



Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com



Original article

Chemometric evaluation of the efficacy of locally administered chlorhexidine in patients with periodontal disease



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ARTICLE INFO

Article history:

Received 17 February 2016

Revised 31 January 2017

Accepted 24 March 2017

Available online 25 March 2017

Keywords:

Treatment efficacy

Chemometric algorithms

Multivariate data analysis

Periodontal disease

Chlorhexidine

ABSTRACT

The process of assessment of drug efficacy produces multivariate data which are difficult to interpret. The interpretation and extraction of relevant data requires application of chemometric algorithms for multivariate data analysis. The aim of our study was evaluation of the efficacy of local treatment with chlorhexidine (CHX) in patients suffering from periodontal disease by chemometric algorithms for multivariate data analysis. Several algorithms were used: principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA). The PCA models identified the examined variables as suitable for monitoring the periodontal disease progression at the same time revealing mutual relationship among them. The developed PLS-DA model successfully distinguished patients treated with CHX from non-treated patients. The OPLS-DA model revealed differences in the mechanism of action of the two widely applied treatments in periodontal disease, local administration of CHX and local administration of doxycycline (DOX). The approach presented in this study opens the possibility of application of chemometric algorithms for multivariate data analysis for assessment of treatment efficacy.

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1. Introduction

The process of drug efficacy assessment encompasses conduction of large controlled clinical trials where relatively homogenous group of subjects are carefully evaluated with respect to predefined clinical and laboratory parameters and closely monitored

Abbreviations: GCF, gingival crevicular fluid; ALP, alkaline phosphatase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; IL-1 β , interleukin -1 beta; TNF, α - tumor necrosis factor-alpha; CAL, clinical attachment loss; PD, pocket depth; GI, index of gingival inflammation; CHX, chlorhexidine; DOX, doxycycline; PCA, principal component analysis; PLS-DA, partial least square discriminant analysis; VIP, variable influence on projection; OPLS-DA, orthogonal projection to latent structures discriminant analysis.

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Peer review under responsibility of King Saud University.

with respect to unexpected effects (Rasmussen et al., 2010; Ren et al., 2012; Cleophas and Zwinderman, 2013). The massive amounts of data generated by these trials are traditionally analyzed using univariate approach *i.e.* considering mean result versus control (Liland, 2011; Cleophas and Zwinderman, 2013).

However, the univariate approach suffers from several disadvantages such as the need for large number of samples as well as the inability to compensate for the missing data points which may require important observations to be discarded from analysis (Helmy et al., 2012).

Compared to the univariate approach, the multivariate approach that uses chemometric algorithms for data analysis represents a powerful tool for exploring large datasets derived from biological systems which contain multiple variables, missing data points or relatively small number of observations (Eriksson et al., 2006; Helmy et al., 2012). Their application provides “multicomponent insight” into treatment effects and therefore the adoption of chemometric algorithms for multivariate data analysis for assessment of treatment efficacy is highly recommendable (Dureckova et al., 2011; Mocak, 2012; Jimenez et al., 2013; Mrazova et al., 2014).



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<http://dx.doi.org/10.1016/j.jsps.2017.03.006>

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Periodontal disease is an inflammatory condition initiated by a bacterial infection which affects the supporting structures of the teeth. The treatment for periodontal disease is known as scaling and root planning and is followed by local administration of antiseptics or antibiotics. The treatment effects can be assessed by: (a) determination of clinical indices: index of gingival inflammation (GI), periodontal pocket depth (PD) or clinical attachment loss (CAL); (b) determination of inflammatory biomarkers in gingival crevicular fluid (GCF), (enzymes alkaline phosphatase (ALP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH)) or cytokines (interleukin - 1β (IL- 1β) and tumor necrosis factor - α (TNF- α)) and (c) determination of concentration of the applied drug in GCF (Perinetti et al., 2008; Aimetti et al., 2012; Ertugrul et al., 2013; Spruill et al., 2014). However, the conclusions regarding treatment efficacy are commonly derived using the conventional approach. The biggest drawback of the univariate approach in dentistry is multicollinearity – mathematical coupling of variables which may lead to greater uncertainty in the result and may require removal of certain variables. One of the possible solutions to the problem lies in the application of chemometric algorithms for multivariate data analysis such as principal component analysis, partial least square regression or extension of these algorithms (Tu et al., 2009).

In our study, we will assess the performance of chemometric algorithms for multivariate data analysis for evaluation of efficacy of local administration of chlorhexidine (CHX) in patients suffering from periodontal disease. Furthermore, the efficacy of local administration of CHX is compared to the efficacy of local administration of doxycycline (DOX), another commonly used drug in periodontal treatment.

2. Materials and methods

2.1. Chemicals and materials

Chlorhexidine digluconate and chlorpheniramine maleate (internal standard, IS) were purchased from Sigma Aldrich (Germany) and Supriya Lifescience Ltd. (India), respectively. HPLC grade methanol and acetonitrile (ACN) were supplied by Carlo Erba (Italy). Sodium phosphate, triethylamine (TEA) and phosphoric acid (p.a grade) were also purchased from Sigma Aldrich (Germany). Throughout the entire chromatographic analysis, HPLC water was used. Whatman 3MM chromatography paper strips, 2×5 mm (Whatman Lab sales Ltd., UK) were used for GCF collection. Chlorhexamed 1% gel (0.5 g chlorhexidine digluconate/50 g gel) was purchased from GlaxoSmithCline, GmbH, Buehl, Germany while ATRIDOX 10% gel (45 mg doxycycline hyclate/0.5 g gel) was supplied by TOLMAR Inc. Fort Collins (USA). Protease inhibitor cocktail for cytokine determination was purchased from Sigma Aldrich (Germany). The assay kits for ALP, LDH and AST activity determination were purchased from Biosystems (Spain). The concentration levels of IL- 1β and TNF- α in GCF samples were analyzed using commercial ELISA kits (Booster Immunoleader, Fremont, CA).

