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Inflammatory markers in dependence on the plasma concentration of 37 fatty acids after the coronary stent implantation

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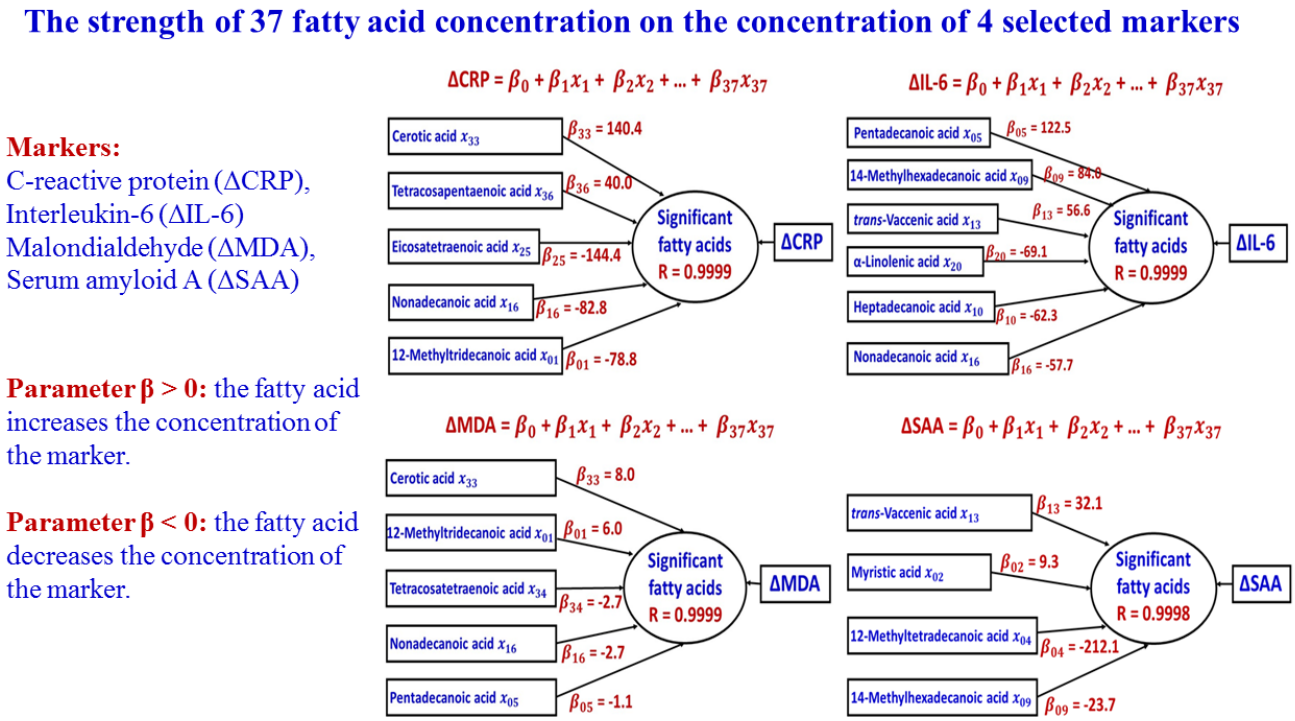
Graphical abstract



Highlights:

- Regression testifies the strength of positive/negative relationship of the fatty acid on markers.
- Selected markers were C-reactive protein, interleukin-6, malondialdehyde and serum amyloid A.
- Factor analysis and cluster analysis separated 37 fatty acids into clusters of similar properties.
- The box plot revealed in fatty acids the largest concentration variation in phospholipid fraction.

Abstract

Using the regression model building the relationships between the concentration of 37 fatty acids of blood plasma phospholipids of 41 patients with coronary artery disease after coronary stent implantation, the inflammatory response and oxidative stress markers were estimated. The dynamics of the inflammatory response and the oxidative stress was indicated by measuring plasma concentrations of highly sensitive C-reactive protein, interleukin-6, serum amyloid A and malondialdehyde before, 24 hours after stent implantation. The multiple linear regression analysis was preceded by an exploratory data analysis, principal component analysis, factor analysis and cluster analysis, which proved a hidden internal relation of 37 fatty acids. The concentration of cerotic acid (C26:0) has been positively associated with an increase of malondialdehyde concentration after stent implantation, while the concentrations of tetracosatetraenoic (C24:4 N6) and nonadecanoic (C19:0) acids were associated with decrease of lipoperoxidation. The increase of interleukin-6 during the 24 hours after implantation was associated with higher levels of pentadecanoic acid (C15:0) and lower levels of α-linolenic acid (C18:3 N3). Regression models found several significant fatty acids at which the strength of the parameter β for each fatty acid on selected markers of C-reactive protein, malondialdehyde, interleukin-6 and serum amyloid A was estimated. Parameter β testifies to the power of the positive or negative relationship of the fatty acid concentration on the concentration of selected markers. The influencing effect of the cerotic acid (C26:0) concentration in plasma phospholipids exhibiting parameter β = 140.4 is, for example, 3.5 times higher than this effect of n-3 tetracosapentaenoic acid (C24:5 N3) with β = 40.0. Composition of fatty acids in plasma phospholipids shows spectrum of fatty acids available for intercellular communication in systemic inflammatory response of organism and should affect clinical outcomes.

Keywords: Fatty acids; Coronary stents; C-reactive protein; Interleukin-6; Malondialdehyde; Multivariate linear regression

1. Introduction

Percutaneous coronary intervention (PCI) with stent is currently a common way to treat significant coronary heart disease. Coronary stent implantation elicits inflammatory response, which negatively affects clinical outcomes. The higher content of some fatty acids, e.g. *n*-3 fatty acids, oleic acid, in plasma or in erythrocyte membranes is supposed to be associated with beneficial cardiovascular effects and a low inflammatory state, whereas *n*-6 or saturated fatty acids and oxidative stress contribute to inflammation.

Fatty acids, which are of the primary interest in relation to coronary heart disease (CHD) include the *n*-6 fatty acids (linoleic, dihomo-γ-linolenic and arachidonic acids), and the *n*-3 fatty acids (α-linolenic, eicosapentaenoic and docosahexaenoic acids). Three highly unsaturated fatty acids – arachidonic (C20:4 N6), dihomo-γ-linolenic (C20:3 N6) and eicosapentaenoic (C20:5 N3) acids - are the original source of a number of eicosanoids (prostaglandins, leukotrienes, prostacyclins, thromboxanes, lipoxins, etc.) [1]. The *n*-3 polyunsaturated fatty acids have shown to exhibit many effects beneficial to

cardiovascular health including anti-arrhythmic, improvement of endothelial function, and down-regulation of blood pressure [2]. Docosahexaenoic (C22:6 N3) and eicosapentaenoic (C20:5 N3) acids' enrichment of membrane phospholipids can increase arrhythmic thresholds [3], electrically stabilize cardiac myocytes by inhibiting sodium and calcium channels [4], and favorably affect autonomic tone [5, 6]. Eicosanoids made from arachidonic acid are generally more potent mediators of inflammation, vasoconstriction, and platelet aggregation than those made from eicosapentaenoic acid [7], the ratio of arachidonic to eicosapentaenoic acid in membrane polyunsaturated fatty acids can theoretically influence biochemical and physiological responses to stress [8]. Further saturated fatty acids, such as myristic or palmitic acid, have been demonstrated to induce inflammatory signaling, e.g. stimulating NFκB [9].

