RESEARCH REPORT

A newborn screening approach to diagnose 3-hydroxy-3-methylglutaryl-CoA lyase deficiency

Jan Václavík1,2 | Lucie Mádrová1,2 | Štěpán Kouřil1,2 | Julie de Sousa1,2,3 | Radana Brumarová1,2 | Hana Janečková1,2 | Jaroslava Jáčová1,2 | David Friedecký1,2 | Mária Knapková4 | Leo A. J. Kluijtmans5 | Sarah C. Grünert6 | Frédéric M. Vaz7 | Nils Janzen8,9 | Ronald J. A. Wanders7 | Ron A. Wevers5 | Tomáš Adam1,2

1Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University Olomouc, Olomouc, Czech Republic
2Laboratory of Inherited Metabolic Disorders, Department of Clinical Chemistry, University Hospital in Olomouc, Olomouc, Czech Republic
3Department of Mathematical Analysis and Applications of Mathematics, Faculty of Science, Palacký University Olomouc, Olomouc, Czech Republic
4Banská Bystrica Children’s University Hospital, Banská Bystrica, Slovakia
5Translational Metabolic Laboratory, Department of Laboratory Medicine, Radboud University Medical Centre, Nijmegen, Netherlands
6Department of General Pediatrics, Adolescent Medicine and Neonatology, Medical Center – University of Freiburg, Faculty of Medicine, Freiburg, Germany
7Laboratory Genetic Metabolic Diseases, Department of Clinical Chemistry, Amsterdam, Netherlands
8Screening-Labor Hannover, Hannover, Germany
9Department of Clinical Chemistry, Hannover Medical School, Hannover, Germany

Correspondence
Tomáš Adam, University Hospital in Olomouc, I.P. Pavlova 6, Olomouc 775 20, Czech Republic.
Email: tomas.adam@fnol.cz

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Abstract
3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency (HMGCLD) is a rare autosomal recessively inherited metabolic disorder. Patients suffer from avoidable neurologically devastating metabolic decompensations and thus would benefit from newborn screening (NBS). The diagnosis is currently made by measuring dry blood spot acylcarnitines (C5OH and C6DC) followed by urinary organic acid profiling for the differential diagnosis from several other disorders. Using untargeted metabolomics (reversed-phase UHPLC coupled to an Orbitrap Elite hybrid mass spectrometer) of plasma samples from 5 HMGCLD patients and 19 age-matched controls, we found 3-methylglutaconic acid and 3-hydroxy-3-methylglutarylcarnitine as

Abbreviations: 3H3MG-A, 3-hydroxy-3-methylglutaric acid; 3H3MG-C, 3-hydroxy-3-methylglutarylcarnitine; 3HIV-A, 3-hydroxyisovaleric acid; 3HIV-C, 3-hydroxyisovalerylcarnitine; 3MC-C, 3-methylcrotonylcarnitine; 3MG-A, 3-methylglutaric acid; 3MG-C, 3-methylglutarylcarnitine; 3MGC-A, 3-methylglutaconic acid; 3MGC-C, 3-methylglutarylcarnitine; C5OH, acylcarnitine with acyl consisting of 5 carbon atoms and hydroxy group; C6DC, acylcarnitine with acyl consisting of 6 carbon atoms and carboxyl group; CID, collision induced dissociation; DBS, dried blood spot; IV-C, isovalerylcarnitine; MCCD, 3-methylcrotonyl-CoA carboxylase deficiency; MGCA, 3-methylglutataconic aciduria; MRM, multiple reaction monitoring; MSI, Metabolomic Standard Initiative; NBS, newborn screening; OPLS-DA, orthogonal partial least squares discriminant analysis; HDI, highest density interval; HMGCLD, 3-hydroxy-3-methylglutaryl-coenzyme A lyase deficiency; RT, retention time; SRM, selected reaction monitoring; VIP, importance in projection.