2.2. Subjects and protocol

The subjects who participated in the study were divided in three groups: experimental group of 34 patients who received local treatment with CHX gel (conventional release gel formulation), experimental group of 25 patients who received local treatment with DOX gel (controlled release gel formulation) and a control group of 9 healthy volunteers, with no previous history of periodontal disease. All patients were recruited from the Department of Periodontology, Faculty of Dentistry in Skopje. The study protocol was approved by the Ethics Committee at the Faculty of Den-

tistry and all the patients provided written informed consent before attending the study.

2.3. GCF sample collection

GCF samples were collected from quadrants consisting of five periodontal pockets before and after the local periodontal treatment. The patients who received the local treatment with CHX were administered 330 mg of gel containing 2 mg of CHX and GCF samples were taken 30 min after the gel application. The patients treated with DOX gel were administered 115 mg gel containing 10 mg of DOX and the GCF samples were taken 7 days after the local treatment. GCF from the selected periodontal pockets was collected using the method proposed by Koss et al. In brief, the paper strips were placed in selected periodontal pockets until mild resistance was felt and left in place for 30 s (Koss et al., 2009).

2.4. Chromatographic conditions for determination of CHX in GCF samples

The HPLC analysis was conducted on Shimadzu Nexera HPLC system with UV diode array detector. The chromatographic separation was achieved using Discovery C18 chromatographic column, 250×4.6 mm, $5 \mu\text{m}$ (Supelco, USA) at 25°C . The mobile phase consisted of ACN, 0.01 mol L^{-1} phosphate buffer adjusted to $\text{pH} = 3.0$ using phosphoric acid and TEA (33: 66:1, V/V/V). The mobile phase flow rate was set at 1 mL/min and the injection volume was $50 \mu\text{L}$. The wavelength of detection was 253 nm and the total runtime for the analysis was 10 min . The method was validated according to EMA Guideline on bioanalytical method validation (EMA, 2011).

2.5. Measurement of clinical indices

The degree of periodontal inflammation/health was assessed by the following clinical indices: pocket depth (PD), clinical attachment level (CAL) and index of gingival inflammation (GI). The indices were recorded by a single examiner, before and after local administration of CHX and DOX. GI was expressed using values from 1 to 3, where value of 1 expresses low inflammation and the value of 3 means high inflammation. PD and CAL were measured in mm. PD value larger than 3 mm indicates existence of periodontal pockets and CAL value larger than 3 indicates periodontal disease.

2.6. Determination of inflammatory biomarkers in GCF samples

For determination of inflammatory biomarker activity/concentration in GCF, the total of five paper strips were placed in $500 \mu\text{L}$ PBS (phosphate buffer saline, $\text{pH} = 7.4$) and the tube was centrifuged for 5 min at $1000 \times g$ (4°C) in a microcentrifuge (Centurion Scientific K3 Series) to elute the GCF component. The solutions containing the GCF component were divided in two aliquots of $250 \mu\text{L}$. The aliquot for determination of enzyme activity was assayed immediately after collection whereas the aliquot for determination of cytokine concentration was added proteinase inhibitor cocktail and kept at -80°C until analysis.

ALP, LDH and AST activity in GCF sample solutions were determined using semiautomatic photometer (Hymalizer Primus, Germany). The determination was performed using commercial kits according to the International Federation of Clinical Chemistry (IFCC) recommendations. The final results were expressed as total enzyme activity per sample (IU/sample).

The concentration levels of IL- 1β and TNF- α in GCF sample solutions were analyzed using commercial ELISA kits, according to manufacturer's instructions. The absorbance was measured by

spectrophotometric ELISA reader (Viktor X3, Perkin Elmer). The total amounts of IL-1 β and TNF- α were expressed as pg mL⁻¹. The analyses were performed in duplicate.

2.7. Chemometric algorithms for multivariate data analysis

2.7.1. Data preprocessing

Prior application of chemometric algorithms, the obtained results were organized in tables (Microsoft Office Excel 2003) where the patients were inserted into rows and the examined variables were positioned in columns. The measured variables were: ALP, AST and LDH activity, IL-1 β and TNF- α concentration in GCF samples before and after local administration of CHX; clinical indices: GI, PD and CAL before and after local administration of CHX, as well as CHX concentration in GCF after the local administration. Data unit-to variance (UV) scaling was performed in order to remove systematic differences among variables due to difference in measurement units and then a logarithmic transformation to each variable was applied.

2.7.2. Chemometric algorithms

Chemometric algorithms for multivariate data analysis: principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were applied to the data sets using SIMCA 13.0.3 software (Umetrics, Umea, Sweden).

PCA is an unsupervised algorithm which performs orthogonal linear transformation of possibly correlated variables into smaller number uncorrelated variables called principal components (PCs). PCA converts data into score plots which are visual representations of the studied samples and enables preliminary investigation of the variability in the data set, correlations among the studied variables and identification of outliers. Next, PLS-DA, algorithm that uses class information to maximize separation between classes, was applied for supervised classification and discrimination between classes of data obtained before and after local administration of CHX. To avoid model overfitting, the obtained PLS-DA model was validated by default 7-round cross-validation with 1/7 of the samples being excluded from the model in each round, as well as 20 times permutation testing. At the final stage, OPLS-DA was used to find the differences and improve separation between classes obtained after local administration of CHX and after local administration of DOX. PLS-DA and OPLS-DA model performance was evaluated using coefficients R² and Q², both of which vary between 0 and 1 (Eriksson et al., 2006). R² (goodness of fit) provides an indication of the extent to which a variation within a data set can be explained by the various components of the model. The value for Q² is more important since it indicates how accurately data, either classes or non-classed can be predicted by the model. A Q² value > 0.5 indicates a good model. To identify variations responsible for separation between classes, the corresponding variable influence on projection (VIP) for the PLS-DA and OPLS-DA model were carefully inspected since they represent the measure of the degree to which a particular variable explains Y variance (class membership in our case).

