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METABOLOMIC STUDY OF ALTERED ENERGY METABOLISM DURING GLOBAL FOREBRAIN ISCHEMIA AND ISCHEMIC PRECONDITIONING IN BLOOD PLASMA IN HOMOCYSTEINE TREATED RATS

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Elevated homocysteine (Hcy) level is a well known risk factor for cardiovascular and neuropsychiatric diseases. In this study, we investigated metabolic changes in blood plasma in Hcy-treated rats. In combination with Hcy injections to induce hyperhomocysteinemia-like state, we used an animal model of global cerebral ischemia to investigate metabolic changes after 24 h reperfusion in rats. We also focused on the endogenous phenomenon known as ischemic tolerance induced by the preischemic treatment. The experiments were carried out on blood plasma samples as they are easily available and metabolically reflect the overall changes in injured organism. We observed significant changes in plasma metabolite levels of: pyruvate, citrate, acetate implicating alterations in energy metabolism, and increase in triacylglycerols, arginine and lysine, in Hcy-treated rats compared with naive animals. Ischemic insult with 24 reperfusion in Hcy-treated rats led to increase in plasma lactate, and decrease in plasma glucose, pyruvate, citrate and acetate. Complementary, an increase in ketone body 3-hydroxybutyrate was observed. The plasma metabolites: alanine, lactate, valine, glucose, leucine, isoleucine, acetate, citrate and 3-hydroxybutyrate were considered to reflect the response induced by ischemic preconditioning in Hcy rats, where the extent of postischemic damage was not as high as in the non-preconditioned rats. Our results provide evidence that nuclear magnetic resonance (NMR) spectra analysis can identify a specific group of metabolites present in plasma with the capability of discriminating between individual groups of animals. Regarding the effect of elevated Hcy level on plasma metabolome, we showed, that acetate, pyruvate and glucose had the excellent discriminatory power between Hcy-treated and naive rats plasma. Concerning ischemic insult in Hcy-treated animals, we also document the ideal discrimination of ischemic from non-ischemic rats by various groups of metabolites, that can be considered as a potential plasma biomarkers.

Key words: *homocysteine, hyperhomocysteinemia, ischemia/reperfusion, ischemic preconditioning, cerebral ischemia, plasma metabolite levels, epigenetics*

INTRODUCTION

Homocysteine is an endogenous sulfur-containing amino acid and is a metabolite of the essential amino acid methionine. It exists at a critical biochemical intersection in the methionine cycle - between S-adenosylmethionine, the indispensable ubiquitous methyl donor, vitamin B_{12} and folic acid. High blood levels of homocysteine signal a breakdown in these vital processes, resulting in far-reaching biochemical and life-threating consequences. It has been demonstrated that gender differences exist in plasma total homocysteine level which may change after extreme physical activity (1). The elevated plasma homocysteine could be *e.g.* a potential marker of acute relapsing- remitting form of multiple sclerosis (2).

Hyperhomocysteinemia (hHcy) is an unfavourable risk factor relating to ischemic stroke (3), but its role in the outcome is controversial (4-6). Its etiopathogenes is associated in particular with the auto-oxidation of homocysteine (Hcy), which results also in the increased formation of free radicals (7-10). The redox imbalance is further caused by the products of Hcy metabolism, homocysteine thiolactone and homocysteic acid, that increase oxidative stress resulting in elevated lipoperoxidation and protein oxidation (9). Interestingly, in spite of the high clinical relevance of elevated Hcy levels in the development of human stroke only a limited number of experimental approaches can be found in the literature to describe the mutual influence of co-morbid hyperhomocysteinemia to ischemic damage on animal models of human stroke (9).

Four-vessel occlusion model in rat induces transient forebrain ischemia that resembles cardiac arrest in clinical situations. Tissue recovery from ischemia is variable and dependent upon a number of factors which include the extent and completeness of ischemia, duration of ischemic insult, preischemic glucose contents, *etc*. The pathogenesis is associated with depletion of cellular energy sources, the release of excitatory amino acids, mitochondrial dysfunction and excessive generation of reactive oxygen and nitrogen species (11-13). Paradoxically, the restored perfusion migh result in

subsequent injury (ischemic/reperfusion injury - (IR)). The adaptation to a sublethal stimuli can induce some level of resistance against subsequent lethal ischemic attack. This protective strategy is known as ischemic tolerance and it is developed by an organism to protect itself from the ever-present risk of ischemic injury (14). Ischemic preconditioning is a method introducing ischemic tolerance in animals, performed by period of short ischemic insult.

The vast majority of physiological and pathophysiological processes in tissues are reflected in the blood, even in intact blood brain barrier conditions. Easy accessibility makes blood very convenient for the clinical use. Metabolic studies of blood plasma in ischemic mice (15) and ischemic rats (16) have shown an increase in ketone bodies in the blood, a state very similar to ketosis, that occured also in ischemically pre-treated rats (IPC) to almost the same extent. Furthermore, there were plasma metabolites found to excellently discriminate between ischemic, respectively ischemic with preconditioning rats and controls (16). This underlines the importance of metabolic studies also towards potential biomarkers, easily detectable in clinical practice.

The aim of this study is to investigate the influence of increased homocysteine level on plasma metabolome without any ischemic insult, after ischemic insults and 24 h reperfusion and after ischemic insults and 24 h reperfusion prior ischemic preconditioning. Based on the obtained relative changes in plasma metabolites, we also focused on identification of potential plasma low molecular biomarkers concerning Hcy treatment, and also in combination with ischemia and ischemic preconditioning.

