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Metabolomic profiling of blood plasma of patients with lung cancer and malignant tumors with metastasis in the lungs showed similar features and promising statistical discrimination against controls

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Targeting metabolomic pathways is a promising strategy for cancer treatment. Alterations in the metabolomic state have also an epigenetic impact, making the metabolomic studies even more interesting. We explored metabolomic changes in the blood plasma of patients with primary and secondary lung cancer and tried to explore their origin. We also applied a discrimination algorithm to the data. In the study, blood samples from 132 patients with primary lung cancer, 47 with secondary lung cancer, and 77 subjectively healthy subjects without any cancer history were used. The samples were measured by NMR spectroscopy. PCA and PLS-DA analyses did not distinguish between patients with primary and secondary lung tumors. Accordingly, no significantly changed levels of plasmatic metabolites were found between these groups. When comparing with healthy controls, significantly increased glucose, citrate, acetate, 3-hydroxybutyrate, and creatinine balanced with decreased pyruvate, lactate, alanine, tyrosine, and tryptophan were found as a common feature of both groups. Metabolomic analysis of blood plasma showed considerable proximity of patients with primary and secondary lung cancer. The changes observed can be partially explained as cancer-derived and also as changes showing ischemic nature. Random Forrest discrimination based on the relative concentration of metabolites in blood plasma performed very promising with AUC of 0.95 against controls; however noticeable parts of differencing metabolites are overlapping with those observed after ischemic injury in other studies.

Key words: lung cancer, NMR metabolomics, blood plasma

Lung malignant tumors have the highest morbidity and mortality rates of any cancer worldwide. A promising therapeutic strategy lies in targeting the altered metabolomic pathways of cancer cells [1]. Studies using the metabolomic approach have shown that there are alterations in the general metabolism in patients with cancer reflecting both the common features and also the disease phenotypes. Tumor entity and secondary malignant tissue communicate with the blood, which finally affects its composition i.e., on metabolomic level [2–5]. In addition, all organs are challenged with the altered levels of basal metabolites what can result in the secondary response. Blood is an important metabolic information carrier and, due to its easy accessibility, very convenient for clinical use. The blood metabolic profiling is thus one of the most substantial sources reflecting metabolic demands and conditions of the whole organism.

Besides essential biological interpretation of metabolomic studies, their importance is underlined by the fact that low molecular metabolites may serve as cancer biomarkers in blood plasma, serum, or urine in various cancer types [6]. Remarkably, the metabolic phenotyping of plasma allows detection of lung cancer even in an early stage. Recent studies by Louis et al. described the possibility to discriminate between lung cancer patients and controls [7], and also between lung cancer and breast cancer patients, where the orthogonal partial least squares discriminant analysis (oPLS-

DA) analysis was performed with very high specificity and sensitivity based on binned NMR spectra of blood plasma [8]. These studies, however, did not determine exactly the particular and plasma metabolites that are of the highest discriminatory power. The great potential of NMR plasma metabolomics to be considered as a screening tool for lung cancer was shown also by Rocha et al. [9] who employed partial least squares discriminant analysis (PLS-DA) modeling of cpmg spectra and Monte Carlo Cross validation. PLS-DA analyses were able also to effectively distinguish the metabolic profile of serum in non-small-cell lung carcinoma patients with or without microwave ablation treatment from that of healthy controls [10]. Recently, Berker et al. published an extended study where NMR spectra of measured matched tissue and serum samples were used also for typing, staging, and survival estimation of early stages of lung cancer [11]. Despite the rapid growth of metabolomics studies, only a little part of them uses the receiver operator characteristics (ROC) curve analysis as a standard method for describing and accessing the performance of medical diagnostic tools [12].

Published NMR studies are predominantly focused on statistical description using multivariate and machine learning techniques rather than biological processes [7–10]. In our work, we focused on the metabolomic alterations in blood plasma in patients with primary and secondary lung tumors and we tried to analyze the origin of observed changes. It was also of our interest whether the origin of malignant structure findings on the lung is manifested in diverse plasma composition on the metabolomic level. Besides broadly used PLS-DA, we used also the ROC curve derived from Random Forest (RF) discrimination algorithm to attempt the differentiation between patients and control groups, between patients with primary and secondary lung cancer as well as among lung cancer subtypes. Unlike published studies using mainly binned NMR spectra [7–10], we used the relative concentration of metabolites in blood plasma as input variables in order to improve the informative value of the study.

