Targeted metabolomic analysis of plasma samples for the diagnosis of inherited metabolic disorders

Hana Janečková a,1, Karel Hron b, Petr Wojtowicz a, Eva Hlídková a, Anna Barešová a, David Friedecký a, Lenka Žídková a, Petr Horník c, Darina Behúlová d, Dagmar Procházková e, Hana Vinohradská f, Karolína Pešková c, Per Bruheim g, Vratislav Smolka a, Sylvie Šťastná c, Tomáš Adam a,d,1

a Laboratory of Inherited Metabolic Disorders and Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University, Tř. Svobody 8, 771 26 Olomouc, Czech Republic
b Department of Mathematical Analysis and Applications of Mathematics, Faculty of Science, Palacký University, 17. listopadu 12, 771 46 Olomouc, Czech Republic
c Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Ke Karlovu 2, 128 08 Praha 2, Czech Republic
d Centre of Inherited Metabolic Diseases, Department of Laboratory Medicine, University Children’s Hospital, Limborův 1, 833 40 Bratislava, Slovakia
e Department of Pediatrics, Medical Faculty of Masaryk University and University Hospital Brno, Černoplně 9, 625 00 Brno, Czech Republic
f Department of Clinical Chemistry, The University Hospital Brno, The Children’s Medical Center, Černoplně 9, 625 00 Brno, Czech Republic
g Department of Biotechnology, Norwegian University of Science and Technology, Sem Sælands vei 6/8, NO-7491 Trondheim, Norway

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A B S T R A C T

Metabolomics has become an important tool in clinical research and diagnosis of human diseases. In this work we focused on the diagnosis of inherited metabolic disorders (IMDs) in plasma samples using a targeted metabolomic approach. The plasma samples were analyzed with the flow injection analysis method. All the experiments were performed on a QTRAP 5500 tandem mass spectrometer (AB SCIEX, USA) with electrospray ionization. The compounds were measured in a multiple reaction monitoring mode. We analyzed 50 control samples and 34 samples with defects in amino acid metabolism (phenylketonuria, maple syrup urine disease, tyrosinemia I, argininaemia, homocystinuria, carbamoyl phosphate synthetase deficiency, orni-thine transcarbamylase deficiency, nonketotic hyperglycinemia), organic acidurias (methylmalonic aciduria, propionic aciduria, glutaric aciduria I, 3-hydroxy-3-methylglutaric aciduria, isovaleric aciduria), and mitochondrial defects (medium-chain acyl-coenzyme A dehydrogenase deficiency, carnitine palmitoyltransferase II deficiency). The controls were distinguished from the patient samples by principal component analysis and hierarchical clustering. Approximately 80% of patients were clearly detected by absolute metabolite concentrations, the sum of variance for first two principle components was in the range of 44–55%. Other patient samples were assigned due to the characteristic ratio of metabolites (the sum of variance for first two principle components 77 and 83%). This study has revealed that targeted metabolomic tools with automated and unsupervised processing can be applied for the diagnosis of various IMDs.

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1. Introduction

Metabolomics has become an important tool in clinical research and diagnosis of human diseases. The first attempt at using metabolomic tools in the diagnosis of inherited metabolic disorders (IMDs) was conducted by Siuzdak’s group [1]. They applied an untargeted metabolomics using reverse phase capillary liquid chromatography-orbitrap technology with exact mass measurements and automatic data processing. This approach was validated with two severe metabolic disturbances – methylmalonic acidemia and propionic acidemia. Data were processed using a nonlinear alignment software (XCMS) and an online database (METLIN) in order to find and identify metabolites differentially regulated in disease.

IMDs are a group of diseases caused by defects in biochemical pathways. These disorders are rare individually (few cases diagnosed worldwide) by a collectively constituted substantial health problem (it is expected that one affected child is born for several hundred healthy newborns [2]). At present these disorders are frequently diagnosed by newborn screening performed with the use of a flow injection analysis-tandem mass spectrometry
The evaluation of the patients’ data was carried out on the basis of reference ranges and cut-off values [5]. The FIA-TMS method was also used in certain metabolomic studies [6–8].

