Metabolomics Identifies Perturbations in Human Disorders of Propionate Metabolism

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Background: We applied untargeted mass spectrometry-based metabolomics to the diseases methylmalonic acidemia (MMA) and propionic acidemia (PA).

Methods: We used a screening platform that used untargeted, mass-based metabolomics of methanol-extracted plasma to find significantly different molecular features in human plasma samples from MMA and PA patients and from healthy individuals. Capillary reversed-phase liquid chromatography (4 μL/min) was interfaced to a TOF mass spectrometer, and data were processed using nonlinear alignment software (XCMS) and an online database (METLIN) to find and identify metabolites differentially regulated in disease.

Results: Of the approximately 3500 features measured, propionyl carnitine was easily identified as the best biomarker of disease (P value 1.3 × 10–18), demonstrating the proof-of-concept use of untargeted metabolomics in clinical chemistry discovery. Five additional acylcarnitine metabolites showed significant differentiation between plasma from patients and healthy individuals, and γ-butyrobetaine was highly increased in a subset of patients. Two acylcarnitine metabolites and numerous unidentified species differentiate MMA and PA. Many metabolites that do not appear in any public database, and that remain unidentified, varied significantly between normal, MMA, and PA, underscoring the complex downstream metabolic effects resulting from the defect in a single enzyme.

Conclusions: This proof-of-concept study demonstrates that metabolomics can expand the range of metabolites associated with human disease and shows that this method may be useful for disease diagnosis and patient clinical evaluation.

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Inborn errors of metabolism can have a severe impact on human health, so comprehensive diagnostic neonatal screening is used for early diagnosis to avoid potentially catastrophic physical and neurological effects (1). These defects can cause a buildup of toxic metabolites, resulting in serious, often fatal, disease early in life (2, 3). Neonatal blood screening with mass spectrometry (MS) is now commonly used to test newborns for a wide array of inborn errors of metabolism using specific metabolites for diagnosis (4). We studied 2 diseases using metabolomics with nontargeted LC-MS to simultaneously profile thousands of metabolites and obtain a more comprehensive metabolic profile of plasma. The platform we developed (Fig. 1) includes a profiling approach, along with a software package that incorporates new nonlinear time correction, peak-finding, and integration methods to allow for semiquantitative comparison between healthy individuals and patient populations (5).

Methylmalonic acidemia (MMA) and propionic acidemia (PA) are inborn errors of amino acid metabolism affecting 1 in 50 000 to 1 in 100 000 individuals (6, 7). PA results from a defect in the enzyme propionyl-CoA carboxylase, which catalyzes the biotin-dependent conversion of propionyl-CoA to methylmalonyl-CoA (8). MMA results from deficiency of the immediately downstream enzyme methylmalonyl-CoA mutase, which catalyzes the vitamin B12-dependent conversion of methylmalonyl-CoA to succinyl-CoA (Fig. 2). Patients have considerable variability in symptoms and clinical prognosis, which are correlated with genetic locus (6, 7). Patients with complete apoenzyme deficiency (mut0) typically present early
in life with severe symptoms, including high mortality and neurologic impairment, whereas those with partial apoenzyme deficiency (mut−) typically have less severe symptoms. Abnormalities in the processing of vitamin B12 to the active coenzyme adenosylcobalamin (cblA, B, C, and D) are amenable to treatment with B12 supplementation, and present later with the best prognosis (9). Within each of these groups there is substantial variability in disease severity and prognosis, for which there is no biomarker. For example, in the complete apoenzyme deficiency form, both MMA and PA often include varying degrees of neurological involvement, the underlying biochemical cause of which is not known (10–14). Perhaps differences in metabolite concentrations correlating with neurological damage could eventually be identified using untargeted metabolomics.

MMA and PA were the 1st human diseases to be diagnosed using MS, originally by GC-MS (15). The level of propionyl carnitine is highly increased in both diseases, arising from the transesterification of propionyl-CoA (Fig. 2), and in MMA methylmalonyl carnitine is also typically increased. Propionyl carnitine is now used as the primary biomarker, screened by tandem electrospray MS after derivatizing bloodspot methanol extracts by butylation (16). Acylcarnitine fragmentation yields a common collision-induced dissociation (CID) fragment with m/z 85, and thus rapid multiple-reaction monitoring with direct injection of the butylated methanol extract has become the method of choice for large-scale neonatal screening (4).

