JOURNAL OF
MOLECULAR AND APPLIED BIOANALYSIS

ORIGINAL RESEARCH ARTICLE
PUBLISHED: 16 August 2017

The canonical correlation of biomarkers in relation to the concentration of 37 fatty acids of erythrocyte membranes after coronary stent implantation

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Abstract

The treatment of significant stenosis of coronary arteries by percutaneous coronary intervention (PCI) with stent implantation elicits an inflammatory response. This study was aimed at evaluating the relationship between the fatty acid profile of erythrocyte membranes and markers of inflammation, oxidative stress and lipid metabolism after PCI. High-sensitivity C-reactive protein, malondialdehyde, HDL-cholesterol, LDL-cholesterol, triacylglycerols and the erythrocyte fatty acid profiles were determined in patients with advanced coronary artery disease undergoing PCI before and 24 hours after drug-eluting stent implantation (n = 45). Using a canonical correlation analysis the relationship of the percentage amount of chosen fatty acids in erythrocyte membranes and the five markers was found. Studied markers included an increase in C-reactive protein and malondialdehyde 24 hours after coronary stent implantation, and the concentration of the triacylglycerols, LDL cholesterol and HDL cholesterol in the plasma. A strong negative relationship to the five selected markers was found with the n-3 polyunsaturated fatty acids, especially α-linolenic acid and eicosapentaenoic acid. On the contrary, fatty acids in the most positive correlation with the five markers were arachidonic, lignoceric, stearidonic, palmitic and *trans*-vaccenic acids. Of the selected markers, the inflammation C-reactive protein marker was in the strongest relation to all chosen fatty acids.

Keywords: C-reactive protein, Canonical correlation analysis, Coronary stents, Erythrocyte membranes fatty acids, Malondialdehyde

Introduction

In coronary heart disease (CHD), ischemia caused by atherosclerotic stenosis of the coronary artery is currently often treated by percutaneous coronary intervention (PCI) with a coronary stent implantation, which elicits local and systemic inflammatory responses. Their intensity and magnitude negatively affect the clinical outcome, and increase risk of stent restenosis. Inflammatory mechanisms play a crucial role in the pathogenesis of neointimal proliferation, which is the main cause of stent restenosis. In order to diminish inflammatory response, anti-inflammatory drug-eluting stents have been used. Therefore, the beneficial effect of these drug-eluting stents might be partly related to a weaker local or systemic inflammatory response after coronary stenting. Many studies have been carried out to assess the beneficial effect of drug-eluting stents, unfortunately with rather contradictory results.

To reduce inflammation, high sensitive C-reactive protein (hsCRP) has proven to be an excellent marker for the inflammatory status after stenting, with a peak 48 hours after PCI [1]. No correlation was found between the post-procedural change in CRP and laboratory, demographic, clinical, and angiographic variables, thus confirming that patient reactivity is the main factor that affects the systemic inflammatory response after coronary stenting. The observed positive correlation between baseline CRP levels and the magnitude of change in CRP suggests that some patients exhibit a tonic proinflammatory state, which is more susceptible to activation by phasic inflammatory stimulation, such as coronary stenting. In an Italian study [2], the risk of cardiovascular events and in-segment restenosis significantly correlated with the post-procedural increase in CRP, regardless of the type of stent used. An important role in the inflammatory response may also be played by oxidative stress.

The putative anti-inflammatory effect of *n*-3 fatty acids

has been demonstrated in several studies. The *n*-3 fatty acids have direct inhibitory effects on the expression of adhesion molecules and chemotaxis [3]. The *n*-3 fatty acids can inhibit the production of inflammatory cytokines by various mechanisms, e.g. [4] observed that an increased intake of α-linolenic acid inhibits IL-6, IL-1β, and TNF-α production in peripheral blood mononuclear cells. In non-fish-eating populations, *n*-6 arachidonic acid is the predominant tissue of highly unsaturated fatty acid (about 80 % of total), and thus the eicosanoids produced in these individuals are primarily from arachidonic acid - [5]. However, in populations consuming high amounts of fish, the tissue content of *n*-3 unsaturated fatty acids rises, reducing the proportion of *n*-6 unsaturated fatty acids to 60-65 % and altering eicosanoid patterns [6]. Because 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 acid to eicosapentaenoic acid in membrane fatty acids can theoretically influence biochemical and physiological responses to stress.

An organism´s response to stress is also affected by the state of lipid metabolism. It has been confirmed that a diet low in saturated fat and cholesterol and high in PUFA, regardless of whether the *n*-3 or *n*-6, significantly reduces the level of serum lipids. One of the main *n*-6 PUFA is linoleic acid, which has the ability to reduce blood total and LDL-cholesterol.

Saturated fatty acids contribute to an increase in total and LDL-cholesterol levels, but not in the same range. Biological effects of monounsaturated fatty acids depend on their *cis* or *trans* configuration. *Cis*-monounsaturated fatty acids are relatively neutral in relation to HDL-cholesterol and LDL-cholesterol, but *trans*-monounsaturated fatty acids provably increase LDL and decrease HDL-cholesterol [8].

Erythrocyte membrane fatty acids

Erythrocyte membranes were chosen to evaluate the status of cell membranes fatty acids for several reasons [9]. They are easily accessible, so that there is only a negligible load of the patient. They well reflect the composition of the other cell membranes less accessible tissues [10]. They have a relatively low biological variability and reflect a relatively long-term profile of fatty acids in the body. Their composition cannot be altered within a few days due to diet. This study was aimed at evaluating the relationship between the fatty acid profile of erythrocyte membranes and markers of inflammation, oxidative stress and lipid metabolism after PCI. Research of the mechanism of inflammatory reaction has a potential in the prevention of complications in patients after the coronary stent implantation. The participation of fatty acids in the process of an inflammatory response after coronary stent implantation is not yet clear. Targeting research to the mediators of inflammation could be mission in the future.

Materials and Methods

Study subjects

The cross-sectional study included 45 patients referred to the PCI with coronary stent implantation for significant coronary stenosis as was described in [11]. Excluded were patients with an initial level of hs CRP>10 mg/l and patients with serious health complications as was also described previously [11]. The appropriate institutional approval of the review board was obtained and the principles outlined in the Declaration of Helsinki for human 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. All intervention was performed with a standard technique, and all patients received drug-eluting stents (Everolimus). Before intervention, patients received weight-adjusted intravenous heparin with a target activated clotting time of 250-350 seconds. The study was approved by the Ethical Committee on Human Research of the Regional Hospital of Pardubice, Czech Republic.

Determination of biochemical markers

Hs CRP, HDL-cholesterol and LDL-cholesterol and triacylglycerols were determined by standard procedures in the Regional Hospital of Pardubice, Czech Republic. Hs CRP was measured with the analytical system VISTA, (Siemens Healthcare Diagnostics Inc., USA).

Determination of malondialdehyde

Malondialdehyde (MDA), a marker of lipid peroxidation, was assessed by HPLC as previously described [12]. Plasma MDA was quantified as the malondialdehyde-thiobarbituric acid complex which was made 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).

Determination of fatty acids in the erythrocyte membranes

Venous blood samples were collected in tubes with EDTA (The Vacuette Detection Tube, No. 455036, Greiner Bio-One GmbH, Kremsmünster, Austria) both before and 24 hours after stent implantation. After 20 min centrifugation of samples at 1500 × g, plasma and the buffy coat were separated into cryotubes. One ml of erythrocytes was taken from the center of the erythrocyte column and immediately stored at -80°C.

The patient sample 200 μl of erythrocytes was added to distilled water. The solution was mixed and placed for 15 minutes in a freezer (-20°C). After centrifugation (1 700

× g, room temperature, 10 min) membranes of erythrocytes were separated. This was followed by removing the supernatant to prevent aspiration pellets. This washing of the membranes of erythrocytes was performed three times. After the last wash, 1 ml of deproteinization solution 2-propanol, *n*-heptane and *ortho*-phosphoric acid (40:20:1, v/v/v) was added to the resulting sediment. The mixture was incubated (room temperature, 10 min), and 400 µl of internal standard (*cis*-13,16,19-docosatrienoic acid) at a concentration of 10 µg / ml, and 300 µl of distilled water was added. *Trans*-esterification and the gas chromatographic separation of the fatty acid methyl esters have been described in [13] and in publication [11]. Fatty acids were labelled x_1 through x_{37} (**Table 1**).

