Scientific paper

Multivariate Data Analysis of Erythrocyte Membrane Phospholipid Fatty Acid Profiles in the Discrimination Between Normal Blood Tissue and Various Disease States

Zdenka Cencič Kodba,[1] Darinka Brodnjak Vončina,[2,*] Marjana Novič[3] and Uroš Potočnik[2,4]

[1] *Public Health Institute, Environmental Protection Institute, Prvomajska 1, 2000 Maribor, Slovenia*

[2] *Faculty of Chemistry and Chemical Engineering, University of Maribor, Smetanova 17, 2000 Maribor, Slovenia*

[3] *National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia*

[4] *Faculty of Medicine, Center for human molecular genetics and pharmacogenomics, Slomškov trg 15, 2000 Maribor, Slovenia*

* *Corresponding author: E-mail: darinka.brodnjak@uni-mb.si*

Received: 17-05-2010

This paper is dedicated to Professor Milan Randić on the occasion of his 80th birthday

Abstract

The investigation presented here aims to compare the fatty acid composition of red blood cells of patients with different disease states and to test the hypothesis that the changes in fatty acid profiles derived from erythrocyte phospholipids might be relevant to various diseases. The study sample consisted of 342 blood donors, among them 135 with inflammatory bowel disease, 53 with uterine leiomyoma, 14 with verified absence of uterine leiomyoma, 52 with asthma, 18 with colon adenomas, and 70 blood samples without any of mentioned diseases that was used as a control group. After the isolation of erythrocytes from blood samples, total extracted lipids were separated by solid-phase extraction (SPE) into non polar lipids and polar phospholipids. After the saponification of phospholipid fraction, the esterification process followed with boron trifluoride-methanol reagent. The fatty acid methyl ester (FAME) composition of the total red blood cell phospholipid fraction was analyzed by gas chromatography (GC) with flame ionization detector (FID). Additionally two fatty aldehyde dimethyl acetals (hexadecanal and octadecanal dimethyl acetals; 16:0 DMA and 18:0 DMA) derived from erythrocyte membrane plasmalogen phospholipids were also determined. The resulting fatty acid and plasmalogen linked fatty acid composition was evaluated by the principal component analysis (PCA). We demonstrated decreased levels of omega-3 polyunsaturated fatty acids (n-3 PUFAs) in red blood cell membrane of patients with colon adenomas. Also, a large negative correlation was observed among all samples between the quantity of saturated acids and arachidonic (20:4n6) acid as well as saturated acids and adrenic (22:4n6) acid. In PCA score plot a group of female donors is distinguished mainly by the content of linoleic (18:2n6) acid; a small subgroup shows its concentration highly above the average value. At the same time, the same subgroup has both dimethyl acetals below the average concentrations. The study demonstrates feasibility of multivariate data analysis in discrimination of patients with different diseases according to fatty acid profile and suggests considerable differences in membrane fatty acid profiles in patients with various disease states.

Keywords: Erythrocyte phospholipids, Cell membranes, Fatty acid profiles, Differentiation, Disease states, Gas chromatography, Principal Component Analysis

1. Introduction

The non-nucleated red cell is unique among human cells in that the membrane, its only structural component, accounts for all of its antigenic, transport and mechanical characteristics. The cell membrane is one of the key factors that have to be explained in details due to its transport function. It provides structure and main-

tains physicochemical properties. The membrane lipid bilayer is composed of equal proportions by weight of cholesterol and phospholipids. While cholesterol is thought to be distributed equally between the layers, the four phospholipids are asymmetrically disposed. Several different types of energy dependent and independent phospholipid transport proteins have been implicated in generating and maintaining phospholipids asymmetry. "Flippases" move phospholipids from the outer to the inner monolayer, while "floppases" do the opposite. Although several different membrane proteins have been said to exert these different lipid transport activities in human red cells, there is still considerable debate about their identity.[1] They are not so well characterized regarding their 3D structure due to difficulties connected to crystallization. Nevertheless, we witness growing information on protein structures available in public databases, which challenges the researchers to apply bioinformatics methods for data exploration. Different topological indices are often used to find correlation between chemical structure and molecular property, branching index being among the most successful one.[2,3] The mathematical methods including graph-theoretical ones have proven to be successful means when comparing different proteins regarding their amino acid sequences.[4–7] Another means of information about proteins is 2D gel electrophoresis for proteomics. These modern experimental methods produce a large amount of data, which need some automated treatment to extract as much information as possible applying bioinformatics. In the recent period Randic et al.[8–12] proposed several methods for extracting collective information on proteomes contained in a two-dimensional gel, so called bubble diagram. A convenient system of classification of lipids, based on the definition of Christie,[13] considers the simple lipids, also referred to as neutral or non-polar lipids, and polar or complex lipids. Simple lipids comprise cholesterol and cholesteryl esters, free fatty acids and glycerides. Complex lipids comprise phospholipids, glycolipids, and sphingolipids. The study of the lipid classes in their natural environment is termed lipidomics. This is a new field of research that focuses on the role specific lipids play in physiological and disease states.

When the membranes are analysed for the composition of phospholipid fatty acid profiles, they can be explored by chemometrics instead of bioinformatics, which is dealing with more complex biological systems. First of all, lipids must be isolated from the sample and separate according polarity. The method for preparation of total lipid extract from biological samples was described by Folch et al. and Bligh et al.[14,15] Separation of phospholipids is performed by silica SPE which was found to be the suitable material.[16] Gas chromatography is usually used for qualitative and quantitative determination of methyl esters of fatty acids.

In this work we analysed 342 blood samples for the fatty acid composition of erythrocyte membranes. The chemometrics methods using multivariate data analysis have been used in order to classify, compare and differentiate patients with different diseases: (0) control group, (1) inflammatory bowel disease – I, (2) asthma, (3) uterine leiomyoma, (4) without uterine leiomyoma, (5) inflammatory bowel disease – II and (6) colon adenomas, according fatty acid composition in erythrocyte membrane. The inflammatory bowel disease – I group represents selected subgroup of inflammatory bowel disease (IBD) patients with Crohn disease, who do not respond to standard IBD treatment with corticosteroids and may develop most severe forms of the disease. The inflammatory bowel disease – II group of patients represent unselected IBD patients. We found that the changes in fatty acid profiles derived from erythrocyte phospholipids are indicative for the disease states considered in this study.