2.7.3. Univariate data analysis

The statistical significance of the differences between the mean of the discriminating variables as identified from the VIP plot of the OPLS-DA model, was assessed by the non-parametric Mann-Whitney U test using SPSS 17 software. A P value < 0.05 (95% confidence level) was considered statistically significant.

3. Results and discussion

CHX is an antiseptic widely used in the treatment of periodontal disease. It shows broad spectrum of topical antimicrobial activity, suitable effectiveness and lack of toxicity (Paolantonio et al., 2008). The local delivery of CHX gel in periodontal pockets enhances the effect of scaling and root planning, thus CHX gel application shows long lasting (up to 90 days) favorable effects (Perinneti et al., 2004).

3.1. Chemometric evaluation of CHX treatment efficacy using multivariate data analysis

3.1.1. PCA

The first step of the chemometric evaluation of treatment efficacy was application of the PCA algorithm to the data set containing results from the control group of patients (Table 1) and periodontal patients before and after the local administration of CHX (Table 2).

The aim of performing PCA was to extract the most important information from the data set and analyze the structure of the observations and the variables. PCA reveals natural grouping of the studied observations (periodontal patients) and the selected variables in a reduced dimensional space. The first principal component (PC1) and further PCs are linear combinations of the original variables, which still preserve maximal variance of the data. Usually, the first two PCs contain most of the variance of the data, whereas the other PCs are unimportant (Cordella, 2012). The results from the application of the PCA algorithm on the data set of the control group vs. diseased patients are shown in Fig. 1.

The inspection of the Hotelling's ellipse of the PCA model reveals that the control group is positioned in a tight cluster in the negative parts of the PC1 axe, whereas the periodontitis patients before local administration of CHX are located at high PC1 values. The discrimination of the data in two groups obtained by the PCA model demonstrates that selected clinical indices and inflammatory biomarkers can be used to monitor the progression of periodontal disease. PC1 accounts for 60.9% and PC2 accounts for 8.86% of the total variance of the data, or together the first two principal components explain around 70% of the data variability. Further inspection of the loading plots of the same PCA model (Fig. 2) was made in order to examine the relative contribution of each of the selected variables to the two principal components.

Four of the selected biomarkers (ALP, AST, LDH and TNF- α) and the clinical indices are located at the positive values of PC1 axe, with ALP, PD and CAL located at the PC2 axe, but also very close to the PC1. This finding suggests that a GCF sample from a patient located at the positive PC1 values indicates not only elevated levels of inflammatory biomarkers but also elevated values for the clinical indices. However, the PCA model did not discriminate between inflammatory biomarkers and clinical indices as distinct groups of parameters for monitoring periodontal disease progression.

In the next step, we aimed to examine applicability of the PCA algorithm for monitoring the therapeutic effects of local administration of CHX based on changes of the selected inflammatory biomarkers and the clinical indices, before and after treatment. The PCA model of resulted only in partial separation, with only 50% of the variability in the data being explained (Fig. 3).

However, the correlation matrix for the selected variables was inspected in order to explore their relationships. The results from this kind of analysis are presented in the form of correlation table, containing the calculated pair (Pearson) correlation coefficient, indicator of the strength of correlation between all pairs of variables. The importance of the correlation is evaluated against the critical value of the sample correlation coefficient, $r = 0.250$. This

Table 1

Results from determination of inflammatory biomarkers in GCF samples and clinical indices in the control group.

N ^a	ALP (IU/L)	LDH (IU/L)	AST (IU/L)	IL-1 β (pg mL ⁻¹)	TNF- α (pg mL ⁻¹)	GI ^b	CAL (mm)	PD (mm)
1	37.3	263.1	26.7	27.3	35.6	2	2	2
2	24.9	251.0	6.7	31.2	15.6	1	1	1
3	7.3	89.5	10.0	15.6	12.4	1	2	1
4	14.9	36.4	26.7	17.4	18.0	1	1	1
5	29.7	99.5	13.3	31.2	18.7	1	2	1
6	27.3	111.0	13.3	15.6	20.4	1	1	1
7	29.6	384.9	16.7	17.4	21.8	1	1	1
8	17.6	99.9	20.0	35.9	18.5	1	1	1
9	19.0	389.6	16.7	29.0	15.6	1	1	1

^a N^o- number of GCF samples from the control group.^b GI – expressed as values ranging from 1 to 3.**Table 2**

Results from determination of inflammatory biomarkers in GCF samples and clinical indices in periodontal patients before and after local administration of CHX.