In this work we focused on the diagnosis of IMDs in plasma samples using a targeted metabolomic approach by FIA-TMS. For ion suppression correction we made use of the deuterated analogues of the studied compounds. In order to view the main patterns in the multivariate data structure, data were processed by a principal component analysis (PCA) and a hierarchical cluster analysis (CA) using R software. Results of these two popular statistical exploratory tools were displayed via compositional biplot and dendrogram.

A dendrogram is a graphical representation of the hierarchical clustering procedure, searching for successive clusters of observations using previous established clusters. Hierarchical methods usually function in an agglomerative manner by first finding the clusters of the most similar items and progressively adding less similar items until all the items have been included into a single large cluster. This idea is reflected by the heights of the links between the clusters (samples) in the dendrogram. The lower the height of the link between the two clusters, the more similar the objects are with respect to an appropriate chosen distance measure. The standard Euclidean distance is usually used. A so-called complete-linkage clustering is typically taken here, where the similarity of the two clusters is represented by the similarity of their most dissimilar members.

The use of Euclidean distance is not meaningful, however, for data carrying relative rather than absolute information, i.e., multivariate observations that quantitatively describe parts of a whole [9]. They are frequently represented in the form of proportions or percentages. Such multivariate observations are usually called compositional data (or compositions for short) [10] and also the analyzed samples represent such a case. The compositions need to be transformed from their sample space, the simplex, with a natural geometry, to the usual actual space with the Euclidean metric using the family of log-ratio transformations before a statistical analysis can be applied. For our purposes, the centred logratio (CLR) transformation seems to be the most appropriate [10]; the resulting new variables correspond to the original compounds. When the CLR transformation is performed, the above described dendrogram provides a reasonable picture of the grouping patterns in the data set [11].

The CLR transformation also needs to be applied before the compositional biplot [12] can be constructed. Generally, the biplot [13] is a planar graph that allows for visualization of information on both samples and variables of a data matrix simultaneously. For this purpose, the scores and loadings of the first two principal components are plotted as points and arrows and the corresponding interpretation of the biplot enables the capturing of the main processes in the multivariate structure of the data set. In case of a compositional biplot, the interpretation of the arrows reflects the nature of the compositional data. In particular, the shorter the link (distance) between the arrows is, the more the ratio between the corresponding compounds tends to be a (fixed) constant throughout the data set. Thus, in general, the links can be used to visualize how strong relations between the (original) compounds are [14]. Moreover, the longer arrow means more importance of the corresponding compound for the explanation of patterns in the data set.

2. Experimental

2.1. Reagents and chemicals

Ethanol (HPLC grade), methanol (LC–MS grade), water (LC–MS grade), pyridine (p.a. grade), ammonium acetate (LC–MS grade), phosphate buffered saline (p.a. grade) and phenylisothiocyanate (≥99.0%; GC) were purchased from Sigma–Aldrich (St. Louis, USA).

2.2. Plasma samples

The control and patient plasma samples were from infants from routine diagnostic processes performed in the authors’ laboratory. The diagnoses had been previously confirmed by biochemical, enzyme or molecular-genetic analyses in all the patients. The collected plasma samples were stored at −20 °C. Prior to preparation, the samples were allowed to thaw at room temperature.

2.3. Targeted metabolomic analysis

This was performed using the AbsoluteIDQ p150 kit (BIOCRATES Life Sciences AG, Austria). The samples were processed as described in detail in the user manual. In brief, the assay preparation was performed on a double-filter 96 well plate containing 27 isotope-labeled internal standards. The plasma samples (10 μL) were derivatized by phenylisothiocyanate and extracted with an organic solvent. The standard flow injection method was applied for all the measurements with two subsequent 20 μL injections (one for the positive and one for the negative detection mode analysis). All the experiments were performed on a QTRAP 5500 tandem mass spectrometer (AB SCIEX, USA) with electrospray ionization. Multiple reaction monitoring detection was used for quantification of 163 endogenous metabolites from different metabolite classes.