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the most discriminating metabolites between the groups. In order to evaluate
the NBS potential of these metabolites we quantified the most discriminating
metabolites from untargeted metabolomics in 23 blood spots from 4 HMGCLD
patients and 55 controls by UHPLC tandem mass spectrometry. The results
provide a tool for expanded NBS of HMGCLD using tandem mass spectrome-
try. Selected reaction monitoring transition 262/85 could be used in a first-tier
NBS analysis to screen for elevated 3-hydroxyisovalerylcarnitine. In a positive
case, a second-tier analysis of 3-hydroxy-3-methylglutaric acid and
3-methylglutaconic acid in a dry blood spot using UHPLC tandem mass spec-
trometry instruments confirms the diagnosis. In conclusion, we describe the
identification of new diagnostic biomarkers for HMGCLD and their application
in NBS in dry blood spots. By using second-tier testing, all patients with
HMGCLD were unequivocally and correctly diagnosed.

**KEYWORDS**
3-hydroxy-3-methylglutaryl-coenzyme A lyase deficiency, acylcarnitines, biomarkers, HMG-CoA
lyase, metabolomics, newborn screening, organic acids

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1 INTRODUCTION

3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency (HMGCLD, OMIM 246450) is a rare autosomal recessively
inherited metabolic disorder caused by mutations in the
HMGCL gene. The mitochondrial enzyme is responsible
for catalyzing the cleavage of HMG-CoA to acetyl-CoA
and acetoacetic acid. This conversion is a common last
step in leucine catabolism and ketogenesis from fatty
acids. Patients with HMGCLD present with a diagnostic
urinary pattern of elevated organic acids such as
3-hydroxyisovaleric acid (3HIV-A), 3-methylglutaconic
acid (3MG-A), 3-hydroxy-3-methylglutaric acid (3H3MG-
A), 3-methylglutaric acid (3MG-A) and in some cases
3-methylcrotonylglycine. Plasma of these patients contains
elevated levels of 3-hydroxyisovalerylcarnitine (3HIV-C),1
3-methylglutarylcarnitine (3MG-C),2 and three isomers of
3-methylglutaconylcarnitine (3MGC-C).3 Patients may suf-
fer from severe attacks of metabolic decompensation with
lethargy, seizures, hypotonia, vomiting and acidosis with
hypoketotic hypoglycemia that may result in irreversible
neurological damage.4 Most patients manifest within the
first year of life. Patients diagnosed at an early stage with
careful dietary management may avoid metabolic crises
and could have good prognosis.

For diagnostics, tandem mass spectrometry based
selective metabolic screening methods on dried blood spot
(DBS) samples are commonly used with a joint selected
reaction monitoring (SRM) transition for C4-DC and
C5-OH acylcarnitines. However, the SRM transition
262/85 used to analyze these acylcarnitines represents
four isobaric acylcarnitines (3HIV-C, 2-methyl-3-hydroxy-
butyrylcarnitine, methylmalonylcarnitine, and succinyl-
carnitine), pointing to many different inborn errors of
metabolism (3-methylcrotonyl-CoA carboxylase defi-
ciency [MCCD], HMGCLD, β-ketothiolase deficiency, 3-methylglutaconic aciduria [MGCA], 2-methyl-3-hydroxy-
butyryl-CoA dehydrogenase deficiency, methylmalonic
acidemia, multiple carboxylase deficiency, succinyl-CoA
ligase deficiency and mutation in genes encoding the
α-subunit and the β-subunit of the ADP-forming succinyl-
CoA synthetase).5 In order to distinguish between these
disorders, a patient must be recalled for urine sampling
and/or analysis of enzyme activities. MCCD has the
highest incidence (1:40 000) among the disorders listed
above. However, it is estimated that about 95% of MCCD
cases are benign6 and require no medical interventions.