N	Results obtained before the local CHX treatment									Results obtained after the local CHX treatment							
	1	2	3	4	5	6	7	8	N	1	2	3	4	5	6	7	8 ^a
1	49.8	267.1	15.0	191.4	26.3	3	5	3	35	22.1	437.1	48.3	214.1	25.9	1	2	3
2	58.0	518.1	33.3	448.2	33.2	2	4	4	36	16.3	342.2	30.7	8.5	20.1	1	2	3
3	53.9	769.0	98.3	445.9	25.2	2	4	5	37	29.0	28.3	200.0	8.5	25.9	1	2	3
4	26.3	445.2	36.7	465.3	22.6	2	3	3	38	41.5	81.0	28.3	623.8	33.4	1	2	3
5	38.7	534.3	36.7	332.9	30.1	1	6	5	39	31.8	388.6	80.0	129.7	49.9	1	3	2
6	42.8	854.0	81.7	416.3	36.3	1	5	4	40	55.3	1447.4	55.0	172.4	45.6	1	2	3
7	56.7	740.7	25.0	356.3	24.2	2	4	3	41	69.0	1094.0	55.0	408.3	45.9	2	3	3
8	59.4	1376.2	61.7	167.5	42.9	2	5	4	42	27.6	696.2	106.7	73.6	21.6	2	2	3
9	53.9	902.6	115	153.3	29.9	2	5	4	43	36.8	562.4	33.3	114.4	21.6	1	4	2
10	47.0	967.4	53.3	103.2	18.9	3	5	3	44	34.2	1461.1	41.7	166.5	28.6	2	4	2
11	48.4	765.0	50.0	143.2	38.2	2	4	2	45	32.8	611.2	26.7	131.2	30.6	1	2	2
12	53.9	1024.1	33.3	125.4	32.9	2	3	4	46	48.4	789.3	56.7	300.3	20.4	2	4	4
13	47.0	655.7	13.3	79.6	40.9	2	4	5	47	29.3	627.5	44.2	173.7	35.6	2	2	2
14	72.6	1255.9	64.9	261.4	43.1	2	4	4	48	25.4	459.3	54.4	200.4	28.9	2	3	4
15	80.0	455.9	98.4	146.1	46.3	2	4	5	49	20.1	168.3	24.2	145.7	25.9	1	2	3
16	39.5	955.4	71.9	407.2	50.9	2	3	3	50	16.3	336.7	29.0	220.0	20.1	1	2	3
17	45.6	1000.7	65.4	294.5	63.1	3	6	5	51	20.0	459.3	31.7	204.5	25.9	1	2	3
18	45.6	1432.9	55.3	228.4	31.0	3	5	4	52	31.5	498.7	28.6	178.3	33.4	1	2	3
19	75.0	851.4	39.0	322.3	30.0	2	4	3	53	31.5	627.5	24.6	322.3	49.9	1	3	2
20	78.6	669.3	43.0	461.7	25.4	2	3	2	54	38.4	269.2	39.2	359.6	33.4	1	1	1
21	62.4	422.0	47.0	271.4	37.3	2	5	4	55	56.2	437.1	52.4	171.2	45.6	1	2	3
22	65.6	536.9	35.9	309.8	41.0	2	4	3	56	26.6	342.2	53.7	309.7	45.9	2	3	3
23	71.4	991.0	40.7	204.0	44.1	2	5	4	57	22.4	28.3	23.7	178.6	21.6	2	2	3
24	76.3	887.9	75.4	125.0	40.3	3	5	4	58	34.2	81.0	35.7	125.0	21.6	1	4	2
25	43.6	731.8	70.2	181.9	26.0	2	4	2	59	42.8	388.6	20.6	157.9	28.6	2	4	2
26	53.9	602.0	45.0	168.7	29.7	2	3	4	60	38.4	157.9	48.3	129.0	30.6	1	2	2
27	78.9	731.7	37.9	300.3	24.1	2	4	5	61	42.8	1447.4	30.7	225.3	20.4	2	4	4
28	61.0	376.7	46.7	287.7	33.7	3	6	5	62	29.3	1094.0	26.7	257.3	26.9	1	3	2
29	65.4	920.5	57.5	259.7	27.5	3	4	3	63	25.4	696.2	28.3	173.7	29.0	1	2	1
30	55.5	554.9	45.4	146.1	33.1	3	2	4	64	29.0	874.9	80.0	103.7	25.1	2	2	2
31	37.3	263.1	26.7	274.4	35.6	2	2	2	65	22.1	437.1	48.3	214.1	25.9	1	2	3
32	24.9	250.9	6.7	31.2	15.6	1	1	1	66	16.3	342.2	30.7	8.5	20.1	1	2	3
33	27.3	263.1	26.7	274.4	35.6	2	2	2	67	29.0	28.3	200.0	8.5	25.9	1	2	3
34	31.8	473.6	31.7	507.3	28.9	2	3	4	68	41.5	81.0	28.3	623.8	33.4	1	2	3

^a 1- ALP activity (IU/L); 2-LDH activity (IU/L); 3- AST activity (IU/L); 4 - IL-1 β concentration (pg mL⁻¹); 5 - TNF- α concentration (pg mL⁻¹); 6- GI index (1–3); 7-CAL (mm); 8-PD (mm).

value depends on the number of degrees of freedom (number of samples minus two) and the selected probability (usually $p < 0.05$). The significant correlations are bolded (Table 3).

The correlation table revealed significant correlations between ALP activity and the clinical indices, GI, CAL and PD before and after local administration of CHX. Considering the fact that the first two principal components of the model insufficiently explained the data variability, application of different chemometric algorithms for multivariate data analysis was needed.

3.1.2. PLS-DA

The next step consisted of analysis of the data before and after the local administration of CHX using the PLS-DA algorithm. PLS-DA is a regression extension of the principal component analysis

that uses class information to maximize the separation between various groups of observations (Helmy et al., 2012). A drug's efficacy is related to its concentration at the site of action (GCF in our case), so in this step the variable concentration of CHX in GCF after local administration was included in order to assess treatment efficacy. In order to quantify CHX concentrations in GCF samples after gel application we developed, optimized and validated a RP - HPLC method with UV detection for determination of CHX in GCF. The results from the validation have been published in our previous paper (Bogdanovska et al., 2014). The concentrations of CHX in GCF samples after the local administration are presented in Table 4 and a representative chromatogram of a patient's GCF sample after the local treatment is shown in Fig. 4.