MATERIALS AND METHODS

Animals

Total 40 adult male Wistar rats at the age of 4 months (having a mean weight of 335.25 g with a standard deviation of 19.07 g) were used in this study. The complete ischemia was not achieved in 6 animals, and 8 animals died during or after surgery. They were housed in a temperature-controlled room 22 ± 2°C on a 12-h light/dark cycle with free access to food and water. All procedures on animals were performed in accordance with ethical and moral principles and approved by the State Veterinary and Food Department of the Slovak Republic.

Introducing hyperhomocysteinemia

We used a model of experimental hyperhomocysteinemia originally developed by Streck *et al*. (17). The homocysteine was administered at a dose of 0.45 μmol/g of body weight subcutaneously twice a day with the 8 h intervals, at strictly controlled dosage times for 14 days.

Induction of ischemic preconditioning and ischemia

Brain ischemia was induced by standard four-vessel occlusion model as described by Kovalska *et al*. (6). On the day 1, bilateral vertebral arteries were irreversibly electrocauterized under anesthesia with sevoflurane (a mixture of 3.5% sevoflurane in 33% O_2 and 66% N_2O). On the day 2, 15 minutes ischemia was induced by occluding the arteries on awakened animals. After 24 h of reperfusion animals were sacrificed.

Ischemic preconditioning: on the day 2, 5 minutes sublethal ischemia was induced by occluding the arteries on awakenied animals. Both common carotids were occluded 48 h later to induce 15 minutes of lethal ischemia. After 24 h of reperfusion animals were sacrificed.

Rats which lost their righting reflex, were unresponsive and whose pupils were dilated during ischemia were selected for the experiments. Rats with seizures after ischemia were excluded from the study. Shame operated rats were sacrificed while anesthetized immediately after surgery.

Rats labeling in the study

naive C rats – rats with sham operation (n = 5)

Hcy C rats – Hcy treated rats with sham operation (n = 8)

Hcy IR rats – Hcy treated rats with 15 min global cerebral ischemia followed by 24 h reperfusion (n = 7), IR, ischemia reperfusion.

Hcy IPC rats – Hcy treated rats with 5 min ischemic preconditioning, after 48 h: 15 min global cerebral ischemia followed by 24 h reperfusion (n = 6), IPC, ischemic preconditioning.

Determination of plasma homocysteine concentration in animal plasma

Total Hcy (tHcy) plasma level was determined by enzymatic two-part reagent system (Hcy Liquid Stable Reagent Kit, Erba Lachema, 50003526) using a chemical analyzer (Siemens ADVIA 1650).

Sample preparation

Stock solution consisted of: phosphate buffer 100 mM, pH 7.4 (pH meter reading), 0.28 mM TMSP-d_4 (trimethylsilylpropionic acid -d_4) as a chemical shift reference in deuterated water.

Blood was collected exclusively in EDTA coated tubes, centrifuged at 4°C, 1500 g , for 20 minutes. Plasma was frozen at –80°C until used.

Deproteinated plasma

The volume of 300 μL of plasma and 600 μL MetOH were mixed for 2 minutes. After storage at –20°C for 20 minutes, the mixture was centrifuged for 15 minutes at 10,000 g. Supernatant (650 μL) was dried out and subsequently carefully mixed with 100 μL of stock solutions and 500 μL of deuterated water. 550 μL of final mixture was transferred into 5 mm NMR tube.

Nuclear magnetic resonance data acquisition

NMR data were acquired on 600 MHz NMR spectrometer Avance III from Bruker equipped with cryoprobe at T = 310 K. Initial settings (basal shimming, receiver gain, water suppression frequency) were done on an independent sample and adopted for measurements. After preparation, samples were stored in a sample jet automatic machine, cooled at approximately 5°C. Before measurement each sample was preheated on the 310 K for 5 minutes. An exponential noise filter was used to introduce 0.3 Hz line broadening before Fourier transform. The proton NMR chemical shifts are reported relative to TMSP-d_4 signal which was assigned a chemical shift of 0.000 ppm.

We modified the standard profiling protocols from bruker as follows: noesy with presaturation: FID size 64k, dummy scans 4, number of scans 128, spectral width 20.4750 ppm; cosy with presaturation: FID size 4k, dummy scans 8, number of scans 1, spectral width 16.0125 ppm; homonuclear J- resolved: FID size 8k, dummy scans 16, number of scans 4; profiling cpmg: FID size 64k, dummy scans 4, number of scans 128, spectral width 20.0156 ppm. All experiments were conducted with a relaxation delay of 5 seconds.

Data analysis

The data analysis was performed in several steps, beginning with preprocessing, binning and normalization. Afterwards, metabolites analysis and prioritization were performed by both the traditional statistical-testing method and by the Random Forest machine learning method.

Binning, preprocessing, normalization

All spectra were binned to bins of the size of 0.001 ppm, starting from 0.000 ppm to 9.000 ppm, with excluded water region: 4.6 – 2.8 ppm and EDTA regions 3.06 – 3.22 ppm and 3.60-3.65 normalized by the quantile- quantile normalization, which appears to be among the best normalization methods in NMR (18). The spectra normalization was performed in MetaboAnalyst 3.0 (19).