Patients and methods

Samples. Blood specimen was sampled from patients on the day of planned surgery in the fasting state. Based on the histological finding, patients were assigned into two subgroups (Tables 1 and 2), patients with primary lung cancer (LC), and patients with secondary lung cancer i.e., metastases in lungs of other origins (LM). As controls, 77 subjectively healthy volunteers without any cancer history, age median 55.0±6.6 years, thereof 40 females and 37 males were used. The blood from controls was also collected in the fasting state. No additional selecting clinical criteria were applied.

Blood was collected exclusively in EDTA-coated tubes, centrifuged at 4 °C 380×g, for 20 min. Blood plasma was stored at –80 °C until used. Plasma deproteination was carried out according to [5, 13], by adding 600 µl of methanol to 300 µl of plasma. Then, the mixture was shortly vortexed, frozen at –24 °C for 30 min, and subsequently centrifuged for 30 min at 14,462×g. Finally, 700 µl of supernatant were dried out. 100 µl of stock solution (0.30 mM TSP-d_4 (3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt) as a chemical shift reference, 0.15 M phosphate buffer pH 7.4, in deuterated water) and 500 µl of deuterated water. For NMR measurement, the 550 µl of the final mixture were transferred into a 5 mm NMR tube.

Ethics. This study was approved by the Ethics Committee of the Jessenius Faculty of Medicine in Martin (registered

Table 1. Classification of patients based on the type of primary lung tumors.

Type of carcinoma	patients	women	men	mean age	median	IQR
All patients with carcinoma	132	44	88	65.6	67.5	10
Adenocarcinoma	55	23	32	65.96	69	11.5
Spinocellular	41	5	36	67.63	68.5	6
Neuroendocrine	34	16	18	62.24	65.5	9
Adenosquamous	2	0	2	71.5	71.5	5

Table 2. Origin of secondary tumors in lungs.

Origin of lung metastases	patients	women	men	mean age	median	IQR
all	47	23	24	63.09	65	12
endometrium	5	5	0	68.6	67	9
kidney	10	6	4	67.1	69	6
breast	4	4	0	58.25	60.5	17.5
esophagus and gastric	2	0	2	63	63	10
intestine	16	4	12	61.94	62.5	6.5
testicles	2	0	2	32	32	14
skin	3	2	1	71.33	73	3.5
bladder	1	0	1	75	75	N
others	4	2	2	62	65	10

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NMR measurements. NMR spectra were acquired on Bruker Avance III spectrometer equipped with TCI cryoprobe. Initial settings were performed on an independent sample and adopted for measurements. Before measurements, the ready samples were stored in a SampleJet machine at ca 6 °C, for not more than 2 hours. Samples were randomly ordered for acquisition. Measurements were carried on at 310 K. For all samples, we kept the half-width of the TSP-d_4 signal under 1.1 Hz. We modified standard Bruker profiling protocols as follows: profiling 1D NOESY with pre-saturation (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=3 ms): FID size 64k, dummy scans 4, number of scans 128, spectral width 20.0156 ppm. For randomly chosen 20 samples, COSY spectra were acquired with NS=24 and j-resolution spectra with NS=32. All experiments were conducted with a relaxation delay of 4 s; all data were once zero filled.

Data processing. NMR spectra were solved with the help of metabolomic database hmbd.ca [14], chenomx software (free trial version), and researching in literature. The multiplicity of peaks were confirmed in j-resolved and cross-peaks in COSY spectra. The metabolites identified are listed in Table 3. From 23 identified metabolites, 22 metabolites (except lysine showing strong peak overlap) were of suitable signals for quantitative evaluation.

We chose spectra subregions with only a single metabolite assigned and summed integrals of selected signals from 0.001 ppm binned spectra. This form of data represents a relative concentration of metabolites in blood plasma. No normalization method was applied to the data. Shapiro-Wilk and Kolmorogov normality tests (OriginPro 2019) were used to test normality. Both tests rejected the normality for about half of the data tested. Statistical analysis was carried on using the Mann-U-Whitney test (Matlab R2018b). Multivariate methods, such as principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and random forest (RF) algorithm resulting in ROC curve were performed using Metaboanalyst 4.0 [15], SPSS software, and Matlab R2018b.