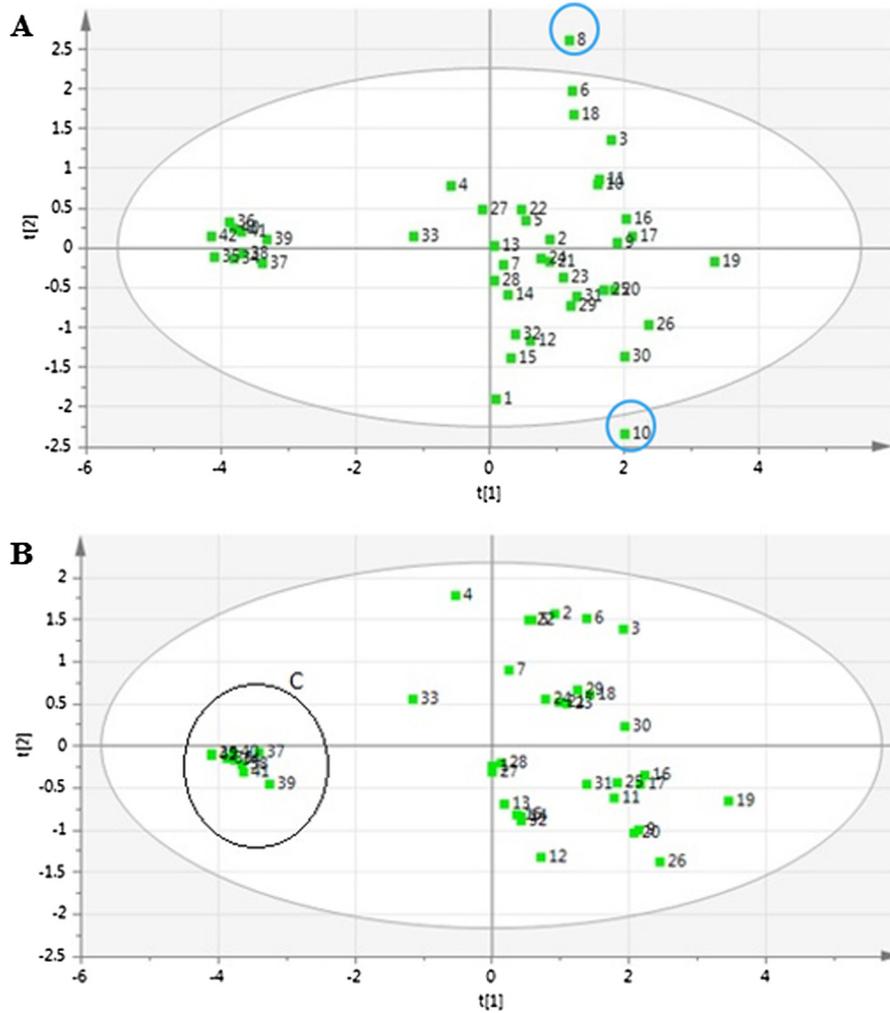


Fig. 1. Score plots of PCA model of data set from the control group of volunteers and periodontal patients before local administration of CHX (Hotelling's ellipse). (A) Identification of outliers by the PCA model (B) PCA model after exclusion of outliers, the left cluster of points belongs to the control group (C), the right cluster to periodontitis patients before local administration of CHX.

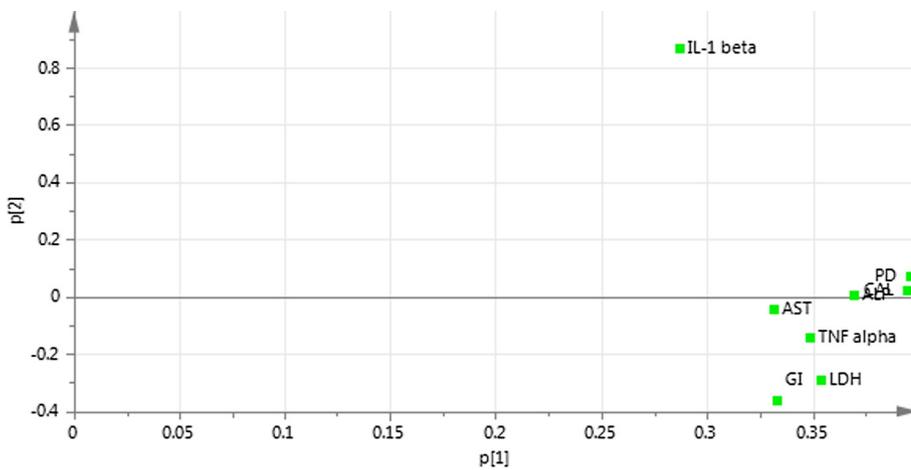


Fig. 2. Loading plot for PC1 and PC2 showing variables that enable discrimination between control group of and periodontal patients before administration of CHX.

The score plot of the three component PLS-DA model showed clear separation between data before and after local administration of CHX, as shown in Fig. 5.

The data before local administration of CHX clustered to the left part of the score plot, whereas data after local administration of CHX clustered to the right. This model provided good group separation, with R^2X (cum) value of 0.572, R^2Y (cum) = 0.894 and Q^2Y

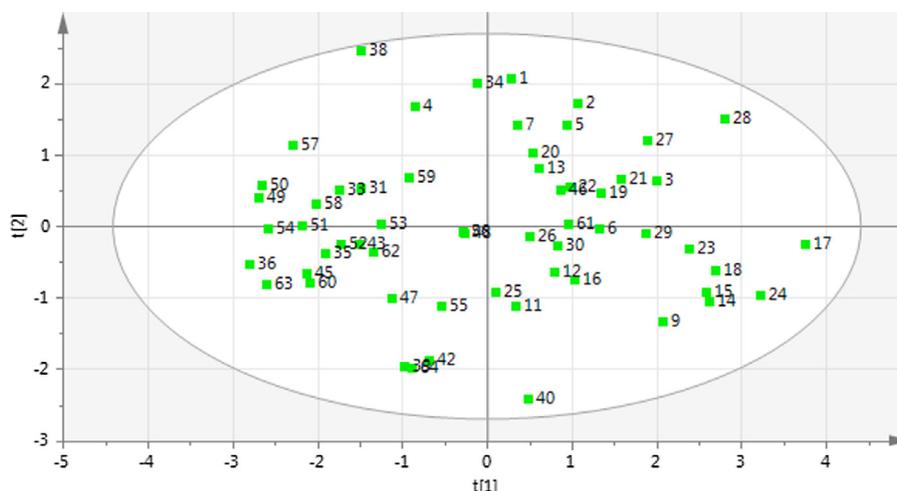


Fig. 3. PCA model of patient's data taken before and after the local administration of CHX. The score plot shows the model's inability for data discrimination.

Table 3

Correlation coefficients among selected variables in patients before and after local administration of CHX. Significant correlations are bolded.

	ALP	LDH	AST	IL-1 β	TNF- α	GI	CAL	PD
ALP	1.000	0.378	0.269	0.169	0.304	0.489	0.539	0.439
LDH		1.000	0.348	-0.028	0.170	0.281	0.341	0.267
AST			1.000	-0.015	0.222	0.192	0.196	0.233
IL-1 β				1.000	0.085	0.052	0.138	0.148
TNF- α					1.000	0.122	0.175	0.137
GI						1.000	0.502	0.438
CAL							1.000	0.592
PD								1.000

Table 4

CHX concentrations in patient's GCF samples after the local treatment.