Spectra were solved with the help of human metabolomics database (www.hmda.ca), chenomx software and literature research (20, 21). For all compounds, the multiplicity of peaks was confirmed in 2D j-resolved spectra and homonuclear cross peaks were confirmed in 2D cosy spectra.

After the metabolites were identified, (*Fig. 1*), we chose spectra subregions with only single metabolite assigned, or minimally affected by other co-metabolites. In 0.001 ppm binned spectra, we summed integrals of selected metabolites. These data were used for a single metabolite analysis. Although this approach did not use all signals of all molecules in full ranges, it was sufficient for tracking relative changes in metabolites quantities. For metabolites having more than one signal, integrals of all appropriate signals were summed. Metabolites showing weak intensive peaks or strong peaks overlap were excluded from this evaluation. Some primary calculations were performed by using Metaboanalyst 3.0 (19). All definite statistical tests were performed in the house made software in Matlab R2015a.

RESULTS

Plasma homocysteine level

Plasma total homocysteine (tHcy) level was determined from plasma collected at time of animals sacrifice, *i.e.* time distance from last Hcy injection cca. 24 h for control rats, cca. 96 h for IR rats and cca. 144 h for IPC rats.

Hcy levels: naive C rats – 5.9 ± 0.7 μmol/l; Hcy C rats 6.4 ± 0.7 μmol/l; Hcy IR rats 8.4 ± 1.4 μmol/l; Hcy IPC rats 5.0 ± 1.3 μmol/l. We did not observe any significant relationship of tHcy plasma level neither between naive and Hcy treated rats, nor in the Hcy group for any combination of C, IR or IPC, also not to body mass.