Table 3. 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-quartet, m-multiple).

Metabolite	Peaks assigned
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
lactate	1.34d, **4.15q**
leucine	0.96d, **0.97d**, 1.72m
isoleucine	0.94t, **1.01d**, 3.67d
valine	0.99d, **1.05d**, 2.28m, 3.62d
alanine	**1.48d**, 3.805q
acetate	**1.92s**
3-hydroxybutyrate	1.20d, **2.31dd**, 2.39dd, 4.15m
pyruvate	**2.37s**
succinate	**2.41s**
citrate	2.55d, **2.66d**
glutamine	2.11m, 2.14m, **2.44m**, 2.47m,
creatine	3.03s, **3.93s**
creatinine	3.04s, **4.05s**
lysine	1.45m, 1.51m, 1.73m, 1.91m, 3.03t
phenylalanine	7.33d, 7.32t, **7.43t**
tyrosine	**6.90d**, 7.15d
tryptophan	7.19t, 7.27t, 7.31s, **7.55d,** 7.74d
2-oxoisocaproate – ketoleucine	0.94d, 2.097m, **2.61d**
3-methyl-2-oxovalerate – ketoisoleucine	0.89t, **1.10d**
2-oxoisovalerate – ketovaline	**1.12d,** (3.01m)
threonine	1.33d, 3.60d, **4.26dq**
proline	1.99m, 2.06m, 2.34m, 3.32m, 3.41dt, **4.14dd**
lipoprotein fraction*	**0.85 - 0.90 m**, 1.20-1.40m, 5.26-5.35m

Note: *the composition of lipoprotein fraction was described by Liu et al. in detail [26]

Results

As applicable in metabolomic studies, data were processed by multivariate methods such as PCA and PLS-DA analyses (Figure 1). As input for both analyses, relative concentrations of metabolites determined by NMR spectroscopy were used. PCA and PLS-DA analyses of the system patients with primary against patients with secondary tumors in lungs showed very strong proximity of both groups (data not shown). Then, we analyzed binary system: all cancer patients against controls, and a system consisting of three groups: patients with primary and secondary lung cancer and controls. In the first system consisting of two groups, cross-validated PLS-DA performed with accuracy = 0.84, R2=0.49 and Q2=0.39 for 5 components and accuracy = 0.86, R2=0.50, and Q2=0.42 for 8 components; most important features were (after VIP score): lactate, lipoprotein fraction, glucose, alanine, acetate, and 3-hydroxybutyrate. In the second system with three groups, PLS-DA performed with accuracy = 0.68, R2=0.42, and Q2=0.32 for 5 components and accuracy = 0.75, R=0.52, and Q2=0.41 for 8 components; most important features were (after VIP score): lactate, alanine, lipoprotein fraction, 3-hydroxybutyrate, acetate, and pyruvate.

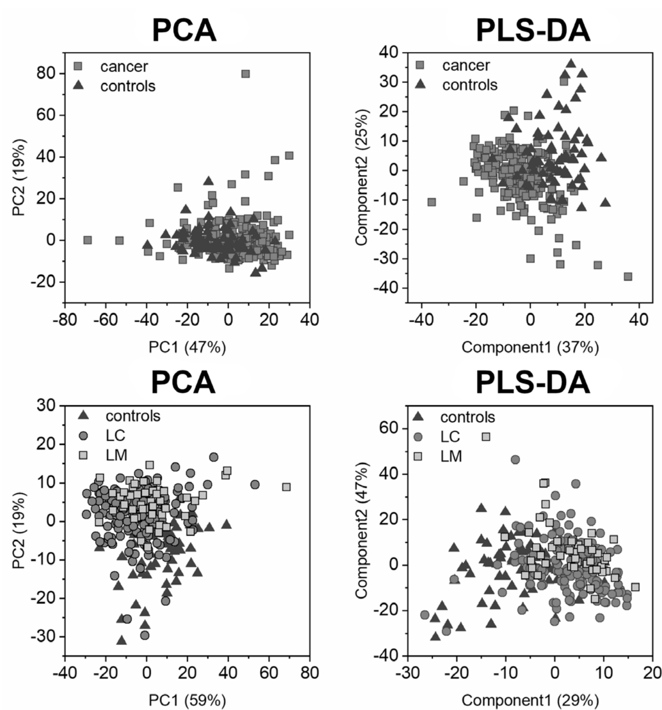

Figure 1. PCA (left) and PLS-DA (right) analyses: patients with malignant findings in lungs against controls (above) and patients with lung cancer (LC), lung metastasis (LM), and controls (below).

