NMR plasma metabolomics study of patients overcoming acute myocardial infarction: in the first 12 h after onset of chest pain with statistical discrimination towards metabolomic biomarkers

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Short title: plasma metabolomics, myocardial infarction, NMR

Summary

Acute myocardial infarction (AMI) is one of the leading causes of death among adults in older age. Understanding mechanisms how organism responds to ischemia is essential for the ischemic patient's prevention and treatment. Despite the great prevalence and incidence, only a small number of studies utilize a metabolomic approach to describe AMI condition. Recent studies have shown the impact of metabolites on epigenetic changes, and plasma metabolites were related to neurological outcome of the patients, making metabolomic studies increasingly interesting. The aim of this study was to describe metabolomic response of an organism to ischemic stress through the changes in energetic metabolites and aminoacids in blood plasma in patients overcoming acute myocardial infarction. Blood plasma from patients in the first 12h after onsent of chest pain was collected and compared with volunteers without any history of ischemic diseases via NMR spectroscopy. Lowered plasma levels of pyruvate, alanine, glutamine and neurotransmitter precursors tyrosine and tryptophan were found. Further, we observed increased plasma levels of 3-hydroxybutyrate and acetoacetate in balance with decreased level of lipoproteins fraction, suggesting the ongoing ketonic state of an organism. Discriminatory analysis showed very promising performance, where compounds: lipoproteins, alanine, pyruvate, glutamine, tryptophan and 3-hydroxybutyrate

were of the highest discriminatory power with feasibility of successful statistical discrimination.

Key points myocardial infarction, plasma, NMR metabolomics

Introduction

Acute myocardial infarction (AMI), or heart attack, is one of the leading causes of death in the developed countries with lower incidence in women than men until midlife (Armeni and Lambrinoudaki 2017, Kittnar 2020). Healthy life-style should be encouraged already in children to avoid the developmet of AMI in future (Linhartová *et al.* 2019). It is most often caused by decrease or cessation of blood flow and the lack of oxygen in the coronary arteries, which results in impaired function of the part of the heart muscle or death (Alaour *et al.* 2018, Barberi and van den Hondel 2018). Atherosclerosis, dyslipidemia, hypertension, oxidation, inflammatory processes, as well as endothelial dysfunction and decreased glucose metabolism are considered to be the main risk factors for AMI (Trebatická *et al.* 2017).

Many studies confirm that the ischemic attack has its own metabolomic response not only in affected tissue but also in the circulation (Sidorov *et al.* 2019, Shah *et al.* 2012). Relatively much is known about blood glucose (Ishihara 2012) and lactate in patients overcoming AMI (Anderssen *et al.* 2013, Lazzeri *et al.* 2015). Interruption of blood supply to a certain region of the myocardium results in increased levels of enzymes in patient's blood such as: lactate dehydrogenase (LDH), creatine kinase (CK) and aspartate aminotransferase (AST) isoenzymes (Mythili and Malathi 2015) which are directly related to metabolites (Ussher *et al.* 2016). In the last years, the importance of metabolomics in cardiovascular diseases increases and the metabolic profiles may serve as diagnostic and/or prognostic tools

that have the potential to significantly alter the management of cardiovascular diseases (Ussher *et al.* 2016). It is relatively novel knowledge that some metabolites and metabolomic pathways have impact on epigenetics (Shimazu *et al.* 2013, Su *et al.* 2016, Ruan and Crawford 2018). Beside this, many clinical and experimental studies suggested the relation between plasma aminoacids levels and neurological outcome (Erdman *et al.* 2011, Coppola *et al.* 2013, Tournissac *et al.* 2018), making metabolomic studies increasingly attractive.

This study was designed to evaluate relative changes in plasma metabolites traceable by NMR spectroscopy which is a robust tool broadly used in untargeted metabolomics. Plasma from patients was sampled within 12h after onset of chest pain and was compared with subjectively healthy volunteers without any history of ischemic diseases. The aim of this work was to describe metabolomic response of an organism to ischemic stress and explore the feasibility of statistical discrimination based on plasma metabolites.

Materials and Methods

Samples

Together 30 plasma samples from patients with hospital-confirmed acute myocardial infarction provided by Division of Invasive Cardiology, Department of Internal Medicine I of University Hospital Martin were used. Plasma was sampled in time of 2.5 - 12h after onset of the chest pain. The patient group consisted of: 10 female, 20 male, aged: mean = 65 ± 11 yrs, median = 64 ± 9 yrs. Except one man, age 81 yrs, all patients survived the heart attack.

As controls, plasma samples from 30 subjectively healthy volunteers free of any medically manifesting diseases and without any history of acute coronary diseases were used, thereof 11 female, 19 male, aged: mean = 57 ± 13 yrs, median = 58 ± 10 yrs. For controls, as well as for patients' selection, no additional criteria were used. Since in AMI it was not possible to plan the blood collection in advance, the blood sampling was carried on not insisting on fasting state for patients as for controls.

Ethics

This study was approved by the Ethics Committee of the Jessenius Faculty of Medicine in Martin (registered under IRB00005636 at Office for Human Research Protection, U.S. Department of Health and Human Services) under the code EK 1859/2016. Informed consent was obtained from all subjects of this study.

