allowed for separate estimation of the therapeutic effect (treatment) and control (placebo) under the "divided dental arches" arrangement.

Additional adjustments in numbers

To offset potential deviations from assumptions of normality (Machin et al., 2007) and the risk of participants dropping out of the study (Pocock, 1983), it was decided to increase the primary sample size by 15% due to the unknown distribution of the variable and another 15% for possible withdrawals (Armitage & Berry, 1994). In total, this gives a roughly 30% increase over the calculated baseline. The final number was rounded up to the nearest whole value (Cohen, 1988), adopting the conservative principle of preventing underestimation.

Characteristics of the statistical tool

Analyses were performed using the statistical language *R* (version 4.3.3; R Core Team, 2024) on Windows 11 Pro 64 bit (compilation 22631), using *pwr* packages (version 1.3.0; Champely S, 2020) and *report* (version 0.5.8; Makowski D et al, 2023).

The results in Table 1 show the estimated number of participants needed in each of the two groups (patients without burdens and smokers) to achieve the expected clinical effects in the periodontal indicators studied. Values include the minimum number needed to show noticeable improvement in parameters.

Table 1. Minimum group sizes of participants depending on the outcome variable

Output variable	*Minimum group size*
IBD	22
PD	10
mSBI	7
PI	5
CAL	7

Final conclusions

The analysis shows that the minimum size of the study group (among both smoking and non-smoking patients) required to observe the assumed therapeutic effect in the defined clinical indicators (IBD, PD, mSBI, PI, CAL) is as follows: IBD (Intrabony Defect): 22 patients in each of the two groups (smokers and non-smokers). Other variables (PD, mSBI, PI, CAL) may require a smaller sample size (5-10), but the target assurance of detecting the desired effects in the IBD area determines the size of the entire study. **In practice, this translates into the need to include at least 22 smoking and 22 non-smoking patients in the study to achieve the expected level of reliability of the results.**

Table A1. Summary of clinical outcomes and effect sizes for IBD outcomes

Author	*Group*	*n*	*baseline*		*FU 3 months*		*FU 6 months*		*Cohen d FU 6m - baseline*
			M	*SD*	*M*	*SD*	*M*	*SD*	
Pankaj (2017)	Placebo	30	5.15	0.35	5.26	0.87	5.18	0.65	-0.05
	Treatment 1	30	5.26	0.87	3.74	0.42	3.08	0.31	2.85
	Treatment 2	30	5.18	0.65	3.63	0.29	3.17	0.24	3.53
Kanoriya (2019)	Placebo	30	4.68	0.16	-	-	4.50	0.21	0.95
	Treatment	30	4.75	0.14	-	-	3.62	0.13	8.35
Rao (2013)	Treatment	33	4.75	0.85	-	-	3.58	0.85	1.38
	Placebo	34	4.86	0.46	-	-	4.73	0.45	0.29
Kumari (2016)	Treatment	33	4.73	0.54	-	-	3.30	0.44	2.87
	Placebo	33	4.71	0.52	-	-	4.58	0.51	0.25

Table A2. Summary of clinical outcomes and effect sizes for Pd outcomes

Author	*Group*	*n*	*baseline*		*FU 3 months*		*FU 6 months*		*Cohen d FU 6m - baseline*
			M	*SD*	*M*	*SD*	*M*	*SD*	
Pankaj (2017)	Placebo	30	6.80	1.21	5.76	0.85	5.26	0.63	1.46
	Treatment 1	30	7.30	1.17	4.20	0.76	2.63	0.63	4.60
	Treatment 2	30	7.40	1.10	4.73	0.78	3.26	0.52	4.34
Kanoriya (2019)	Placebo	30	7.65	1.15	6.56	1.67	5.96	1.06	1.53
	Treatment	30	7.83	1.12	5.73	1.11	4.63	1.18	2.77
Rao (2013)	Treatment	33	7.87	0.90	5.57	1.10	4.50	1.08	3.36
	Placebo	34	7.93	1.12	6.70	1.06	6.03	1.30	1.56
Kumari (2016)	Treatment	33	6.97	1.44	5.42	0.79	4.30	0.88	2.12
	Placebo	33	7.03	1.38	6.15	1.09	6.03	0.91	0.82