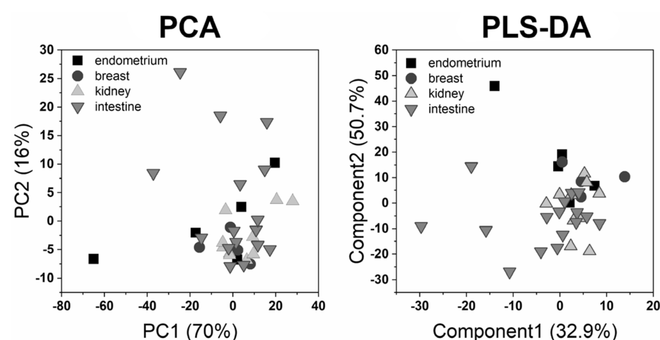

Figure 2. PCA (left) and PLS-DA (right) analyses for patients with lung metastasis based on NMR metabolomic data from blood plasma.

Although this was not the primary aim of this study, we ran PCA and PLS-DA analyses for lung metastasis patients divided into subgroups by the primary origin of the tumor. Only groups of size n>4: endometrium, breast, intestine, and kidney were used (Figure 2). PLS-DA performed with accuracy = 0.42, R2=0.56 and Q2=0.28 for 8 components; the most important features were (after VIP score): glucose, alanine, lipoproteins, and lactate. Other metabolites were of low importance.

Next, we employed Random Forest discriminatory algorithm to obtain a more realistic estimation of the discriminatory power of the system in the context of biomarker analysis. The main advantage of RF against PLS-DA is that RF is more robust to overfitting and outliers [16] and the used RF algorithm includes cross-validation via balanced subsampling. Similarly, to PCA and PLS-DA, an algorithm was fed by relative concentrations of metabolites in plasma expressed by integrals of specific NMR regions. In the case of highly correlating predictors, the RF algorithm may label one of them as unimportant. To avoid this, we ran the algorithm ten times. As an output from the analysis, ROC curves were created. ROC curve analysis is the established method for the evaluating specificity and sensitivity of diagnostic – discrimination tests. It is created by plotting the true positive rate against the false-positive rate at various threshold settings. An important output is the parameter area under the curve (AUC) that 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) [12]. Unlikely PCA and PLS-DA, the ROC curve is defined only for binary systems. The results from discriminatory analysis via RF of selected systems represented by AUC value and by the list of the most important variables (metabolites) are summarized in Table 4. Within the repeated RF runs, metabolites slightly permuted in the importance order.

Shapiro-Wilk and Kolmogorov-Smirnov tests rejected normality for approximately half of the data. With respect to, Mann-U-Whitney test was used to test significant difference between two groups. We additionally performed the Bonferroni correction to avoid the type I error and the p-value of 0.0023 was the cut-off value to claim statistical significance. Plasma metabolites: leucine, isoleucine, valine, 3-methyloxovalerate, 2-oxoisovalerate, glutamine, lysine, phenyl-

Table 4. Result from Random Forest discriminatory analysis applied on binary systems, algorithm fed by relative concentration of metabolites in plasma.

System	Nr. of variables	AUC	features (rel. conc. metabolites in plasma) ordered by importance
Lung cancer/Controls	2 5 10	0.824 0.898 0.957	lactate, 3-hydroxybutyrate, creatine, glucose, citrate, acetate, lipoprotein fraction, 2-oxoisocaproate, succinate, pyruvate,
Lung metastasis/Controls	2 5 10	0.899 0.937 0.956	lactate, acetate, citrate, 3-hydroxybutyrate, creatinine, pyruvate, 2-oxoisocaproate, succinate, creatine, glucose
All patients/Controls	2 5 10	0.86 0.899 0.938	lactate, 3-hydroxybutyrate, citrate, creatine, glucose, pyruvate, acetate, 2-oxoisocaproate, succinate, alanine
Lung cancer/Lung metastasis	2 5 10	0.542 0.562 0.562	all features were of very low importance

alanine did not show significant changes after Bonferroni correction. Significant results are summarized in Table 5 and visualized in Figure 3.