2. Experimental

2. 1. Reagents and Standards

Bond Elut Silica solid phase extraction cartridges (SPE, 3 mL/500 mg) were purchased from Varian (P/N 14102037). Chloroform, methanol, n-hexane and potassium hydroxide were obtained from J. T. Baker while boron trifluoride in methanol (20% w/v), sodium chloride and sodium sulphate were purchased from Merck. Phosphate buffered saline (PBS) was purchased from Medicago (Uppsala, Sweden). The Ficoll-Paque PLUS reagent (a sterile density gradient centrifugation medium for separation of mononuclear cells from human blood) was obtained from GE Healthcare (Uppsala, Sweden).

AOCS Standard 3 (Restek, P/N 35024), cis / trans oleic fatty acid methyl ester mix (Restek, P/N 35079), Supelco 37 component fatty acid methyl ester mix (Supelco, P/N 47885-U), linoleic (Supelco, P/N 47791) and linolenic (Supelco, P/N 47792) fatty acid methyl ester isomer mix were prepared by dissolving fatty acid methyl esters in n-hexane, stored at –20 °C and were used for peak identification.

2. 2. Sample Preparation

Human erythrocyte samples were obtained from clinical genetic studies.[17–19] Characteristics of the 342 subjects are shown in Table 2. The isolation of erythrocytes was performed as follows: 9 mL of PBS and 9 mL of Ficoll-Paque PLUS reagent was added to 9 mL of peripheral blood and centrifuged at 1800 rpm for 30 min at 18 °C. After centrifugation, upper layer containing serum, leukocytes and Ficoll reagent were removed from the tube and erythrocytes were collected as the lower layer. All samples were stored at –80 °C prior to analyses.

2. 2. 1. Lipid Extraction and Isolation of Phospholipids

Lipid extraction was necessary to remove any other constituents such as proteins, sugars or other small molecules that might interfere the chromatographic steps. Human erythrocytes were stored in PTFE screw-capped Pyrex tubes. Before extraction, samples were thawed at room temperature and homogenized thoroughly. Lipids were extracted according to modified Folch procedure[14] to get the final volume ratio (0.9% NaCl solution: chloroform : methanol; 0.2 : 2 : 1, v/v/v). Tubes were centrifuged at 2500 rpm at 20 °C for 15 min to facilitate phase separation. The lower chloroform phase was carefully transferred through the protein disc to another test tube and left drying with anhydrous sodium sulphate. Then it was evaporated to dryness with a steam of nitrogen. Extracted lipids were re-dissolved in about 500 µL chloroform, vortex mixed at high speed and applied to the SPE Silica cartridges previously conditioned with 5 mL methanol followed by 5 mL chloroform. After sample loading, the column was washed with 5 mL chloroform followed with 5 mL chloroform : methanol (49 : 1, v/v) to remove the neutral lipids. Then phospholipids were eluted with 5 mL of methanol.

2. 2. 2. Preparation of Fatty Acid Methyl Esters

Preparation of fatty acid methyl esters was carried out according to standard method.[20] The identities of sample methyl ester peaks were determined by comparison of their relative retention times with those of FAME standards. Quantification was accomplished by standard normalization and expressed in relative amounts (wt. % of total fatty acids). No correction factors or internal standards were used.

2. 3. Gas Chromatography

GC analyses were performed on an AT6890N model (Agilent Technologies, USA) equipped with a split/splitless injector and FID. Separation of FAME was carried out on a fused silica capillary column (Supelco, P/N 23348-U, 75 m × 0,18 mm i.d., 0,14 µm film thickness), coated with a very polar bis-cyanopropyl SP-2560 stationary phase. Helium was used as a carrier (0.4 mL min^{-1}) and nitrogen 6.0 as a makeup (10 mL min^{-1}) gas. Hydrogen flow was set to 35 mL min^{-1} and the airflow to 300 mL min^{-1}. The amount injected was 1 µl and the split ratio was 100:1.

The temperature of the injector and the detector were 230 °C and 300 °C, respectively. The oven temperature was held at 140 °C for 1 min, then programmed to 180 °C at 10 °C min^{-1} and held there for 5 min. Afterwards the temperature was increased to 230 °C at 1.5 °C min^{-1} and held there for 1.1 min, then increased to 235 °C at 5 °C min^{-1} and held for 12.57 min. The total run takes 58 min.

3. Results and Discussion

3. 1. Impact of Phospholipid Composition on Membrane Structure and Function

The membrane proteins are incorporated in the bilayer composed mainly of lipids. Plasmalogens as a phospholipids subclass, also found in cell membrane are thought to be very sensible to oxidation reactions and may have a role as a natural marker of oxidative stress.[21,22] Lipid peroxidation contributes to the loss of cellular functions through the inactivation of membrane-bound enzymes which may be associated with changes in the fatty acid profiles in erythrocytes of hypertensive and normal subjects.[23] Essential fatty acids and their longer chain unsaturated derivatives, are structural components of cell membrane phospholipids and as such important determinants of membrane function. Phospholipids constitute 60% of the lipid mass of an eukaryotic cell membrane.[24] Therefore, changes in phospholipids may lead to changes in the membrane that might have major consequences on cell function in health and disease state. The ubiquitous nature of lipids in the biological system establishes them as a key player in many cellular functions. The ability to differentiate the remarkably similar phospholipids in biological samples might be used as diagnostic tool for disease states.