The power of untargeted metabolomics lies in its potential to broaden our understanding of disease biochemistry, identify new biomarkers, and provide finer disease categorization and treatment. Although originating in the disturbance of a single gene, inborn errors of metabolism produce highly diverse phenotypes and complex downstream metabolic effects, the result of the interplay of many biochemical pathways and the individual’s interaction with the environment (17–19). We hypothesized that a metabolomics study may reveal additional differences between disease and normal plasma, and thus a more complete biochemical profile. This study demonstrated the application of a new toolbox of methods to clinical chemistry, an approach with the potential to provide new insights into the biochemical mechanisms of disease.

**Materials and Methods**

**Sample Preparation**

Plasma samples were stored frozen at −80 °C until use, at which point the samples were thawed on ice. We performed metabolite extraction and protein precipitation by adding 100 μL cold methanol to 50 μL plasma. Samples were then vortex-mixed and stored at −20 °C for 1 h. The pellet was removed by centrifuging at 16 000g for 10 min. The supernatant was removed to a clean tube and the centrifugation step was repeated, and the samples were dried in a SpeedVac to dryness. We added 50 μL water, followed by vortex-mixing and sonication in a bath soni-
cator for 5 min. The samples were maintained at 4 °C in the autosampler and analyzed by LC-MS.

**PATIENTS AND METABOLOMICS WORKFLOW**

Existing samples referred to a clinical laboratory for prior testing were anonymized, and the investigation was in accordance with the University of California–San Diego Human Research Protection Program. In an initial experiment, plasma samples from individuals with MMA and healthy individuals were collected, comparing 9 MMA samples to 10 normal plasma samples. After finding significantly increased concentrations of several metabolites in MMA, including various acylcarnitines, the sample size was expanded. Samples from patients with PA were included, along with healthy adults before and after supplementation with l-carnitine (TwinLabs), 1000 mg twice a day for 1 week. The final study included samples from healthy children (n = 12), healthy adults with (n = 3) and without (n = 3) carnitine supplementation, healthy children with carnitine supplementation (n = 3), children with MMA (n = 15), and children with PA (n = 9). The patient group was restricted to patients with complete apoenzyme deficiency (termed mut0 in MMA).

We used a metabolomics workflow (Fig. 1) to find significantly different molecular features in human plasma, followed by metabolite identification. Briefly, the plasma samples were extracted with methanol to remove proteins and extract the maximum number of metabolites. We injected 8 μL extracted plasma onto a reversed-phase capillary-flow HPLC interfaced to an electrospray ionization TOF mass spectrometer (ESI-TOF). Data were collected and converted to a common data format for MS. We used the program XCMS to integrate the chromatographic peaks and assign the peaks into groups, followed by nonlinear alignment of the grouped data in the time domain.

**CHROMATOGRAPHY**

We injected 8 μL processed plasma for each run. Reversed-phase chromatography was performed using either a 150 by 0.32 mm (diameter) endcapped C18 column (Aquasil, Thermo) with 5-μm particles, at a flow rate of 4 μL/min or a 150 by 0.5 mm (diameter) Zorbax C18 column (Agilent) with 5-μm particles at a flow rate of 8 μL/min. The LC system was an Agilent 1100 with a capillary pump. Buffer A was water with 0.1% formic acid, and buffer B was acetonitrile with 0.1% formic acid. For the endcapped C18 column, the column was equilibrated in 100% A, and the gradient was 0%–20% B over 20 min, 20%–60% B over 20 min, and 60%–80% B over

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**Fig. 2.** (A), simplified pathway illustrating the metabolic blockage points of MMA and PA disease. The mut0 form of MMA results in a complete blockage of methylmalonyl-CoA mutase, whereas PA results from a defect in propionyl-CoA carboxylase. Both produce a buildup of toxic upstream acyl-CoA precursors, and their corresponding acylcarnitines, propionyl-carnitine, and methylmalonyl-carnitine. (B), simplified pathway illustrating conversion of toxic acyl-CoA intermediates to the corresponding acylcarnitines and cleared into the plasma. Long-chain acylcarnitines require carnitine acetyltransferase (CAT) to pass through the membrane, whereas shorter-chain acylcarnitines can pass directly through the cell membrane. The direct precursor of carnitine is γ-butyrobetaine, which was found in this study to be highly increased in some patients, without a corresponding increase in carnitine concentrations.
5 min. For the C18 (Zorbax) column, the column was equilibrated in 5% B, and the gradient was 5%–95% B over 50 min.