In this paper we describe the detection of plasma
acylcarnitines and organic acids related to the leucine
degradation pathway in patients suffering from
HMGCLD using untargeted metabolomics. 3-Hydroxy-3-
methylglutarylcarmitnine (3H3MG-C), previously

**SYNOPSIS**
In this article we describe new blood biomarkers
of 3-hydroxy-3-methylglutaryl-CoA lyase defi-
ciency that allow newborn screening from initial
dried blood spots without the necessity of addi-
tional sampling.
considered undetectable in plasma of HMGCLD patients, was found for the first time. Furthermore, organic acid counterparts of elevated acylcarnitines were detected in plasma of HMGCLD patients as the most discriminating metabolites. Determination of these metabolites could be implemented in newborn screening (NBS) programs in order to detect patients suffering from HMGCLD. A following experiment of targeted analysis of the most discriminating metabolites between patient and control groups using DBS samples showed applicability of these metabolites in second-tier LC-MS/MS method within NBS programs to specifically diagnose HMGCLD, which will facilitate early recognition, appropriate treatment and prevention of metabolic decompensations in this disease.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Methanol, ethanol, water, and formic acid (LC-MS quality) were purchased from Sigma-Aldrich (St. Louis, Missouri). The following chemical standards were used for feature identification/quantification: isovaleryl-carnitine (IV-C), 3-methylcrotonylcarnitine (3MC-C), and 2-methylbutyrylcarnitine were purchased from Sigma-Aldrich (Switzerland). Adipoylcarnitine and tiglylcarnitine from Sigma-Aldrich (Austria). 3MG-C from Avanti (Massachusetts). 3MGC-A and 3H3MG-A from Sigma-Aldrich (Germany). Adipate from Fluka (Germany) and 3MG-A from Sigma-Aldrich (India).

2.2 | Samples

The study was conducted in accordance with the Declaration of Helsinki and adhered to Good Clinical Practice guidelines. Approval for the protocol was obtained from the joint ethics committee of the Medical Faculty of Palacký University and University Hospital Olomouc. Plasma samples for untargeted metabolomics were obtained from five HMGCLD patients—three girls (age 4, 17 days and 8 years) and two boys (1 and 5 years)—whose diagnosis was confirmed by enzyme and genetic testing. Control plasma samples were obtained from 19 children (9 boys and 10 girls between 2 and 17 years of age) into 5 mL vacuum sampling tube K3EDTA Vacuette, Greiner (Germany) at room temperature and centrifuged for 10 minutes at 3000g. Separated plasma was stored at −80°C until analysis. Shipping of samples between collaborating laboratories was conducted via 48 hours-courier on sufficient dry ice.

For targeted LC-MS/MS analysis, three types of control DBS samples were used: healthy newborns (ConA, n = 14) sampled a maximum of 3 weeks before analysis (representing controls for comparison with newborns suffering from HMGCLD), healthy newborns (ConB, n = 14) sampled approx. Six years before analysis (representing controls for comparison with the aged patient DBS samples we managed to acquire for this study) and disease-free individuals of age between 1 month and 15 years old (ConC, n = 27) sampled a maximum of 2 months before analysis (representing controls matching the age of HMGCLD patients that we managed to acquire for this study). All control samples were collected in the Laboratory for Inherited Metabolic Disorders (Department of Clinical Biochemistry, University Hospital Olomouc, Czech Republic).

DBS samples of genetically confirmed HMGCLD patients, were obtained anonymized from co-working laboratories from Freiburg (Germany) (Pt1—a treated girl with low protein diet and l-carnitine supplementation, 20 DBS sampled at age 4.4 to 15.2 years) and Banská Bystrica (Slovakia) (Pt4—a newborn sample from a boy diagnosed at 10th day of age treated since with low protein diet and l-carnitine supplementation). Two more DBS samples of suspected HMGCLD patients (Pt2 and Pt3—two girls, 5.6 and 10.0 years old) from Hannover (Germany) were included in the study, even though their diagnoses were not confirmed by genetic testing yet.

2.3 | Methods

Plasma samples of five HMGCLD patients were analyzed together with a group of controls using untargeted metabolomics via liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) to define discriminating compounds. Detailed information about performed untargeted metabolomics and data processing is enclosed in Supporting information S1. After data processing and statistical evaluation, physiological concentrations of the most elevated metabolites were determined using 55 control DBS samples using LC-MS/MS analysis. Also, pathological concentrations of these metabolites were calculated in DBS samples of HMGCLD patients. An overview of performed experiments is depicted in Supporting information S2. All data are reported in accordance to Metabolomic Standard Initiative (MSI) reporting requirements.