3. 2. Data Analysis

The methyl esters of 28 fatty acids and 2 dimethyl acetals determined in this study are presented in Table 1. The 342 samples of human erythrocytes are characterized by the composition of 28 fatty acids, 2 dimethyl acetals, 6 groups of fatty acids grouped by their properties (S, M, P, T, n3, n6, see Table 1, six bottom rows) and 3 ratios (M/S, M/P, n3/n6). The levels of 28 fatty acids and 2 dimethyl acetals represent 30 variables, components of the vector representation of each sample, further used in chemometrics analysis.[25–27] The erythrocyte samples belong to one control group and six groups of patients with a different disease: (0) control group, (1) inflammatory bowel disease – I, (2) asthma, (3) uterine leiomyoma, (4) without uterine leiomyoma, (5) inflammatory bowel disease – II and (6) colon adenomas. From 342 samples, 135 belong to patients with inflammatory bowel disease, 53 with and 14 without uterine leiomyoma, 52 with asthma, 18 with colon adenomas, while 70 of them served as a control group without any of mentioned diseases as shown in Table 2. The mutual correlation coefficients between all fatty acid regarding their percentage levels were calculated. The PCA was applied for grouping of 342 samples according to the measured fatty acid composition. All the calculations and plots in the following (PCA) section were done with the Teach/Me software[26] using Teach/Me Data Analysis option, which is one of the applications of the

Table 1: A list of fatty acids and dimethyl acetals determined in erythrocyte phospholipid samples.

Fatty acids	Common name	Numeric name
Saturated fatty acids (S, SatFAs)		
	Myristic acid	14:0
	Pentadecanoic acid	15:0
	Palmitic acid	16:0
	Margaric acid	17:0
	Stearic acid	18:0
	Arachidic acid	20:0
	Behenic acid	22:0
	Lignoceric acid	24:0
Monounsaturated fatty acids (M, MUFAs)		
n7		
16:1 (9c)	Palmitoleic acid	16:1n7
18:1 (11c)	Vaccenic acid	18:1n7
n9		
18:1 (9c)	Oleic acid	18:1n9
20:1 (11c)	Eicosenoic acid	20:1n9
22:1 (13c)	Erucic acid	22:1n9
24:1 (15c)	Nervonic acid	
Polyunsaturated fatty acids (P, PUFAs)		24:1n9
n6		
18:2 (9,12)c	Linoleic acid	18:2n6
18:3 (6,9,12)c	Gammalinolenic acid (GLA)	18:3n6
20:2 (11,14)c	Eicosadienoic acid	20:2n6
20:3 (8,11,14)c	Dihomo gamma linolenic acid (DGLA)	20:3n6
20:4 (5,8,11,14)c	Arachidonic acid (AA)	20:4n6
22:4 (7,10,13,16)c	Adrenic acid	22:4n6
22:5n6 (4,7,10,13,16)c	Docosapentaenoic acid	22:5n6
n3		
18:3 (9,12,15)c	Alpha linolenic acid (ALA)	18:3n3
18:4 (6, 9, 12, 15)c	Stearidonic acid (STD)	18:4n3
20:5 (5,8.11.14,17)c	Eicosapentaenoic acid (EPA)	20:5n3
22:5 (7,10,13,16,19)c	Docosapentaenoic acid (DPA)	22:5n3
22:6 (4,7,10,13,16,19)c	Docosahexaenoic acid (DHA)	22:6n3
Trans fatty acids (T, TransFAs)		
18 :1 (6+9+11)t		
18 :3 (9t, 12c, 15t)		
Fatty aldehyde dimethyl acetals (DMAs)		
	Hexadecanal dimethyl acetal	16:0 DMA
	Octadecanal dimethyl acetal	18:0 DMA
		Abbreviation
Sum of saturated fatty acids (SatFAs)		S
Sum of monounsaturated fatty acids (MUFAs)		M
Sum of polyunsaturated fatty acids (PUFAs)		P
Sum of trans fatty acids (TransFAs)		T
Sum n-3 PUFAs		n3
Sum n-6 PUFAs		n6

Teach/Me system, providing very flexible tools for most fields of data analysis.

A typical chromatogram of a human erythrocyte membrane phospholipid fatty acid profile can be seen in Fig. 1. Twenty-eight fatty acid methyl esters and two dimethyl acetals were identified. There are seven major compounds: 16:0, 18:0, 18:0 DMA, 18:1n9, 18:2n6, 20:4n6 and 22:4n6 besides two omega-3 ones: 22:5n3 and 22:6n3.

3. 3. Statistical Screening of Data

The cross correlation matrix between the quantities of 30 variables was calculated. Besides obvious mutual correlations between a group of fatty acids, such as M, P, T, S, n3 and n6 (see Table 1), and individual members of these groups (see Table 1), some additional interesting correlations were found. A large negative correlation coefficient (–0.964) was observed between the quantity of saturated acids (S) and arachidonic (20:4n6) acid.

Table 2: The characteristics and classification of blood donors.

Number of samples	Age (year)	Sex (female, %)	Disease	Label
70	42 ± 12	53	no disease (control)	0
60	34 ± 9	71	inflammatory bowel disease – I	1
52	14 ± 3	43	asthma	2
53	42 ± 6	100	uterine leiomyoma	3
14	62 ± 12	100	without uterine leiomyoma	4
75	40 ± 15	56	inflammatory bowel disease – II	5
18	65 ± 13	44	colon adenomas	6