Table 5. Plasma metabolites significantly changed (p-value <0.0023 after Bonferroni correction) between patients with lung cancer and lung metastasis of other origin against controls. Percentual change derived from medians.

	Lung cancer/Controls		Lung metastasis/Controls	
	p-value	change/%	p-value	change/%
glucose	<0.0001	10	<0.01	11
lactate	<0.0001	−27	<0.0001	−35
alanine	<0.0001	−15	<0.0001	−15
acetate	<0.0001	7	<0.0001	15
3-hydroxybutyrate	<0.0001	28	<0.0001	41
pyruvate	<0.05	−8	<0.001	−15
succinate	<0.0001	−15	<0.0001	−18
citrate	<0.0001	21	<0.0001	22
creatinine	<0.0001	9	<0.0001	18
tyrosine	<0.001	−10	<0.001	−11
tryptophan	<0.0001	−13	<0.005	−11
2-oxoisocaproate	<0.0001	20	<0.0001	21
threonine	<0.001	−10	<0.005	−6
proline*	<0.0001	18	<0.0005	22
lipoproteins	<0.0001	−22	n.s.	–
creatine	<0.005	27	<0.001	21

Note: *NMR signal was slightly overlapped

Discussion

Multivariate analyses PCA and PLS-DA, Random Forest. There are two main approaches when evaluating metabolomic data. Commonly used p-value serves rather to explain biology since the biomarker discovery requires a different analysis, evaluation, and validation. In the second access, data analysis is usually performed using multivariate statistical methods such as PLS-DA and oPLS-DA [12, 17].

Firstly, we employed PCA analysis as a method serving to 2D visualization of multidimensional data. The visual interpretation suggested rather a similarity of the metabolomic data among patients' groups and controls. When dividing patients into subgroups LC and LM, in PCA analysis, controls were slightly separated from the patients (Figure 1).

Unlike PCA, PLS-DA is an analytical method including also the discriminatory algorithm. Very convincing results via PLS-DA analysis regarding high discrimination power between lung cancer patients and controls were obtained with binned NMR spectra as input variables by Louis et al. [7, 8]. Although the method used in general does not omit any part of the NMR spectrum; it is possible that it defined as important some bins which cannot be associated with a biologically meaningful feature, i.e., metabolite. In contrast to these works, we fed the algorithm by the particular integral of NMR spectra exactly selected to be corresponding to only one metabolite. In this way, the input variables expressed the relative concentration of metabolites in blood plasma. We used this approach with the aim to describe the system on 'metabolites level', (by biologically interpretable features) in order to be more understandable and usable also for non-NMR users. PLS-DA results suggested better, but not ideal discrimination between patients and controls. Having divided patients into subgroups LC and LM, the partial shift of controls from LC and LM patients was obvious; however, the discrimination was also not ideal (Figure 1).

Out of curiosity, we ran PCA and PLS-DA analyses in patients with lung metastasis divided by cancer origin (Figure 2). Both of these analyses showed proximity among the groups based on relative concentrations of plasmatic metabolites via NMR. Since cancer cells have common, but also disease-specific metabolomic features, this finding, although surely suffering from low sample sizes, is noteworthy and will be discussed in the next section.