Systemic inflammation is a key component in the development and progression of atherosclerosis and also determines the occurrence of complications after PCI. A serum fatty acid pattern exhibiting a high content of monounsaturated fatty acids and a low level of linoleic acid has been linked to elevated CRP [10]. In a Spanish study, CRP was inversely associated with linoleic acid and *n*-3 fatty acids whereas IL-6 correlated positively with myristic (C14:0) and palmitic (C16:0) acids [11]. The Italian study showed that lower proportions of arachidonic, eicosapentaenoic and docosahexaenoic acids were associated with higher IL-6 concentrations and that α-linolenic acid was inversely related to CRP [12]. Also Farzaneh-Far et al. [13] found that CRP and IL-6 were inversely associated with docosahexaenoic and eicosapentaenoic acid, in a large cross-sectional study of patients with stable coronary artery disease.

The induction of the pro-inflammatory cytokine IL-6 is regulated with several pathways. An important role in the regulation of expression of cytokines, such as IL-6, may be played by reactive oxygen species, generated by vascular enzymes and by other sources. Elevated reactive oxygen species contribute significantly to oxidative stress, which further induces the inflammation pathways [14]. Oxidative stress and inflammation were reported to create a self-perpetuating cycles of oxidation and inflammation. Čermák et al. [15] found a correlation between fatty acid profile of erythrocyte membranes and an increase in inflammatory reactions after PCI with stenting. The aim of our study was to decide if inflammatory response after PCI, which is highly individual, is affected by plasma phospholipid fatty acid profile, and quantitatively express to what extent by individual fatty acids in plasma phospholipids.

2. Material and methods

2.1 Study subjects

This cross-sectional study was approved by the Ethical Committee on Human Research of the Regional Hospital of Pardubice, Czech Republic, and all participants provided written informed consent. The study included 41 patients referred to the PCI with coronary stent implantation for significant coronary stenosis, *i.e.* at least 50% stenosis of the left main coronary artery or 70% stenosis of the epicardial coronary artery according to coronarographic examination. Excluded from the study were patients with an initial level of hs CRP>10 mg/l, patients with serious health complications, ST Segment Elevation Myocardial Infarction

(STEMI), heart failure according to New York Heart Association (NYHA) II-IV, renal failure, thyroid dysfunction, hepatic or oncology disease or patients that regularly consume alcohol. The appropriate institutional approval of the review board was obtained as well as the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations have been followed. Participants received a description of the study and signed an informed participation consent that included permission to conduct analyses on the biological specimens collected and stored. All intervention was performed with a standard technique, and all patients received drug-eluting stents (Everolimus). Before intervention, all patients received weight-adjusted intravenous heparin with a target activated clotting time of 250-350 seconds.

2.2 Blood sample collection

Venous blood samples were collected in tubes with EDTA (The Vacuette Detection Tube, No. 455036, Greiner Bio-One GmbH, Kremsmünster, Austria) before and 24 hours after stent implantation. After 20 min centrifugation of samples at 1500 × g, plasma was stored at -80 °C.

2.3 Determination of inflammatory markers

Inflammatory markers (high sensitivity C-reactive protein, interleukin-6 and serum amyloid A) were determined by standard procedures in the Regional Hospital of Pardubice, Czech Republic. High sensitivity C-reactive protein was measured with the analytical system VISTA, interleukin-6 with an immunochemistry analyzer Immulite and serum amyloid A with a BN ProSpec laser nephelometer (Siemens Healthcare Diagnostics Inc., USA).

2.4 Determination of malondialdehyde

Malondialdehyde was assessed by HPLC as previously described [16]. Plasma malondialdehyde was quantified as the malondialdehyde-thiobarbituric acid complex which was accomplished using an isocratic elution on a LiChroCart 250 × 4 mm, Purospher Star RP-18e, 5 μm, analytical column fitted with a LiChroCart 4 × 4 mm, Purospher Star RP-18e, 5 μm, guard column (Merck, Darmstadt, Germany).

2.5 Determination of plasma phospholipid fatty acids

Plasma fatty acids were determined using the thin layer chromatography (TLC Silica Gel 60 Glass plates 20 × 20 cm) technique with a subsequent analysis of individual lipid fractions containing phospholipids, free fatty acids, cholesterol esters, diacylglycerols and triacylglycerols by gas chromatography with flame ionization detection (Agilent Technologies 7890 GC System, USA). The plasma samples were briefly deproteinized with 2-propanol, *n*-heptane and ortho-phosphoric acid (40:20:1, v/v/v) mixture. Then a mixture of methanol and toluene (4:1, v/v) and water were added and, after centrifugation (1700 × g, room temperature, 5 min), the upper organic phase was evaporated under nitrogen to dryness.

After adding chloroform-methanol mixture (2:1, v/v) to the residue, the content of the tube was transferred on the silica gel chromatography plate. Lipids were separated using a mobile phase composed of *n*-hexane, diethyl ether and acetic acid (8:2:0.3, v/v/v). The phospholipid fraction were scraped off the TLC plate, transferred to screw-capped tube, and dissolved in a mixture of methanol and toluene (4:1, v/v) containing *cis*-13,16,19-docosatrienoic acid as an internal standard. Trans-esterification and the gas chromatographic separation of the fatty acid methyl esters have been described in a publication of Čermák et al. [15].

We used HP-88 capillary column (100 m in length, 250 μm in id, 0.25 μm in film thickness). The injector temperature was set at 250°C, the flame ionization detector at 280°C, and a programmed temperature ramp was used. The analysis time was 75 min. The flow rate of helium as the carrier gas was 3 mL/min and the inlet split ratio was set at 10:1. The injection volume of the sample was 1 μL.

2.6 Multivariate data analysis

At first, it was necessary to verify some assumptions made on the data. The dependent variable formed successively selected markers CRP, IL-6, SAA and MDA which represented the function on the phospholipid plasma concentration of 37 fatty acids. Matrix of the independent variables contained concentrations of 37 fatty acids in columns for 41 examined patients P1 to P41 placed in rows. Fatty acids in a regression model were labelled x_1 through x_{37} (Table 1). Mean Value (Median) of analysed concentration [μmol/l] of 37 fatty acids in group of 41 patients is in Table 2.

Table 1

Table 2

An assumption of the independent variables in the source data matrix is a demand for independence, originality and uniqueness of all fatty acids x_1 to x_{37}. Statistically significant correlation means that the signs are bound with a hidden internal relationship expressed by the correlation, which can cause biased estimates of the regression parameters. When such multicollinearity in independent variables is detected, it is necessary to use in regression analysis the Welsch robust M-estimation method which leads to unbiased parameter estimates.

The correlation matrix showed that most Pearson correlation coefficients are statistically significant in concentrations of fatty acids. Correlation allows for an application of the principal components method and factor analysis but in a regression analysis brings some problems in evaluating the true parameter estimates for 37 fatty acids.

Fig. 1

Fig. 2

Factor analysis provides a graph of factor loadings (Fig. 1a) after a varimax rotation. The factors were named according to clusters of fatty acids located near factor axes. The graph of factor scores (Fig. 2a) enables an explanation of some features concerning all patients. The similarity of objects can also be examined with cluster analysis which classifies similar objects into clusters. The Ward hierarchical clustering method was used. In addition to the dendrogram of fatty acids, the dendrogram of patients seems to be highly important (Fig. 1b and Fig. 2b).

Multiple linear regression analyzed an influence of the concentration of 37 fatty acids on four selected biological markers, such as a change in CRP at 24 hours (denoted ΔCRP), a change of IL-6 after 24 hours (denoted ΔIL-6), a change SAA after 24 hours (denoted ΔSAA) and the MDA change after 24 hours (denoted ΔMDA). Multiple linear regression with the use of regression triplet examines the quality of input data (Table 3). Analysis of the relationship for various biological markers and fatty acids was performed with the use of the concentrations of fatty acids in the plasma fraction of phospholipids. Concentrations were calculated by the linear calibration of fatty acids in plasma.