MASS SPECTROMETRY
We collected data in continuum mode from m/z 75 to 1000 using an Agilent ESI-TOF. The capillary voltage was 3500 V, with a nebulizer gas flow of 13 L/min. The instrument was calibrated immediately before use. The data files were converted from the instrument format (.wiff) to the common data format, using the PESciEX data translator. We used the XCMS program (5) to align and analyze the LC-MS data. To confirm the identification of significant ions, we used a linear ion trap (Thermo LTQ), with a custom nanospray interface operated at a voltage of 2 kV. The Aquasil column was used with the identical chromatography parameters as for the metabolomics runs, with a passive postcolumn split, to reduce the flow rate from 4 µL/min to 300 nL/min for nanospray. Masses for CID were targeted from a mass list and fragmented during the chromatography run.

Results

PROPYIONYL CARNITINE
The most significant difference between normal and disease samples in this study was an ion with m/z 218.14 at a retention time of 16 min, with a t-test P value of 1.3 × 10⁻¹⁸, comparing all disease and normal samples, and an ANOVA P value of 1.8 × 10⁻¹⁸. Tandem MS (MS/MS) fragmentation in a linear ion trap produced fragments of m/z 159 and 85. The common fragment of 85 and the neutral loss of 59 are both characteristic CID fragmentation patterns of acylcarnitines. This process, in combination with accurate mass data from the ESI-TOF, was used to identify the compound as propionyl carnitine. In addition, the retention time of a propionyl carnitine calibrator was checked against the retention time of the endogenous peak in patient plasma. The raw integrated intensity data from XCMS indicates a mean intensity ratio of approximately 2000 between disease and normal plasma (Table 1, Fig. 3). The endcapped Aquasil column provided better retention of propionyl carnitine, with a retention time of 16 min, than a standard C18 column, on which it is not retained and elutes at 5.5 min.

INCREASE IN ACYLcARNITINES IN DISEASE
In the initial experiment comparing MMA and normal plasma, a number of compounds were found to be significantly increased in MMA plasma, several of which were identified as acylcarnitines. To test whether treatment with carnitine produced this effect, samples from 3 healthy children treated with carnitine and from 3 healthy adults before and after carnitine supplementation were included in the experimental controls. The acylcarnitine levels of the 6 supplemented individuals were not significantly increased relative to the unsupplemented controls, and levels were within the general range of the other normal samples. Comparing supplemented vs unsupplemented normal samples, only the ion at m/z 244 (identified as pentenoyl or C5:1 carnitine) differed by more than a factor of 2 (2.2), whereas this ion is increased by a factor of 6.6 in disease vs normal plasma.

After propionyl carnitine and its related ions (isotopes, the sodium adduct, a dimer, and fragments), the most significant feature differentiating disease from normal plasma has an m/z of 258, with a retention time of 32.7 min. It was identified as an acylcarnitine based on fragmentation pattern (characteristic CID fragments with

<table>
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<tr>
<th>Table 1. Identification and significance of compounds.⁴</th>
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<tr>
<td><strong>Molecule</strong></td>
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<tr>
<td>Propionyl carnitine (C3)</td>
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<tr>
<td>Fragment of propionyl carnitine</td>
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<tr>
<td>Hexanoyl carnitine (C6)</td>
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<tr>
<td>Acetyl carnitine</td>
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<tr>
<td>Pentenoyl carnitine (C5:1)</td>
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<tr>
<td>γ-Butyrobetaine</td>
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<tr>
<td>Hexenoyl (C6:1) or methyl C₅:1 carnitine</td>
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<tr>
<td>Myristoyl carnitine</td>
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<td>Carnitine</td>
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⁴ A longer retention time for the acyl carnitines on the reverse-phase column correlates with a longer acyl chain length. The ratio of the average intensity of all disease samples to normal samples is shown in column 8. Shown are the P value of the t-test between disease and normal samples and the P value of an ANOVA test comparing MMA, PA, and normal samples. NA, not applicable; ND, not determined.
m/z 199 and 85), corresponding to the molecular formula C_{13}H_{24}NO_{4}, which could be either a straight-chain hexenoic (C6:1) or a branched-chain methyl-pentenoic carnitine; these compounds cannot be differentiated by mass or fragmentation pattern, because the elemental composition and general acylcarnitine structure are the same. Although neither compound is known to be associated with MMA or PA as a biomarker, 2-methyl-branched acylcarnitines have been reported in MMA and PA patient urines during acute illness (21), including 2-methyl-2-pentenoic carnitine, which could correspond to the C6:1 acylcarnitine compound identified in our study. In another recent study, novel branched-chain acylcarnitines were recently identified in the urine of 1 patient with another metabolic disorder, medium-chain CoA dehydrogenase disorder (22).