Canonical correlation analysis

The objectives of Canonical Correlation Analysis (CCA)

CCA is a multi-dimensional approach to examining the linear relationship between two sets of variables [14,15]. The first of the two sets is considered as *the left set of variables x* and the other set as *the right set of variables y*. This division is purely circumstantial and has no effect on problem solving. The canonical correlation analysis technique is best understood by considering it as an extension of multiple regression and correlation analysis. In the multiple regression analysis we find the best linear combination *of p* variables, $x_1, x_2,..., x_p$, to predict only one variate *y*. The multiple correlation coefficient *R* represents the *simple correlation* between *y* and its predicted value y_{calc} which can be expressed with the use of the Pearson correlation coefficient, page 638 in ref. [14]. In CCA we examine the linear relationships between a set of ***x*** variables $x_1, x_2,..., x_p$, i.e. here fatty acids on the left side, expressed as the relation $U_1=a_1x_1 + a_2x_2 +... + a_px_p$, and a set of more than one ***y*** variable $y_1, y_2,..., y_q$, i.e. here markers on the right hand side, expressed as the relation $V_1=b_1y_1 + b_2y_2 +... + b_qy_q$. Those linear composites U_1 and V_1 are known as the *canonical variates,* and the correlations between the corresponding pairs of canonical variates are called the *canonical correlation* R_i. Once found, then a search for the next canonical variates U_2 and V_2, which have the second largest correlation coefficient under the condition that U_2 and V_2 are uncorrelated with the first canonical variates U_1 and V_1. Both canonical variates form a new coordinate system of mutually orthogonal components [16].

Deriving the Canonical Functions

For any particular choice of the coefficients (*a*'s and *b*'s) we can compute values of U_1 and V_1 variates for each individual patient in the sample. From the *n* patients in the sample we can then compute the simple correlation between the *n* pairs of U_1 and V_1 variates in the usual manner. The resulting correlation depends on the choice of the slopes *a*'s and *b*'s. In CCA we select slope values of *a* and *b* so as to *maximize* the correlation between U_1 and V_1 variates. With this particular choice the resulting linear combination U_1 is called the *first canonical variate* of the **x**'s and V_1 is called the *first canonical variate* of the **y**'s. The resulting correlation between U_1 and

Table 1: Analysed 37 fatty acids with PubChem CID denoted in ID as x_1 through x_{37}.

ID	PubChem CID	Fatty acid	x_{19}	10467	Arachidic
x_1	520298	12-Methyltridecanoic	x_{20}	5280934	α-Linolenic
x_2	11005	Myristic	x_{21}	5312508	Stearidonic
x_3	151014	13-Methyltetradecanoic	x_{22}	5280581	Dihomo-γ-linolenic
x_4	21672	12-Methyltetradecanoic	x_{23}	8215	Behenic
x_5	13849	Pentadecanoic	x_{24}	444899	Arachidonic
x_6	985	Palmitic	x_{25}	11722594	Eicosatetraenoic
x_7	5312419	Sapienic	x_{26}	446284	Eicosapentaenoic
x_8	445638	*cis*-Palmitoleic	x_{27}	11197	Lignoceric
x_9	22207	14-Methylhexadecanoic	x_{28}	5282844	Docosatetraenoic
x_{10}	10465	Heptadecanoic	x_{29}	5281120	Nervonic
x_{11}	21859	16-Methylheptadecanoic	x_{30}	6441454	Docosapentaenoic *n*-6
x_{12}	5281	Stearic	x_{31}	5282850	Docosapentaenoic *n*-3
x_{13}	5281127	*trans*-Vaccenic	x_{32}	445580	Docosahexaenoic
x_{14}	445639	Oleic	x_{33}	10469	Cerotic
x_{15}	5282761	*cis*-Vaccenic	x_{34}	52921800	Tetracosatetraenoic
x_{16}	12591	Nonadecanoic	x_{35}	14505435	Tetracosapentaenoic *n*-6
x_{17}	5280450	Linoleic	x_{36}	52921801	Tetracosapentaenoic *n*-3
x_{18}	5280933	γ-Linolenic	x_{37}	53481586	Tetracosahexaenoic

V_1 is called the *first canonical correlation*. The first canonical correlation R_1 thus represents the highest possible correlation between a linear combination of the **x**'s and a linear combination of the **y**'s. The canonical importance of each variate is evaluated from two perspectives:

a) It specifies the intensity of the linear relationship between the canonical variate U and the original variable x or canonical variate V and variable y.

b) It expresses the intensity of the relationship between the canonical variates U and V.

Since the canonical correlation analysis assumes only a linear dependence between variables, it is necessary to examine the graphs of each pair of variates and examine the linearity of data and outliers. It should respect several of the following points:

1. Determining the number of canonical variates pairs to use: The number of pairs possible is equal to the smaller number of variables in each set.

2. The canonical variates themselves must also be named and interpreted. As in factor analysis, they are also working with mathematically constructed hypothetical variates, which are usually difficult to physically interpret and name.

3. The importance of each variate should be evaluated from two aspects: It must estimate the intensity of the linear relationship between the canonical variate U and original variables ***x*** from which it was created and between the canonical variate V and original variables ***y*** from which it was created. It must also express the intensity of the linear relationship between the corresponding canonical U and V variates.

Graphical tools

Two kinds of graphical representations are displayed to visualize and interpret the results of CCA: Scatter plots of the variates U and V and scatter plots of variables. Graphical representations can be drawn for every pair of the variates U and V. It is advocated to choose a small value for the *number of significant pairs of variates d* from an interval (1, p). In practice, this p value is very often 2 or 3. Note that small canonical correlations are not relevant: they do not express linear relationships between columns of U and V and therefore can be neglected. The Cattel scree plot is the plot of canonical correlations versus the index; a clear gap between two successive values suggests selecting for d the rank of the greatest one. The original variables plot is of interest because it allows to discern the structure of correlation between the two sets of variables **x** and **y**.

A useful option available in some programs is a plot of the canonical variate scores U_i versus V_i. For multivariate normal data the graph would approximate an ellipse of concentration. Such a plot can be useful in highlighting unusual cases in the sample as possible outliers or blunders. The plot of V_1 versus U_1 does not result in an apparently nonlinear scatter diagram, nor does it look like bivariate normal distribution (elliptical in shape). Canonical correlation creates linear combinations of variables, canonical variates, that represent mathematically viable combinations of variables. However, although mathematically viable, they are not necessarily interpretable. A major task for the researcher is to discern and explain, if possible, the meaning of pairs of canonical variates. Interpretation of reliable pairs of canonical variates is based on the slopes, **a** and **b**.

Another useful optional output is the set of correlations between the canonical variates and the original variables used in deriving them. This output provides a way of interpreting the canonical variates when some of the variates within either the set of independent or the set of dependent variables are highly intercorrelated. These correlations are called *canonical structural coefficients*. Since the canonical variate coefficients can be interpreted as simple correlations between each variable and the canonical variate, they are useful in understanding the relationship between the original variables and the canonical variates.

Useful graphical diagnostics include a scatterplot of scores of the canonical variate V_i on the canonical variate U_i. The graph is useful in uncovering cases of unusual choices such as outliers. Dependence V_1 on U_1 can seemingly appear as nonlinear scatterplot or can have an elliptical shape which is typical for two-dimensional normal distribution. An important criterion is the sample size and a sufficient number of patients related to one variable x (or y). Very small samples of patients do not describe the data matrix correlation well as they obscure a meaningful relationship.

Small samples of patients tend to indicate statistical significance in all cases, since it may be found in a perfect linear combination due to a low degree of freedom. It should be therefore convenient to get at least 5 or 10 patients on 1 variable x (or y) fatty acid and prevent problems from arising due to a too small sample.

Classification symbols V_i and U_i on the left and right sets of a variable in estimating the canonical function are not important. Canonical correlation analysis weighs both canonical variates to maximize the correlation, and does not find any special emphasis on some of the canonical variates.