Sample preparation

Stock solution consisted of: 150 mM phosphate buffer and 0.30 mM TSP-d_4 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt as a chemical shift reference in deuterated water. Blood was collected in EDTA coated tubes, centrifuged at 4 ^{o}C, 2000 rpm, for 20 minutes. Plasma was deproteinated by adding 600 μL of methanol to 300 μL of plasma. The mixture was vortexed for a few seconds and storaged at -20 ^{o}C for 20 minutes. Then, the mixture was centrifuged for 30 minutes at 14000 rpm. Finally, 700 μL of supernatant were dried out and subsequently carefully mixed with 100 μL of stock solution and 500 μL of deuterated water. 550 μL of final mixture were transferred into 5 mm NMR tube.

NMR data acquisition

NMR data were acquired on 600 MHz NMR spectrometer Avance III from Bruker equipped with TCI (triple resonance) cryoprobe. Initial settings were done on an independent sample and adopted for measurements. Samples were stored in Sample Jet at cca. 6 ^{o}C and randomly ordered for acquisition. Measurements were carried on at 310 K. An exponential noise filter was used to introduce 0.3 Hz line broadening before Fourier transform. We used standard Bruker profiling protocols that we modified as follows: profiling 1D NOESY with presaturation (noesygppr1d): FID size 64k, dummy scans 4, number of scans 128, spectral width 20.4750 ppm; COSY with presaturation (cosygpprqf): FID size 4k, dummy scans 8, number of scans 1, spectral width 16.0125 ppm; homonuclear *J*-resolved (jresgpprqf): FID size 8k, dummy scans 16, number of scans 4; profiling CPMG with presaturation (cpmgpr1d, L4 = 126, d20 = 3ms): FID size 64k, dummy scans 4, number of scans 128, spectral width 20.0156 ppm. All experiments were conducted with a relaxation delay of 4 s; all data were once zero filled.

Data analysis

TSP-d_4 signal was assigned a chemical shift of 0.000 ppm. All spectra were binned to bins of the size of 0.001 ppm, starting from 0.500 ppm to 9.000 ppm, with excluded water region 4.6 - 4.9 ppm. Spectra were solved with the help of human metabolome database (www.hmdb.ca), chenomx software and by researching in metabolomics literature. For all compounds, the multiplicity of peaks was confirmed in J-resolved spectra and homonuclear cross peaks were confirmed in cosy spectra.

Metabolites showing weak intensive peaks or peaks overlap were excluded from the evaluation. After the metabolites were identified (Table 1), we chose the spectra subregions with only single metabolite assigned. In 0.001 ppm binned spectra, we summed integrals of selected signals. These data representing relative concentration of metabolites in plasma were used for statistical analysis. No normalization method was applied on NMR data. After normality test by Shapiro-Wilk and Kolmorogov test (OriginPro 2019), statistical analysis was carried on using Mann-*U*-Whitney test (Matlab R2015a). Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and receiver operating characteristic (ROC) curves derived from random forest (RF) algorithm were performed by using Metaboanalyst 4.0 (Chong *et al.* 2019) and Matlab R2015a.

Here Table 1

Results

Together 20 plasma metabolites from 21 identified (except threonine, Table 1) were quantified. Statistically significant (p value < 0.05, Mann-*U*-Whitney test) changes in plasma metabolite levels in patients´ group against controls are summarized in Table 2. Other metabolites were without significant changes. Lipoproteins fraction contained mainly VLDL, LDL, HDL and IDL as described in the work by Liu *et al.* (2002).

Here Table 2

PCA, PLS-DA analysis

Firstly, the results were visualized by PCA and PLS-DA methods (Figure 1). PCA is a tool for exploratory data analysis and, in addition to other uses, provides a 2D plot of the multivariate data. Unlike PCA, PLS-DA includes also a discrimination algorithm. We used as an input for the PCA and PLS-DA algorithm relative concentrations of plasma metabolites determined by NMR spectroscopy. Both analyzes suggested that good discrimination between patients and controls is attainable (Figure 1). In order to show the reliability of PLSDA, the PLSDA results are presented along with the results of permutation test and leave-one-out cross-validation (LOOCV). In permutation test, we permuted the y values 1000 times and showed the resulting Rsquare (value calculated based on the difference between the real outcome and the outcome predicted) distribution together with Rsquare value determined on true labeled samples (Figure 2). In the each iteration of LOOCV, one sample was left out and the multivariate model was constructed by the rest of the samples. Then the predicted value of omitted sample was calculated based on model created. The entire procedure was repeated until each case had been omitted once. Finally, we calculated Rsquare, Q square and accuracay that were (in the mentioned order): 0.72, 0.306 and -0.11 for 5 components and 0.74, 0.52 and -3.98 for 8 components. The results from PCA and PLSDA analysis based on spectral NMR bins of 0.001 ppm are shown in the Supplementary material.