The complete analytical process was performed using the MetIQ software, which is an integral part of the AbsoluteIDQ kit. A total of 163 metabolites were measured. The metabolomics data set contains 14 amino acids, a sum of hexoses, 41 acyl carnitines, 15 sphingolipids and 92 glycerophospholipids.

The method has been shown to have been in conformity with the “Guidance for Industry – Bioanalytical Method Validation” published by the FDA (Food and Drug Administration) [15], which implies the proof of reproducibility within a given error range.

2.4. Statistical analysis

Prior to statistical analysis the centred logratio (CLR) transformation was applied. Data were evaluated using PCA (compositional biplots) and CA based on hierarchical clustering with a complete-linkage method (dendrograms). All statistical calculations were performed using the R statistical software.

3. Results and discussion

3.1. Plasma samples

Control (n = 50) and patient (n = 34) plasma samples including various inherited metabolic disorders (Table 1) were analyzed. The diagnoses had been previously confirmed by biochemical, enzyme or molecular-genetic analyses in all the patients (Tables 2A and 2B). The samples MSUD 1 and 2, MSUD 3 and 4 and PA 2 and 3 were from the same patients taken in different time.

3.2. Multivariate analysis

All the patient samples were discriminated from the controls by appropriate metabolites in the PCA analysis. Patients with identical disease were recognized using the PCA approach and also clustered together. Some details are given by appropriate cases below.
3.3. Repeatability

The repeatability of the analyses was determined on the 16 aliquot samples of pooled plasma. The concentrations of particular metabolites (n = 163) over each pair of technical replicates were compared using the Spearman correlation coefficient. The range of coefficients was higher than 0.92 (Fig. 1).

Another view of the repeatability is visible with the PCA analysis where an overlap of three different measurements of patients with PKU is marked (Fig. 2).

3.4. Amino acid defects

The patient samples (n = 20) included 8 different defects in amino acid metabolism (Table 2A) – phenylketonuria (PKU), maple syrup urine disease (MSUD), tyrosinemia I (Tyr I), arginemia (Arg), homocystinuria (Hcy), carbamoyl phosphate synthetase deficiency (CPS), ornithine transcarbamylase deficiency (OTC) and nonketotic hyperglycinemia (NKH).

### Table 1
Summary of studied aminocaridurias, organic acidurias and mitochondrial diseases with their plasma primary and secondary markers.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Primary markers</th>
<th>Secondary markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKU</td>
<td>↑ Phe</td>
<td>↑ Phe/Tyr</td>
</tr>
<tr>
<td>MSUD</td>
<td>↑ Leu</td>
<td>↑ Leu/Ala</td>
</tr>
<tr>
<td>Tyr I</td>
<td>↑ Tyr</td>
<td>↑ Tyr/Phe</td>
</tr>
<tr>
<td>Hcy</td>
<td>↑ Met</td>
<td>↑ Homocyst(e)ine</td>
</tr>
<tr>
<td>OTC</td>
<td>↑ Glu</td>
<td>↓ Cit</td>
</tr>
<tr>
<td>CPS</td>
<td>↑ Glu</td>
<td>↓ Cit</td>
</tr>
<tr>
<td>Arg</td>
<td>↑ Arg</td>
<td>↑ Glu</td>
</tr>
<tr>
<td>NKH</td>
<td>↑ Gly</td>
<td>↓ C3-DC-M</td>
</tr>
<tr>
<td>MMA</td>
<td>↑ C3</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>↑ C3</td>
<td></td>
</tr>
<tr>
<td>HMG</td>
<td>↑ C5-M-DC</td>
<td></td>
</tr>
<tr>
<td>IV A</td>
<td>↑ C5</td>
<td></td>
</tr>
<tr>
<td>GA I</td>
<td>↑ C5-DC</td>
<td></td>
</tr>
<tr>
<td>MCAD</td>
<td>↑ C8</td>
<td></td>
</tr>
<tr>
<td>CPT II</td>
<td>↑ (C16 + C18:1)/C2</td>
<td>↑ C16</td>
</tr>
</tbody>
</table>