The goodness-of-fit achieved

The *strength of linear relationship* between canonical variates U_i and V_i is expressed in the canonical correlation coefficient R [17]. The square canonical correlation R^2 represents the size of the shared variance between the two canonical variates U_i and V_i. These squares of canonical correlation are called the *canonical roots*. Statistically, it is appropriate to analyse the canonical variates, of which the canonical correlation coefficients are statistically significant. When other independent canonical variates are statistically insignificant then the corresponding relations between the variables are not explained.

Results

Fatty acids of erythrocyte membranes - EDA

The exploratory data analysis EDA was applied on the concentrations of fatty acids in the erythrocyte membranes. The Box-and-whisker plot represents a graphical measure of the variability of the concentrations of 37 fatty acids examined in the erythrocyte membranes for 45 patients. (Figure 1).

Some publications state that the fatty acids of the erythrocytes membranes better reflect the long-term intake of fatty acids in the diet. However, the box plot of fatty acids in plasma phospholipids fraction and the box plot of fatty acids in erythrocytes membranes were similar.

Canonical Correlation Analysis

Canonical correlation analysis (CCA) generally quantifies *the strength of the linear relationship* in the form of the Pearson correlation coefficient R between two groups of variables of the data matrix while the objective of the principal component analysis (PCA) is the representation of the objects coming from m-dimensional space of properties (here fatty acids) in the new principal component PC-coordinate space usualy two-dimensional [14]. The left data set contains particularly selected fatty acids and the right set contains five given markers. Selections of fatty acids were assessed according to the quality and quantity of their impact on the specified markers. CCA seeks the linear combination of fatty acids which best correlate with the linear combination of specified markers. A linear combination of variables in two sets forming the hypothetical canonical variates U_1 and V_1 is sought, which shows that the maximum value of the Pearson correlation coefficient R. CCA is particularly useful in situations where variables are in each set intrinsically correlated, so that it makes no

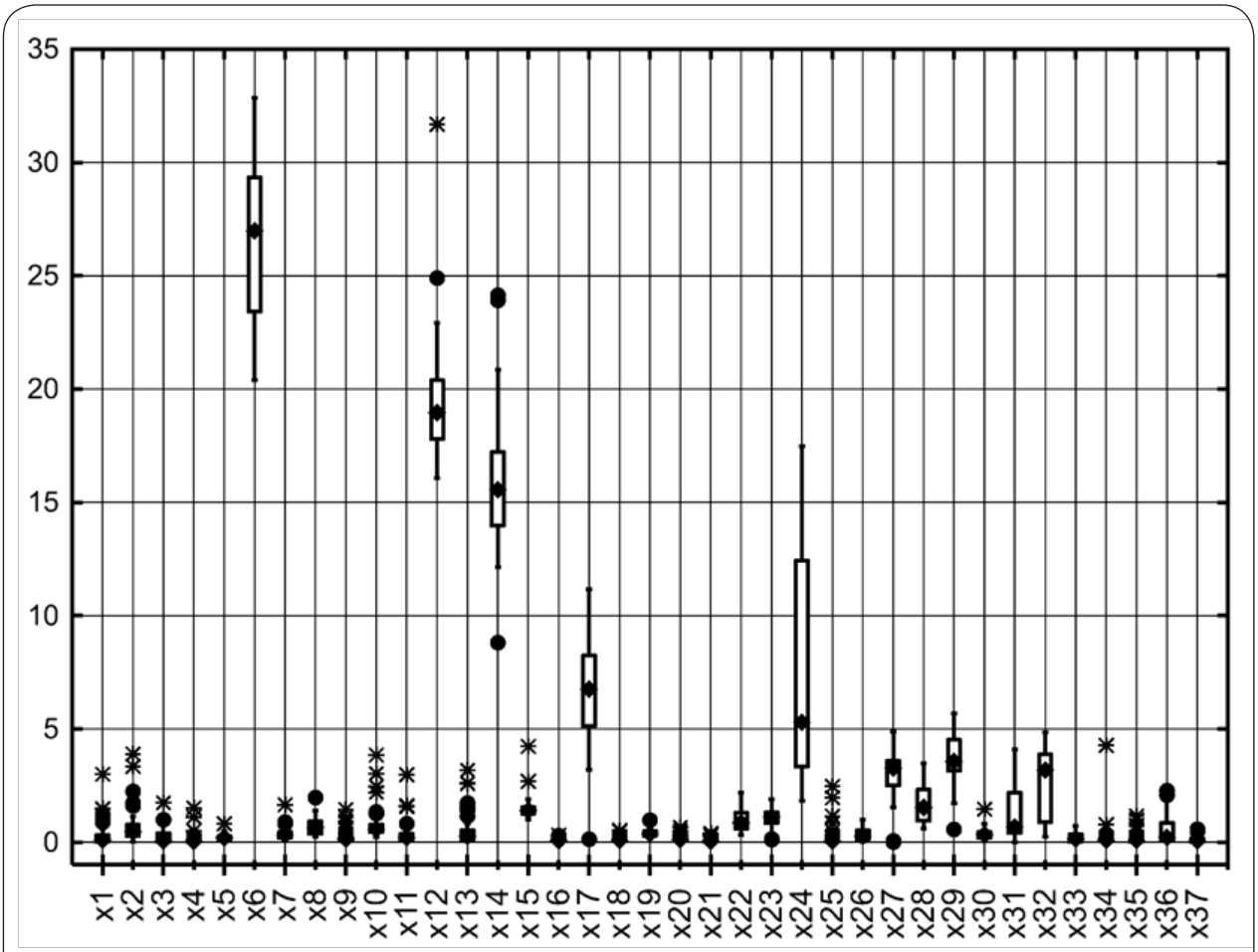

Figure 1. The Box-and-whisker plot represents a graphical measure of the variability of the concentration of 37 fatty acids examined in the erythrocyte membranes for 45 patients. Here the x-coordinate represents the index of an actual fatty acid and y-coordinate is value of the relative concentration in percents. The circles in graph concern the outliers, the stars concern the leverages, the square is for the median, the box states for two limits: the lower quartile and the upper quantile, (STATISTICA, StatSoft, Origin).

sense to evaluate them separately since they are neglecting their intrinsic correlation. CCA binds together two canonical variates to maximize their correlation R, but performs it without any attention to any of the canonical variates U_1 and V_1.

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In an exploratory analysis the box plot proved that variables in both sets generally exhibit sufficient volatility and also correlation between variables in columns so that the data are suitable for canonical correlation analysis. Both canonical variables U and V are symmetrical and equal. Variables of both canonical variates U and V can therefore be freely interchanged. The criterion for considering the number of pairs (U_i, V_i) variates is like in PCA and the Cattel scree plot (**Figure 2**) confirms that the first canonical root U_1 and V_1 will describe data sufficiently.

In **Figure 2** the table of the Pearson Chi^2-test of the statistical significance of correlation coefficient R for pairs of canonical variates (U_1, V_1), (U_2, V_2), (U_3, V_3) is examined. While for (U_1, V_1) $R_1 = 0.8350$ exhibits the calculated a probability $p = 0.0002$ which is smaller than the given $\alpha = 0.05$, the calculated probability p for another two pairs (U_2, V_2), (U_3, V_3) is much greater than $\alpha = 0.05$. The higher pair of canonical variates with correlation coefficients, R_2 and R_3 are therefore statistically insignificant, which concurs with the conclusion of the Cattel scree plot.

The remaining two parts of **Figure 2** (U_2, V_2), (U_3, V_3) show that the scatter diagrams of canonical correlation exhibit the slopes of straight lines statistically insignificant. Therefore, it is appropriate to consider only the first canonical root (U_1, V_1), which leads to the highest canonical correlation. **Figure 2** delivers estimated slope values called in CCA as the *structural coefficients* which represent the correlation coefficients between the original variables in each set and canonical root. It is important to keep in mind that the canonical variate is created in each set of variables as their weighted sum. Another interesting property of the scatter diagram of canonical correlation is the possibility to indicate clusters of similar patients. Some patients are placed at the low values of both canonical variates U and V while the other is at high values, which is related to an intensity of the inflammatory response. The patients with similar diseases will be located on this scatter diagram mutually close. The occurrence of remote patients is also interesting, which is likely related to the occurrence of associated diseases such as diabetes mellitus of the 2nd type, dyslipidemia or hypertension in these patients.