Probably mostly used chemometrics algorithm PLS-DA has some shortcomings, i.e., tendency to overfitting and

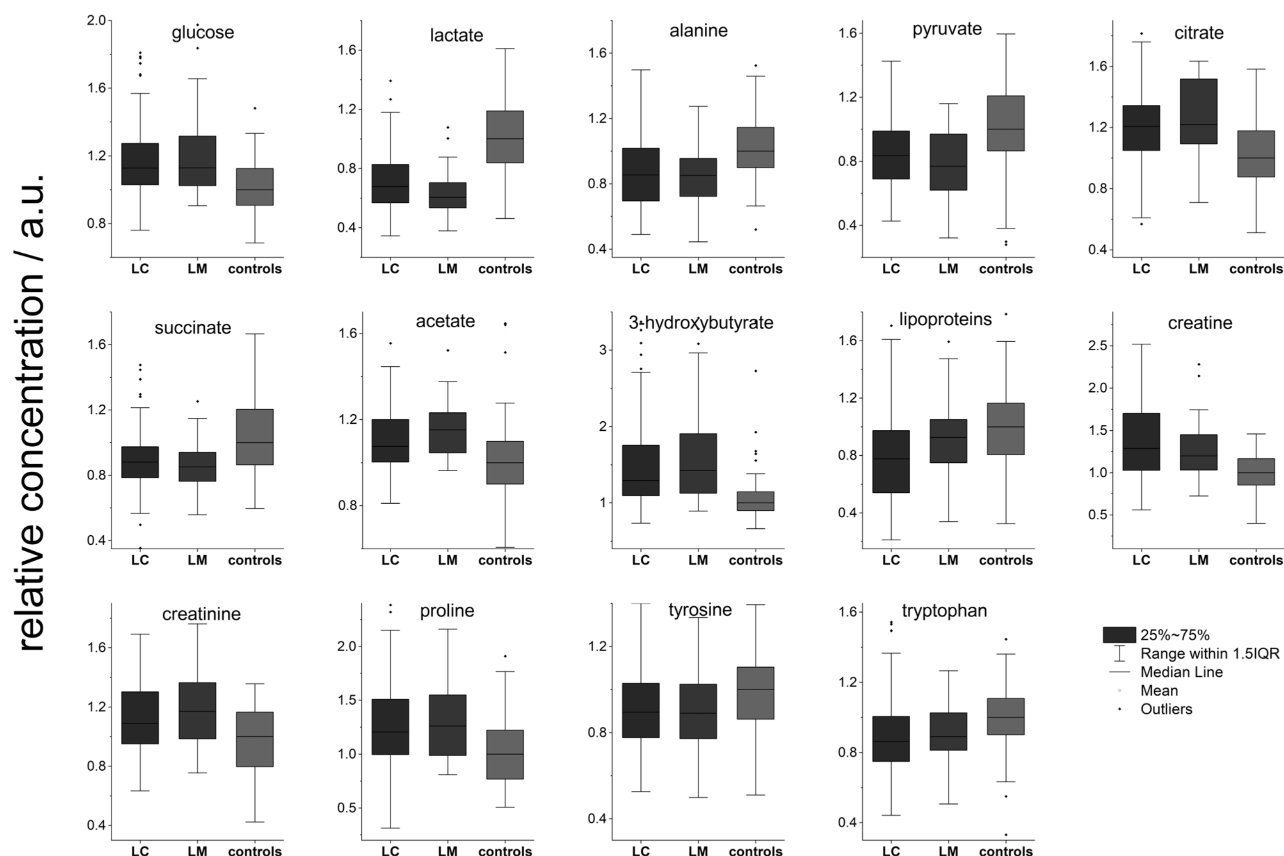

Figure 3. Relative concentrations of plasmatic metabolites in patients with lung cancer, lung metastasis, and controls expressed in arbitrary units (a.u.); abbreviations: LC-lung cancer, LM-lung metastasis. For each metabolite, data were scaled to the median of controls = 1.

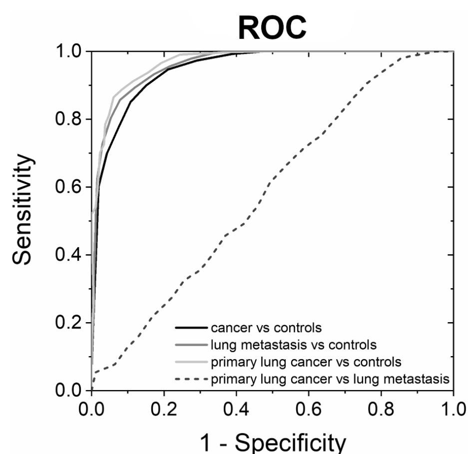

Figure 4. ROC curve derived from RF classification for selected systems, calculated always for 10 variables (details in Table 4).