Here Figure 1

Here Figure 2

Random Forest classification

In the same way as in PCA and PLS-DA, the input variables for RF were the relative plasma concentration of metabolites. After RF run, ROC curve was created. ROC curve analysis is the standard method for describing and assessing the performance of diagnostic - discrimination tests. It is produced by plotting the true positive rate against the false positive rate at various threshold settings. An important parameter, area under curve (AUC) represents ranking quality. The AUC of a ranking is 1 (the maximum of AUC value) when all samples are truly assigned into the groups. An AUC of 0.5 is equivalent to randomly classifying subjects as either positive or negative (the classifier is of no practical utility). When evaluated AMI patients against controls, an excellent classification with AUC of 0.97 for n=2 features (alanine and lipoproteins) and AUC of 0.985 for n=5 features (alanine, lipoproteins, pyruvate, tryptophan and glutamine) was obtained. By including further metabolites n=10, we obtained AUC of 0.989 and with n=20, AUC of 0.992 was achieved.

Discussion

Biochemical aspects related to heart attack

Significantly increased plasma glucose level found in AMI patients was expected, as acute hyperglycemia is a common feature during the early phase after acute myocardial infarction, regardless of diabetes status (Ishihara 2012). As a result of tissue hypoxia and accelerated anaerobic glycolysis, lactate plasma level increases. Elevated plasma lactate level in AMI patients can vary dependent on extent of injury (Andersen *et al.* 2013) and can serve as a

predictor of early mortality (Lazzeri *et al.* 2015). During shock, lactate is the most important fuel for the heart, and rather than its absolute value the lactate clearance has been reported as clinically more reliable (Abramson *et al.* 1993, Zhang and Xu 2014). It has been demonstrated in previous studies that after ischemic attack, the amount of lactate gradually decreases during reperfusion time, both in the brain parenchyma (Liu and Li 2016) as well as in blood (Rehncrona *et al.* 1981, Kliegel *et al.* 2004). In our study, we did not observe significantly increased lactate level in blood plasma in cardiac patients (Figure 3), as it can be expected in early time after onset of the chest pain. As the lactate is rapidly consumed in stress condition by heart, brain and other tissues, the time of blood collection plays an important role. Normalization of lactate level after AMI was observed within 24h (Kliegel *et al.* 2004), and in patients who underwent therapeutic hypothermia within 48h respectively (Lee *et al.* 2013). The fact that 29 from 30 patients examinated in our study survived the AMI indicates better general outcome in studied group what is related to faster lactate clearance. It is to note, that there were no special criteria in selecting the control group regarding diabetes or other metabolomic disorders, which may cause e.g. lactic acidosis in uninjured group and also influence the statistical result.

Here Figure 3

Lactate metabolism is closely interconnected with pyruvate and alanine, where the mutual conversion of these metabolites occurs in Cori and Callih cycles. Unlike lactate, AMI patients showed significantly decreased plasma levels of aerobic glycolysis intermediate pyruvate and amino acid alanine (Table 2, Figure 3). In the liver, the carbon skeleton of alanine is through pyruvate reconverted to glucose and released into the bloodstream. Circulating glucose is available for uptake by muscle and resynthesis of alanine in pyruvate-alanine cycle. The

decreased plasma levels of alanine and pyruvate suggest the modifications in alanine- and pyruvate- related metabolism induced by AMI injury.

At the time of altered glucose utilization, alternative substrates such as ketone bodies could support metabolic requirements. Ketone bodies can provide more than two thirds of the brain energy demands more efficiently than glucose (Puchalska and Crawford 2017). Very recent study showed that failing heart utilizes 3-hydroxybutyrate as a metabolic stress defence (Horton *et al.* 2019). In our study, we observed significantly increased plasma levels of ketone 3-hydroxybutyrate and its redox partner acetoacetate. The increase in plasmatic ketone bodies in blood after brain ischemic injury has been already described in rodents (Baranovicova *et al.* 2018a, Baranovicova *et al.* 2018b). It is to note, that the levels of glycolytic intermediates and ketone bodies in blood plasma are strongly dependent on diet and time elapsed from last food intake. 3-hydroxybutyrate level in starving subjects starts to increase prominently after 4-8h (Cahill 2006). After the onset of chest pain, no food intake by patients can be assumed, although, in this study, no exact data were collected to confirm it. With respect to these facts, we cannot recognise the define origin of increased plasma ketone bodies and this may result from combination of both, starvation and ischemic attack. Parallel to increased plasmatic ketone bodies, a significant decrease in lipoprotein fraction containing LDL, HDL and VLDL was observed. This fraction, besides others, contains the main substrates for ketone bodies production, such as triacylglyceroles (Liu *et al.* 2002). Detailed profile of plasma lipids in AMI patients was already described, showing decrease in LDL and HDL after AMI (Kumar *et al.* 2019).

Ketone bodies, besides serving as fuel in metabolic pathways, coordinate cellular function via epigenomic regulation (Ruan and Crawford 2018). There is evidence that increased level of ketone bodies, above all of 3-hydroxybutyrate, may influence epigenetic histone acetylation

(Shimazu *et al.* 2013, Su *et al.* 2016). The ketonic state with increased plasma ketone bodies 3-hydroxybutyrate and acetoacetate is interfering with the level of NAD and in further may influence eventual histone acetylation (Su *et al.* 2016). Histone deacetylase inhibitors have provide beneficial cardiac and vascular protective effects in rats with pressure overload cardiac hypertrophy (Jung *et al.* 2019). As next, methylation status is sensitive to oxygen and TCA-related metabolism (Su *et al.* 2016). Lai et al. (2014) showed that TCA cycle intermediates in heart failure patients are reduced (Aubert *et al.* 2016). It is to consider that the metabolic enzyme expression has the potential to impact DNA methylation and histone acetylation in mammals (Su *et al.* 2016), what makes detailed metabolomic and enzymatic studies all the more interesting and useful.