Table 3

In the data criticism the outlying patients were detected and were removed from the data. Some graphic diagnostics indicated influential points at the significance level $\alpha = 0.10$. In the regression model ΔMDA patients P19 and P39 were removed; in the regression model ΔCRP the patient P12 was removed, in the regression model ΔSAA patients P12, P16 and P30 were removed; and lastly in the regression model ΔIL-6 patients P26 and P27 were removed.

In the regression model criticism $y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_{37} x_{37}$, $i = 1, \ldots 41$, for 41 patients and for concentration of 37 fatty acids x_j listed in each Table 4 through Table 7 the parameters have been estimated $\beta_0, \beta_1, \beta_2, \ldots \beta_{37}$. The final number of patients n after outliers removal was always mentioned with the number of statistically significant estimates β of fatty acids and the reliability criteria of a found regression model. Many various combinations of a number of patients, the number of fatty acids and modification of the method of least squares under monitoring resolution criteria such as the correlation coefficient R, the coefficient of determination R^2, mean square error of prediction MEP, Akaike information criterion AIC. The residual sum of squares RSS and the standard deviation of residuals $s(e)$ belong among the extensive resolution criteria of the regression model building. Both are dependent on the number of patients n [17, 18].

The positive value of the parameter estimates β_i describe a positive attitude with increasing concentrations of fatty acids x_j the value marker y is increased, or negative value when for increasing concentrations of fatty acids the marker y is reduced. Besides point estimates of parameters, their standard deviation s followed by their interval estimates $[L_L, L_U]$ are calculated. An interesting variable is the variability of the fatty acid concentrations [μmol/l], from which the parameters estimate was calculated.

The relative standard deviation of the variability of the fatty acid expresses the relative standard deviation called the coefficient of variation CV and expressed in %.

Table 4

Table 5

Table 6

Table 7

3. Results

3.1 Exploratory data analysis of the plasma phospholipid fraction

From icon plots the patients show that the composition of fatty acids in the phospholipid fraction of plasma in patients very much differs. Based on the shapes of polygons, the patient groups with similar fatty acid composition were ascertained. The group of patients P2, P20, P29 and P25 has one of the smallest stars and therefore can be comprehensively described as a group of patients with low concentrations of fatty acids in plasma phospholipids. A strong counterpoint to this group is the group of patients with the biggest stars. Patients P17, P14, P15 and P18 can be described as a group of patients with the greatest concentrations of fatty acids in the phospholipid fraction of plasma.

Significantly high concentrations of fatty acids can also be observed in patients P17 with the biggest star. The expectation that the concentration of fatty acids may be related to the amount of adipose tissue with the incidence of obesity in a patient in this case was refuted. Patient P17 suffers from, in addition to an ischemic heart disease and chronic obstructive pulmonary disease of the IV. step (COPD) also asthma bronchiale, which leads to respiratory distress with a respiratory acidosis. Moreover, two weeks prior to surgery he underwent antibiotic treatment for respiratory infection and worsening dyspnea. When comparing the physical parameters of the patient with all the others, we can conclude that this patient was clearly the smallest figure, the smallest stature (160 cm) and low weight (48 kg).

The factor loadings plots (Fig. 1a) are used to name factors. Naming is based on factor-pure variables that are located in this plot near the factor axes and also at the ends of the factor axes having high factor loadings. The most beneficial is always the factor loading plot FA1-2 of loading Factor 1 and Factor 2. When naming Factor 1 we focused on the fatty acids oleic x_{14} (C18:1 N9), α-linolenic x_{20} (C18:3 N3), palmitic x_6 (C16:0), stearic x_{12} (C18:0), linoleic x_{17} (C18:2 N6), *cis*-palmitoleic x_8 (C16:1 N7-cis) and heptadecanoic x_{10} (C17:0) and also their significant contrasts due to a Factor 1, or to the fatty acids 12-methyltridecanoic x_1 (12-Me C13:0), *n*-6 tetracosapentaenoic x_{35} (C24:5 N6) and possibly *n*-3 tetracosapentaenoic x_{36} (C24:5 N3) and tetracosahexaenoic x_{37} (C24:6 N3). Based on the placement of fatty acids Factor 1 was named as "Concentration of fatty acid variability".

The concetration of fatty acids with a large variability of plasma concentrations was therefore highly positive in Factor 1 and, conversely, those fatty acids whose variation is not so significant were

located near the beginning of the chart (0, 0) or were slightly negative. The factor loading plot FA2-3 was used for naming Factor 3. The fatty acids which are located at the negative part of Factor 3 are contained particularly in nuts, seeds and other natural plant products. Fatty acids that do not lie in the factor loading plot at the ends of the factor axes or which are factor-dirty are quite common in all kinds of food. Fatty acids found in the positive range of Factor 3, are particularly of animal origin.

The factor graph feedback score, showing the placement of patients in the graph, was used for naming Factor 2. The distribution of fatty acids in Factor 2 using the location of degradation in an organism, where the fatty acids in the positive range are degraded in peroxisomes, is probably indicated. Many patients suffer from concomitant diseases, such as hypercholesterolemia, diabetes mellitus, hypertension, psoriasis etc. Some patients take various drugs, such as statins which interfere with lipid metabolism and reduce blood cholesterol. Given these enumerated factors it seems difficult to unambiguously define clusters of patients with similar features.

Named factors were taken off the factor loading plots and transferred into factor score plots. A positive finding of this analysis was an agreement between information obtained from the icon plots and the results obtained by the principal component analysis and the factor analysis. The last step was to compare the results achieved so far with the results of cluster analysis of the concentration of the fatty acids. The concentration of the dendrogram of fatty acids clusters shows the selected variables into two main clusters (Fig. 1b). This is group J and group K, wherein group J coalesces fatty acids with the largest variability in the organism, which are also in the highest concentrations in plasma phospholipids. All the fatty acids of group J were indicated with black circles in the factor loading plot FAW1-2 in Factor 1 and Factor 2 while the fatty acids of cluster K have been marked with open circles. In group J fatty acids such as arachidonic x_{24}, stearic x_{12}, linoleic x_{17}, palmitic x_{6}, docosahexaenoic x_{32}, nervonic x_{29}, *cis*-vaccenic x_{15}, dihomo-γ-linolenic x_{22} and oleic x_{14} were found. The significant variability of concentration of these fatty acids was also confirmed with the box-and-whisker plot.

The concentration of the fatty acids in cluster K achieves in plasma phospholipids significantly lower concentrations than the fatty acids in cluster J. When comparing the results of the dendrogram of fatty acids concentration with the factor loading plot of Factor 1 and Factor 2 no unanimity was found. It turned out that the overwhelming majority of fatty acids from group J are strongly positive for Factor 1, which was named as “Concentration of the fatty acid variability” Two completely different statistical methods succeeded in good agreement in classifying fatty acids according to a variability of their concentrations.

A more important clusters presentation seems to be the dendrogram of patients (Fig. 2b), which divided the patients into clusters of similar characteristics. Clusters were denoted in dendrogram by letters A to I. Subsequently, similar clusters were found in the plot of factor scores. When comparing both

graphs, each based on different statistical method, good agreement between the indicated clusters was proven.