Patient N°	CHX ($\mu\text{g mL}^{-1}$)	Patient N°	CHX ($\mu\text{g mL}^{-1}$)
1	154.12	18	135.39
2	122.05	19	187.84
3	133.07	20	156.96
4	135.39	21	126.05
5	167.84	22	122.92
6	156.96	23	164.39
7	136.05	24	145.36
8	164.39	25	139.82
9	139.82	26	149.82
10	139.82	27	126.79
11	126.79	28	135.21
12	130.21	29	137.82
13	147.82	30	159.25
14	169.25	31	100.36
15	174.12	32	112.69
16	125.05	33	135.39
17	123.07	34	187.24

(cum) = 0.823, which indicates potent statistical model according to the cross-validation.

Further confirmation of no overfitting in the obtained PLS-DA model was made by permutation testing. Permutation testing provides the statistical significance of the estimated predictive power of the model by comparing the R^2Y and Q^2Y values of the original model with those of the reordered model, created by random permutation of y – data. Models with R^2Y intercept < 0.4 and Q^2Y intercept < 0.05 indicate valid models. As shown in Fig. 6, the PLS-DA model had R^2Y intercept of 0.0743 and Q^2Y intercept of -0.361, indicating there is no overfitting.

The most important variables accountable for the separation between the classes were extracted using the VIP statistics (vari-

able influence on projection) of the PLS-DA model. VIP statistics ranks the overall contribution of each variable to the generation of the model and points out most significant variables. It is generally accepted that a VIP value between 0.8 and 1.2 yields relevant variables (Chong and Jun, 2005).

According to the VIP criterion, three examined variables in the model (AST, IL-1 β and TNF- α) did not influence separation significantly. The rest of the variables showed significant influence on the separation in classes, with change in CHX concentration after treatment being the most significant. The PLS-DA model identified the change in ALP activity after treatment as second most important variable for class separation suggesting that ALP activity may be used in assessment of treatment efficacy. At the same time, as revealed by the VIP plot, the treatment induced small changes on the studied cytokines which might be due to the antibacterial mechanism of action of CHX (see Fig. 7).

3.1.3. OPLS-DA

The final step in our study consisted of comparison between effects of local administration of CHX and local administration of DOX, commonly applied drugs in the periodontal treatment, with distinct mechanism of action. The results from determination of inflammatory biomarkers and clinical indices after local administration of DOX are presented in Table 5. In order to understand the differences, a sophisticated chemometric algorithm, OPLS-DA, was applied on the data sets containing results after local treatment with CHX and after local treatment with DOX.

The OPLS-DA algorithm is a modification of the traditional PLS-DA algorithm with integral orthogonal signal correction filter (Wiklund et al., 2008; Smilde et al., 2010). The separation of Y-predictive (discriminating variation) and Y-orthogonal variation (variation not contributing to class separation) yields in better

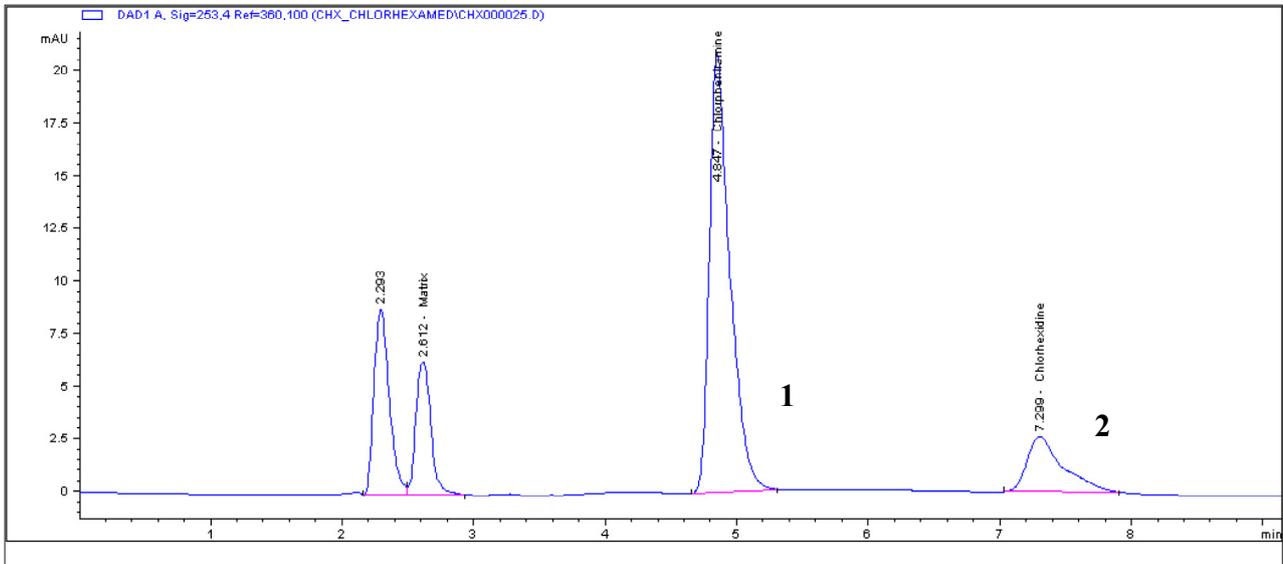


Fig. 4. Representative chromatogram of a patient's GCF sample after the local administration of CHX (1-chlorpheniramine (internal standard), 2-CHX).