Glutamine is conditionally essential in humans and in some instances of stress the demand of body for glutamine increases. It serves as fuel for lymphocytes and macrophages (Parry-Billings *et al.* 1992), is involved in modulating immune cells function and protecting them from apoptosis (Chang *et al.* 2002). Glutamine also plays a crucial role in the production of cytokines (Shah *et al.* 2020). During catabolic/hypercatabolic situations glutamine can become essential for metabolic function, but its availability may be compromised due to the impairment of homeostasis in the inter-tissue metabolism of amino acids. Reduced plasma level of glutamine was observed in various emergency conditions including surgical interventions (Parry-Billings *et al.* 1992). In cardiac patients, intracellular myocardial glutamine concentrations were reduced (Suleiman *et al.* 1997). Interestingly, experimental and clinical studies have demonstrated the cardio protective effect of glutamine. For example, the beneficial effect of glutamine on ischemic rat heart was shown by Khogali *et al.* (2002). Consistently with current knowledge, we observed significantly lowered glutamine plasma levels in patients overcoming AMI when compared with controls.

Tryptophan is an essential amino acid and besides participating in the biosynthesis of proteins serves as a precursor to kynurenine metabolites, neurotransmitter serotonin and others. The role of tryptophan metabolism in cardiovascular diseases was summarized by Lenhert *et al.* (2014). It is i.a. involved in endothelium derived blood pressure control and microvascular reactivity in stroke (Mangge *et al.* 2014). Tyrosine is conditionally essential amino acid for patients with chronic renal failure having various relations to vascular system. In our study, both, tyrosine and tryptophan plasma levels were observed to be decreased in AMI patients against controls, similarly as it was in stroke patients in study by Ormstad *et al.* (2013), suggesting increased rate of their metabolic conversion. These findings indicated that the proinflammatory response may be responsible for a reduced capacity for the biosynthesis of brain catecholamines and mediate neurotoxic effects. Results of many studies support this concept, e.g. patients after AMI suffered cognitive and somatic depressive symptoms with depression recognition (Smolderen *et al.* 2009), they have also a higher risk of anxiety and depressive disorders (Feng *et al.* 2016), cognitive impairment (Gharacholou *et al.* 2011) and so on.

As methodological note: some metabolites, such as lactate or tryptophan bind to serum albumine (Cunningham *et al.* 1975) what influences the concentration of 'free' metabolites in blood plasma. During deproteinization procedure proteins are denaturized and non-specifically bound substances are likely to be released. We did not run additional experiments to examine this process in detail, however would not expect much impact on the results.

Biomarkers discovery

There are two main approaches in data evaluation and interpretation in the metabolomics. The first of them is focused on gaining the improved biological understanding through analyzing metabolite profiles. Here, p values from hypothetical statistical testing are used as essential statistical tools. While lists of compounds found to be significantly changed are sometimes referred as 'putative biomarkers', they are not really useful as clinical biomarkers which require different analysis, evaluation and validation (Xia *et al.* 2013). The second fundamental metabolomic approach is oriented towards biomarkers that are not intended to explain biology but they are rather designed to discriminate with an optimal sensitivity/specificity (Xia *et al.* 2013). Whereas PCA serves to visualise data in 2D format, supervised PLS-DA includes also a discrimination algorithm. Both, PCA as well as PLS-DA suggested the good potential of the system to obtain sufficient discrimination (Figure 1ab). However, PLS-DA models have a very strong propensity to overfit to training data mainly under high dimensionalities and small sample conditions (Rodríguez-Pérez *et al.* 2018). We run additional tests to confirm the reliability of PLSDA results. As can be seen from Figure 2, the permutation test shows a relatively good reliability of PLSDA analysis to discriminate between cases and controls with Rsquare = 0,8326. In cotrast to, the Qsquare values from LOOCV cross validation suggest overfitted PLSDA model.

To estimate the more realistic discriminatory power of the system, we decided to employ random forest discriminatory analysis. Cross validated RF algorithm picks up two-third data for training and rest for testing for regression and almost 70% data for training and rest for testing during classification in order to overcome the training and testing on the same data. Although this approach does not substitute the clinical validation, it may lead to encouraging results in exploratory studies. The further advantage of RF algorithm is identifying the most important features that are responsible for success of discrimination. Random Forest