A search for similar patient characteristics is not easy due to the number of possible influences. Clusters of patients located far from three relatively large clusters identified by letters A, E and H containing 23 patients were analyzed. These large clusters in the dendrogram of patients were indicated with a plot of factor scores as the cluster of overlapping patients and it is apparent after color designation that such clusters overlap. Therefore, it was not possible to determine the main reason for patient dislocation and their inclusion in clusters in the objects dendrogram.

For smaller clusters, located off the main group of patients, cluster C, which includes several times mentioned the patients P17 and P15 is somewhat distant. The characteristic of patient P17 were cited above, attention was focused on patient P15. This patient was admitted for coronary artery disease, acute anterior wall NSTEMI, treated for hypertension, recently demonstrated a high level of TAG for which the statin Torvacard was used to treat. He is a smoker of smaller stature. According to the plot of component scores PCS1-2 for the first and second principal component patients P17 and P15 are similar. The plot of the component score in this cluster also ranks the patient P14, who was also adopted for CHD, acute NSTEMI inferolateral, treated for hypertension, dyslipidemia (statin therapy), and has impaired glucose tolerance diagnosed two months ago. Patient P14 is a slightly overweight non-smoking female.

Another found cluster of outlier patients was cluster B with patients P2 and P29. Both patients suffer from an ischemic heart disease with hypertension and they are treated for type 2 diabetes mellitus, it is interesting that in comparison with other patients it is quite exceptional that both suffer from psoriasis. Conformity has both patients in the values of the ejection fraction, which is 55% for both. The ejection fraction characterizes the pumping function of the left ventricle, which is on the lower limit of the normal values. Both patients are nonsmokers treated with statins, patient P2 for hypercholesterolemia and patient P29 for the overall dyslipidemia.

Cluster F has patients P39, and P34, who suffer from coronary heart disease. Both are relatively young smokers several years after myocardial infarction at the age of 44 and 48 years, respectively, with dyslipidemia treated with statins and moderate obesity. Two other clusters, which partly overlap, but are outside the main area of patient distribution, are clusters D and G.

3.2 Multiple linear regression model building

The intensity of the influence of each fatty acid x_i concentration on the individual marker, which was selected as dependent variable y is defined as the magnitude of an estimate of parameter β in the multiple linear regression model $y = \beta_0 + \beta_1 x_1 + \ldots + \beta_m x_m$. The high and positive value of the estimate β of actual fatty acid concentration in comparison with estimates β of other fatty acids concentration means that the

actual fatty acid acts most strongly to the selected marker. The effect of the fatty acid concentration is involved in the growth of the plasma concentration of the marker in patients. Therefore it has the strongest relationship to the inflammatory process that the marker indicates the intensity of an inflammatory response. The strongest negative effect on the reference marker have fatty acids whose estimates β are the most negative, and therefore act intensively on the decrease in plasma concentration of the reference marker. In our case they have the strongest effect on reducing the inflammatory response and thereby also on reducing the rate of the oxidative cell damage.

The group of fatty acids whose estimated values β not assessed in the multiple linear regression to be statistically significant, because they are close to zero should not have significant changes in the plasma concentrations of these markers in patients.

Results of the multiple linear regression show that a concentration of the fatty acid which strongly supports an increase of the plasma C-reactive protein is the cerotic acid, as well as *n*-3 tetracosapentaenoic and docosapentaenoic acids, while a concentration of eicosatetraenoic acid has an inverse relationship to the concentration of C-reactive protein.

An increase of malondialdehyde is associated with the higher concentrations of cerotic acid while a negative relationship to an increase of malondialdehyde during 24 hours after stent implantation was exhibited by the plasma fosfolipid concentration of tetracosatetraenoic and nonadecanoic acids. A positive relationship to increasing the level of the plasma concentration of serum amyloid A during 24 hours after coronary stents implantation was found by linear regression particularly for the concentration of *trans*-vaccenic acid, whereas an inverse correlation was found with the concentration of 12-methyltetradecanoic acid. The plasma phospholipid concentration of pentadecanoic acid was found in positive correlation to the increase of plasma interleukin-6 during the first 24 hours after implantation and conversely the negative correlation exhibited the concentration of α-linolenic acid. Multiple linear regression was able to quantitatively examine which fatty acids participated in increasing or decreasing the plasma concentrations of the selected markers and which force this was done.

4. Discussion

Exploratory analysis of the concentrations of fatty acids in plasma phospholipids fraction was proved that the hidden strong internal relationships exist among fatty acids. Principal component analysis, factor analysis and cluster analysis of the concentration of the fatty acids divided them into groups of similar characteristics. Based on the division of fatty acids into clusters with similar properties, patients can be classified into clusters with similar characteristics.

C-reactive protein, interleukin-6 and serum amyloid A are markers of the inflammatory response that were observed for 24 hours after percutaneous coronary intervention with a coronary stent implantation followed in patients with a coronary heart disease. These markers can also be considered as independent risk factors for the coronary heart disease [19, 20]. A product of lipid peroxidation was used for monitoring of the oxidative cell´s damage the malondialdehyde. Oxidative stress is often associated with the inflammatory processes in the organism [21]. The increase of C-reactive protein, interleukin-6 and serum amyloid A and malondialdehyde was maximal after 24 hours and therefore it was used as an image of development of inflammatory reactions and the oxidative damage resulting in patients.

Fatty acids with the largest concentration variability according to the exploratory data analysis are palmitic x_6, stearic x_{12}, oleic x_{14}, *cis*-vaccenic x_{15}, linoleic x_{17}, dihomo γ-linolenic x_{22}, arachidonic x_{24}, nervonic x_{29} and docosahexaenoic x_{32} acids. This finding is in agreement with the fact that currently a majority of these fatty acids are monitored and studied in the literature. Exploratory data analysis revealed the least variable to be methylated fatty acid 12-methyltridecanoic x_1, 13-methyltetradecanoic x_3, *n*-6 docosapentaenoic x_{30}, eicosatetraenoic x_{25}, tetracosatetraenoic x_{34} and *n*-6 tetracosapentaenoic x_{35}, which is related to their very low concentrations.

Linear regression examined the relationship between the concentration of the fatty acids and the individual markers. The results of multiple linear regression discovered that the increase of C-reactive protein most strongly potentiates the plasma phospholipid concentration of cerotic acid x_{33} (C26:0). This finding is consistent with the Japanese study published in 2014, which shows that the saturated fatty acids of very long chain and coronary heart disease may be in correlation, and cerotic acid in particular can be considered as a potential risk factor for developing coronary heart disease [22, 23]. A quite opposite effect on C-reactive protein have concentrations of acid such as eicosatetraenoic x_{25}, nonadecanoic x_{16}, 12-methyltetradecanoic x_4 that counteract increasing levels of C-reactive protein.

The frequently mentioned strong anti-inflammatory effect of docosahexaenoic acid presented in some studies [24] at C-reactive protein levels was not monitored in our study. This is in agreement with a study [25], which suggests that supplementation of fish oil rich in docosahexaenoic and eicosapentaenoic acids did not decrease of markers of systemic inflammation, as the concentration of C-reactive protein nor interleukin-6 in serum. However, that study was performed on a group of healthy individuals and docosahexaenoic and eicosapentaenoic acids had been artificially administered. The reason why we did not observe an anti-inflammatory effect of docosahexaenoic acid, may be that patients and generally the Czech population's intake of *n*-3 polyunsaturated fatty acids is low, which is reflected by low amounts of these fatty acids in both plasma and erythrocyte membranes.