### Table 2A
Summary of studied patients with inborn error of amino acid metabolism.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Type</th>
<th>Gender</th>
<th>Age</th>
<th>Mutation</th>
<th>Clinical status</th>
<th>Previous biochemical findings in plasma sample (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKU 1</td>
<td>Class.</td>
<td>M</td>
<td>18</td>
<td>p.R158Q/p.a395P</td>
<td>Discontinued</td>
<td>↑ Phe 1674</td>
</tr>
<tr>
<td>PKU 2</td>
<td>Mild HPA</td>
<td>M</td>
<td>28</td>
<td>p.E306V/unk</td>
<td>Normal diet</td>
<td>↑ Phe 441</td>
</tr>
<tr>
<td>PKU 3</td>
<td>Class.</td>
<td>F</td>
<td>22</td>
<td>p.R408W/p.R408W</td>
<td>Low-phenylalanine diet</td>
<td>↑ Phe 805</td>
</tr>
<tr>
<td>PKU 4</td>
<td>Mild</td>
<td>M</td>
<td>20</td>
<td>p.R408W/unk</td>
<td>Low-phenylalanine diet</td>
<td>↑ Phe 629</td>
</tr>
<tr>
<td>PKU 5</td>
<td>Class.</td>
<td>F</td>
<td>19</td>
<td>p.R408W/c.1066-3C&gt;T</td>
<td>Low-phenylalanine diet</td>
<td>↑ Phe 272</td>
</tr>
<tr>
<td>PKU 6</td>
<td>Class.</td>
<td>M</td>
<td>14</td>
<td>p.R261Q/p.R65ST</td>
<td>Low-phenylalanine diet</td>
<td>↑ Phe 1168</td>
</tr>
<tr>
<td>MSUD 1</td>
<td>Class.</td>
<td>F</td>
<td>20</td>
<td>unk</td>
<td>Mild MR</td>
<td>↑ Leu 1130, Ile 285, Val 532</td>
</tr>
<tr>
<td>MSUD 2</td>
<td>Class.</td>
<td>F</td>
<td>20</td>
<td>unk</td>
<td>Mild MR</td>
<td>↑ Leu 1314, Ile 302, Val 789</td>
</tr>
<tr>
<td>MSUD 3</td>
<td>Intermittent</td>
<td>M</td>
<td>18</td>
<td>unk</td>
<td>Hepatopathy</td>
<td>↑ Leu 312, Ile 152, Val 1064</td>
</tr>
<tr>
<td>MSUD 4</td>
<td>Intermittent</td>
<td>M</td>
<td>18</td>
<td>unk</td>
<td>Hepatopathy</td>
<td>↑ Leu 379, Ile 226, Val 930</td>
</tr>
<tr>
<td>MSUD 5</td>
<td>Serious neonatal</td>
<td>F</td>
<td>20</td>
<td>unk</td>
<td>Attacks of decomp., moderate MR</td>
<td>↑ Leu 836, Ile 142, Val 393</td>
</tr>
<tr>
<td>Tyr I</td>
<td>Class.</td>
<td>F</td>
<td>8</td>
<td>c. 1062 + 5G &gt; A/c. 1210G &gt; a</td>
<td>HSM without nodules, borderline intellect</td>
<td>↑ Tyr 828</td>
</tr>
<tr>
<td>Tyr I</td>
<td>Class.</td>
<td>M</td>
<td>6</td>
<td>c. 554-1G &gt; T/c. 680G &gt; T</td>
<td>HSM without nodules, pruritus, normal MD</td>
<td>↑ Tyr 328</td>
</tr>
<tr>
<td>Hcy</td>
<td>Class. B6 nonresp.</td>
<td>M</td>
<td>31</td>
<td>unk</td>
<td>Mild MR</td>
<td>↑ Hcy 159, Met 109</td>
</tr>
<tr>
<td>Hcy</td>
<td>Class. B6 nonresp.</td>
<td>M</td>
<td>30</td>
<td>unk</td>
<td>MR</td>
<td>↑ Hcy 62, Met 508</td>
</tr>
<tr>
<td>NKH</td>
<td>Atypical</td>
<td>M</td>
<td>14</td>
<td>unk</td>
<td>Epilepsy, serious MR</td>
<td>↑ Gly 987</td>
</tr>
<tr>
<td>Arg</td>
<td>Moderately serious</td>
<td>M</td>
<td>27</td>
<td>unk</td>
<td>Epilepsy, diplegia</td>
<td>↑ Arg 363, Glu 669</td>
</tr>
<tr>
<td>CPS</td>
<td>Serious neonatal</td>
<td>F</td>
<td>2</td>
<td>unk</td>
<td>Good</td>
<td>↑ Gln 945; ↓ Cit 3</td>
</tr>
<tr>
<td>OTC</td>
<td>Serious</td>
<td>F</td>
<td>15</td>
<td>IIV7 + 1G &gt; T</td>
<td>Good</td>
<td>↑ Gln 1011, Gln 631; ↓ Orn 42</td>
</tr>
<tr>
<td>OTC 2</td>
<td>Serious</td>
<td>F</td>
<td>18</td>
<td>unk</td>
<td>Borderline MR</td>
<td>↑ Gln 1370; ↓ Cit 0</td>
</tr>
</tbody>
</table>