performed very well with AUC of 0.97 for only two variables: lipoproteins and alanine. With increasing number of variables the AUC was getting closer to 1. It is to note, that the obtained performance could be achieved by various combination of variables that permuted in importance order. For example, if alanine was excluded from the variables, RF performed also very well with AUC very close to 1 for two metabolites lipoproteins and tryptophan. The discriminatory power of this system was not dependent exclusively on one particular metabolite but offered various combinations to achieve very good results. As examples, the very good discrimination was achieved by using relative concentration of plasma metabolites: lipoproteins and alanine (AUC = 0.97), lipoproteins and pyruvate (AUC = 0.97), alanine and pyruvate (AUC = 0.92), lipoproteins and tryptophan (AUC = 0.96), lipoproteins and glutamine (AUC = 0.97). Having run RF with various combinations of variables as an input, we observed that following metabolites combined together gave the best discriminatory performance and behaved in our study as potential plasma biomarkers of AMI: lipoproteins, alanine, pyruvate, glutamine, tryptophan and 3-hydroxybutyrate. In addition to metabolomics, proteomics approach seems to be also a suitable method to identify reliable specific novel biomarkers in heart failure. Rehulkova *et al.* (2016) found a number of proteins with unique changes in plasma levels. More studies are required for complete the understaning and clarify the the pathophysiology of serious complications (Lacko *et al.* 2018) of acute cardiogenic shock, from which the patient would ultimately benefit.

Here Figure 4

Conclusion

In patients overcoming AMI, besides expected hyperglycemia, the decrease in plasma level of pyruvate, alanine, glutamine, tyrosine and tryptophan was found. The elevated plasma levels of ketone bodies 3-hydroxybutyrate and acetoacetate, parallel to decrease in plasma lipoprotein fraction point out on the ongoing ketogenic state. PCA and PLS-DA analyzes separated favourably but not ideally patients from controls. By employing cross-validated Random Forest algorithm, we obtained almost ideal discrimination patients from controls, where following metabolites combined together gave the best discriminatory performance: lipoproteins, alanine, pyruvate, glutamine, tryptophan and 3-hydroxybutyrate. Based on the obtained results, we would like to emphasize the very promising possibility to discriminate between AMI patients and controls on the basis of principal plasma metabolites.

Conflict of Interest: There is no conflict of interest.

Acknowledgments : This work was supported by Scientific Grant Agency of the Slovak Republic grant no. VEGA 1/0277/18 and no. VEGA 1/0289/12, project "Competence Center for Research and Development in Diagnosis and Therapy of Oncological Diseases." code 26220220153 co-financed from EU sources and European Regional Development Fund, the Slovak Research and Development Agency under contract no. APVV-0224-12 and APVV-16-0020, Ministry of Health Slovakia under the grant nr. 2018/11-UKMT-7.

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Tables

Table 1 Plasma metabolites: ^{1}H NMR chemical shifts used for identification, in bold chemical shifts used for quantification (all or the part of, s-singlet, d-doublet, t-triplet, q-quarte, m-multiplet).

metabolite	**peaks assigned**
threonine	1.33d, 3.60 d, 4.26dq
lactate	1.34d, **4.15q**
formate	**8.46 s**
alanine	**1.48d,** 3.81q
valine	**0.99d, 1.05d,** 2.28m, 3.62d
glucose	3.25dd, 3.40t, 3.41dd, 3.47m, 3.49m, 3.53 dd, 3.71t, 3.72m, 3.76m, 3.83m, 3.84m, **3.90dd,** 4.63d, 5.23d
leucine	**0.96d, 0.97d,** 1.72m
isoleucine	0.94t, **1.01d,** 3.67d
acetoacetate	**2.28s**
acetate	**1.91s**
pyruvate	**2.37s**
citrate	**2.55d**, 2.66d
2-oxoisocaproate	0.95d, 2.1m, **2.61d**
phenylalanine	**7.33d, 7.38t, 7.42t**
tryptophan	7.19t, 7.27t, 7.31s, **7.55d, 7.74d**
tyrosine	**6.90d, 7.15d**
creatine	3.03s, **3.93s**
creatinine	3.04s, **4.05s**

glutamine	2.11m, 2.14m, **2.44m, 2.47m,**
3-hydroxybutyrate	1.20d, 2.31dd, **2.39dd,** 4.15m
lipoproteins	**0.8-0.87m, 1.19-1.33m,** see ref. Liu *et al.* (2002)

Table 2 Relative changes in plasma metabolites in patients after AMI against subjectively healthy controls, p value derived from Mann-*U*-Witney test, % change derived from median.

metabolite	**p-value**	**% change in patients against controls**
glucose	$p < 0.005$	26
pyruvate	$p < 0.0005$	-34
alanine	$p < 0.0005$	-17
tryptophan	$p < 0.000005$	-28
tyrosine	$p < 0.05$	-11
glutamine	$p < 0.005$	-18
3-hydroxy-butyrate	$p < 0.00005$	57
acetoacetate	$p < 0.0005$	40
lipoproteins	$p < 0.000005$	-45