An increase of malondialdehyde levels in plasma during 24 hours after stent implantation correlates mainly with the concentration of cerotic acid x_{33} (C26:0), which is similar to the association with an

increase in the plasma concentrations of C-reactive protein during 24 hours after an implantation of the coronary stent with the difference that the β estimation is at C-reactive protein twenty times greater than that of malondialdehyde. Together with the cerotic acid the positive effect also manifested 12-methyltridecanoic acid x_1 (12-Me C13:0). Conversely, the negative, i. e. reducing effect on an increase of malondialdehyde in plasma during 24 hours after stent implantation induce tetracosatetraenoic x_{34} (C24:4 N6) and nonadecanoic x_{16} (C19:0) acids. Their impact, however, can not be overvalued, because of their β estimates in linear regression. With the raising concentration of serum amyloid A after stent implantation an increase of the concentration of *trans*-vaccenic acid x_{13} (C18:1 N7, trans) appeared. Together with the *trans*-vaccenic acid the positive effect was detected in concentration of myristic x_2 (C14:0) and arachidic x_{19} (C20:0) acids. The effect of *trans*-vaccenic acid led to some controversy.

Since *trans*-vaccenic acid is a precursor to conjugated linoleic acid in humans [26], which has, for example, according to the results of an Irish study [27] the anti-inflammatory effects presented by reducing levels of the tumor necrosis factor-α and an interleukin-6, observed on cells of the human epithelial colorectal adenocarcinoma, is presumed that the *trans*-vaccenic acid can also possess anti-inflammatory traits. These results are supported by the study [28], which monitored anti-inflammatory effects in animal models and came to the conclusion that the *trans*-vaccenic acid along with signaling lipid molecule oleoyl-ethanolamide can act lipopolysacharids induced anti-inflammatory activity through PPAR.

The inverse relationship to the plasma concentration of serum amyloid A during 24 hours after stent implantation was observed for the concentration of 12-methyltetradecanoic x_4 (12-Me C14:0) acid and 14-methylhexadecanoic acid x_9 (14-Me C16:0). The last reference marker on which we have focused was the interleukin-6. The increase of interleukin-6 after stent implantation was associated with higher levels of pentadecanoic acid x_5 (C15:0) and negatively correlated with the plasma phosphoplipid concentration of α-linolenic acid x_{20} (C18:3 N3). A favorable effect of the α-linolenic acid is in accordance with the American studies [29], which summarizes the effects of α-linolenic acid so that the α-linolenic acid has a cardioprotective effect, is capable of reducing the risk of cardiovascular diseases and has a favorable impact against vascular inflammation and endothelial dysfunction.

Plasma phospholipids and their constituents - fatty acids - are in interaction with components of biological membranes and play an important role in signal transduction and intercellular communication. Cerotic (C26:0) acid has been found to be of great interest in the development of inflammatory reaction after coronary stent implantation, because its relationship to the increase of inflammatory markers has been found the most statistically significant. The fact that this fatty acid occurs in blood in relatively low concentration does not mean that the meaning of this fatty acid is negligible, but precise mechanism of these interactions should be another subject of research.

4. Conclusion

Using statistical data treatment the multiple linear regression model was built based on the studied fatty acids of plasma phospholipids in groups of patients after coronary stent implantation and markers of inflammatory response and oxidative stress. The dynamics of the inflammatory response and oxidative stress was monitored by measuring blood plasma concentrations of C-reactive protein, interleukin-6 and serum amyloid A and malondialdehyde before and 24 hours after surgery. Parameter estimates are proven with the goodness-of-fit test expressed with the correlation coefficient *R* and the coefficient of variance *CV* (%). CRP, a non-specific marker of inflammation that also has a direct inflammatory activity in atherosclerosis has been associated with adverse cardiovascular outcomes in patients with coronary artery disease seems to be the most sensitive marker. The following conclusions were reached (Fig. 3):

Fig. 3

1. The principal component analysis, factor analysis and cluster analysis separated fatty acids into clusters of similar properties. Similar properties seem to be the concentration variability, the natural resource degradation and the localization in the cell. Based on the division of fatty acids into clusters the patient also can be classified into clusters with similar characteristics, such as the presence of other related diseases, e.g. hypertension, psoriasis, dyslipidemia, or diabetes mellitus.

2. Regression models found several significant fatty acids at which the strength of the parameter β for each fatty acid on selected markers of C-reactive protein, malondialdehyde, interleukin-6 and serum amyloid A was estimated. Parameter β testifies to the power of the positive or negative relationship of the fatty acid concentration on the concentration of selected markers. The influencing effect of the cerotic acid concentration in plasma phospholipids exhibiting parameter $\beta = 140.4$ is, for example, 3.5 times higher than this effect of *n*-3 tetracosapentaenoic acid with $\beta = 40.0$.

3. The increase of malondialdehyde is associated with higher concentrations of cerotic acid. Conversely, the concentration of tetracosatetraenoic and nonadecanoic acids had a negative relation to an increase of malondialdehyde within 24 hours after stent implantation.

4. A positive relation to an increase of the plasma concentration of serum amyloid A during 24 hours after stent implantation was found for the concentration of *trans*-vaccenic acid, whereas an inverse correlation was found for the concentration of 12-methyltetradecanoic acid. A positive correlation to an increase in the plasma concentration of interleukin-6 during the first 24 hours after stent implantation was found for the concentration of pentadecanoic acid and conversely a negative correlation was found for the concentration of α-linolenic acid. Multiple linear regression was able to quantitatively examine which fatty acid, and which force was involved in increasing or decreasing the plasma concentrations of the selected markers. Levels of CRP were found to be associated with future vascular events, such as atherothrombosis, in greater extent than levels of IL-6 or SAA. It was found systemic production of CRP, but local production

of both IL-6 and SAA in culprit coronary lesions. Malondialdehyde, a stable product of lipoperoxidation, is a marker of oxidative stress, which plays minor role than inflammation.

5. Research of the inflammatory reaction has a great potential in the prevention of complications in patients after the coronary stent implantation. The mechanisms of the involvement of certain fatty acids in a process of the inflammatory response is not yet clear, but participation of fatty acids in the process of an inflammatory response after coronary stent implantation is obvious. Targeting research to the mediators of inflammation, products of the metabolism of some fatty acids, could be mission in the future in this issue.

6. Analysis of composition of fatty acids in plasma phospholipids could be a promising tool for estimation of patient´s clinical prognosis and occurrence of clinical complications. Determination of fatty acid profile contributes to prediction how lipids can react in the individual biological system, providing a powerful tool for elucidation of the pathophysiological mechanisms of diseases connected with systemic inflammation, screening for novel biomarkers.

Ethical conduct of research

The appropriate institutional approval of the review board was obtained as well as the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations have been followed. Participants received a description of the study and signed an informed participation consent that included permission to conduct analyses on the biological specimens collected and stored.