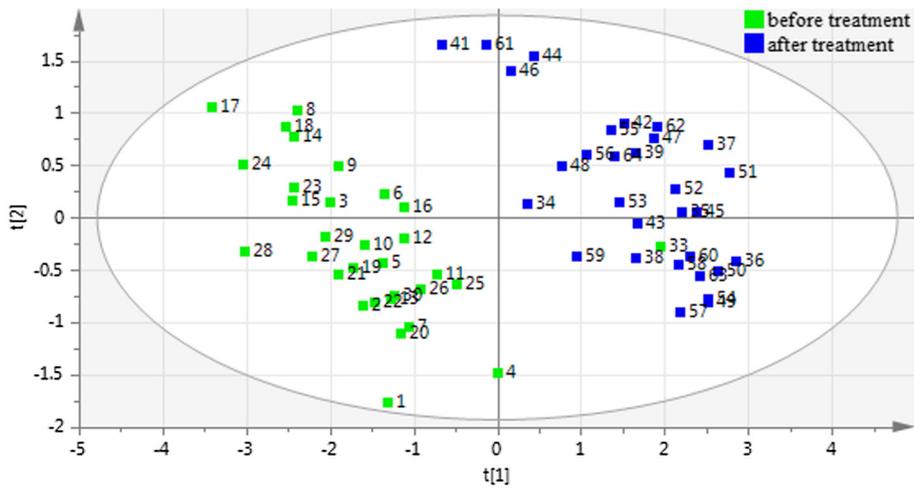


Fig. 5. PLS-DA score plot of data (GCF inflammatory biomarkers, clinical indices and CHX concentration in GCF) before and after the local administration of CHX. Squares clustered to left represent data before treatment, whereas squares clustered to the right represent data after treatment with CHX.

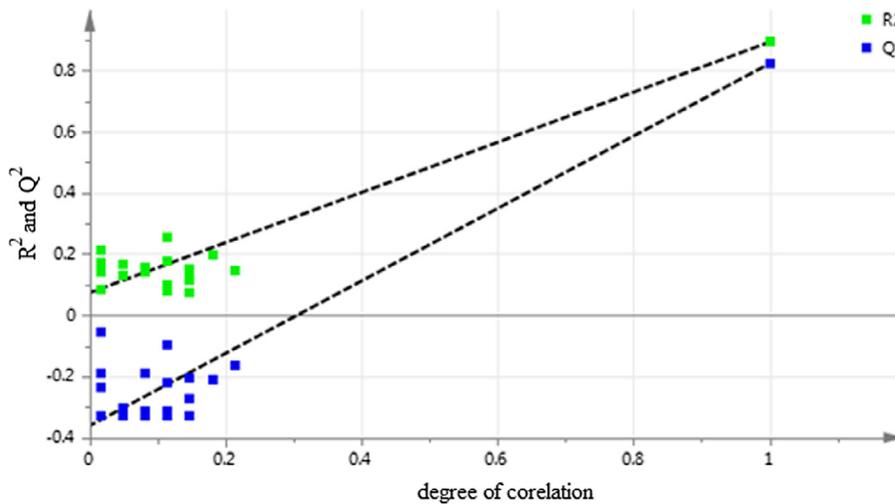


Fig. 6. Validation of the PLS-DA model using permutation testing. Each symbol represents a permutation result, R² is represented by green squares and Q² by blue squares.

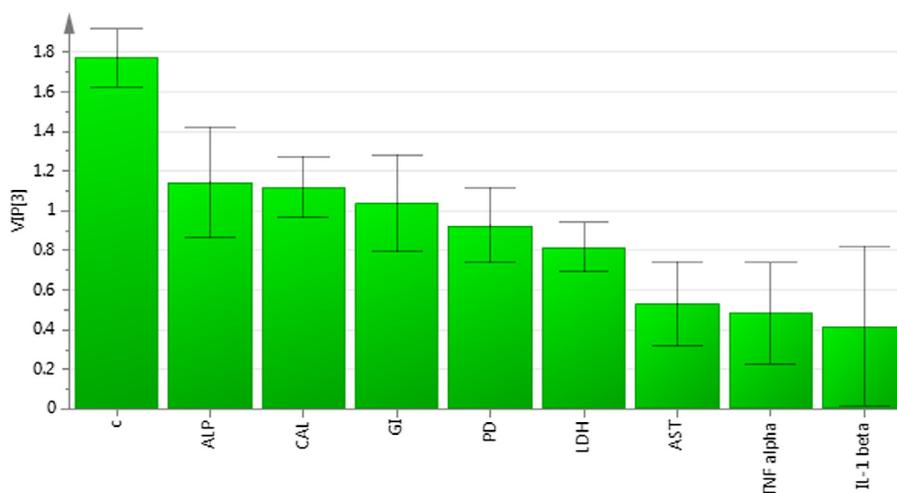


Fig. 7. VIP statistics plot of the X-variables of the three component PLS-DA model for efficacy evaluation of local administration of CHX.

Table 5

Results from determination of inflammatory biomarkers in GCF samples and clinical indices in periodontal patients after the local treatment with DOX.

N°	1	2	3	4	5	6	7	8 ^a
1	41.5	226.7	8.3	15.6	21.9	1	2	2
2	16.6	137.6	20.0	33.6	23.3	1	1	1
3	6.9	668.8	16.7	24.0	22.9	1	1	1
4	22.1	741.6	16.7	89.0	29.0	1	1	1
5	18.0	429.0	16.7	55.4	15.6	1	2	2
6	38.7	416.9	16.7	62.5	23.7	1	1	1
7	24.9	53.3	2.3	31.8	7.8	1	1	1
8	27.6	315.7	7.8	42.2	17.7	1	2	2
9	42.8	267.1	15.0	162.0	22.9	1	1	1
10	52.5	1003.1	73.3	37.6	19.6	1	2	3
11	38.7	505.9	56.7	95.5	30.6	1	3	2
12	19.3	1044.3	25.0	146.8	35.2	1	3	2
13	34.6	1291.2	35.0	136.4	15.6	2	3	1
14	31.8	1894.9	103.3	56.9	35.6	1	2	2
15	52.5	918.8	70.0	47.8	23.2	1	2	3
16	33.2	323.8	16.7	221.8	33.9	1	4	2
17	66.3	1076.2	30.0	301.4	42.9	1	3	1
18	24.9	344.0	56.7	156.4	15.6	1	2	1
19	16.0	429.0	16.7	125.4	15.6	1	2	2
20	35.7	416.9	16.7	62.5	23.7	1	2	3
21	20.9	789.3	28.3	31.8	7.8	1	4	2
22	21.7	315.7	7.8	221.8	17.7	1	3	1
23	38.7	267.1	15.0	162.0	22.9	1	2	1
24	52.5	283.1	73.3	137.6	19.6	1	3	2
25	38.7	505.9	56.7	95.5	30.6	2	2	2