Class, classical; F, female; HSM, hepatosplonemegaly; HPA, hyperphenylalaninemia; Ile, isoleucine; M, male; MD, mental development; MR, mental retardation; nonresp., nonresponsive; unk, unknown.

* The samples MSUD 1 and 2 were taken from the same patient in different time.
* The samples MSUD 3 and 4 were taken from the same patient in different time.
3.4.2. MSUD

Maple syrup urine disease (OMIM 248600) is caused by a deficiency of the branched-chain alpha-keto acid dehydrogenase complex. The enzyme defect results in marked increases in branched-chain 2-amino acids (e.g., leucine, isoleucine, valine) in plasma.

A total of 5 plasma samples from patients with MSUD were analyzed. Using the PCA analysis all of them were clearly discriminated and clustered by XLeu (the sum of leucine, isoleucine and alloisoleucine) and valine (Fig. 3). The same results were obtained using hierarchical clustering (Fig. 4).

In order to increase the power of minor metabolites, the samples with PKU and MSUD were deleted in the PCA analysis for other defects evaluation (Fig. 5).

3.4.3. Tyr 1

Tyrosinemia I (OMIM 276700) is caused by fumarylacetoacetate hydrolase (EC 3.7.1.2) deficiency and results in a high accumulation of tyrosine.

We analyzed 2 plasma samples from patients with Tyr 1. Both were distinguished from the controls in the PCA analysis (Fig. 5) and clustered together in CA (Fig. 4) due to the high plasma concentrations of tyrosine in comparison with the controls.

![Fig. 1. Repeatability of the metabolomic profiling platform. Metabolite concentrations in plasma samples over two technical replicates are shown. The Spearman correlation coefficient between the technical replicates was higher than 0.92. This plot shows the two replicates with the weakest correlation.](image)

![Fig. 2. Repeatability of metabolomic profiling using the PCA analysis of all plasma samples and all the analyzed amino acids. Three different measurements of patients with PKU are marked.](image)
3.4.4. Hcys
Homocystinuria (OMIM 236200) is caused by cystathionine beta-synthase deficiency (EC 4.2.1.22). Its diagnosis is based on markedly increased concentrations of plasma homocystine, total homocysteine, homocysteine–cysteine mixed disulfide, and methionine. In this study we used methionine as a marker for this disease.

Both patients with Hcys were clearly discriminated from the controls using the PCA analysis due to a high concentration of methionine (Fig. 5). We also noticed an unambiguous clustering of both samples in the dendrogram (Fig. 4).

3.4.5. OTC
Ornithine transcarbamylase (EC 2.1.3.3) deficiency (OMIM 311250) is characterized by increased glutamine and decreased citrulline and ornithine concentrations.

Two samples from patients with OTC were distinguished from the controls in the PCA analysis (Fig. 5) and clustered together in the dendrogram (Fig. 4) due to enhanced glutamine concentrations. An increased concentration of glycine was previously found in these samples (Table 2A), with this corresponding with our results, while the score of glycine correlates with glutamine in the biplot (Fig. 5).