2.3.1 | Identification of discriminating metabolites

Following MSI principles for metabolite identification, the identity of acylcarnitines and organic acids related to leucine degradation pathway intermediates was determined by comparing the accurate mass of precursor ions,
fragmentation spectra and RTs with commercially available standards (MSI level 1). In absence of commercial standards, discriminating metabolites were putatively annotated based on accurate masses and comparison of fragmentation spectra and a region of elution from chromatographic column with a similar compound class metabolite for which a commercial standard was available (MSI level 2). Not all discriminating metabolites could be annotated and those were marked as unknowns (MSI level 4).

### 2.3.2 Targeted LC-MS/MS analysis of DBS

In order to evaluate the diagnostic potential of discriminating metabolites from untargeted metabolomic analysis for NBS, 23 DBS samples of 4 HMGCLD patients (one patient provided 20 samples over the course of 11 years of life) were analyzed together with 60 controls by LC system Sciex Exion AD which was coupled to a mass spectrometer (QTrap 6500+; Sciex, Framingham, Massachusetts) in scheduled multiple reaction monitoring (MRM) mode. The targeted LC-MS/MS method consisted of MRM transitions representing metabolites found by untargeted metabolomic experiment and MRM transitions for metabolites that could be theoretically expected (acylglycines, acylcarnitines and organic acids related to the affected pathway). Targeted LC-MS/MS method was modified based on original work from Körver-Keularts et al.\(^9\) and is described in Supporting information S3.

Sample preparation involved dissection of one disk (3.0 mm) from each DBS sample and extracted in methanol (100 \(\mu\)L) containing isotopically labeled internal standards; isovalerylcar nitine-D9 and methylmalonate-D3. After 20 minutes shaking at 405 RPM, extracts were lyophilized, reconstituted in 0.1% formic acid (100 \(\mu\)L), centrifuged at 21 300 g for 10 minutes at 4°C and 90 \(\mu\)L of supernatants were transferred into UHPLC vials. A six-point calibration mixture of three standards (3HIV-A, 3MGC-A and 3H3MG-A) was used to calculate their concentrations in blood using a dilution factor of 62.5 as a 3.0 mm DBS punch contains 1.6 \(\mu\)L of blood\(^10\) which was extracted with 100 \(\mu\)L of solvent.

### 3 RESULTS

#### 3.1 Untargeted metabolomic analysis

Plasma samples of five HMGCLD patients were analyzed together with controls via untargeted metabolomic analysis. After data processing and filtering, a total of 429 unique \(m/z\) features were found. PCA as unsupervised statistical

| Metabolite   | [M+H]\(^{+}\) | RT (min) | VIP score | HDI distance | Fold-change\(^{a}\) | MSI identification |
|--------------|--------------|----------|-----------|--------------|-------------------|-------------------|
| 3MG-C        | 290.1598     | 4.38     | 3.04      | 5.1          | 63.2              | Level 1           |
| 3MGC-A       | 127.0383\(^{b}\) | 5.49     | 2.68      | 3.4          | 151.4             | Level 1           |
| 3HIV-C       | 262.1649     | 3.65     | 2.47      | 2.9          | 137.1             | Level 2           |
| 3H3MG-A      | 163.0601     | 3.00     | 2.47      | 2.0          | 359.3             | Level 1           |
| 3MGC-C       | 288.1441     | 4.42     | 2.24      | 2.4          | 50.5              | Level 2           |
| Unknown      | 247.0391     | 5.48     | 2.18      | 2.2          | 55.2              | Level 4           |
| Unknown      | 321.0854     | 3.97     | 2.03      | 0.6          | 135.8             | Level 4           |

\(^{a}\)Calculated as a mean of patient’s peak area divided by mean of ConC peak area.
\(^{b}\)In-source fragment with larger peak area than molecular ion.