Figure legends

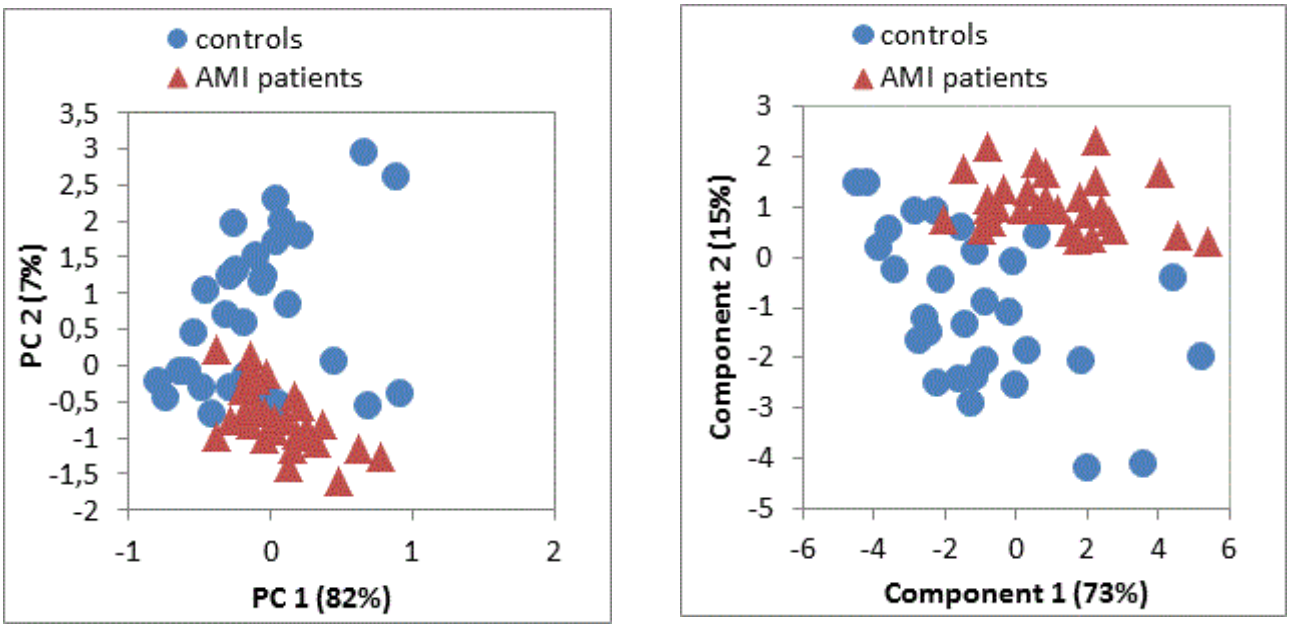

Figure 1 PCA (left) and PLS-DA (right) analysis of plasma metabolites in patients after ischemic attack in comparison to controls.

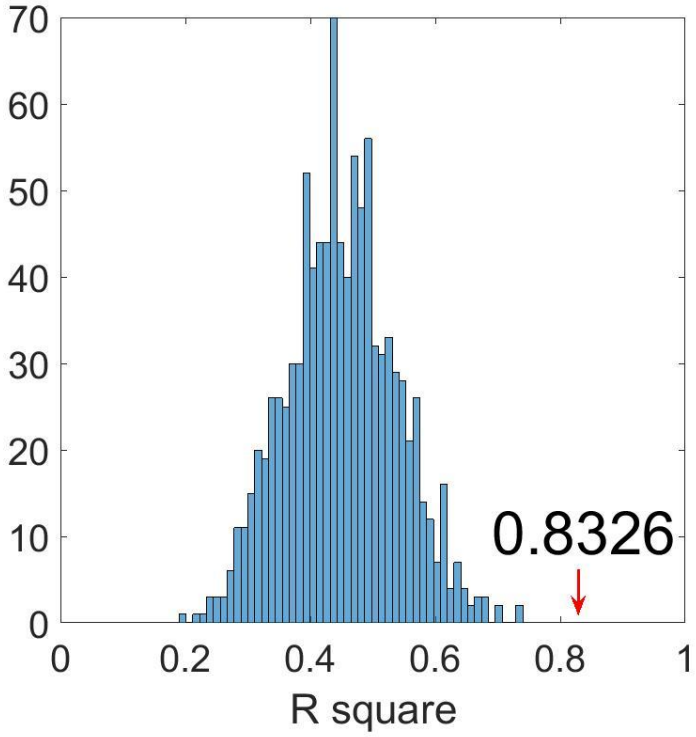

Figure 2 PLSDA permutation test: expressed by histogram of Rsquare values with marked Rsquare value after PLSDA on truely labeled samples.