In the diagram on **Figure 2** the estimates of structural coefficients $a_1, ..., a_6$ and of structural coefficients $b_1, ..., b_5$ can be explained as a strength of the linear relationship of markers b_i on the five opposing selected fatty acids. Similarly, one can also consider the strength of the correlation or linear dependence on the concentration of the fatty acid a_i on the five selected opposite markers.

The percentage of selected fatty acids in erythrocyte membranes formed a key to how to sort the fatty acid into six groups according to their similar structural and biochemical properties. Received groups were named according to the fatty acids which bring them together: for example, the group of the methylated fatty acids, saturated fatty acids to C18, saturated fatty acids with a long chain (from C19), n-3 polyunsaturated fatty acids, n-6 polyunsaturated fatty acids and, finally, a group of unsaturated fatty acids with different positions and number of double bonds. Each of these groups was subjected to a canonical correlation analysis and each group of fatty acids was examined in relation to the same set of five markers. The following markers were analysed: increasing CRP after 24 hours (labelled ΔCRP), increase of MDA for 24 hours (labelled ΔMDA), the concentration of TAG in mmol/l (labelled TAG), concentrations of LDL cholesterol in mmol/L (labelled LDL-chol), and finally the concentration of HDL cholesterol in mmol/L (labelled HDL-chol).

A strong dependence of the relationship between the six selected n-3 polyunsaturated fatty acids and selected the five markers was detected, indicating with a high value of the Pearson correlation coefficient R=0.8350 in **Figure 2**. The negative change of the selected five mark-

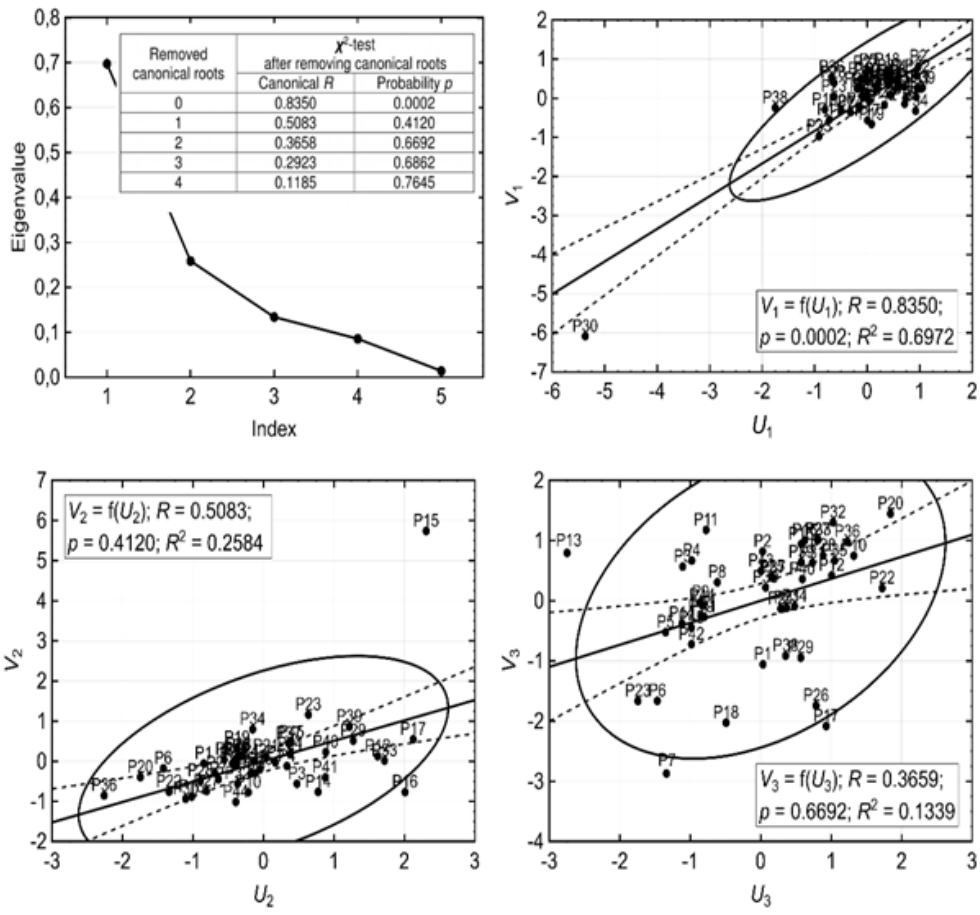

Figure 2. The CCA scatterplot of the first three pairs of canonical variates (U_1, V_1), (U_2, V_2), (U_3, V_3), describing the association of original variables n-3 PUFA on the other five original markers ΔCRP, ΔMDA, HDLchol, LDLchol and TAG indicate that the first pair of canonical variates U1 and V1 sufficiently describe this canonical association for n=45 patients. The first pair of canonical variates U1 and V1 sufficiently describe the canonical association of six original variables n-3 PUFA on other five original markers ΔCRP, ΔMDA, HDLchol, LDLchol and TAG. The magnitude of estimated parameters a and b indicates the strength of the actual variate (i.e. the slope) on the group of opposite variates, (STATISTICA, StatSoft, Origin).

ers was observed in α-linolenic acid with a high value of its structural coefficient a_1=-0.957, and then the effect of eicosapentaenoic acid with a_4=-0.365, which is consistent with published conclusions on anti-inflammatory action of metabolites of *n*-3 fatty acids. The strongest positive effect on the five markers exhibited stearidonic acid of its structural coefficient a_2=0.781 and docosapentaenoic acid with a_5=0.370. While the effect of docosahexaenoic acid and eicosatetraenoic was significantly weaker, it is regarded as insignificant.

Of the selected markers the particularly inflammatory reaction marker ΔCRP with its structural coefficient b_1 = -0.996 was shown in the stronger relation to the set of *n*-3 PUFA. The much weaker relationship was the set of fatty acids with HDL-cholesterol with the lower structural coefficient b_3 = -0.270. For other markers ΔMDA, LDL-cholesterol and TAG levels, the relationship for a very low value of structural coefficient was negligible.

The second highest correlation coefficient *R*, and therefore another most significant relationship was found between the set of five saturated fatty acids of chain length up to C18 and the set of five markers *R* = 0.8175, (**Figure 3**). On the test set of five markers the significantly negative impact of pentadecanoic acid with structural coefficient a_2 = -1.250 was indicated and also the positive effects of palmitic acid with a_3 = 0.672, heptadecanoic acid with a_4 = 0.279. Palmitic acid is described in literature as a pro-inflammatory factor acting harmfully to cells with a number of mechanisms which, for example, causes an endoplasmic reticulum stress of cells. The effect of the myristic acid and stearic acid exhibited a low value of structural coefficient and was insignificant. Another significant and positive relationship was observed in the relation of TAG to the selected group of saturated fatty acids with its structural coefficient b_5 = 0.300. The relationship with LDL-cholesterol was significantly smaller and therefore less important and the relationship ΔMDA and HDL-cholesterol levels against the compared set of saturated fatty acids was according to its low structural factors considered insignificant.

Highly significant correlations with a higher value *R*=0.8031 showed the relationship of five methylated fatty acids tested against a set of same markers as previously (**Figure 4**). The strongest negative impact on a set of tested markers exhibited 12-methyltridecanoic acid with a_1=-0.755, and also 13-methyltetradecanoic acid with a_2=-0.477. Conversely, a significant positive effect of fatty acid had a 14-methylhexadecanoic acid with a_4=0.753. Of the group tested markers the ΔCRP was significant only with its negative value of structural coefficient b_1=-0.981. A significant relationship with a higher correlation coefficient *R*=0.7888 was found between the set of fatty acids which have a different position and number of double bonds against the tested group of markers (**Figure 5**). Of these fatty acids, the greatest negative impact had sapienic acid with a_1=-0.886, followed by *cis*-palmitoleic acid with a_2=-0.438. Conversely, a positive effect on the five markers exhibited *trans*-vaccenic acid with a_3=0.529. The effect of nervonic, oleic and *cis*-vaccenic acids on a set of tested markers was due to the effects of other fatty acids in this group less significant. Of the group of tested markers, a strong relationship at the value of ΔCRP was demonstrated with its structural coefficient b_1=-0.971.