^a 1- ALP activity (IU/L); 2-LDH activity (IU/L); 3- AST activity (IU/L); 4- IL-1 β concentration (pg mL⁻¹); 5- TNF- α concentration (pg mL⁻¹); 6- GI index (1–3); 7-CAL (mm); 8- PD (mm).

interpretability of the data, especially in cases where subtle differences among classes are present. Thus, in this study, OPLS – DA was used to extract statistically significant variables responsible for class separation using the VIP statistics of the model. As it can be seen from the score plot (Fig. 8), the OPLS-DA model divided samples from the two treatment groups in different clusters. According to the results from cross-validation of the model, R^2X (cum) = 0.527, R^2Y (cum) = 0.507 and Q^2X (cum) = 0.530, indicating a good model.

The VIP statistics, as with the PLS-DA model, was used to identify variables which differ significantly between both treatment groups. The VIP statistics plot is shown in Fig. 9. As it can be seen from the VIP plot, four examined variables contributed significantly for separation between treatment groups (PD, AST, IL-1 β and TNF- α).

In order to get confirmation for the statistical significance of the identified differences, we used non-parametric Mann Whitney U

test, as the data were not normally distributed. Compared to the local administration of CHX, the local administration of DOX resulted in greater decrease of AST activity and cytokine concentration ($p < 0.05$) which might be due to the anti-inflammatory action of DOX and its ability to stabilize cell membranes, thus decreasing the intracytoplasmic activity of enzymes such as AST.

4. Conclusion

In this study, we performed chemometric evaluation of the efficacy of local administration of CHX in patients with periodontal disease. The PCA models showed that the selected inflammatory biomarkers and clinical indices can be used for monitoring periodontal disease progression due to their discriminative power. Although, the PCA algorithm could not identify changes as a result of CHX administration, the PLS-DA model successfully classified

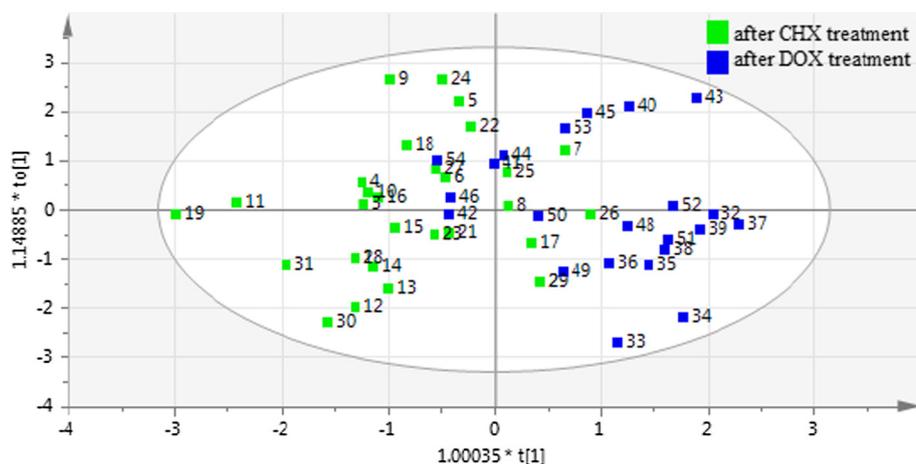


Fig. 8. Score plot of the OPLS-DA model of data obtained after the local administration of CHX and after local administration of DOX in periodontal patients.

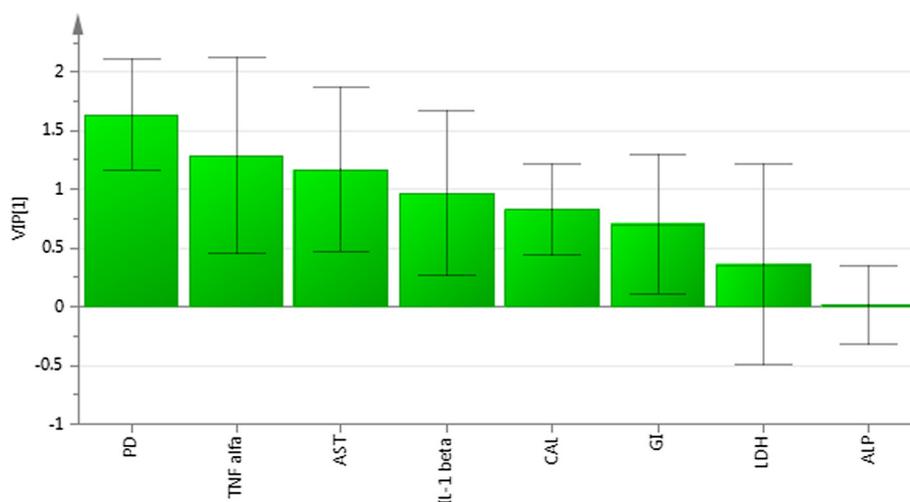


Fig. 9. VIP statistics plot of the X-variables of the OPLS-DA model for comparative evaluation of efficacy of local administration of CHX and local administration of DOX.

data before and after local administration of CHX and identified the most influential variables responsible for class separation. The comparison of efficacy of local administration of CHX and local administration of DOX using the OPLS-DA algorithm identified differences in treatment effects thus providing insights into mechanisms of action of the two treatments. In summary, the application of chemometric algorithms for multivariate data analysis (PCA, PLS-DA and OPLS-DA) may provide useful approach for evaluation of treatment efficacy.

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