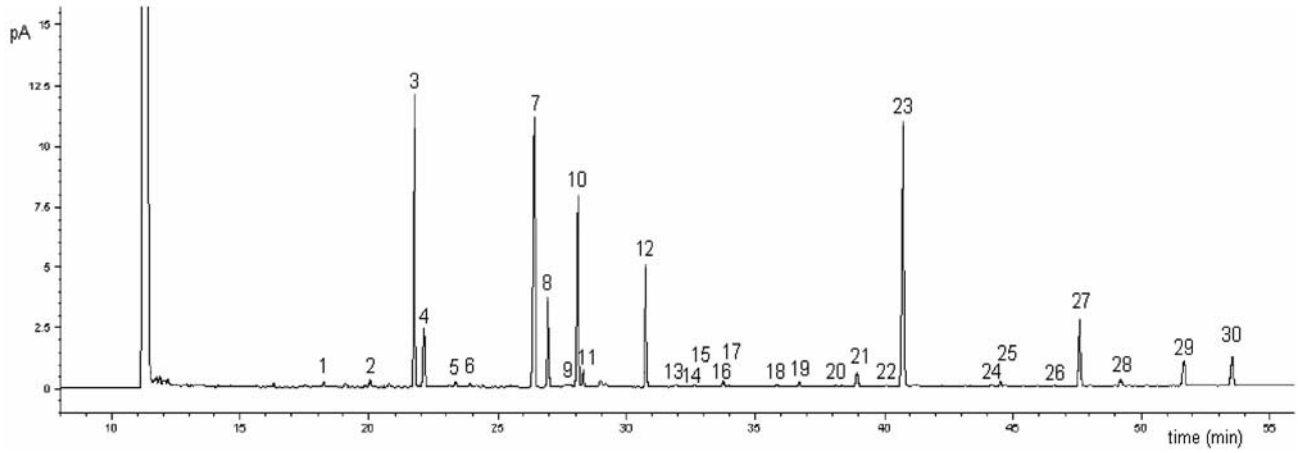

Figure 1. GC chromatogram of FAMEs and DMAs from phospholipid human erythrocytes. Peak identification: (1) 14:0, (2) 15:0, (**3**) 16:0, (4) 16:0 DMA, (5) 16:1n7, (6) 17:0, (**7**) 18:0, (**8**) 18:0 DMA, (9) sum18:1 trans, (**10**) 18:1n9, (11) 18:1n7, (**12**) 18:2n6, (13) 20:0, (14) 18:3*(9t, 12c, 15t)*, (15) 18:3n6, (16) 20:1n9, (17) 18:3n3, (18) 18:4n3, (19) 20:2n6, (20) 22:0, (21) 20:3n6, (22) 22:1n9, (**23**) 20:4n6, (24) 20:5n3, (25) 24:0, (26) 24:1n9, (**27**) 22:4n6, (28) 22:5n6, (**29**) 22:5n3, (**30**) 22:6n3.

From Figs. 2 and 3 it can be seen that the correlations between the saturated acids (S) and arachidonic (20:4n6) acid, and saturated acids (S) and adrenic (22:4n6) acid are highly negative (r = –0.964 and –0.903, respectively). Similar observations were found in the studies of diabetic patients.[28,29]

Different correlations of specific fatty acids were observed is the study of Sepulveda et al.,[30] where the aut-

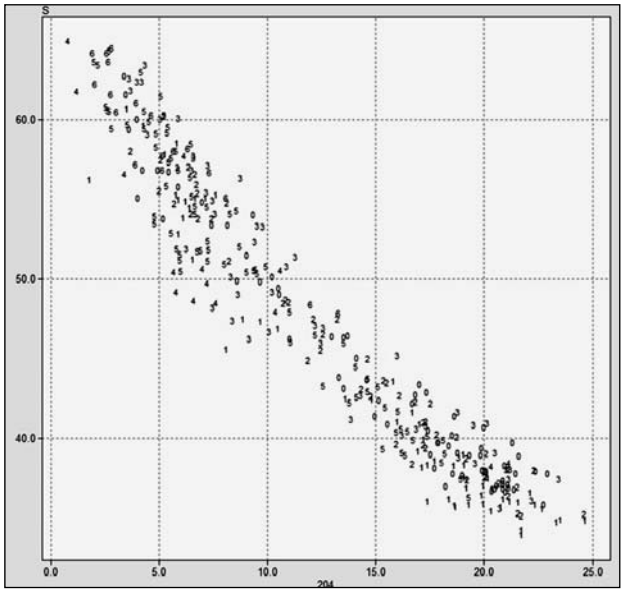

Figure 2. Relationship between percentage levels of saturated acids (S) and arachidonic (20:4n6) acid in 342 samples.

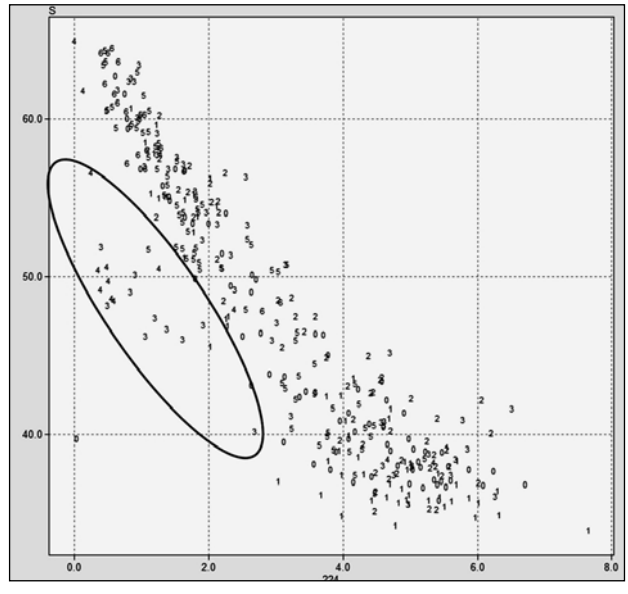

Figure 3. Relationship between percentage levels of saturated acids (S) and adrenic (22:4n6) acid in 342 samples.

hors concluded that the erythrocyte accumulation of unsaturated fatty acids may be a useful indicator of vascular disease. A significant increase in n-3 PUFA content, in the ratio of n-3 PUFA against arachidonic acid, was correlated to bone mineral density.[31]

In Fig. 3 it is shown that already on the basis of one of two variables, namely S and adrenic (22:4n6) acid, two groups of samples are distinguished. Individual samples belonging to female patients with and without uterine leiomyoma, namely classes 3 and 4, encircled in Fig. 3 form one of these groups. They can be found considerably below the majority of samples, so we can say that there are two sets of regression parameters for these two groups. Negative correlation has also been found by Rodriguez et al.[28] between fasting insuline and arachidonic (20:4n6) or n-6 fatty acids, which can be used as surrogate measure of insuline resistance. While value of the parent linoleic (18:2n6) acid was found to be higher in diabetic patients, values of arachidonic (20:4n6) acid was reported to be lower.[29]

An expected correlation between the levels of arachidonic (20:4n6) acid and its elongation product adrenic (22:4n6) acid is present (r = 0.932) and does not show the imbalance within these n-6 polyunsaturated fatty acid pathway except for a small group of female patients shown in Fig. 4 below.