A less significant effect on five markers was found in the *n*-6 polyunsaturated fatty acids with *R*=0.5837, (**Figure 6**). Of this group of fatty acids, the greatest positive impact had arachidonic acid with a_4=2.817, which is consistent with fact that it represents the starting material from which a whole group of highly pro-inflammatory eicosanoids arises. A significant negative impact was detected in docosatetraenoic acid with a_5=-2,004, then also for linoleic acid with a_1=-0.590 and dihomo-γ-linolenic acid with a_3=-0.590. The influence of γ-linolenic acid and docosapentaenoic acid was weak. Of the group tested markers a significantly negative effect was shown in relation ΔCRP with b_1=-0.752, TAG with b_5=-0.411 and then ΔMDA with b_2=-0.277 with the *n*-6 polyunsaturated fatty acids, i.e. an accretion inflammation, oxidative stress and TAG levels in serum is associated with the lower levels of *n*-6 PUFA. On the contrary, a positive influence of HDL-cholesterol was shown with the coefficient b_3=0.339. The effect of LDL-cholesterol was not significant.

The last group of fatty acids which have been investigated in relation to the group of markers tested, is the selected group of saturated fatty acids with a long chain up C19 (**Figure 7**). Its influence on the aforementioned five tested markers was weaker with a lower correlation *R*=0.5473. Of this group of fatty acids, lignoceric acid showed the greatest positive effect on the five markers with a_4=1.445 while behenic acid had the largest negative influence of with a_3=-1.838. The remaining fatty acids (such as cerotic, arachidic and nonadecanoic acids) were due to the behenic and lignoceric acids considered less important, particularly because of the relatively lower correlation coefficient *R*.

The next step in the canonical correlation analysis was a search for the ideal combination of fatty acids that ensure the highest possible value of the Pearson´s correlation coefficient *R*. The following combination of fatty acids compiled according to different criteria were tested:

The first combination of fatty acids was the fatty acids selected according to their greatest variability in erythrocyte membranes. These were fatty acids as palmitic x_6, stearic x_{12}, oleic x_{14}, linoleic x_{17}, arachidonic x_{24} and docosahexaenic acids x_{32}, wherein *R*=0.6496.

The second group of fatty acids was chosen according to the results of linear regression, i.e. 12-methyltetradecanoic x_4, *trans*-vaccenic x_{13}, nonadecanoic x_{16}, arachidic x_{19}, eicosatetraenoic x_{25}, cerotic x_{33} and tetracosapentaenoic

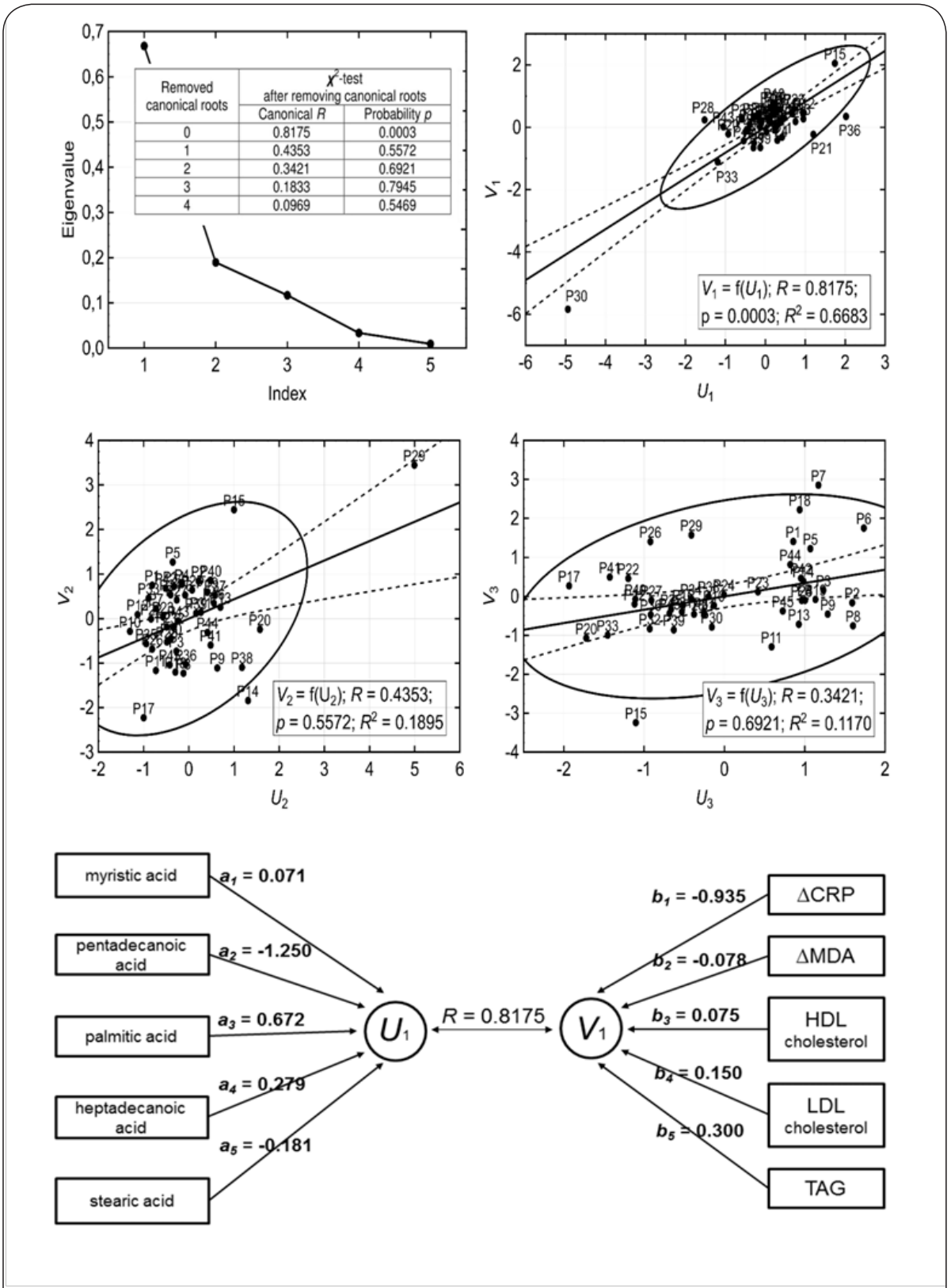

Figure 3. The same as Fig. 2 but describing the association of saturated fatty acids (to C18).