Table A3. Summary of clinical outcomes and effect sizes for MSBI outcomes

Author	*Group*	*n*	*baseline*		*FU 3 months*		*FU 6 months*		*Cohen d FU 6m – baseline*
			M	*SD*	*M*	*SD*	*M*	*SD*	
Pankaj (2017)	Placebo	30	1.96	0.61	1.16	0.59	0.83	0.64	1.81
	Treatment 1	30	2.03	0.61	0.86	0.73	0.50	0.50	2.71
	Treatment 2	30	2.10	0.66	0.70	0.65	0.46	0.57	2.64

Table A4. Summary of clinical outcomes and effect sizes for PI outcomes

Author	*Group*	*n*	*baseline*		*FU 3 months*		*FU 6 months*		*Cohen d FU 6m – baseline*
			M	*SD*	*M*	*SD*	*M*	*SD*	
Gaekwad (2015)	Treatment	20	1.897	0.37	0.697	0.15	0.422	0.13	4.54
	Placebo	20	1.909	0.28	0.786	0.25	0.561	0.11	5.51
Pankaj (2017)	Placebo	30	1.98	0.33	0.79	0.13	0.59	0.09	4.70
	Treatment 1	30	1.94	0.31	0.81	0.11	0.53	0.11	5.18
	Treatment 2	30	1.96	0.35	0.76	0.12	0.53	0.09	4.54

Table A5. Summary of clinical outcomes and effect sizes for CAL outcomes

Author	*Group*	*n*	*baseline*		*FU 3 months*		*FU 6 months*		*Cohen d FU 6m - baseline*
			M	*SD*	*M*	*SD*	*M*	*SD*	
Gaekwad (2015)	Treatment	20	6.250	0.71	4.400	0.68	3.050	0.75	4.37
	Placebo	20	6.200	0.76	4.900	0.78	3.00	0.65	4.50
Pankaj (2017)	Placebo	30	6.06	1.17	4.83	0.74	4.60	0.62	1.44

Author	*Group*	*n*	*baseline*		*FU 3 months*		*FU 6 months*		*Cohen d FU 6m - baseline*
			M	*SD*	*M*	*SD*	*M*	*SD*	
	Treatment 1	30	6.23	1.01	2.56	0.56	2.00	0.45	4.82
	Treatment 2	30	6.20	1.12	3.43	0.97	2.30	0.59	4.01
Kanoriya (2019)	Placebo	30	5.90	1.29	4.93	1.11	4.26	1.14	1.34
	Treatment	30	6.06	1.11	3.93	1.20	3.10	0.92	2.88
Rao (2013)	Treatment	33	6.20	1.16	4.07	0.87	2.57	0.63	3.61
	Placebo	34	6.13	0.94	4.47	1.11	4.67	1.56	1.07
Kumari (2016)	Treatment	33	6.90	1.40	4.63	1.17	3.30	1.01	2.88
	Placebo	33	6.94	1.41	5.45	0.97	5.03	0.72	1.56

VII STATISTICAL ANALYSIS PLAN – GENERAL INFORMATION

Statistical analyses will be carried out at a significance level of $\alpha = 0.05$. In the case of the presence of missing data, considered random (MCAR – Missing Completely at Random), appropriate imputation methods will be used (including predictive mean matching, PMM)

GAMLSS model

In the planned analysis, Generalized Additive Models for Location, Scale, and Shape (GAMLSS) will be used to assess the effects associated with the intervention (drug vs. placebo) within smoking and non-smoking patients for each of the measured outcome variables (IBD, PD, mSBI, PI, CAL). The method will allow simultaneous modeling of the expected value of a variable (location), its dispersion (scale), and additional parameters of the shape of the distribution, offering a flexible approach to complex relationships between variables, including nonlinear relationships resulting from multiple measurements (Generalized Additive Model for Location, Scale, and Shape, GAMLSS) (Rigby et al. 2019; Stasinopoulos and Rigby, 2007; Stasinopoulos et al, 2017, 2018, 2024).

The approach will take into account random effects in the form of random slope (time) and intersection (patient id) to account for the variation of individual baseline levels and the different dynamics of change in individual patients in the longitudinal design of the study.

In addition, a correlation structure based on an autoregressive of order one (AR (1)) is expected to be introduced to reflect the fact that measurements taken from the same participant at similar time intervals are more strongly correlated.

In the part of the model describing location, age and gender will be included so as to control the influence of demographic factors on the level of the outcome variable, while the time will be included in the component describing the variance, which will make it possible to identify any changes in the dispersion of the data at successive stages of observation.

In addition, in order to examine the effect of both treatment or placebo group assignment and smoking status (smokers vs. non-smokers) on the values of the outcome variable, their interaction will be introduced to assess whether and how these factors together shape the observed effects.