**γ-BUTYROBETaine**

The observed ion with m/z of 146.18 and retention time of 6.9 min was identified as γ-butyrobetaine, based on accurate mass, retention time, and MS/MS in a linear ion trap. The average concentration of γ-butyrobetaine in patient plasma was increased 13-fold (P value 3.3 × 10^{-3}), with 1 PA patient having a 55-fold increase over the upper
reference limit value (Table 1, Fig. 3). Some MMA and PA samples were within the reference interval.

**DIFFERENTIATING BETWEEN MMA AND PA**

Many ions differed significantly between MMA and PA (Table 2). At a significance of 0.001, 76 features differed between MMA and PA. One of the most significant differentiating features had an m/z of 246.17 and was identified as isovaleryl carnitine (C₁₅H₂₅NO₄) based on accurate mass and MS/MS data (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue12). Isovaleryl carnitine was increased in MMA relative to normal samples by a factor of 5.1 (P value 1.66 × 10⁻⁶) and was increased relative to PA samples, which had isovaleryl carnitine concentrations similar to normal samples. The P value differentiating MMA and PA was 9.7 × 10⁻³. Also found to differentiate MMA and PA was an ion with m/z 177.10 at a retention time of 48.5 min, which was increased 40-fold in PA compared to MMA. The P value was 5.8 × 10⁻⁶ for PA vs normal samples and 1.6 × 10⁻⁵ for PA vs MMA samples. Methylmalonyl carnitine was not observed in the untargeted metabolomic analysis, although it is usually increased in MMA.

**OVERALL DIFFERENCES BETWEEN DISEASE AND NORMAL SAMPLES AND UNIDENTIFIED IONS**

We observed a several statistically significant differences between disease and normal plasma. At a t-test α of 0.001 (P value threshold), there were 153 features, or 4.4% of the total ions detected, that differed between disease and normal samples, and at an α of 0.005 there were 317 features, or 9.1% of the total that differ (Table 2). According to either significance criterion, about 72% of those differences were the result of an increased concentration of the ion in plasma in the disease state vs normal samples, indicating a clearly skewed distribution.

**Discussion**

The advantage of capillary chromatography for untargeted metabolomics is the sensitivity arising from the low flow rate (4 μL/min), allowing for the detection of many compounds. Using a 300-μm ID C18 column (Zorbax; Agilent) with a total run time of 75 min, 4313 features were detected when the equivalent of only 8 μL plasma was injected. Using an endcapped C18 column (Aquasil; Thermo) with a run time of 75 min, 3473 features were detected. The simultaneous detection and profiling of many (hundreds to thousands) metabolites increases the probability of identifying new compounds associated with disease using untargeted metabolomics.

The target compound for newborn screening by MS/MS in both MMA and PA is propionyl carnitine (Fig. 2) (4, 16). Indeed, the most significant feature in the untargeted metabolomic analysis that distinguished disease patient vs normal plasma was propionyl carnitine (P value 1.3 × 10⁻¹⁸). This result validates the untargeted metabolomics approach for identification of biomarkers, and is the most important finding, demonstrating proof-of-concept application of metabolomics to clinical chemistry.

Many other compounds were significantly altered in disease vs normal samples, with 317 features differing at an α level of 0.005 (Table 2), approximately 72% of which represent increased plasma metabolite concentrations. Among the identified compounds were C5:1 and C6:1 carnitine. These compounds do not seem to be the result of nonspecific transesterification of CoA esters, because they are not increased in samples from metabolically normal children on carnitine supplementation or the adults treated with carnitine. The C5:1 carnitine species (m/z 244) may be tiglyl carnitine arising from an intermediate in the catabolism of isoleucine; increase of tiglylglycine occurs in PA (23).