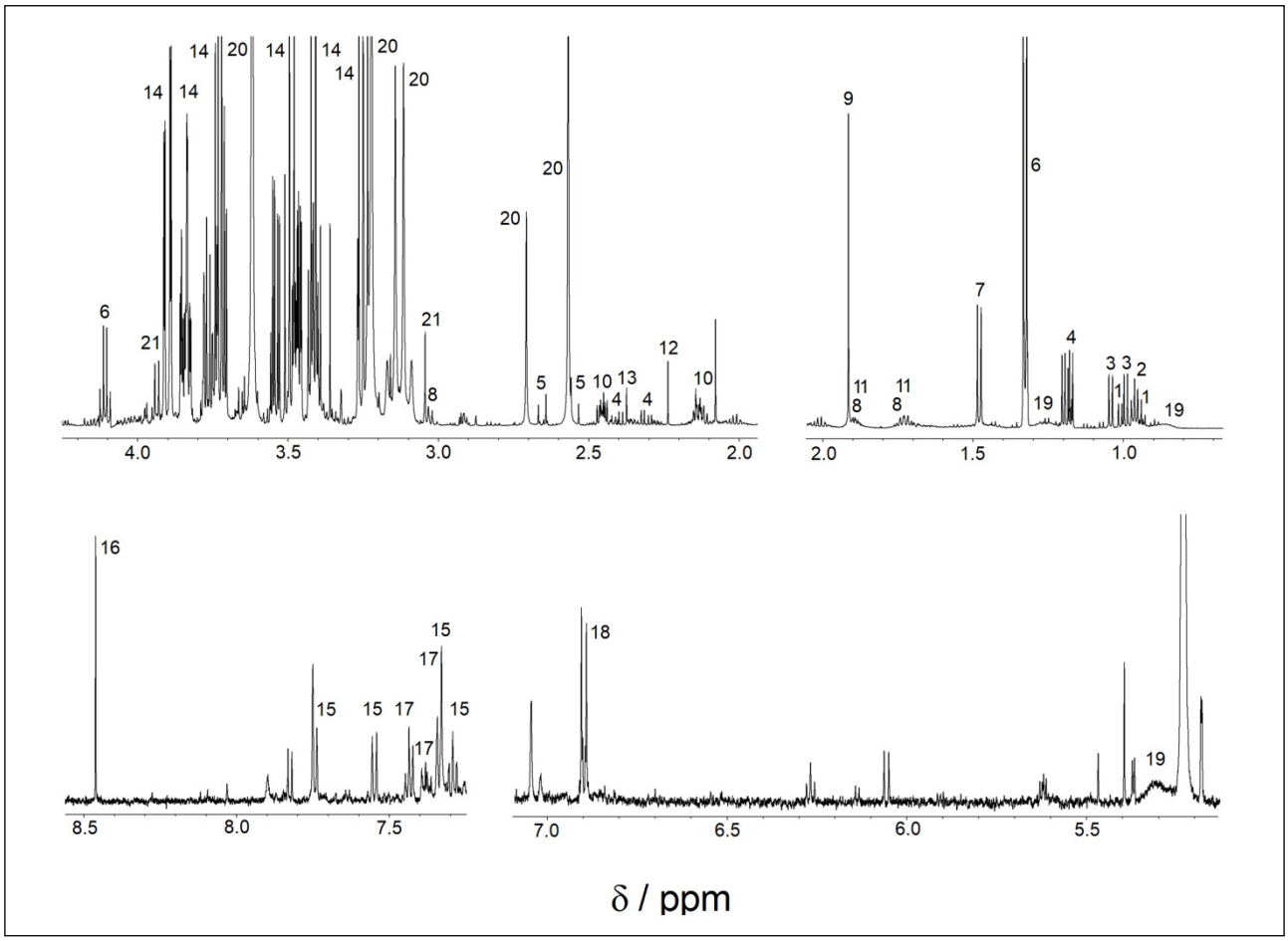

Fig. 1. Representative 600-MHz ^{1}H-nuclear magnetic resonance (NMR) spectra of deproteinated blood plasma, noesy acquisition with typical peaks for selected metabolites: 1: isoleucine; 2: leucine; 3: valine; 4: 3-hydroxybutyrate; 5: citrate; 6: lactate; 7: alanine; 8: lysine; 9: acetate; 10: glutamine; 11: arginine; 12: acetone; 13: pyruvate; 14: glucose; 15: tryptophan; 16: formate; 17: phenylalanine; 18: tyrosine; 19: lipoproteins; 20: EDTA and its chelates; 21: creatine.

Table 1. Relative changes in metabolite levels in blood plasma between naive rats (C) and homocysteine (Hcy) treated rats: without ischemic insult (Hcy C), after 24-h reperfusion (Hcy IR) and after 24-h reperfusion with ischemic preconditionning (Hcy IPC).

	Hcy C/ naive C	% change	**Hcy IR/ Hcy C**	% change	**Hcy IPC/ Hcy C**	% change	**Hcy IR/ Hcy IPC**	% change
lactate	0.19	9	0.00023	111	0.068	21	0.000045	–43
formate	0.018	23	0.56	–12	0.81	–3	0.75	10
alanine	0.068	–3	0.22	–11	0.30	4	0.33	16
valine	0.0089	–11	0.00075	31	0.61	–1	0.00064	–24
glucose	0.000083	–8	0.00023	–22	0.000083	–10	0.000045	18
leucine	0.19	–2	0.00075	35	0.44	3	0.0034	–23
isoleucine	0.00065	–10	0.0059	38	0.44	–2	0.0034	–29
acetate	0.000083	196	0.068	–10	0.037	9	0.0034	21
acetone	0.000083	–56	0.22	8	0.068	–18	0.037	–24
pyruvate	0.000083	–52	0.014	–8	0.004	–5	0.33	4
citrate	0.0016	–15	0.00023	–29	0.004	–20	0.18	14
phenylalanine	0.81	–0.7	0.00023	16	0.000083	13	0.18	–3
tryptophan	0.068	–4	0.13	–8	0.0089	9	0.012	18
tyrosine	0.018	–7	0.068	64	0.00065	10	0.18	4
creatine	0.44	–6	0.00023	83	0.000083	65	0.000045	–10
glutamine	0.30	0	0.13	–5	0.19	3	0.00064	8
arginine	0.000083	17	0.37	–2	0.00065	18	0.00064	20
lysine	0.00024	11	0.0022	12	0.000083	24	0.089	11
lipoproteins	0.00024	21	0.0022	–14	0.000083	–23	0.54	4
3-hydroxybutyrate	0.12	–3	0.00023	32	0.018	–29	0.0034	–46

Table 2. Area under the curve (AUC) and out of bag error (OOB) parameters derived from random forest classification, algorithm fed by the relative concentrations of plasma metabolites determined by NMR.

	AUC	**OOB**	**metabolites**
Naive C versus **Hcy C**	1	0	acetate, pyruvate, glucose
Hcy IR versus **Hcy C**	1	0	creatine, citrate, 3-hydroxybutyrate or glucose
Hcy IPC versus **Hcy C**	0.992	0	lysine, creatine, phospholipids or lysine, glucose, creatine
Hcy IPC versus **Hcy IR**	0.915	0	glucose, lactate, creatine

Metabolite levels in plasma

Statistical evaluation of relative changes in plasma metabolite levels was performed by Wilcoxon's test with a P value < 0.05 considering significant. The results are summarized in *Table 1* and visualized in *Fig. 2*. Lipoproteins were representing components of very low density and low density lipoproteins, consisting mainly of apolipoproteins, triacylglycerols, cholesterol and phospholipides (22).

Random forest classification

Having calculated relative metabolite concentrations in samples, random forest (RF) classification with nested cross validation was run in order to classify the system at the metabolite level. The calculated reciever operator characteristic curves (ROC) are often summarized into a single metric known as the: area under the curve (AUC), what is the probability that a diagnostic test or a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one. Out of bag error (OOB) is a method of measuring the prediction error of RF classiffier. The higher AUC (range 0 – 1) and the lower OOB (range 0 – 1), the better is the discriminatory power. The results are summarized in *Table 2*.

DISCUSSION

Plasma homocysteine level

Several forms of total Hcy are present in blood plasma under physiological conditions as a results of Hcy metabolic conversion and oxidation as well as bindig to the plasma protein. There are plasma forms include: free Hcy; protein-bound Hcy S-linked, and N-linked); oxidized forms of Hcy; and Hcy-thiolactone. Reduced, oxidized and protein bound Hcy was correlated with tHcy (23), and Hcy-N-protein has been correlated with plasma tHcy (24). In human beings, the majority of plasma tHcy (80 – 90%) is N-linked and S-linked to γ-globulins or serum albumines (24-25), but in rats only about 30% of tHcy is bound to proteins (26).

As we have observed, estimated levels of Hcy in plasma in the Hcy-treated animals exceeds only slightly the level in controls. One of the explanations comes from the rapid Hcy phamacokinetics, conversion and Hcy elimination. Secondarily, the analytical method used in the study is based on the reduction of oxidized Hcy forms back to Hcy and its irreversible conversion to cystathion, which is probably unable to detect all metabolized forms of Hcy.