| Metabolite (\(\mu\)M) | ConA   | ConB   | ConC   | Mean Pt1 | Pt2 | Pt3 | Pt4 | Fold-change\(^{a}\) | Fold-change\(^{b}\) |
|-----------------------|--------|--------|--------|----------|-----|-----|-----|-------------------|-------------------|
| 3MGC-A                | 4.1 ± 2.5 | 4.6 ± 4.2 | 4.2 ± 3.7 | 55.5 ± 51.7 | 195.9 | 343.2 | 126.9 | 13.3             | 53.2              |
| 3H3MG-A               | 1.2 ± 0.2 | 1.3 ± 0.3 | 0.9 ± 0.4 | 5.6 ± 3.6 | 40.0 | 64.9 | 16.1 | 6.3              | 45.2              |
| 3HIV-A                | 9.5 ± 2.1 | 19.3 ± 6.2 | 8.6 ± 3.6 | 66.2 ± 43.9 | 158.5 | 659.8 | 95.8 | 7.7              | 35.4              |
| 3HIV-C                | 1.3 ± 1.0 | 0.8 ± 0.7 | 1.9 ± 1.5 | 14.6 ± 13.6 | 45.7 | 47.5 | 31.7 | 7.6              | 21.8              |

\(^{a}\)Calculated as a mean of patient 1 (treated patient) concentrations divided by mean of ConC concentration.
\(^{b}\)Calculated as a mean of patient 2, 3 and 4 (untreated at a time of sampling) concentrations divided by mean of ConC concentration.
method with cumulative variance explained by the first two principal components of 62.3% showed clear separation of clusters pointing to distinct metabolite differences between patient and control groups (see Supporting information S4).

Supervised statistical methods such as Bayesian volcano plot (see Supporting information S5) with colored HDI distance levels and VIP plot from OPLS-DA were used to determine the most elevated metabolites in a

**FIGURE 1**  A, Boxplots of the 3MGC-A, 3H3MG-A, 3HIV-A, and 3HIV-C concentration of patients (Pt) and controls (ConA, ConB, and ConC) from targeted LC-MS/MS analysis of dried blood spot samples. A logarithmic scale was used for the y-axis, allowing better comparability between the different groups of controls. B, The concentration of the relevant metabolites in blood of patient 1 was followed over time between ages 4.4 and 15.2 years.
group of patients, see Table 1. The list of discriminating metabolites contains all known plasma biomarkers of the disease (3HIV-C, 3MG-C, 3MGC-C). Furthermore, it also comprises two free organic acids (3MGC-A and 3H3MG-A) derived from accumulated acyl-CoAs prior to the metabolic block that are known biomarkers of the disease in the urine, although they were never reported as being elevated in blood samples from affected patients. Also, 3HIV-A is listed in Supporting information S6 alongside 3MGC-A and 3H3MG-A as it is the organic acid counterpart to a known plasma biomarker of HMGCLD (3HIV-C).

Apart from known plasma biomarkers of HMGCLD patients (3HIV-C, 3MG-C, and 3MGC-C) other acylcarnitine species derived from intermediates in the leucine degradation pathway turned out to be elevated when compared to controls (see Supporting information S6). Especially, the identification of 3H3MG-C deserves attention as its CoA analogue is an intermediate of the leucine degradation pathway which is, as of yet, known as the single substrate for the HMGCL enzyme and was previously considered undetectable.7

3.2 Targeted LC-MS/MS analysis

The concentration of metabolites in blood calculated (described in Section 2.3.2) from DBS samples of HMGCLD patients and controls is provided in Table 2. Box plots show a clear separation between the healthy population and HMGCLD patients without any overlap (ie, statistically significant difference between the respective groups) for 3MGC-A, 3H3MG-A, 3HIV-A, and 3HIV-C, see Figure 1.