This study also suggests that MMA and PA can be differentiated, and 2 of these molecules were identified (Table 2, Supplemental Data Table 1, and Fig. 4). The ion with m/z 258.17 and a retention time of 33 min (Table 1) was identified as a C6:1 (or methyl-C5:1) carnitine ester. It is significantly increased in PA relative to MMA (P value 1.6 × 10⁻⁴) (Supplemental Data Table 1). There are many more significant differences, however, and C6:1 ranks only at number 54 when sorted using a t-test. More significantly, isovaleryl carnitine was increased by a factor of 2.8 in MMA vs PA, with a P value of 9.7 × 10⁻³⁸ (Fig. 4). There are other significant ions differentiating MMA and PA, which are clearly not acylcarnitines based on fragmentation pattern but have not yet been identified. For compounds such as the ion at m/z 177.1, which is increased in PA vs MMA (P value 1.6 × 10⁻⁷) (Supplemental Data Table 1, Fig. 4), identification of this molecule may be biochemically informative. The task of de novo identification of metabolites can be very time-consuming.

<table>
<thead>
<tr>
<th>Test criteria (P &lt; value)</th>
<th>Features differentiating disease vs normal samples, n (%)</th>
<th>Features increased in disease vs normal samples, n (%)</th>
<th>Features differentiating MMA vs PA, n (%)</th>
<th>Features increased in PA vs MMA, n (%)</th>
</tr>
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<tbody>
<tr>
<td>0.001</td>
<td>153 (4.4)</td>
<td>110 (72)</td>
<td>76 (2.2)</td>
<td>49 (64.5)</td>
</tr>
<tr>
<td>0.005</td>
<td>317 (9.1)</td>
<td>225 (71)</td>
<td>154 (4.4)</td>
<td>96 (62.3)</td>
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</table>
however, and is an open-ended process; identification of additional compounds awaits further study and validation with additional samples. By combining multiple ions, it is possible to clearly differentiate between the 2 diseases, but larger numbers of patient samples are needed for validation. Methylmalonyl carnitine was not identified in the untargeted analysis, although it was 15-fold increased in MMA vs PA and normal individuals in an independent targeted analysis (data not shown). Methylmalonyl carnitine may not have been observed because of its high polarity and lack of retention on the reversed-phase column.

An intriguing result of this study is the significant increase in γ-butyrobetaine concentrations in patients with MMA and PA, which has not been previously reported. γ-Butyrobetaine is converted to l-carnitine by γ-butyrobetaine hydroxylase, localized in liver, kidney, and brain in humans (24). Although studies are limited, normal plasma concentrations of γ-butyrobetaine were mean (SD) 1.8 (0.23) μmol/L at rest in normal adults (25) and 0.98 (0.08) μmol/L in long-term hemodialysis patients (26). Increase of γ-butyrobetaine may arise from disruption of carnitine synthesis, for example by inhibition of γ-butyrobetaine hydroxylase. Alternatively, propionyl carnitine inhibits γ-butyrobetaine transport across the plasma membrane in liver (27), and thus high propionyl carnitine concentrations in MMA and PA may affect γ-butyrobetaine transport dynamics and thus plasma concentrations. The increase of γ-butyrobetaine could also be a direct result of carnitine supplementation. Although there was no significant difference in free carnitine concentrations in patient samples compared to normal samples (Table 1), each of the patients was receiving high doses (100–200 mg/kg/day) of supplemental carnitine. γ-Butyrobetaine was not increased in carnitine-supplemented children or adults from this study. Plasma γ-butyrobetaine can increase with carnitine supplementation in dialysis patients (26) and in patients with medium-chain CoA dehydrogenase deficiency (28) and via carnitine metabolism by gut flora (29, 30). Further study is needed to separate effects of carnitine supplementation and correlate γ-butyrobetaine concentrations with clinical variables.

Application of metabolomics to MMA and PA has revealed metabolites that differentiate between disease and normal patients, and between MMA and PA. Propionyl carnitine was identified as a biomarker using a completely untargeted approach, illustrating the usefulness of metabolomics for identifying biomarkers of disease. New metabolites continue to be discovered even in well-studied diseases (21, 22, 31), and the metabolomics approach permits analysis of an unprecedented range of compounds. In this study, metabolites such as unsaturated acylcarnitines, isovaleryl carnitine, and γ-butyrobetaine were associated with MMA and PA. The finding of many significant differences underscores the evolving understanding of metabolic disease as having complex, diverse phenotypes that transcend simple concepts of a single-locus genotype (17, 19).

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References