The selection of the optimal model structure, taking into account random effects, correlation over time, or both, along with the appropriate distribution and decomposition, will be evaluated on the basis of global deviation measures, including **logarithm of reliability (-2 × logL)** (Rigby and Stasinopoulos, 2005), the **Akaike Information Criterion (AIC)** (Akaike, 1974) and the **Bayesian Information Criterion (BIC/SBC)** (Schwarz, 1978).

Analysis of model residuals

The adequacy of the model will be assessed by analyzing the quantile residuals, taking into account their mean, variance, skewness, kurtosis and normality. To assess the fit of the assumed distribution, the Filliben correlation coefficient (Filliben, 1975) will be estimated to assess the degree of fit of the model.

The residuals diagnostics will include a comprehensive graphical analysis, including a plot showing the relationship of the residuals to the observation index or selected explanatory variable, a residual density plot, and a normal quantile-quantile plot (Q-Q plot). In addition, a de-trended Q-Q plot, visualized with a worm plot (van Buuren and Fredriks, 2001), will be used to assess possible deviations from normality.

For an in-depth evaluation of the distribution of residuals, the transformed skewness and transformed moment kurtosis will be presented using the moment bucket plot (De Bastiani et al., 2022), which will enable visualization of the results of the Jarque-Bera test (Jarque and Bera, 1980). The point cloud obtained by nonparametric bootstrapping will allow graphical interpretation of the consistency of the distribution of residuals with model assumptions.

Analysis of changes within the group (smoking status)

In order to assess changes in the value of the outcome variable at subsequent time points (known as follow-up) in relation to baseline values, contrasts between Estimated Marginal Means (EMMs) at baseline and corresponding values at subsequent measurements will be made in each group (smokers and non-smokers). Each contrast defines a difference:

$$Contrast = EMM_{time,i} - EMM_{baseline},$$

Where *i* denotes consecutive measurement points. The statistical significance of each contrast was assessed using a two-sided t-test for dependent samples.

Treatment efficacy at the patient level (placebo vs drug comparison)

The effectiveness of treatment in each intervention group (placebo, drug) at *t* will be estimated as the difference between EMMs at time point *i* and baseline, according to the formula:

$$Time\ change_{t,j} = EMM_{t,i} - EMM_{baseline}$$

where: *t* - time point (follow-up), *j* - intervention group (placebo or drug).

The difference in changes between the drug group and the placebo group at a given time point *t* (considered within the same patient) will be calculated as follows:

$$Time\ variation\ (at\ patient\ level) = (EMM_{(t)treatment} - EMM_{treatment,\ baseline}) - (EMM_{(t)placebo} - EMM_{placebo,\ baseline})$$

Analysis of differences by smoking status (z-test)

To assess whether the treatment effect differ between smokers and non-smokers, a two-sided *z* test will be used. First, the change (difference from baseline) in the smoking group and the non-smoking group will be calculated, followed by their difference:

$$Difference\ in\ changes = (EMM_{(t),\ treatment\ non\text{-}smokers} - EMM_{treatment\ non\text{-}smokers,\ baseline}) -$$

$$- (EMM_{(t),\ treatment\ smokers} - EMM_{treatment\ smokers,\ baseline})$$

To estimate the standard error of the difference in changes, **combined standard error (SE)** was calculated, assuming **independent variances** between the smoking and non-smoking groups. The total standard error will be estimated according to the following formula:

$$SE_{pooled\ (t)} = \sqrt{SE^2_{placebo\ (t)} + SE^2_{t,\ treatment\ (t)}}$$

The *z* statistic will be estimated according to the formula:

$$z_{(t)} = \frac{\text{Differences in changes}}{SE_{pooled}}$$

and *p* value will be calculated based on the standard distribution of the z distribution.

Characteristics of the statistical tool

Statistical analyses will be carried out using the statistical language *R* (version 4.3.3; R Core Team, 2024) in IDE RStudio version 2023.12.1 (build 402) on Windows 11 Pro 64 bit (compilation 22631).

VIII OUTLINE OF EXPECTED RESULTS:

After 6 months, a significant improvement in all analyzed outcome parameters (IBD, PD, mSBI, PI, CAL) compared to baseline are expected in both the treatment and placebo groups. Treatment efficacy is significantly higher in the treatment group than in the placebo group, and non-smoking patients show a better response to treatment than smokers.