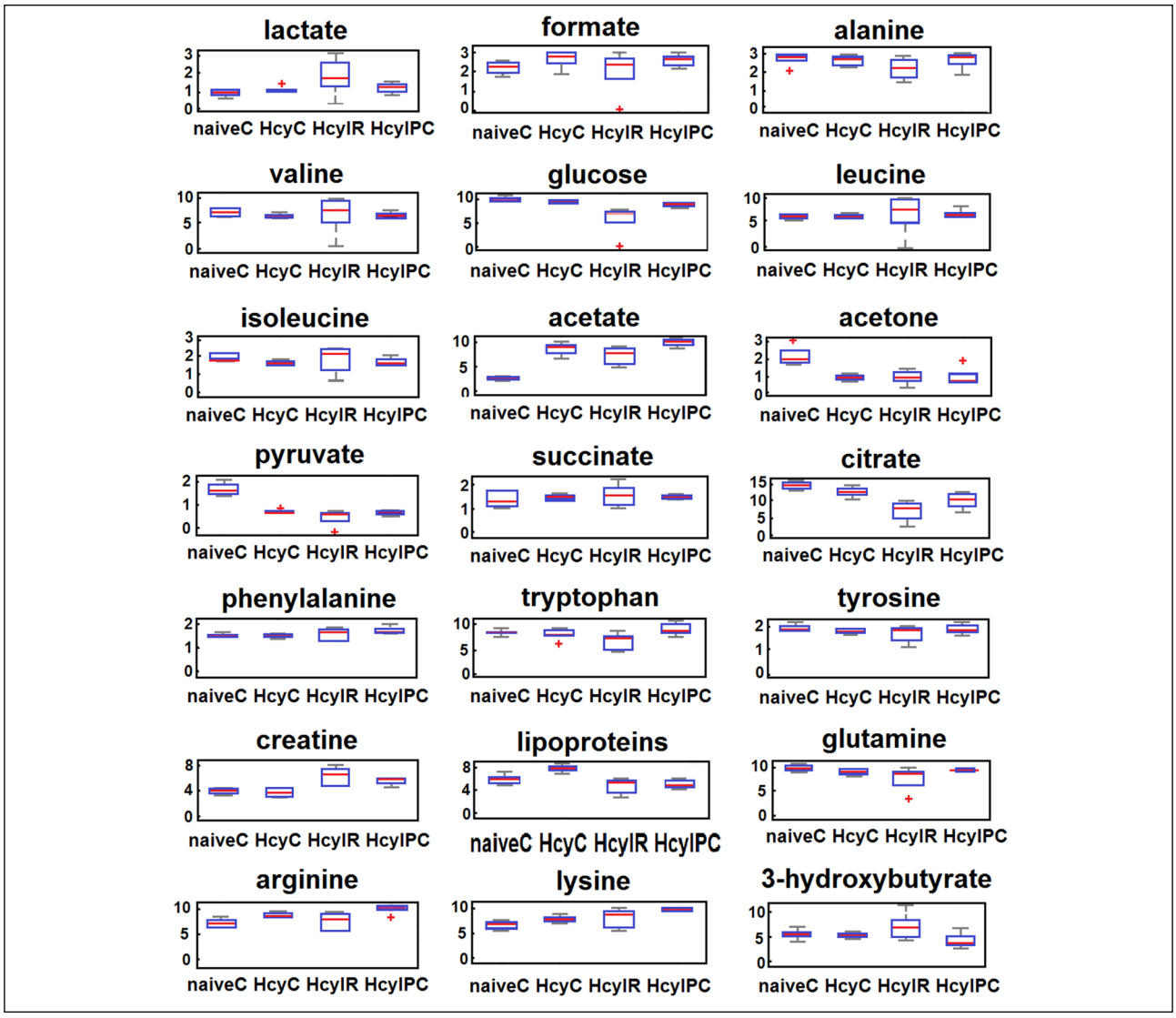

Fig. 2. Relative levels of plasma metabolites, (not having the common scaling factor) in naive-sham group (naive-C); and homocysteine-treated rats: sham group (Hcy-C); 24-h reperfusion (Hcy-IR); ischemically preconditioned with 24-h reperfusion (Hcy-IPC).

Influence of homocysteine treatment on plasma metabolome

Development of hyperhomocysteinemia-liked state in rats was manifested by changes in plasma metabolites levels compared to the control sham-operated animals (*Table 1*). Blood glucose decreased in Hcy-C rats only slightly compared to naive controls, however, rats markedly differed in plasma glycolytic intermediate pyruvate, that was found significantly decreased. Together with increased plasma formate level it can indicate an increased activity of pyruvate dehydrogenase, similarly to the observation made by Jin and Brennan (27) in cultured astrocytes treated with homocysteine. A key intermediate of tricarboxylic acid (TCA) cycle, citrate was found significantly decreased in Hcy-C rats compared to naive-C animals, implicating possible alterations in mitochondrial amphibolic pathways. Significantly increased acetate plasma level also supports the concept of general alterations in energy metabolism induced by Hcy.