Another confirmation of the n-6 polyunsaturated fatty acid pathway can be seen in Fig. 5. The same individual samples have much higher content of linoleic (18:2n6) acid. This might imply that formation of n-6 PUFA from the parent linoleic (18:2n6) acid through the desaturation-elongation cascade is reduced.

The metabolic pathways for polyunsaturated fatty acids explains the transformation of linoleic (18:2n6) acid through GLA (18:3n6), DGLA (20:3n6), arachidonic (20:4n6), and adrenic (22:4n6) to docosapentaenoic acid (22:5n3). All patients from the small subgroup formed in Figs. 2–5 show a high content of linoleic (18:2n6) acid, which indicates the corrupted n-6 polyunsaturated pathway. Further investigation of correlations between individual acids from the above pathway did not show the imbalance except for the plot of the adrenic *versus* arachidonic acid shown in Fig. 4. Thus we can anticipate that the n-6 polyunsaturated fatty acid pathway is interrupted between the arachidonic and adrenic acids.

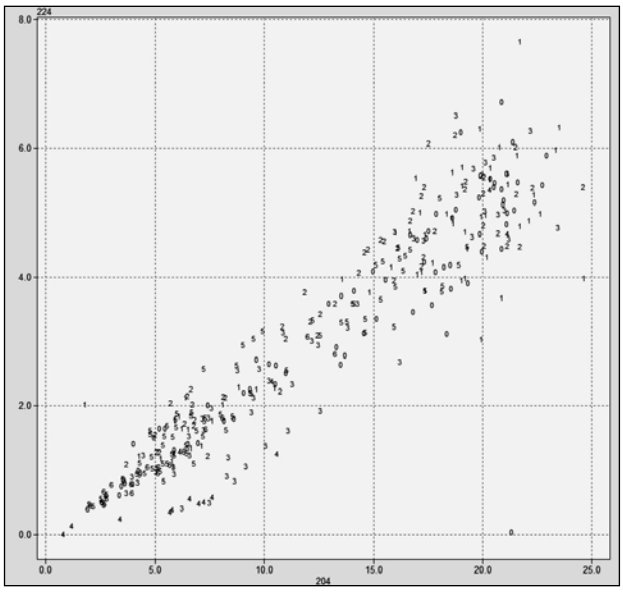

Figure 4. Relationship between percentage levels of adrenic (22:4n6) and arachidonic (20:4n6) acid in 342 samples.

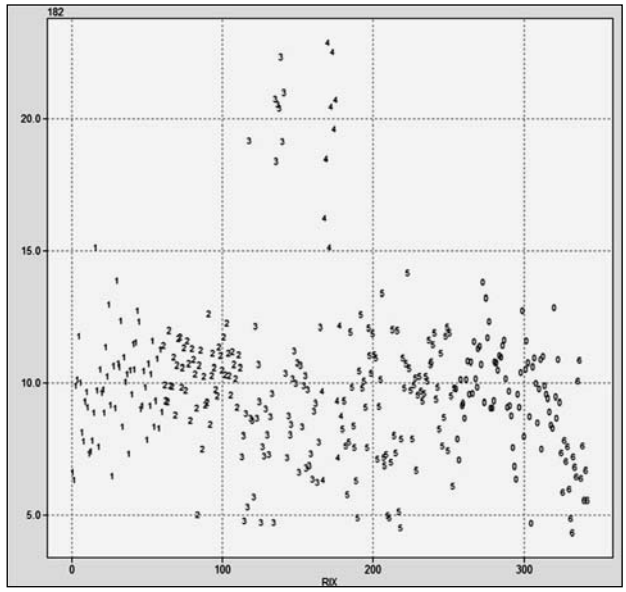

Figure 5. Concentrations of linoleic (18:2n6) acid in the patients with a different disease: (0) control group, (1) inflammatory bowel disease – I, (2) asthma, (3) uterine leiomyoma, (4) without uterine leiomyoma, (5) inflammatory bowel disease – II and (6) colon adenomas.

3. 4. Parametric Tests

We have performed two types of parametric tests, namely t-test and analysis of variance (ANOVA) to compare variables.[25,27] The reproducibility of measurements was assessed by paired t-test.[25,27] The data for the paired t-test were collected by duplicate analyses of the same samples analysed by two analysts in different time period. For this test we selected 23 samples from class 5, which represented patients with inflammatory bowel disease. The test showed no significant difference regarding different analyst and different time of analysis for 24 variables. Only in 6 variables (fatty acids 14:0, 15:0, 16:0, 17:0, 20:0 and 24:1, see Table 1), which were present in a very low percentage, mainly less than 1% (except for the palmitic (16:0) acid), the differences were significant within 95% confidence level (P = 0.05). On the basis of these test results we concluded that we reached a satisfactory reproducibility of measurements.

In the next step, we assessed the biological variation between groups of samples. ANOVA was performed for all 342 samples belonging to seven different classes labe-

led from 0 to 6. The results of ANOVA performed for each of the variables at 95% confidence level confirmed that the fatty acid composition considered in our research enabled the discrimination between the samples belonging to different classes characterized by a particular disease (see Table 2). For all but one out of 30 variables there is a significant difference between averages of individual measured percentage values for all 7 different classes. Only for one variable, i.e. the eicosadienoic (20:2n6) acid the ANOVA test gives no difference between the classes. Another test, which was performed, was the comparative t-test as demonstrated in Table 3. It compares the average values of individual variables between two groups of samples, in particular between the control group (class 0) and all other groups (classes 1–6), and two additional comparisons are given in Table 3.

Table 3: The comparison of different groups with the COMPAR-t-test.