3.4.6. Other amino acid diseases – NKH, Arg and CPS
We analyzed only one patient with nonketotic hyperglycinemia (OMIM 605899) caused by a glycine cleavage system defect, one patient with argininemia (OMIM 207800) having arginase (EC 3.5.3.1) deficiency and one patient with carbamoyl phosphate synthetase (EC 6.3.4.16) deficiency (OMIM 237300). The markers of these diseases are shown in Table 1.

The patient with NKH was also clearly distinguished by a high glycine concentration. Although it was not unambiguous, patients with Arg and CPS were slightly discriminated from the controls by appropriate metabolites (Fig. 5).
3.5. Organic acidurias and mitochondrial defects

A total of 14 patient plasma samples with 7 various organic acidurias and mitochondrial defects were analyzed (Table 2B) – methylmalonic aciduria (MMA), propionic aciduria (PA), glutaric aciduria I (GA I), 3-hydroxy-3-methylglutaric aciduria (HMG), isovaleric aciduria (IV A), medium-chain acyl-coenzyme A dehydrogenase deficiency (MCAD) and carnitine palmitoyltransferase II deficiency (CPT II).

3.5.1. MMA and PA

Methylmalonic aciduria (OMIM 251000) is caused by methylmalonyl CoA mutase (EC 5.4.99.2) apoenzyme deficiency and propionic aciduria (OMIM 606054) by propionyl-CoA carboxylase (EC 6.4.1.3) deficiency. Propionyl-CoA and methylmalonyl-CoA are intermediates in the metabolism of certain amino acids (valine, isoleucine, methionine, threonine), cholesterol, and odd chain fatty acids. Both disorders are characterized by increased propionylcarnitine accumulation in the blood (Table 1).

We analyzed 3 plasma samples with MMA and 4 samples with PA. All the samples correlate in the PCA analysis (Fig. 6) and are clustered together in CA (Fig. 7) due to the same marker propionylcarnitine (C3). Identical results were observed in the case of the evaluation of metabolite ratios (Fig. 8), all the patient samples were clustered by the C3/C2 ratio.

Other compounds were minor and had a low variability in comparison with C3, therefore the samples with PA and MMA were deleted from the PCA analysis for other defects evaluation (Fig. 9). Note that with metabolite ratios the logarithmic transformation was used with data before the biplots were applied in order to obtain log-ratios and avoid undesirable effects coming from the distorted covariance structure of the raw ratio variables.
3.5.2. MCAD
Medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) deficiency (OMIM 2014450), the most frequently occurring fatty acid oxidation disorder, is mainly characterized by elevated octanoylcarnitine (C8). All 83 samples from patients with MCAD were clustered (Fig. 7), although C8 was not sufficiently specific for this disease. It was determined that the evaluation of acylcarnitine ratios was more important. Patients with MCAD were discriminated due to the C8/C2 ratio (Fig. 9).

3.5.3. Other diseases – CPT II, IV A, HMG, GA I
Other defects were studied analyzing only one plasma sample per for every disease. We analyzed only one patient sample with carnitine palmitoyltransferase II (EC 2.3.1.21) deficiency (OMIM 255110), isovaleric aciduria (OMIM 243500) having isovaleryl CoA dehydrogenase deficiency (EC 1.3.99.10), 3-hydroxy-3-methylglutaric aciduria (OMIM 246450) defined by 3-hydroxy-3-methylglutaryl CoA lyase (EC 4.1.3.4) deficiency and glutaric aciduria I (OMIM 231670) with glutaryl CoA dehydrogenase (EC 1.3.99.7) deficiency. All the 7 samples were distinguished due to the characteristic ratio of acylcarnitines. The (C16+C18:1)/C2 ratio was specific for CPT II, both the ratios of C5/C8 and C5/C2 for IV A, less significant C5-DC/C16 ratio for GA I and C5-DC/C8 for HMG (Fig. 9).

4. Conclusions
Our work was focused on the testing of the method of targeted metabolomics in combination with unsupervised data processing for detection of patients with IMDS (amino acids defects, organic acidurias, and mitochondrial defects). This approach was successful in all cases and detected all 34 patients with IMDS against the 50 healthy controls.

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