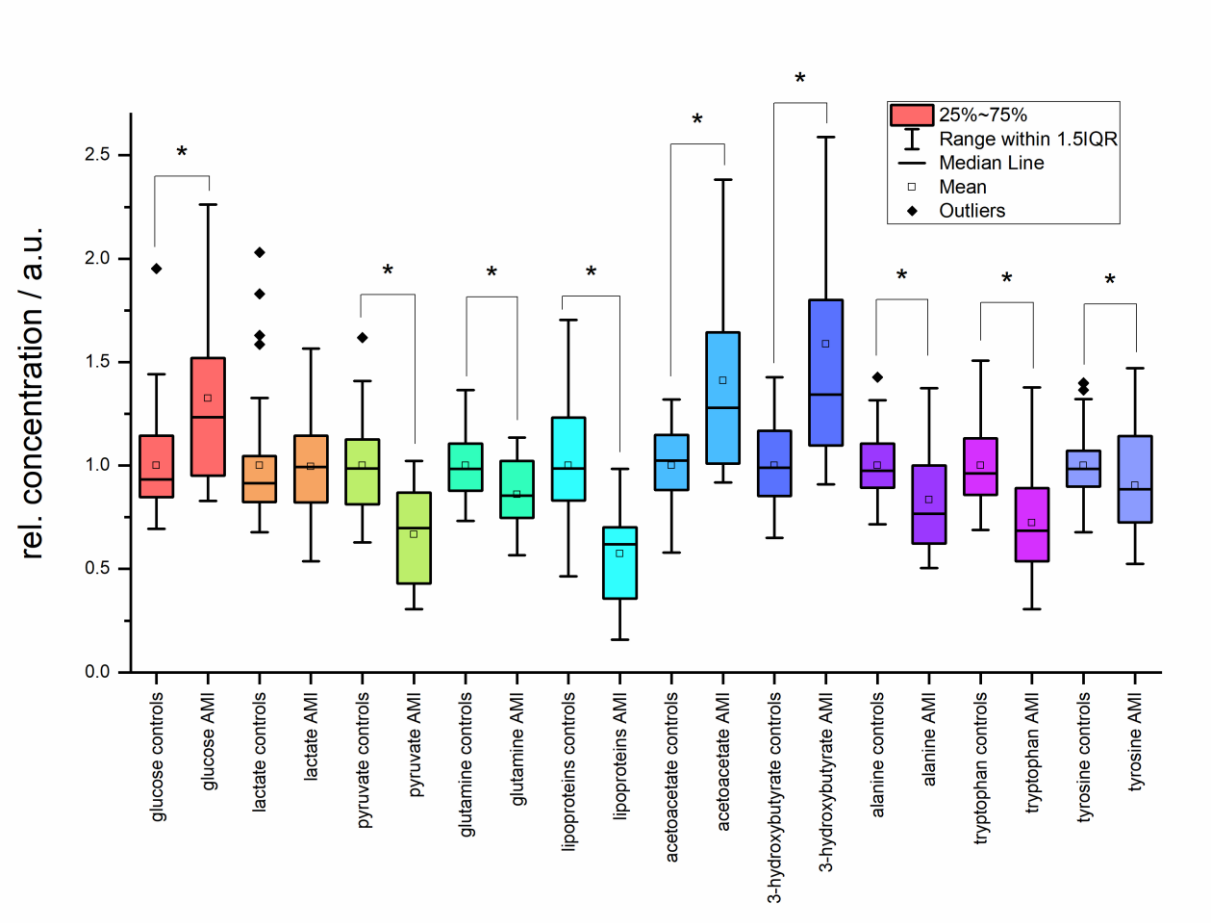

Figure 3 Relative plasma concentration of selected metabolites determined via NMR.