Acknowledgments

Conflict of Interest: none

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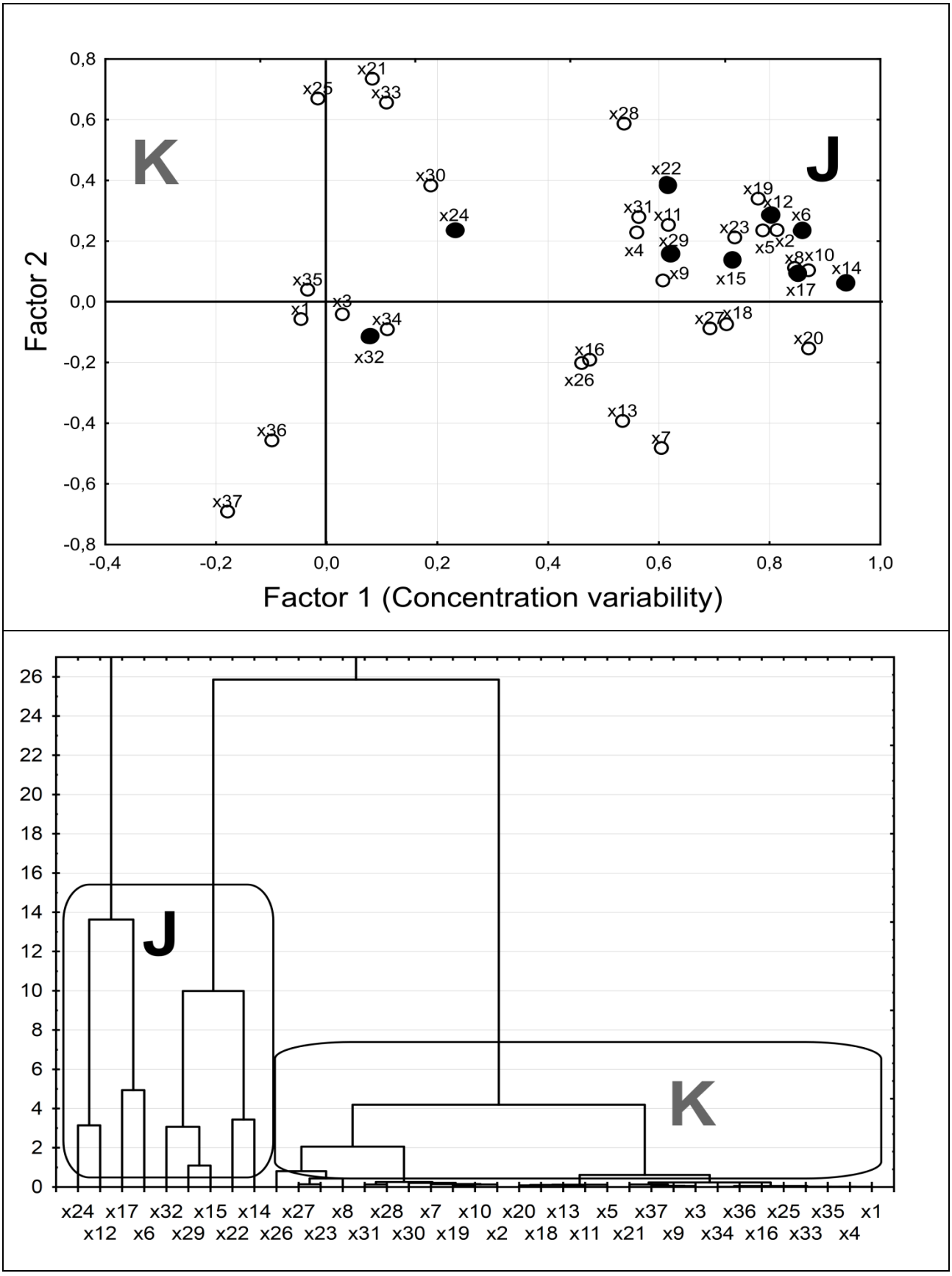
Captions

Fig. 1 The similarity and correlation of 37 fatty acids are examined in the form of (a) the graph of factor loadings Factor 1 – Factor 2 after normalized varimax rotation, and (b) the dendrogram of clusters with use of Ward method, (STATISTICA, StatSoft).

Fig. 2 The similarity and correlation of 41 patients are examined in the form of (a) the graph of factor scores Factor 1 – Factor 2 after normalized varimax rotation, and (b) the dendrogram of patients when clusters were built using the Ward method, (STATISTICA, StatSoft).

Fig. 3 The resulting flow-chart of statistically significant fatty acids estimated in the multiple linear regression models of four inflammatory markers ΔCRP, ΔMDA, ΔSAA and ΔIL-6. Parameter estimates are proven with the goodness-of-fit test expressed with the correlation coefficient *R* and the coefficient of variance *CV* (%). CRP, a non-specific marker of inflammation that also has a direct inflammatory activity in atherosclerosis has been associated with adverse cardiovascular outcomes in patients with coronary artery disease seems to be the most sensitive marker.



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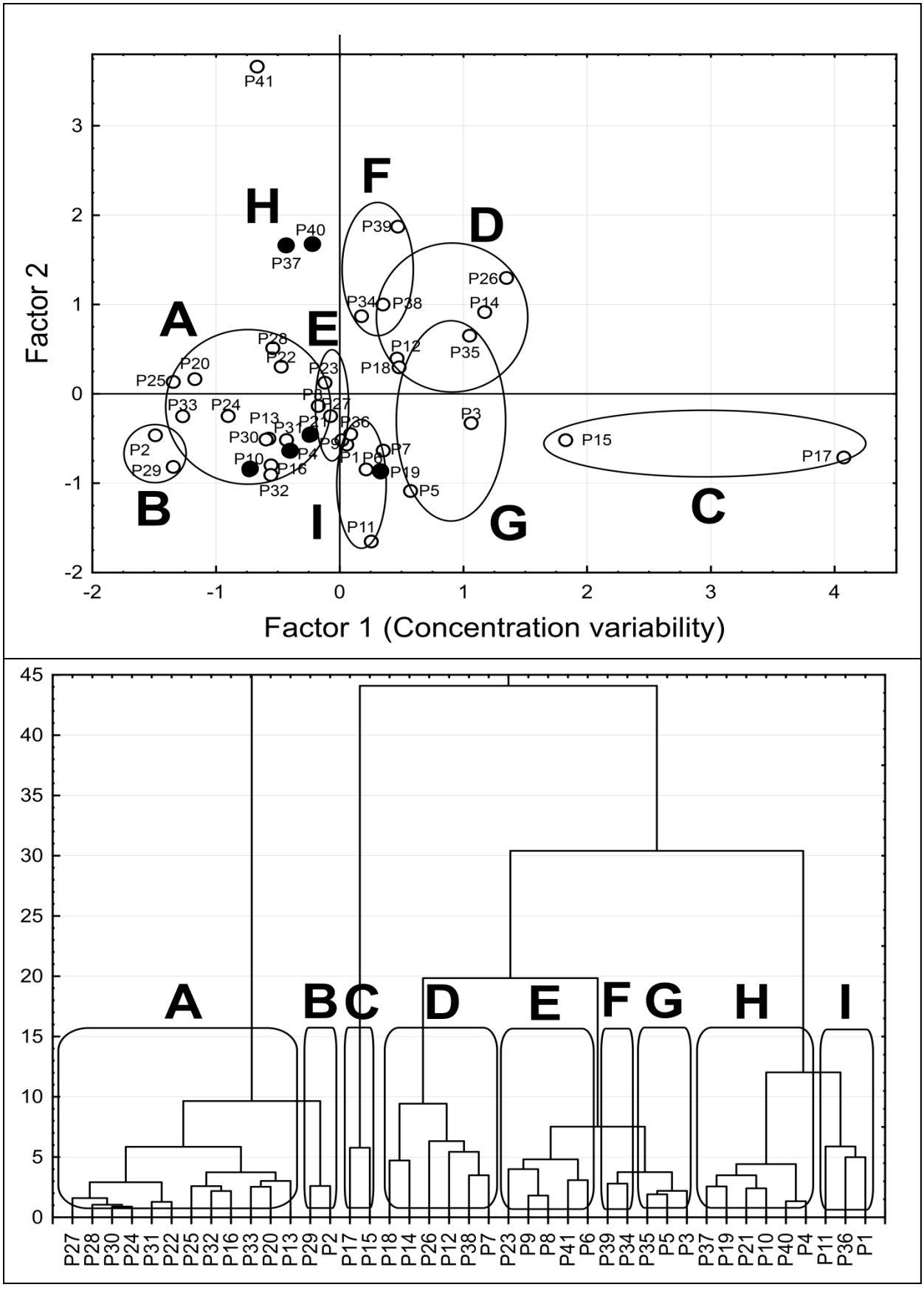