Groups to compare	S	M	P	T	n-3 PUFAs	n-6 PUFAs
1–0	–	–	–	–	–	–
2–0	–	–	–	–	x	–
3–0	x	–	x	–	–	x
4–0	x	–	x	–	x	–
5–0	x	x	x	x	x	x
6–0	x	x	x	x	x	x
1–5	x	x	x	x	x	x
3–4	–	–	–	–	x	–

x ……significant difference on 0.05 P-level of confidence
– …….no differences on 0.05 P-level of confidence

The comparative t-test has proven that the female patients with uterine leiomyoma (class 3) and without uterine leiomyoma (class 4) can be distinguished only by the content of n-3 PUFAs as demonstrated in Table 3. The content of n-3 PUFAs can also distinguish the control group (class 0) from the following groups: asthma (class 2), without uterine leiomyoma (class 4), inflammatory bowel disease – II (class 5), and colon adenomas (class 6). The content of n-6 PUFAs can distinguish control group without any of mentioned diseases (class 0) from the group of female patients with uterine leiomyoma (class 3), inflammatory bowel disease – II (class 5), and colon adenomas (class 6). Inflammatory bowel disease – I (class 1) and inflammatory bowel disease – II (class 5), can also be distinguished by the content of n-6 PUFAs (Table 3). For a comparison, the values of n-3 PUFAs for all 342 samples are given in Fig. 6.

It is evident from the Fig. 6 that the concentrations of n-3 PUFAs were evenly distributed between 1.0 and 10.0 wt. % for all class samples except for the class 6 representing the patients with colon adenomas, whose blood samples contain significantly lower concentration of this variable, up to 4.0 wt. % of omega-3 polyunsaturated fatty acids.

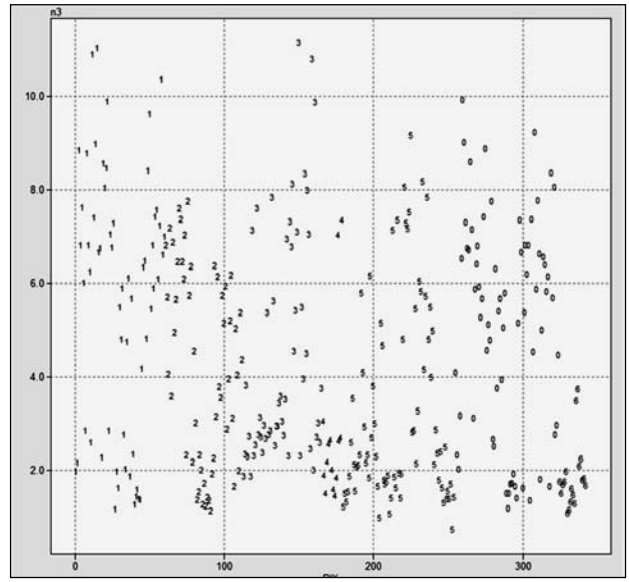

Figure 6. Concentrations of n-3 PUFAs in the patients with a different disease: (0) control group, (1) inflammatory bowel disease – I, (2) asthma, (3) uterine leiomyoma, (4) without uterine leiomyoma, (5) inflammatory bowel disease – II and (6) colon adenomas.

3. 5. Principal Component Analysis (PCA)

PCA was performed in order to get an overall impression about the correlation of 342 samples of erythrocytes. PCA was applied on the matrix composed of 342 × 39 elements. In 342 rows samples of erythrocytes represented by 39 variables are given. The data were additionally reprocessed by »column centering« (mean centering), which means that the mean value of each column was subtracted from individual (342) elements. In the first two principal components of transformed data 96.4% of variance was explained.

It is evident that we do not lose a considerable amount of information by keeping only the first two principal components. The 96.4% of information is gathered in the first two principal components. Fig. 7 shows the scores (PC1 *vs*. PC2) resulting from PCA of the 342 samples represented with 39 variables.

It is evident from Fig. 7 that three clusters are formed. The lower cluster contains samples only from two classes, female patients with and without uterine leiomyoma, namely classes 3 and 4. Only 16 out of 67 samples of these two classes were distinguished. We cannot associate this separated group with any disease of our study, however, comparing with Figs 2–5 it can be seen that this group has high content of linoleic (18:2n6) and low content of adrenic (22:4n6) and arachidonic (20:4n6) fatty acids. The samples labeled with "0" belong to the control group without any declared diseases, and are all grouped in the upper part of PCA. One may distinguish two subgroups in this upper part of PCA score plot, partially separating classes 1 and 2 (mainly

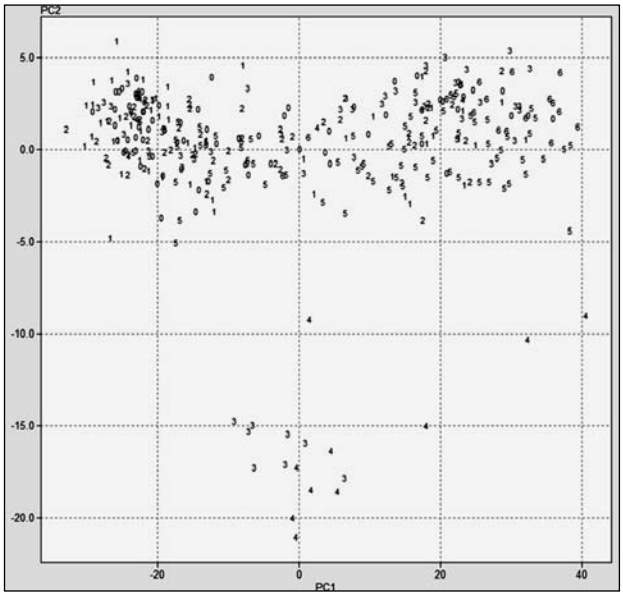

Figure 7. Scores of 342 samples and 39 variables in the PC1-PC2 co-ordinate system.

in the left cluster) from classes 5 and 6 (mainly in the right cluster).

In order to find the influence of original variables on the PC axes and consequently the influence of original variables on the formed clusters, the loading plot is shown in Fig. 8.