Hcy-treated rats showed a strong and statistically significant increase in plasma levels of phospolipids fraction, which also contains a large part of triacylglycerols. This is in accordance with the finding of Frausher *et al*, who reported about increased plasma triacylglycerols after oral administration of homocysteine (28) related to the additional role of triacylglycerols - linked lipoproteins for vascular pathology.

Arginine level was found significantly increased in blood plasma of Hcy-treated rats. It is, besides participating in other metabolic pathways such as protein synthesis and conversion to urea and ornithine, the only source for nitric oxide (NO) production, where endothelial NO has vasoprotective and antiatherosclerotic properties. Supplementation of the NO synthase substrate L-arginine can improve endothelial dysfunction in animals and human. Therefore, arginine is considered as a metabolic protector against cardiovascular disease (29, 30). Its increased plasma level in Hcy treated rats may signal either insufficient arginine utilization, or its overproduction as a response to the elevated Hcy level. Plasma levels of aminoacid lysine, important building block for all proteins, was also found significantly increased in Hcy rats. Lysine can affect NO production *via* competition for the shared intracellular transport protein with arginine. Therefore, it may be suggested that the excess of lysine may inhibit NO biosynthesis. In a rat model of heart failure, the effect of arginine on NO

production could be blocked by lysine, suggesting that resticted arginine transport might play an important role in enhanced NO production in these conditions (30).

The plasma isoleucine levels were found to be decreased in Hcy-treated rats compared to the naive controls. Besides functioning as a gluconeogenetic substrate and potential energy stabilizer and regulator, it has been found to be deficient in people suffering from several mental and physical disorders (31). Here it is important to note that metabolites which are included primarilly in energy metabolism are generally linked with several metabolic pathways, that are not linear but often interconnected and causal. Secondarily, the found changes in metabolic intermediates confirm the multifactorial impact of hyperhomocysteinemia, which also affects metabolites, which do not directly participate in the homocysteine metabolism.

Twenty four-hour ischemia in homocysteine-treated rats

Koch *et al*., demonstrated an onset of the hepatic ketogenesis after middle cerebral artery occlusion (MCAO) in mice in tissues and blood with 90 minutes reperfusion (15). Further, 24-h global cerebral ischemia causes an increased level of ketone bodies and decreased content of glycolytic products in the rats in the blood (16). During global cerebral ischemia, the critical variables are restricted delivery of the main energy metabolites and oxygen. The lack of oxygen activates residual glucose metabolism by anaerobic glycolysis with an increased lactate production. In Hcy-treated rats, the decrease in plasma glucose and the increase in plasma lactate with 24-h reperfusion correspond to this, already generally described state. The plasma levels of pyruvate and acetate significantly decreased 24 hours after ischemia, which is comparable to naive rats (16). When oxygen is in short supply, the pyruvate can be converted to lactate. Acetate in blood of rats has been decreased during starvation (32). Remarkably in our conditions, we noticed a decreased food intake in animals after surgery, as it was shown in mice after MCAO previously (15). Decreased appetite was more noticeable in animals without preconditioning; however, we did not run behavioral tests to confirm it. Acetate functions metabolically in a similar manner to the ketone bodies, acetoacetate and 3-hydroxybutyrate. All three compounds are important in the redistribution of carbon substrates throughout the body (32).

Both groups, naive and Hcy rats subjected to ischemia with 24-h reperfusion exhibited common features of metabolic pattern with the naive rats (16), such as a slowdown of the TCA cycle rate by significant downregulation of the energy metabolite citrate. It is generally accepted that TCA cycle is used by all aerobic organisms to release stored energy, and citrate restriction might have a great impact on the metabolism. The level of glutamine, the potential energy source in TCA cycle did not change in 24-h reperfusion in naive (16) as well as in Hcy treated rats. The plasma level of ketone body 3-hydroxybutyrate seem to follow the same profile in Hcy rats as in naive rats (16), unlike another ketone body component – acetone that stayed unchanged. The quantitative evaluation of acetoacetate was not performed due to poor peak quality. Interestingly, the increase in 3-hydroxybutyrate may be a sign of an incipient and ongoing ketosis. 3-hydroxybutyrate is possibly utilized as an alternative energy substrate and may ameliorate the disruption of cerebral energy metabolism after hypoxia, anoxia, and ischemia, where the anaerobic glycolytic pathway is active (33). The decreased amount of triacylglycerols and phospholipides after 24-h reperfusion is very similar to that found in naive rats (16). Remarkably, the utilization of triacylglycerols as a metabolic source for future ketone bodies is demonstrated by statistically significant reduction in triacylglycerols in Hcy-IR/IPC rats compared to Hcy controls. Plasma creatine levels were significantly increased after ischemic insult when compared to naive rats. Creatine serves mainly for recycling of adenosine triphosphate, primarily in muscle, heart and also brain tissue. It may act as a neuroprotective supplement under the conditions of compromised cellular oxygenation and bioenergetics in ischemic conditions.

Comparison twenty four-hour ischemia with and without ischemic preconditioning in homocysteine-treated rats

The phenomenon of ischemic preconditioning has been recognized as one of the most potent mechanisms to protect tissues against ischemic injury (14). In general, one would expect the weaker metabolic changes caused by ischemia in already preconditioned rats. This was the case in levels of some metabolites, where the changes after ischemia with preconditioning were less pronouced (*Fig. 2*). Taking this as a criterion, the levels of plasma metabolites: alanine, lactate, valine, glucose, leucine, isoleucine, acetate, citrate and 3-hydroxybutyrate are good candidates reflecting the protective effect of preconditioning in Hcy-IPC rats (*Fig. 2*).