associated overoptimistic results [16, 18]. Another machine learning algorithm that is more robust to overfitting and may offer a more realistic estimation of the discriminatory power of the system is supervised Random Forest [16]. In our study, it was, similarly to PCA and PLS-DA, fed by the relative concentrations of concrete metabolites in plasma as variables. Based on RF output, the ROC curve was created (Figure 4). Patients were very well discriminated from controls with an AUC of 0.957 for lung cancer and controls, 0.956 for lung metastasis and controls, and 0.938 for all patients and controls, results obtained for 10 features. Further increase in the number of features did not improve the AUC values. Metabolites: lactate, 3-hydroxybutyrate, creatine, glucose, citrate, acetate, lipoprotein fraction, 2-oxoisocaproate, succinate, pyruvate, and alanine were classified as the most important. In the repeated RF runs, the metabolites slightly permuted in the importance order. If we liked to define metabolites of highest discriminatory power as biomarkers, we should be aware, as stated by Liu et al. [19], that it is too optimistic to define the features as biomarkers only based on the ROC curve without clinical validation. Used RF algorithm included cross-validation meaning that the algorithm picks up 2/3rds of data for training and the rest for testing for regression and almost 70% of data for training and the rest for testing during classification in order to overcome training and testing at the same data. Naturally, this approach does not substitute clinical validation; however, it may lead to encouraging results.

Metabolomic changes in blood plasma in the presence of tumor on the lung. Lactate is one of the most discussed metabolites related to cancer. Lactate was identified as the main fuel for tumor cells, being able to diffuse from hypoxic areas to the oxygenated area to be converted to pyruvate. Pyruvate is further oxidized by pyruvate dehydrogenase complex and by TCA cycle enzymes providing reduced coenzymes for mitochondrial oxidative phosphorylation. The preference of oxygenated tumor cells for lactate allows the spare glucose to reach hypoxic tumor cells and so fuel the glycolysis [20]. The importance of lactate as a carbon source for human non-small-cell lung cancers (NSCLC) was shown by Faubert et al. [21]. The circulatory turnover flux of lactate is the highest of all metabolites [22]. In three cancer cases, the contribution of circulating lactate to tumor TCA intermediates exceeded that of glucose by about two-fold, consistent with glucose contributing to the tumor TCA cycle primarily through circulating lactate [22]. Hyperlactatemia with or without type B lactic acidosis is a rare complication of cancer, previously observed most often in hematological malignancies [23]. Clinically, increased blood lactate is a negative prognostic factor in metastatic lung cancer [23]. In our study, where the lung cancer patients of various progression and severities were grouped together, lactate was observed to be significantly decreased in plasma of lung cancer patients against controls. The same result was obtained in the study by Louis et al. [7]. Lactate is a key metabolite of the Cori cycle, where lactate produced in the muscle cells, instead of accumulating, is taken up by the liver and converted back to glucose. This is closely interconnected with the Cahill cycle, where alanine takes away the residual amino group from the muscles and via bloodstream is transported into the liver where it is through intermediate pyruvate converted to glucose. The mutual conversion of lactate, pyruvate and alanine may explain their common decrease in plasma level, causing alteration in the circulation of mentioned metabolites in the body.

In blood plasma of patients with the malignant structure findings in the lungs, we observed slightly, but statistically significant increased glucose level. Mild hyperglycemia and insulin resistance are common phenomena related to advanced cancer [24, 25]. At the time of altered glucose utilization, alternative substrates such as ketone bodies could support metabolic requirements. In our study, we observed a strong significantly increased level of 3-hydroxybutyrate in blood plasma in the patients' group. This finding is accompanied by decreased lipoprotein fraction plasma level that contains about 40% of triacylglycerols [26] as the main substrate for the ketone bodies synthesis. In further, regarding TCA metabolites, we found increased levels of plasmatic citrate and decreased level of plasmatic succinate what suggests TCA cycle imbalance.

The importance of basal metabolites, as mentioned above, is not only to fulfill the demands on energy and intermediate for the growth of tumors. As it has been shown in recent studies, metabolomic enzyme expression has the potential to impact DNA methylation and histone acetylation in mammals [27]. Increased level of 3-hydroxybutyrate may influence cellular function via epigenomic regulation by influencing histone acetylation [27, 28]. As a further example, methylation status is sensitive to oxygen and TCA-related metabolism [27]. The arising knowledge about the multiple effects of particular metabolites makes metabolomics studies much more interesting and useful.