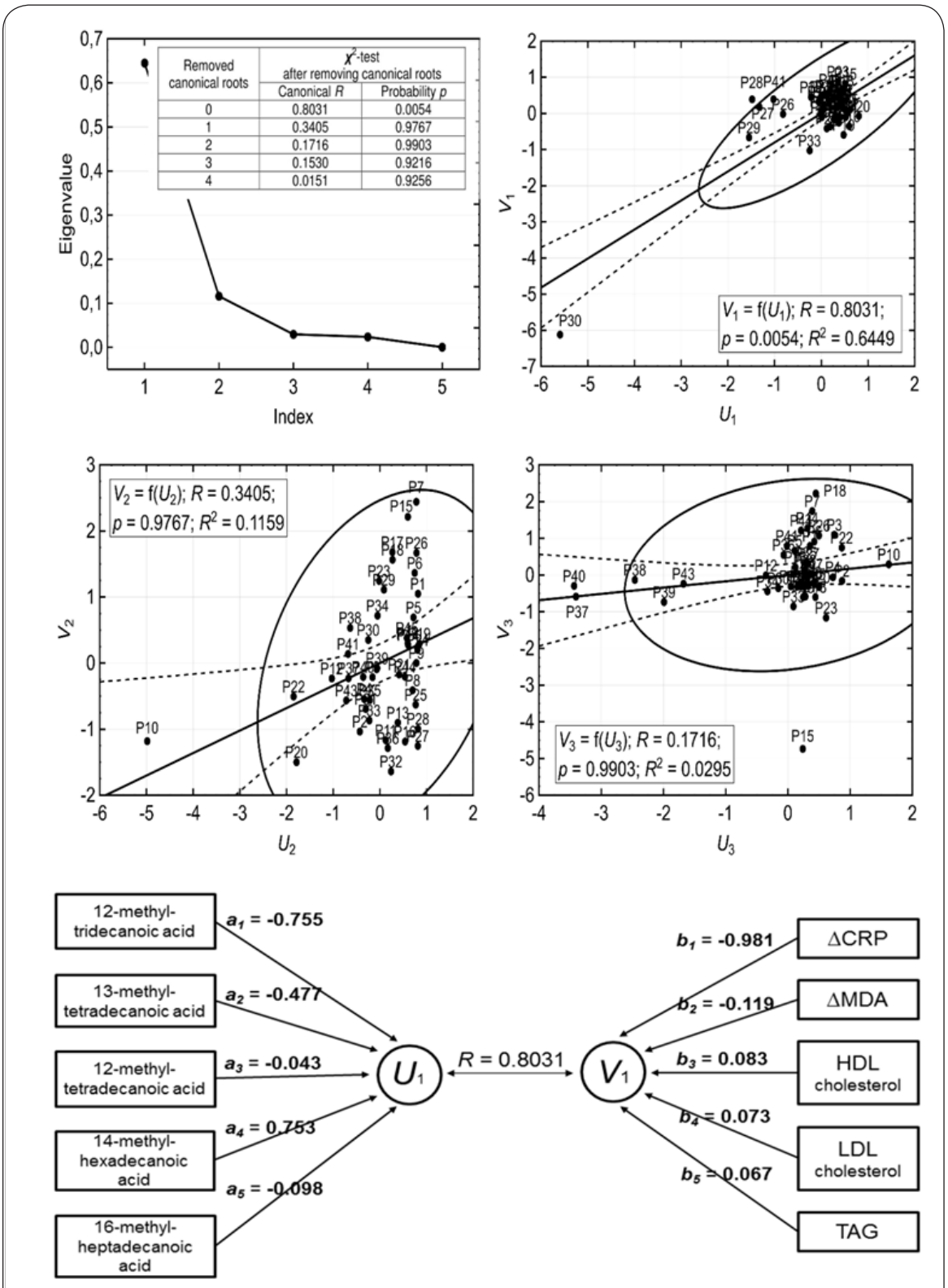

Figure 4. The same as Fig. 2 but describing the association of methylated fatty acids.

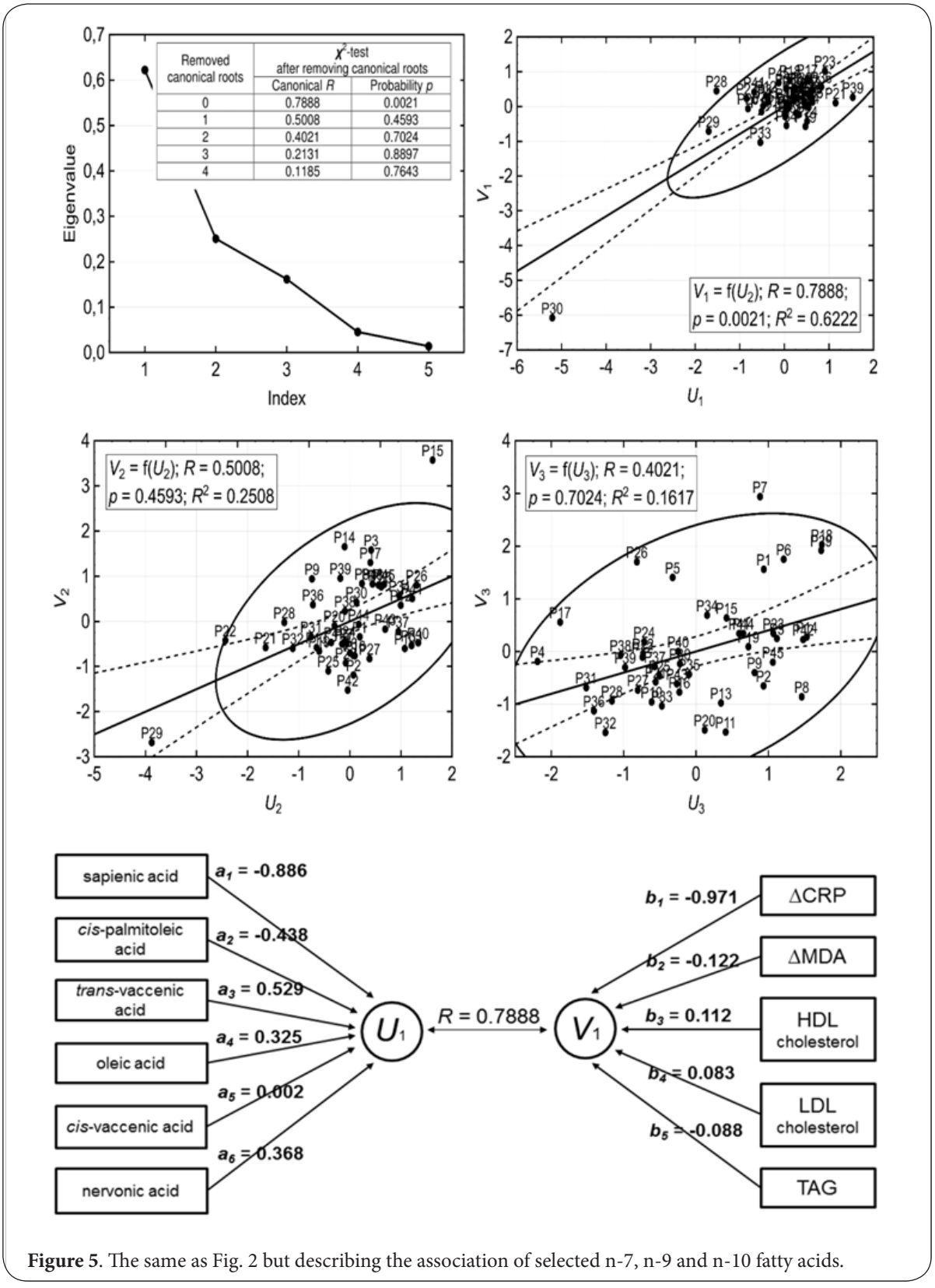

Figure 5. The same as Fig. 2 but describing the association of selected n-7, n-9 and n-10 fatty acids.