Metabolism and epigenetics

Interestingly, changes in the plasma metabolites can also be interpreted in terms of novel impact of metabolism on the tissue epigenetic regulation. There is evidence, that ketosis, *i.e*. increased level of ketone bodies in plasma, above all 3-hydroxybutyrate which reflects the metabolic state of brain, may influence the tissue epigenetic programming through the histone acetylation (34, 35). Ketonic state, which is observed after ischemic insult (15, 16) and also found in our study, is interfering with the level of reduced form of NAD (NAD^+), as well as it changes the ratio between S-adenosyl methionine/S-adenosyl homocysteine, all changes which may have impact an on the level of histone acetylation (31). Further, another epigenetic mark such as methylation status is broadly sensitive to oxygen concentration, one-carbon, and TCA-related metabolism (34). Taking all this to consideration, the metabolic enzyme expression has the potential to influence DNA methylation and histone acetylation in mammals. It can sufficiently drive an important biological outcome such as stem cell fate or possibly tissue adaptation to preischemia (34), what makes detailed metabolomic and enzymatic studies more interesting and useful.

Altered energy metabolism scenario and its mitigation via preconditioning

Based on obtained relative changes of plasma metabolites it is possible to point out the main metabolic processes, where the metabolites with altered plasma levels play a key or one of the crucial roles (*Fig. 3*). However, having evaluated only a limited number of metabolites this scenario is derived exlusively from the results of this study.

Random forest classification

It is important to recognize two different approaches when analyzing the metabolomic data. Those that aim to understand biological processes and those that aim for biomarkers. With a focus on gaining improved biological understanding through the analysis of metabolite profiles, P-value derived from Wilcoxon's test was used in the previous text. 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 somewhat different analysis,

evaluation and validation procedures. Perhaps the most important difference is that biomarker models are not intended to help explain biology, they are rather designed to discriminate with an optimal sensitivity/specificity (36).

As described above, hyperhomocysteinemia in rats was manifested in changes of various plasma metabolite levels. Due to the rapid metabolism of Hcy in rats and the fact, that hyperhomocysteinemia when inducted according to Shreck (17) is not easily detected if the blood is taken several hours after last Hcy injection, the combination of other, easily detectable plasma metabolites can be of use. There was found a combinations of plasma metabolites: acetate, pyruvate and glucose, that discriminated the Hcy-treated rats from the naive ones with 100% sensitivity and specificity. Based on these results, named metabolites served together as plasma biomarkers. Regarding ischemia, in the Hcy-treated rats, the metabolites: citrate, 3-hydroxybutyrate and creatine or glucose were responsible for an excellent discrimination between Hcy-C and Hcy-IR. The ischemically preconditioned Hcy-IPC rats were almost idealy discriminated from Hcy-C animals by combinations of metabolites: lysine, creatine a lipoproteins, or by lysine, glucose and creatine combination (both with AUC of 0.992). By adding other metabolites, the discriminatory ability was enhanced and AUC reached the level of 1. To complete the scheme, the discrimination between Hcy-IPC and Hcy-IR rats performed very well for three metabolites: glucose, lactate and creatine (AUC of

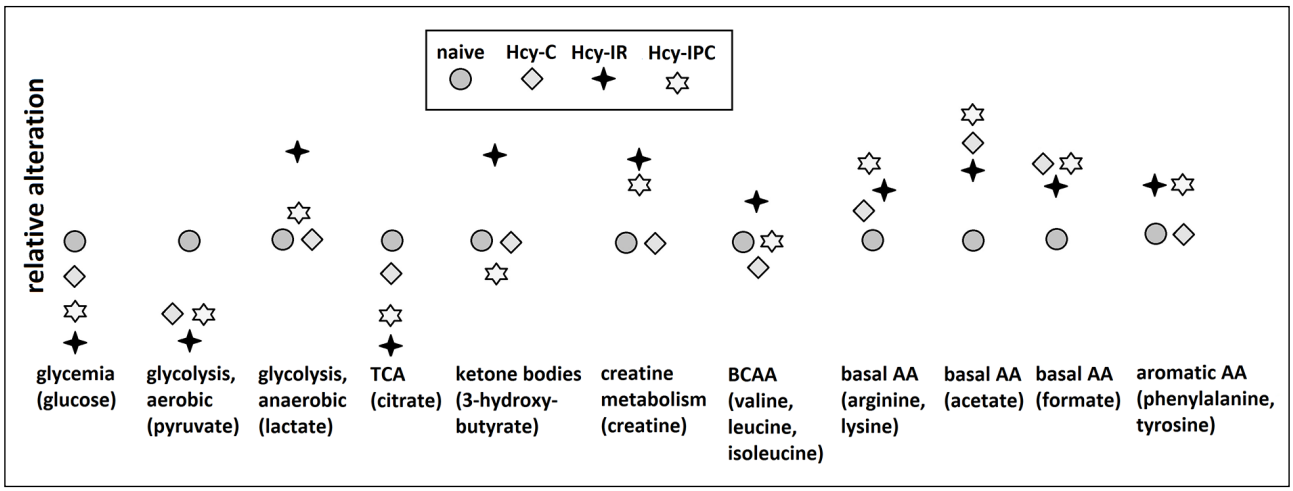

Fig. 3. Simplified visualisation of proposed alterations in the selected metabolic states and processes based on relative change in plasma level of crucial metabolite (in brackets), naive: rats with sham operation; Hcy-C: Hcy-treated rats with sham operation; Hcy-IR: Hcy-treated rats with 24-h reperfusion; Hcy-IPC: Hcy-treated rats with ischemic preconditioning; BCAA: branched chain amino acids. (The alterations are related to metabolites levels, non having common scale factor.)