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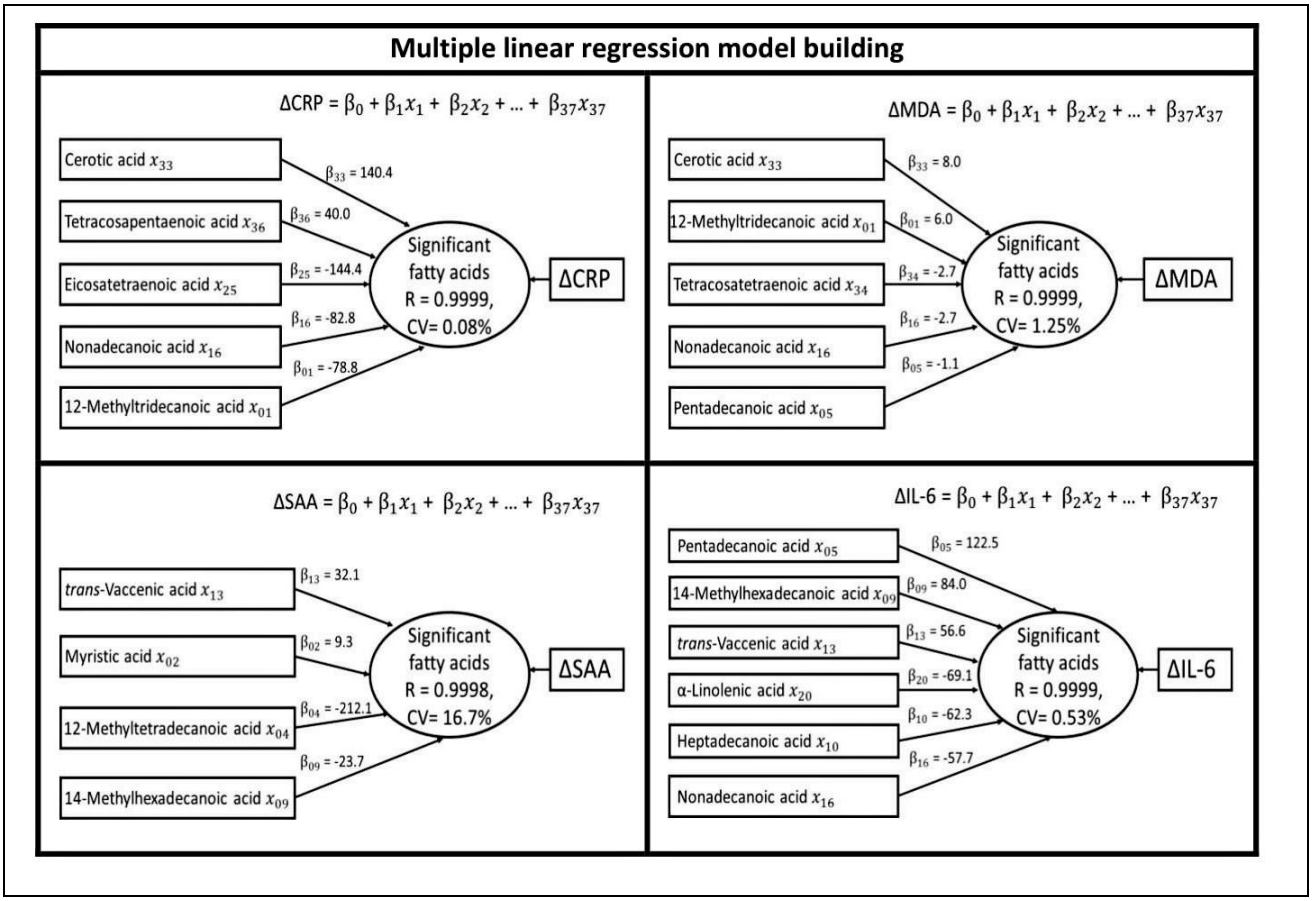

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Table 1 Analysed 37 fatty acids with molecular formula denoted in ID as x_1 through x_{37}.

ID	Molecular formula	Fatty acid			
x_1	12-Me C13:0	12-Methyltridecanoic	x_{19}	C20:0	Arachidic
x_2	C14:0	Myristic	x_{20}	C18:3 N3	α-Linolenic
x_3	13-Me C14:0	13-Methyltetradecanoic	x_{21}	C18:4 N3	Stearidonic
x_4	12-Me C14:0	12-Methyltetradecanoic	x_{22}	C20:3 N6	Dihomo-γ-linolenic
x_5	C15:0	Pentadecanoic	x_{23}	C22:0	Behenic
x_6	C16:0	Palmitic	x_{24}	C20:4 N6	Arachidonic
x_7	C16:1 N10	Sapienic	x_{25}	C20:4 N3	Eicosatetraenoic
x_8	C16:1 N7-cis	*cis*-Palmitoleic	x_{26}	C20:5 N3	Eicosapentaenoic
x_9	14-Me C16:0	14-Methylhexadecanoic	x_{27}	C24:0	Lignoceric
			x_{28}	C22:4 N6	Docosatetraenoic

x_{10}	C17:0	Heptadecanoic	x_{29}	C24:1 N9	Nervonic
x_{11}	16-Me C17:0	16-Methylheptadecanoic	x_{30}	C22:5 N6	Docosapentaenoic *n*-6
x_{12}	C18:0	Stearic	x_{31}	C22:5 N3	Docosapentaenoic *n*-3
x_{13}	C18:1 N7, trans	*trans*-Vaccenic	x_{32}	C22:6 N3	Docosahexaenoic
x_{14}	C18:1 N9	Oleic	x_{33}	C26:0	Cerotic
x_{15}	C18:1 N7, cis	*cis*-Vaccenic	x_{34}	C24:4 N6	Tetracosatetraenoic
x_{16}	C19:0	Nonadecanoic	x_{35}	C24:5 N6	Tetracosapentaenoic *n*-6
x_{17}	C18:2 N6	Linoleic	x_{36}	C24:5 N3	Tetracosapentaenoic *n*-3
x_{18}	C18:3 N6	γ-Linolenic	x_{37}	C24:6 N3	Tetracosahexaenoic

Table 2 Analysed concentration [µmol/l]of 37 fatty acids in group of 41 patients.