4 DISCUSSION

Patients suffering from HMGCLD may exhibit abnormal plasma levels of ammonia, lactate, 3MG-C,2 3HIV-C,1 and three isomers of 3MGC-C.3 None of these metabolites is a selective biomarker for the disease making the differential diagnosis of HMGCLD directly from DBS impossible. The diagnosis of HMGCLD relies on urinary organic acid profile characterized by elevated concentrations of 3HIV-A, 3H3MG-A, 3MGC-A, 3MG-A, adipic acid, and in some cases 3-methylcrotonylglycine.11 Urine sampling of these patients usually occurs during a (first) metabolic decompensation with manifestation of symptoms, such as recurrent vomiting, seizures, and impaired vigilance. Common laboratory findings of the metabolic crisis include hypoglycemia, acidosis, hyperammonemia, an increased anion gap, and elevated transaminase activities. The majority of HMGCLD patients (92%) become symptomatic within the first year of life and about half of them within the neonatal period. Over 70% of patients exhibit brain MRI abnormalities8 presumably due to metabolic crises that occur early in their lives. Recognition of the HMGCLD diagnosis at (very) early age may lead to early start of appropriate clinical management, and the number of future metabolic decompensations may, therefore, be limited. Most HMGCLD patients show a favorable outcome with normal psychomotor development, which demonstrates the need for early recognition and diagnosing the disease. As in other organic acidurias, a timely diagnosis is crucial for overall quality of life of HMGCLD patients and demonstrates that this disease is a perfect candidate for NBS programs. The diagnosis is usually confirmed by enzyme and/or genetic testing.

To the best of our knowledge, there is no reliable source of information about worldwide incidence of HMGCLD, nevertheless, it seems to be extremely rare. There are only few studies reporting higher frequencies in certain populations such as in Saudi Arabia, Brazil, Portugal and Spain, however, all less than 1/100 000 live births.11-13 According to the Uniform Screening Panel,14 HMGCLD scored 16th from the top of 84 inherited metabolic disorders reconsidered for implementation into NBS programs in the United States. The low incidence of the disease may limit scientific progress in the development of specific analytical methods useful for timely diagnosis of HMGCLD without the need for confirmation analysis on urine samples.

Using untargeted metabolomic analysis of HMGCLD patient plasma, 3MGC-A and 3H3MG-A were found among the most discriminating metabolites between patient and control group. Subsequent LC-MS/MS analysis of DBS samples from HMGCLD patients and controls was used to determine the physiological concentration range of 3MGC-A (4.1 ± 2.5 μM), 3H3MG-A (1.2 ± 0.2 μM), 3HIV-A (9.5 ± 2.1 μM), and 3HIV-C (1.3 ± 1.0 μM) in blood spots of newborns and show significantly increased pathological levels of these metabolites in HMGCLD patients. It is worth noticing that elevations of proposed diagnostic metabolites in newborn patient 4 (although milder than in the other three symptomatic patients) are approximately one order of magnitude higher than the highest value in controls.

In conclusion, our results provide a tool for expanded NBS of HMGCLD using tandem mass spectrometry. The SRM transition 262/85 could be used in a first-tier NBS analysis to screen for elevated 3HIV-C. In a positive case, a second-tier analysis of 3H3MG-A and 3MGC-A using an LC-MS/MS instrument may confirm the diagnosis.
Given the rarity of the diseases a larger study is warranted to prove the practicality of this approach and to develop an optimal screening algorithm for HMGCLD. The methodology used in the article could be repeated for others disorders characterized by the elevated CSOH, leading to more diagnostically precise second tier tests that would facilitate timely diagnosis in the blood spot. Authors are looking for collaboration in this respect.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

ETHICS STATEMENT
All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. All patient and control samples were pseudonymized in the study. This article does not contain any studies with animal subjects performed by any of the authors.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

Data S1. Supporting information.

Data S2. An overview of performed experiments and data analysis.

Data S3. LC-MS/MS analysis of DBS.

Data S4. Two-dimensional score plot of unsupervised PCA analysis of HMGCLD patient plasma (Pt, blue) and controls (Con, pink). The tight green cluster of QC samples show a good stability of the analysis. Circled areas represent 75% confidence ellipses.

Data S5. Bayesian volcano plot with colored HDI ellipses. Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Data S1. Supporting information.
upper left corner for controls, respectively. *No metabolites detected in these HDI distance levels.

**Data S6.** Acylcarnitine profile of five HMGCLD patients compared to 19 healthy controls in plasma. Elevated organic acids found in plasma samples of HMGCLD patients compared to 19 healthy controls.