Plasmatic level of proline was observed to be increased in patients' groups against controls. Proline is a key player in ROS signaling, and its metabolism may play a role not only in suppressing tumors but also in augmenting tumor growth what may make it a possible therapeutic target [29]. Further, we found an increased creatine and creatinine level in the blood plasma of tumor patients against controls. Elevated plasma creatine level suggests its suppressed uptake by creatine-phosphate utilizing tissues. The renal dysfunction cannot be ruled out as well. Patients' groups showed also a statistically strong significant increase in acetate. Acetate may serve, besides as energy fuel or for lipid synthesis also as a signaling molecule for cancer cells. These and many other relations of acetate to cancer were reviewed in detail by Schug et al. [30].

Decreased plasmatic levels of tyrosine and tryptophan in lung cancer patients were found in our study similar to the study by Ren et al. [31]. Accelerated tryptophan catabolism has been described in several malignant diseases e.g. [32, 33]. Interestingly, tryptophan breakdown relates to fatigue and impaired quality of life in patients with lung cancer [34]. A decrease in tryptophan suggests an enhanced cytokine-induced degradation of tryptophan and an activated IDO mechanism that is a part of the malignant transformation process and plays a key role in suppressing the anti-tumor immune response in the body [35]. All observed changes are visualized in Figure 3.

The origin of all observed changes can be assumed but is not entirely clear and understood in the concept of metabolism of cancer cells. From another point of view, the patients with primary lung cancer showed almost identical metabolomic changes in plasma compared with patients with lung metastasis, as it seems that the cancer origin had not played a substantial role (Figure 1). This trend was also confirmed in the comparison of plasma samples from patients with lung metastasis of different origins by PCA and PLS-DA analyses that showed considerable metabolomic proximity (Figure 2). There were not also any significantly changed metabolites found between these groups. It is remarkable that observed metabolomic changes, in particular, increased glucose and decreased pyruvate and alanine, tryptophan, and tyrosine balanced with increased ketone bodies are very similar to those found in rats after ischemia [36–38] and in patients after myocardial infarction [39]. (Details about the consequence of ischemic injury on plasma metabolites are described in mentioned studies). It could be suggested,

that the patients with malignant findings on the lungs may suffer from hypoxia. This opinion should be supported by clinically relevant data, e.g., astrup, DLCO, or others. We do not have this data available from all patients, therefore, these correlations are unfortunately missing and we can state only a hypothesis. However, this explanation could be also supported by the fact that the metabolomic changes in patients with lung malignancies differ from those found in plasma in patients with primary brain tumors that are not generally associated with secondary metastatic lung tumors [5]. As another example can be used the above-mentioned study by Louis et al. who showed successful discrimination among lung cancer patients, breast cancer patients (stage I and II = not metastatic) via NMR metabolomics of blood plasma [8]. Taking together, observed metabolomic changes in patients with primary and secondary lung cancer are the superposition of changes caused by the presence of cancer and changes similar to those observed after ischemia, where the second ones appear to be dominant in the statistical discrimination. Here it is important to note, that metabolites that were of the highest discrimination power (section 3) cannot be connected primarily to lung cancer, as they were strongly overlapping with those differencing subjects after ischemic injury from controls [36–39].

In conclusion, NMR analysis of the blood plasma showed considerable metabolomic proximity of patients with primary and secondary lung cancer. The metabolomic similarity was also observed within the group of secondary lung cancer patients regardless of cancer origin. By comparing with healthy controls, the part of observed changes can be explained in terms of cancer presence, and another part of observed changes is very similar to those observed after ischemic injury. Random forest performed the discrimination of both groups from controls almost ideally with an AUC above 0.95 when relative concentrations of metabolites in the blood plasma were used as input variables. Regarding the metabolites evaluated, we would conclude that malignant findings on the lungs caused metabolomic changes in blood plasma that were not correlated with the origin of cancer cells. The changes in plasmatic metabolites levels in patients with malignant findings on the lungs showed features similar to those observed in relation to ischemia.

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