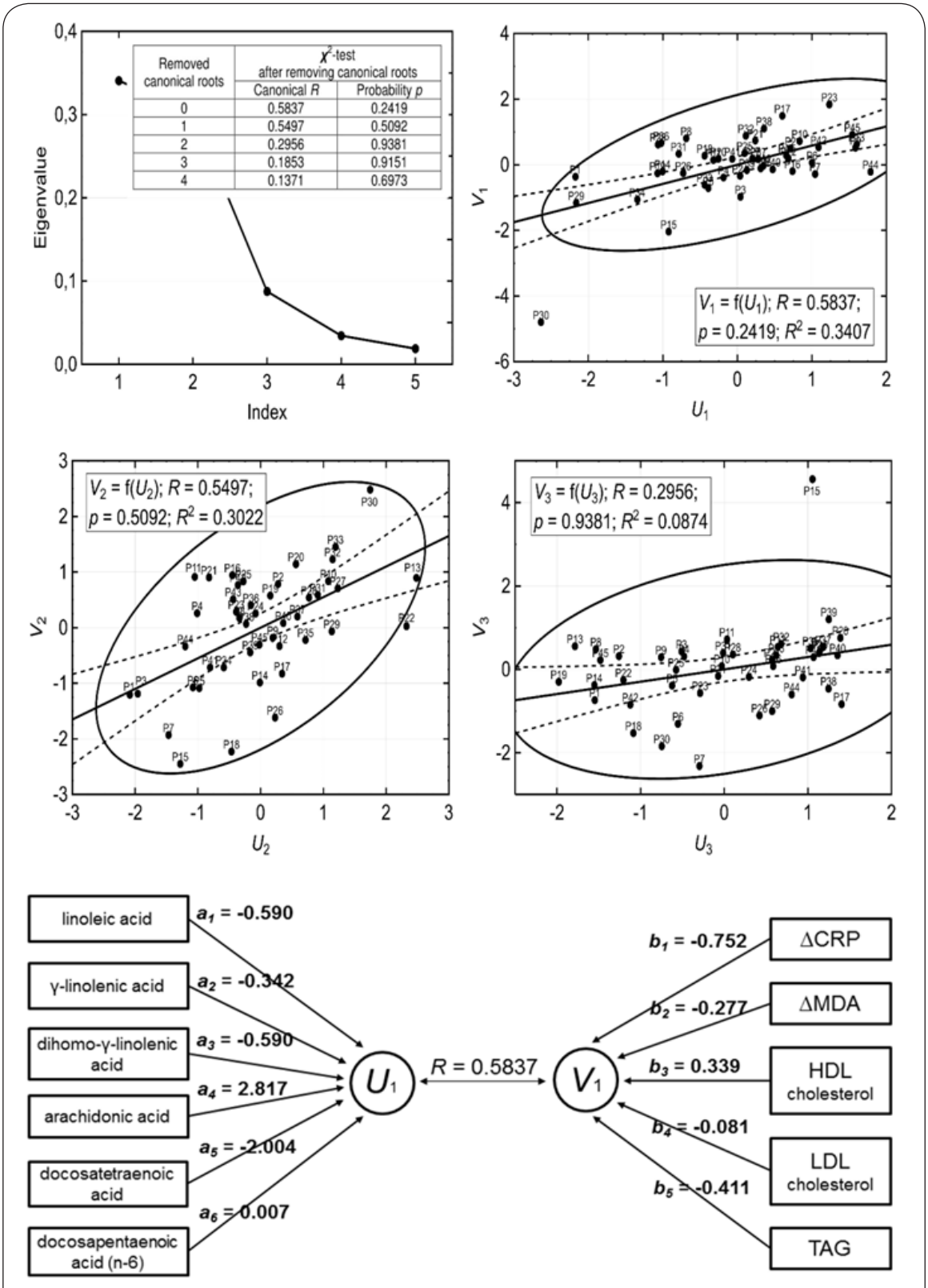

Figure 6. The same as Fig. 2 but describing the association of selected n-6 PUFA.

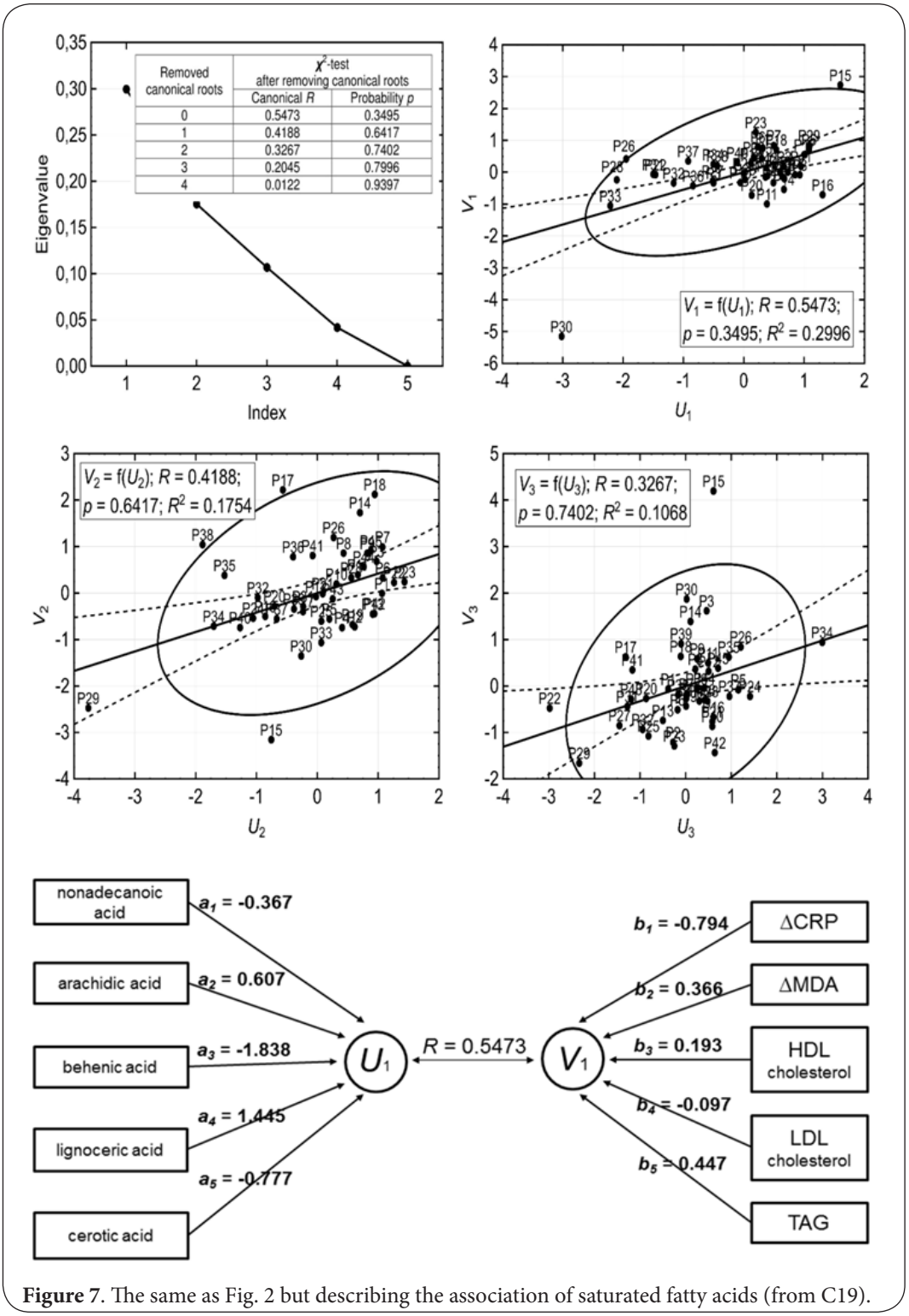

Figure 7. The same as Fig. 2 but describing the association of saturated fatty acids (from C19).

x_{36} acids. Their R=0.7800.

Other groups were the combination of n-3 and n-6 polyunsaturated fatty acids linoleic x_{17}, γ-linolenic x_{18}, dihomo-γ-linolenic x_{22}, arachidonic x_{24}, eicosapentaenoic x_{26}, docosapentaenoic x_{31}, docosahexaenoic x_{32} wherein R = 0.5633 and docosapentaenoic x_{30}, docosapentaenoic x_{31}, tetracosatetraenoic x_{34}, tetracosapentaenoic x_{35}, tetracosapentaenoic x_{36}, tetracosahexaenoic x_{37} wherein R=0.6082.

In the last group there are selected fatty acids according to the most negative and the most positive slope value ***a*** evaluated in the canonical correlation analysis. The most positive group of the fatty acid contains 12-methyltetradecanoic x_4, palmitic x_6, *trans*-vaccenic x_{13}, stearidonic x_{21}, arachidonic x_{24} and lignoceric x_{27} acids with R=0.5781.

By far the most interesting seems to be a group of fatty acids selected according to the most negative slope values ***a***, thus 12-methyltridecanoic x_1, pentadecanoic x_5, sapienic x_7, α-linolenic x_{20}, behenic x_{23} and docosatetraenoic x_{28} acids, wherein R=0.8696. The results of this last analysis are shown in **Figure 8**.

Discussion

Canonical correlation analysis showed fatty acid groups determined in membranes of erythrocytes, which most strongly affect the five selected markers. It regarded an increase in C-reactive protein and malondialdehyde after 24 hours, triacylglycerols, HDL-cholesterol and LDL-cholesterol in plasma. Further, the positive and negative power of each fatty acid in their group was defined, which affects the five said markers.