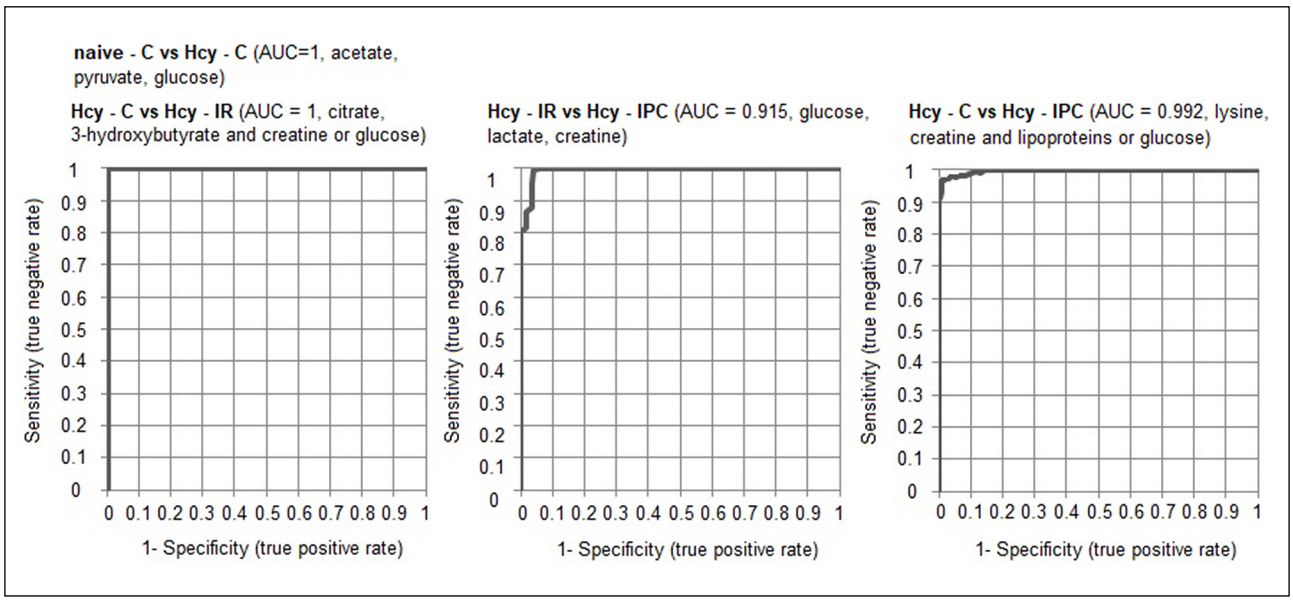

Fig. 4. ROC curves derived from random forest classification. The algorithm was fed by the relative concentrations of plasma metabolites. In the legend, there are listed three metabolites responsible for achieving drawn curve. Note: An important parameter, area under curve (AUC) represents ranking quality. The AUC of a ranking is 1 (the maximum of AUC value) if no positive example precedes any negative example. An AUC of 0.5 is equivalent to random classifying subjects as either positive or negative (*i.e.* the classifier is of no practical utility). Having very high AUC values (near or equal to 1), we consider the system as a one with excellent discrimination power.

0.915). The further improving of discriminatory ability was achieved by involving next four metabolites: glutamine, arginine, acetate and 3-hydroxybutyrate. In this case, the algorithm performed the discrimination with AUC of 1. Corresponding ROC curves are showed in *Fig. 4*. The listing of more than one metabolite combination showed that there are more metabolite groups that can be used as potential biomarkers, confirming the great discrimination power of the system. These results are in a logical relationship with results obtained by Wilcoxon's test used for the understanding of biological processes.

Our paper documents that NMR spectra analysis can identify a specific group of metabolites present in blood plasma with the capability for discrimination between between HCy-treated and naive rats: acetate, pyruvate and glucose. In addition, study also shows an ideal discrimination between ischemic from non-ischemic rats achieved by particular metabolite groups. With the aim to gain an improved biological understanding through the analysis of metabolite profiles, when comparing Hcy-treated animals with naive ones, we observed significant changes in plasma metabolite levels: pyruvate, citrate, acetate implicating alterations in energy metabolism, as well as increase in the triacylglycerides, arginine and lysine. Global cerebral ischemia with 24 reperfusion in Hcy-treated rats led to the increase in plasma lactate, and decrease in plasma glucose, pyruvate, citrate and acetate. Moreover, an increase in ketone body 3-hydroxybutyrate was observed. The protective effect of ischemic preconditioning was reflected in the reduction of extent changes in several plasma metabolites.

Authors' contributions: E. Baranovicova – animal and all experimental measurements, data processing, manuscript preparation; D. Kalenska – animal and all experimental measurements; A. Tomascova – animal preparations and experimental measurements; J. Lehotsky – experimental design, coordination of the experimental measurements and data, final preparation and approval of data and manuscript.

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