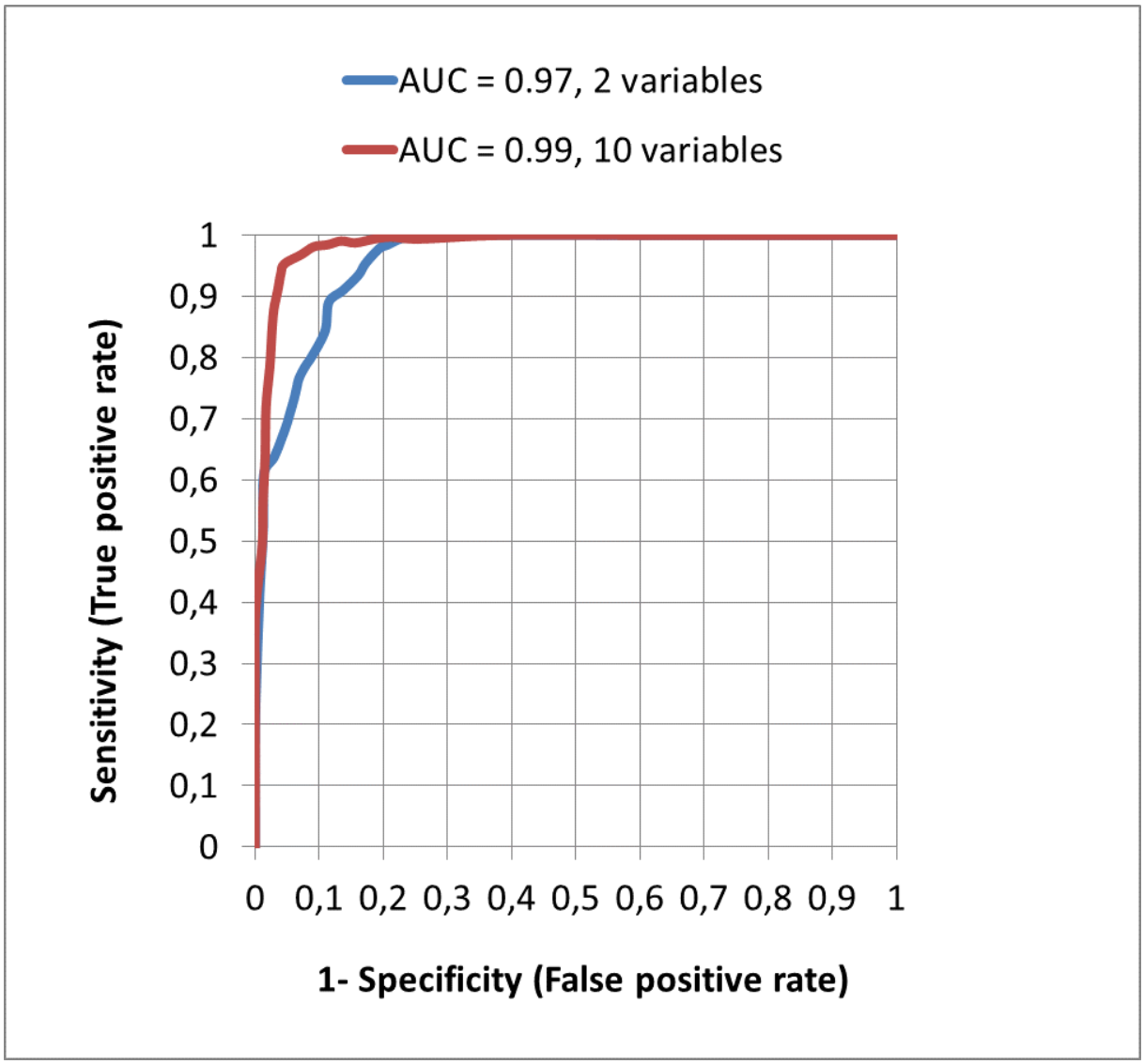

Figure 4 ROC curve based on Random Forest classification for plasma metabolites determined by NMR is showing very good performance with AUC very close to 1 for system AMI patients - controls.

Supplementary material

Multivariation analyzes based on binned NMR spectra

We performed PCA and PLSDA analyzes based on NMR spectra binned to bins of 0.001 ppm. We left out water region 4.6-5.0 ppm, and used NMR spectra from 0.5 ppm to 9.0 ppm. The result of PCA and PLSDA analyzes are shown on Figure S1.

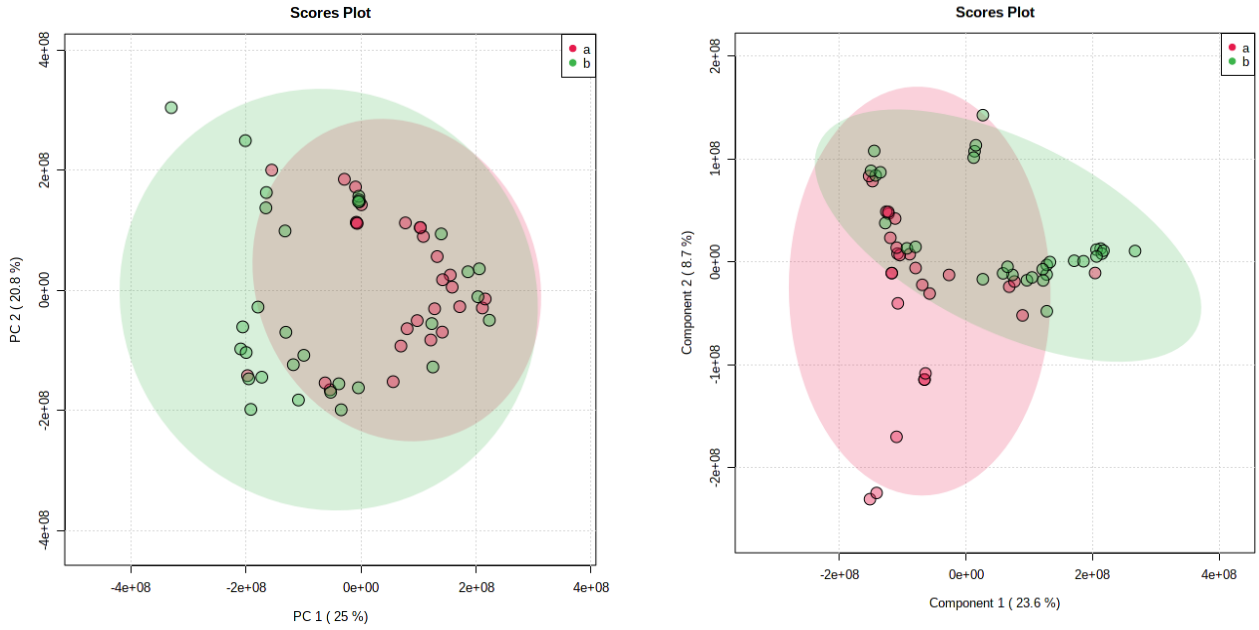

Figure S1. PCA (left) and PLSDA (right) analyzes based on NMR spectral bins of 0.001 ppm, a - AMI patients, b- controls, performed online using Metaboanalyst 4.0.

To show the reliability of PLS-DA method, we used leave-one-out cross-validation (LOOCV) and permutation test. The validation results are summarized in table S1. The permutation test performed with $p<0.01$ (0/100).

Table S1. Details from LOOCV PLS-DA validation.

Measure	1 comps	2 comps	3 comps	4 comps	5 comps	6 comps	7 comps	8 comps
Accuracy	0.76271	0.77966	0.77966	0.79661	0.79661	0.77966	0.79661	0.77966
R2	0.30297	0.44752	0.4922	0.53222	0.56052	0.58769	0.62336	0.65369
Q2	0.16529	0.25991	0.29227	0.30281	0.28668	0.28188	0.27459	0.23037