Of the selected markers, the inflammation C-reactive marker showed the strongest relation to all the selected groups of the fatty acid. The relationship of C-reactive protein and selected fatty acids is mentioned, for example, in the American study, confirming that the n-3 polyunsaturated fatty acids and especially α-linolenic acid significantly reduce levels of C-reactive protein. Inversely proportional to the C-reactive protein levels were eicosapentaenoic acid and docosahexaenoic acids [18]. The relationship between saturated fatty acids and C-reactive protein is described in the Polish study which found that the total saturated fatty acids and monounsaturated fatty acids are positively correlated with serum levels of C-reactive protein, in this case considered in pathologically obese women [19].

Using the canonical correlation analysis our results showed a strong dependence between the group n-3 polyunsaturated fatty acids and the five selected markers. The strongest inverse relationship was demonstrated between the acids α-linolenic x_{20}, eicosapentaenoic x_{26} due to the C-reactive protein, and the rest of four markers, which is consistent with published conclusions about inflammatory action metabolites of n-3 fatty acids. Consistent results also were also shown by the afore mentioned American study [18]. In a Japanese study in 2008 the relationship of n-3 polyunsaturated fatty acids and C-reactive protein in serum was examined. The results show that the intake of n-3 polyunsaturated fatty acids is higher, and the level of C-reactive protein in healthy patients is lower: therefore the inflammatory process is attenuated with the n-3 polyunsaturated fatty acid [20]. The conclusion of this study supports our conclusions about the effects of eicosapentaenoic and α-linolenic acid. Other markers evaluated were triacylglycerols concentration, HDL-cholesterol and LDL-cholesterol levels and an increase of malondialdehyde after 24 hours from the time of stent implantation. All these markers were in a ten-times smaller relation to all particular groups of fatty acids than was an increase of the plasma concentrations of the C-reactive protein 24 hours after stent implantation.

As a certainly strong dependence, the relationship between a group of six selected fatty acids having the most negative value of the slope ***a*** was proven namely 12-methyltridecanoic, pentadecanoic, sapienic, α-linolenic, behenic, and docosatetraenoic acids with the five selected markers. Fatty acids with a positive value of the slope ***a***, and therefore with the largest positive effect on the five markers, were fatty acids such as arachidonic, lignoceric, stearidonic, 14-methylhexadecanoic, palmitic and *trans*-vaccenic. Of particular interest among these is the arachidonic acid, which is the basis for the formation of inflammatory mediators such as prostaglandins and leukotrienes [21]. In our case, the resulting effects were pro-inflammatory but not to such an extent that significantly exceeded all other fatty acids.

Conclusion

In the canonical correlation analysis the relationship between the percentage of selected groups of the fatty acids in membranes of erythrocytes and a set of five markers was assessed: An increase in C-reactive protein 24 hours after stent implantation, the increase of malondialdehyde after 24 hours, plasma triacylglycerols, LDL-cholesterol and HDL-cholesterol. Canonical correlation analysis showed that the inverse correlation with the said markers are α-linolenic and eicosapentaenoic acid of the examined group of n-3 polyunsaturated fatty acids and of other selected groups there are acid pentadecanoic, docosatetraenoic, behenic, sapienic and 12-methyltridecanoic. The strongest positive relationship and a positive correlation with the observed markers exhibit fatty acids such as arachidonic, lignoceric, stearidonic, 14-methylhexadecanoic, palmitic and *trans*-vaccenic.

The the marker of inflammation C-reactive protein was indicated to have the strongest relation to all the selected groups of the fatty acid. While the other tested markers, i.e. an increase of malondialdehyde after 24 hours, the plasma concentrations of LDL-cholesterol, HDL-cholesterol and triacylglycerols were up to ten times smaller in relation

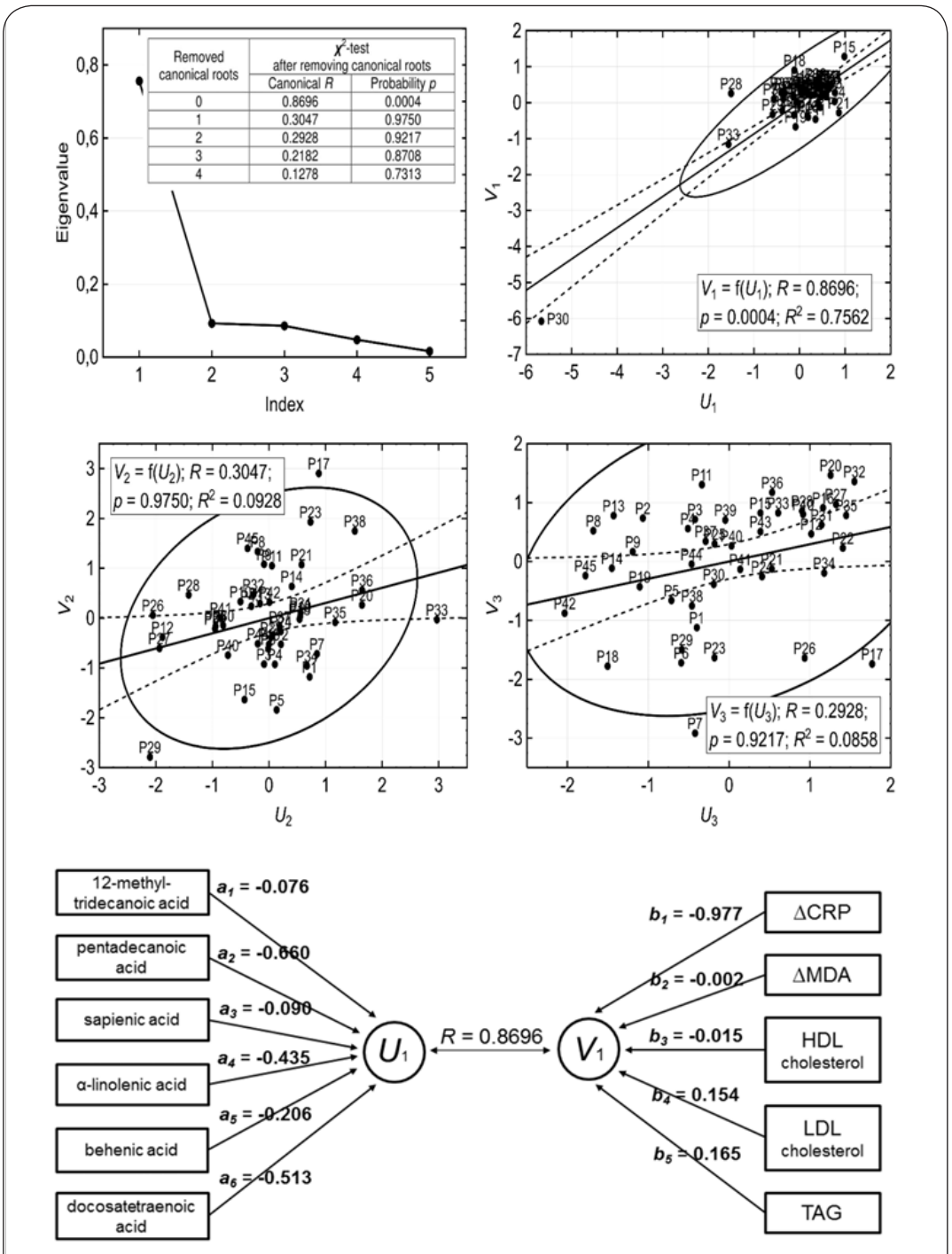

Figure 8. The same as Fig. 2 but describing the association of selected fatty acids according the most negative values.

to particular groups of fatty acids than an increase of the plasma concentrations of C-reactive protein 24 hours after stent implantation.

Abbreviations

CCA: Canonical correlation analysis
CHD: Coronary heart disease
CRP: C-reactive protein
EDA: Exploratory data analysis
EDTA: Ethylenediaminetetraacetic acid
HDL: High density lipoproteins
HDL-chol: HDL cholesterol

hsCRP: high sensitivity C-reactive protein
IL-1β: Interleukin-1β
IL-6: Interleukin-6
LDL: Low Density Lipoproteins
LDL-chol: LDL cholesterol
MDA: Malondialdehyde
PCA: Principal component analysis
PCI: Percutaneous coronary intervention
PUFA: Polyunsaturated fatty acids
TAG: Triacylglycerols
TNF-α: Tumor necrosis factor-α

Competing interests

The authors declare that they have no competing interests.

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