It can be seen from Fig. 8 that the first component, PC1, is associated mainly with the variables 31 (S), 33 (P) and 38 (n6). S (sum of saturated fatty acids) shows high values in the upper right cluster (associated with classes 5 and 6), while P (sum of polyunsaturated fatty acids) and n6 (sum of omega-6 polyunsaturated fatty acids) groups

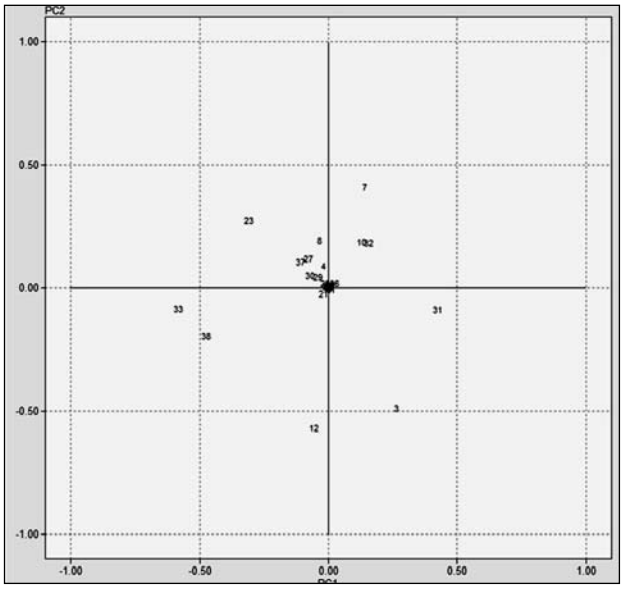

Figure 8. Loadings of 342 samples and 39 variables in the PC1-PC2 co-ordinate system.

of acids show high values in the upper left cluster associated with classes 1 and 2. The second component, PC2, represents mainly the stearic (18:0), linoleic (18:2n6) and palmitic (16:0) acids (variables no. 7, 12, and 3, respectively). Thus the samples from classes 3 and 4, located in the lower part of the PC score plot, can be associated with high values of linoleic (18:2n6) and palmitic (16:0) acids, and with low values of stearic (18:0) acid.

4. Conclusions

The goal of the research presented in this work was to find the possible correlations between the classes, which represent different diseases and the fatty acid composition. The reproducibility of measurements was assessed by paired t-test. For 24 variables (fatty acids) there was statistically no difference regarding different analyst and different time of analysis. From the remaining 6 variables, 5 were present at a very low percentage, mainly less than 1%. On the basis of these test results we concluded that we reached a satisfactory reproducibility of measurements.

The results of ANOVA performed for each of the variables at 95% confidence level confirmed that the fatty acid composition considered in our research enabled the discrimination between the samples belonging to different classes characterized by a particular disease.

Red blood cell membrane phospholipid omega-3 polyunsaturated fatty acids content was more than twice lower in the patients with colon adenomas (class 6) than in average of all other patients (classes). The study suggests that DPA (22:5n3) and DHA (22:6n3) might be important fatty acids in the formation of colon adenomas. The mutual negative correlation between the saturated acids and arachidonic (20:4n6) acid and saturated acids and adrenic (22:4n6) acid, respectively, is high. An expected correlation between the levels of arachidonic (20:4n6) acid and its elongation product adrenic (22:4n6) acid is present and does not show the imbalance within these n-6 polyunsaturated fatty acid pathway except for a small group of female patients. It is evident that already on the basis of one of these two groups of fatty acids, namely saturated acids and adrenic (22:4n6) acid, the groups of different types of erythrocyte samples are distinguished; these are individual samples belonging to female patients with and without uterine leiomyoma, namely classes 3 and 4.

Comparative t-test for comparing each individual class with a control one gives the possibility to distinguish two intestine-disease classes (class 1 and 5) and 2 gynecologic diseases classes (class 3 and 4).

Using PCA it is evident that three clusters are formed. One cluster contains samples only from two classes, female patients with and without uterine leiomyoma, namely classes 3 and 4. The samples labeled with "0" are samples of control group without any declared diseases,

and are distributed into two groups – clusters, which have shape of two circles (like ∞). PCA group of female donors (class 3 and 4) is distinguished mainly by the content of linoleic (18:2n6) acid; a small subgroup shows its concentration highly above the average value while at the same time the reduced values of both dimethyl acetals suggests an increased oxidative stress. This might imply that formation of n-6 PUFA from the parent linoleic (18:2n6) acid through the desaturation-elongation cascade is reduced. Method showed to be robust and reliable for identification in routine analysis.

Considerable differences in membrane fatty acid profiles were observed in this study and further investigations are planned. One of the promising directions would be the proteome investigation of blood samples in addition to the determination of fatty acid composition. Efficient exploration of proteomic maps would enable further insight into the biological response on various diseases.[8–12,32,33] Besides some rational explanations of the corrupted n-6 polyunsaturated fatty acid pathway in a small subgroup of female patients this investigation might have a potential for diagnostics and population screening for diseases.

5. Acknowledgement

The authors thank the Ministry of Higher Education, Science and Technology of the Republic of Slovenia, contract numbers P1-017 and P2-006 for financial support.