ID	Molecular	*s*(e)	Median				
x_1	12-Me C13:0	0,06	**0,16**	x_{19}	C20:0	2,08	**6,81**
x_2	C14:0	2,86	**6,64**	x_{20}	C18:3 N3	1,72	**3,12**
x_3	13-Me C14:0	0,87	**1,34**	x_{21}	C18:4 N3	1,44	**1,85**
x_4	12-Me C14:0	0,06	**0,09**	x_{22}	C20:3 N6	42,70	**131,07**
x_5	C15:0	1,69	**3,34**	x_{23}	C22:0	4,61	**17,10**
x_6	C16:0	115,51	**432,39**	x_{24}	C20:4 N6	52,60	**250,01**
x_7	C16:1 N10	2,15	**6,91**	x_{25}	C20:4 N3	0,15	**0,16**
x_8	C16:1 N7-cis	7,81	**13,97**	x_{26}	C20:5 N3	11,24	**19,51**
x_9	14-Me C16:0	0,38	**1,40**	x_{27}	C24:0	4,60	**15,58**
x_{10}	C17:0	2,38	**6,78**	x_{28}	C22:4 N6	2,97	**8,82**
x_{11}	16-Me C17:0	1,53	**2,27**	x_{29}	C24:1 N9	12,92	**50,24**
x_{12}	C18:0	65,86	**277,43**	x_{30}	C22:5 N6	1,97	**5,07**
x_{13}	C18:1 N7, trans	1,11	**2,24**	x_{31}	C22:5 N3	2,39	**8,24**
x_{14}	C18:1 N9	62,13	**163,04**	x_{32}	C22:6 N3	28,79	**76,51**
x_{15}	C18:1 N7, cis	7,33	**33,00**	x_{33}	C26:0	0,09	**0,11**
x_{16}	C19:0	0,20	**0,54**	x_{34}	C24:4 N6	0,18	**0,31**
x_{17}	C18:2 N6	119,19	**352,91**	x_{35}	C24:5 N6	0,07	**0,12**
x_{18}	C18:3 N6	1,60	**2,19**	x_{36}	C24:5 N3	0,20	**0,61**
				x_{37}	C24:6 N3	0,67	**0,74**

Table 3 Critique of the method used in the regression triplet

	Classical least-squares	Welsch method of M-estimates
Fisher-Snedecor test of the regression model *F* criterion vs. *F*(1-alfa, *m*-1, *n*-*m*), Probability *p* Conclusion: Regression model is	4750.2 vs. 62.47, *p* = 0.0115 Significant	8552.9 vs.62.47,*p* = 0.0086 Significant
Scott criterion of multicollinearity *SC* criterion, Probability *p* Conclusion: Regression model is	-0.079 Correct	-0.060 Correct.
Cook-Weisberg test heteroscedasticity *CW* criterion vs. *Chi*^*2*(1-alfa,1), Probability *p* Conclusion: Residuals exhibit	0.001 vs. 2.706, *p* = 0.978 Homoscedasticity	0.079 vs. 2.706, *p* = 0.778 Homoscedasticity
Jarque-Berra test normality *JB* criterion vs. Chi^2(1-alfa,1), Probability *p* Conclusion: Residuals exhibit	1.036 vs. 4.605, *p* = 0.596 Normal distribution	1343.3 vs. 4.605, *p* = 0 No normal distribution
Wald test of residuals autocorrelation *WA*criterion vs. *Chi*^*2*(1-alfa,1), Probability *p* Conclusion: Residuals exhibit	0.0030 vs. 2.706, *p* = 0.956 No autocorrelation	0.0087 vs. 2.706, *p* = 0.926 No autocorrelation
Sign test of residuals *Sg* criterion vs. *N*(1-alfa/2) Conclusion: In residuals is	1.017 vs. 1.645, *p* = 0.309 No trend	1.017 vs. 1.645, *p* = 0.309 No trend

Table 4 Build regression model $\Delta MDA = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \ldots + \beta_{37} x_{37}$ of concerning a dependence of ΔMDA 24 on the concentration of all 37 fatty acids are used when outlying patients P19 and P39 are excluded. In table the only most significant fatty acids with negative and positive estimate β are used. The Welsch method of M-estimates is applied (QCEXPERT, TriloByte). Reliability of estimates found is proven with statistics: *R* = 0.9999; R^2 = 0.9999; *MEP* = 9.500E-05; *AIC* = -491.20; *RSC* = 1.88E-05; *s(e)* = 0.004.

Fatty acid	**Estimate β (*s*)**	***p* (α=0,05)**	**Concentration variability (μmol/l)**	
			Min	**Max**
Cerotic acid	8.0 (08)	6.22E-03	0.02	0.36
12-Methyltridecanoic acid	6.0 (06)	5.93E-03	0.09	0.42
Tetracosatetraenoic acid	-2.7 (04)	9.89E-03	0.08	0.98
Nonadecanoic acid	-2.7 (03)	7.10E-03	0.24	1.13

Pentadecanoic acid	-1.1 (01)	8.65E-03	1.60	9.84

Table 5 Build regression model $\Delta CRP = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \ldots + \beta_{37} x_{37}$ of concerning a dependence of ΔCRP 24 on the concentration of all 37 fatty acids are used when outlying patient P12 is excluded. In table the only most significant fatty acids with negative and positive estimate β are used. Classical LS method of estimates is applied (QCEXPERT, TriloByte). Reliability of estimates found is proven with statistics: R = 0.9999; R^2 = 0.9999; *MEP* = 0.013; *AIC* = -531.4; *RSC* = 6.71E-06; *s(e)* = 0.003, g_1 = 0.07; g_2 = 3.13.

Fatty acid	**Estimate *β* (*s*)**	***p* (α=0,05)**	**Concentration variability (μmol/l)**	
			Min	**Max**
Cerotic acid	140.4 (04)	1.96E-04	0.02	0.36
n-3 Tetracosapentaenoic acid	40.0 (01)	1.95E-04	0.15	1.00
Eicosatetraenoic acid	-144.4 (03)	1.28E-04	0.06	0.92
Nonadecanoic acid	-82.8 (02)	1.42E-04	0.24	1.13
12-Methyltridecanoic acid	-78.8 (03)	2.54E-04	0.03	0.30

Table 6 Build regression model $\Delta SAA = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \ldots + \beta_{37} x_{37}$ of concerning a dependence of ΔSAA 24 on the concentration of all 26 selected and the most important fatty acids are used. Outlying patients P12, P16 and P30 are excluded. The Welsch method of M-estimates is applied (QCEXPERT, TriloByte). Reliability of estimates found is proven with statistics: R = 0.9998; R^2 = 0.9995; *MEP* = 2.106; *AIC* = -38.35; *RSC* = 1.2; *s(e)* = 0.775, g_1 = 24.38; g_2 = 25.78.

Fatty acid	**Estimate *β* (*s*)**	***p* (α=0,05)**	**Concentration variability (μmol/l)**	
			Min	**Max**
trans-Vaccenic acid	32.1 (96)	8.83E-04	0.52	5.27
Myristic acid	9.3 (40)	1.81E-03	2.63	13.79
12-Methyltetradecanoic acid	-212.1 (13.42)	3.98E-03	0.03	0.30
14-Methylhexadecanoic acid	-23.7 (2.15)	8.14E-03	0.87	2.41

Table 7 Build regression model ΔIL-6 = $\beta_0 + \beta_1 x_1 + \beta_2 x_2 \ldots + \beta_{37} x_{37}$ of concerning a dependence of ΔIL-6 24 on the concentration of all 26 selected and the most important fatty acids with negative and positive estimate β were used. Outlying patients P26 and P27 are excluded. Welsch method of M-estimates was applied (QCEXPERT, TriloByte). Reliability of estimates found is proven with statistics: R = 0.9999; R^2 = 0.9999; *MEP* = 0.004; *AIC* = -212.69; *RSC* = 0.003; *s(e)* = 0.038, g_1 = 26.13; g_2 = 27.05.

Fatty acid	**Estimate β (*s*)**	***p* (α=0,05)**	**Concentration variability (μmol/l)**	
			Min	**Max**
Pentadecanoic acid	122.5 (46)	1.38E-05	1.60	9.84
14-Methylhexadecanoic acid	84.0 (30)	1.25E-05	0.87	2.41
trans-Vaccenic acid	56.6 (14)	5.76E-06	0.52	5.27
α-Linolenic acid	-69.1 (22)	1.01E-05	1.03	9.84
Heptadecanoic acid	-62.3 (27)	1.91E-05	3.61	16.73
Nonadecanoic acid	-57.7 (17)	8.57E-06	0.24	1.13