6. References

1. N. Mohandas, P.G. Gallagher, *Blood*, **2008**, *112*, 3939–3948.
2. M. Randić, *J. Am. Chem. Soc.* **1975**, *23,* 6609–6615.
3. M. Randić, *Acta Chim. Slov.* **1997**, *44,* 1, 57–77.
4. M. Randić, *J. Math. Chem*. **2008**, *43*, 756–772.
5. M. Randić, *Chem. Phys. Lett*. **2007**, *444*, 176–180.
6. M. Randić, Quantitative characterization of proteomics maps by matrix invariants. P. M. Conn (editor): Handbook of proteomic methods, Totowa, Humana Press, NJ, **2003**, pp. 429–450.
7. M. Randić, *J. Chem. Inf. Comput. Sci*. **2001**, *41*, 1330–1338.
8. M. Randić, M. Novič, M. Vračko, *J. Proteome Res.* **2002**, *1(3)*, 217–226.
9. M. Randić, A. Balaban, M. Novič, A. Založnik, T. Pisanski, *Period. biol.* **2005**, *107(4)*, 403–413.
10. M. Randić, M. Novič, M. Vračko, *J. Chem. Inf. Mod*. **2005**, *45(5)*, 1205–1213.
11. M. Randić, F. A. Witzmann, V. Kodali, S. Basak, C.Subhash, *J. Chem. Inf. Mod*. **2006**, *46(1)*, 116–122.
12. T. Brosche, *Arch. Gerontol. Geriatr*. **1997**, *25*, 73–81.
13. W. W. Christie, Lipid Analysis, Pergamon Press, Oxford, **1982**.
14. J. Folch, M. Lees, G. H. Sloane Stanley, *J. Biol. Chem*. **1957**, *226(1)*, 497–509.
15. E. G. Bligh, W. Y. Dyer, *J. Biochem. Physiol.* **1959**, *37(8)*, 911–917.
16. V. Ruiz-Gutierrez, M.C. Perez-Camino, *J. Chromatogr. A*, **2000**, *885*, 321–341.
17. U. Potocnik, I. Ferkolj, D. Glavac, M. Dean, *Genes Immun*. **2004**, *5*, 530–539.
18. V. Berce, K. Repnik, U. Potocnik, *J. Asthma* **2008**, *45*, 780–784.
19. M. Pakiz, U. Potocnik, I. But, *Fertil Steril.* **2010,** (Article in press).
20. Animal and vegetable fats and oils, ISO 5508:1990 (E), ISO 5509:2000 (E).
21. P. Stenvinkel, U. Diczfalusy, B. Lindholm, O. Heimburger, *Nephrol. Dial. Transplant*. **200**4, *19*, 972–976.
22. J. Lessid, B. Fuchs, *Curr. Med. Chem*. **2009**, *16*, 2021–2041.
23. R. Rodrigo, J. P. Bächler, J. Araya, H. Prat, W. Passalacqua, *Mol. Cell Biochem.* **2007**, *303*, 73–81.
24. X. Han, R. W. Gross, *Anal. Biochem.* **2001**, *295(1)*, 88–100.
25. J. C. Miller, J.N. Miller (5^{th}): Statistics and Chemometrics for Analytical Chemistry, Ellis Horwood, New York, **2005,** pp. 39–67.
26. Teach/Me, SDL – Software Development Lohninger; Teach/Me DataLab 2.002 © **1999** Springer, Berlin, Developed by H. Lohninger and the Teach/Me people.
27. D. L. Massart, B. G. M. Vandeginste, L. M. C. Buydens, S. De Jong, P. J. Lewi, J. Smeyers-Verbeke: Handbook of Chemometrics and Qualimetrics: Part A, Elsevier, Amsterdam, **1997,** pp. 519–556.
28. Y. Rodriguez, A.B. Christophe, *Clin. Chim. Acta* **2005**, *354*, 195–199.
29. T. Decsi, H. Minda, R. Hermann, A. Kozari, E. Erhardt, I. Burus, Sz. Molnar, Gy. Soltesz, Prostaglandins, *Leukotrienes and Essential Fatty Acids* **2002**, *67*(4), 203–210.
30. J. L. Sepulveda, Y. C. Tanhehco, M. Frey, L. Guo, L. J. Cropcho, K. M. Gibson, H. C. Blair, *Arch. Pathol. Lab. Med*. **2010**, *134*, 73–80.
31. B. Baggio, A. Budakovic, A. Ferraro, S. Checchetto, G. Priante, E. Musacchio, E. Manzato, M. Zaninotto, M.C. Maresca *Transplantation,* **2005**, *80*, 1349–1352.
32. H. Kim, S. Eliuk, J. Deshane, S. Meleth, T. Sanderson, A. Pinner, G. Robinson, L. Wilson, M. Kirk, S. Barnes, *Methods Mol. Biol*. **2007**, *371*, 349–391.
33. M. Oh-Ishi, T. Maeda, *J. Chromatogr. B* **2007** *849*, 211–22.
34. O. O. Aalen, *Statistics in Medicine,* **2010,** *29,* 1159–1160

Povzetek

V prispevku je predstavljena študija primerjave sestave maščobnih kislin iz fosfolipidov človeških membran rdečih krvnih celic bolnikov z izbranimi bolezenskimi stanji z namenom testiranja hipoteze, da je sprememba v sestavi lahko povezana z različnimi boleznimi. V študijo je bilo vključenih 342 posameznikov, od tega 135 bolnikov s kronično vnetno črevesno boleznijo, 53 bolnic z miomi maternice in 14 žensk dokazano brez miomov maternice, nadalje 52 bolnikov z astmo, 18 bolnikov s črevesnimi polipi in 70 zdravih posameznikov (krvodajalcev), ki so služili kot kontrolna skupina. Izolaciji rdečih krvnih celic je sledilo ločevanje celokupnih ekstrahiranih lipidov z ekstrakcijo na trdni fazi (SPE) na nepolarne in polarne lipide. Po umiljenju fosfolipidne frakcije je sledila esterifikacija z bor trifluorid metanolnim reagentom. Sestavo metil estrov maščobnih kislin skupne fosfolipidne frakcije rdečih krvnih celic smo določali s plinsko kromatografijo s plamensko ionizacijskim detektorjem. Dodatno smo iz membran rdečih krvnih celic fosfolipidnih plazmalogenov določili tudi dva dimetil acetala (heksadekanal in oktadekanal dimetil acetal; 16:0 DMA in 18:0 DMA). Z metodo glavnih osi smo karakterizirali vzorce glede na sestavo maščobnih kislin in dimetil acetalov, povezanih s plazmalogeni. Ugotovili smo zelo nizke vsebnosti omega-3 polinenasičenih maščobnih kislin v celičnih membranah rdečih krvnih celic pri pacientih s črevesnimi polipi. Velik negativni korelacijski koeficient je bil ugotovljen med vsebnostjo nasičenih maščobnih kislin in arahidonsko (20:4n6) kislino in med vsebnostjo nasičenih maščobnih kislin in adrensko (22:4n6) kislino. Metoda glavnih osi je pokazala manjšo izstopajočo skupino pacientov ženskega spola z različnim bolezenskim fenotipom, ki se je ločila glede na vsebnost linolne (18:2n6) kisline, ki je bila pri tej podskupini precej nad povprečno vrednostjo vseh 342 vzorcev. Študija je pokazala, da obstajajo razlike v sestavi maščobnih kislin rdečih krvnih celic pri pacientih z različnimi boleznimi.