A New Approach for Fast Metabolic Diagnostics in CMAMMA

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Abstract *Background:* The presence of increased urinary concentrations of both methylmalonic acid (MMA) and malonic acid (MA) is assumed to differentiate combined malonic and methylmalonic aciduria (CMAMMA), due to mutations in the ACSF3 gene, from other causes of methylmalonic aciduria (classic MMAemia). Detection of MA in urine, however, is challenging since excretion of MA can be easily missed. The objective of the study was to develop a method for quantification of MA in plasma to allow differentiation between CMAMMA and classic MMAemia.

Methods: Compound heterozygosity for mutations in the ACSF3 gene was detected in two female siblings using diagnostic exome sequencing. Urine (MMA and MA) was analyzed with GC/MS, while plasma was analyzed with UPLC-MS/MS. MA/MMA ratios were calculated.

Results: Both patients had a severe psychiatric presentation (at the age of 6 years and 5.5 years, respectively) after a viral infection. MA excretion in the patients was only just above the highest control value in several samples. MA concentrations in plasma from the two patients were clearly above the highest value observed in control subjects. However, MA concentrations in plasma from patients with classic MMAemia were also elevated. Additional, calcu-

tion of MA/MMA ratio in plasma allowed to fully differentiate between CMAMMA and classic MMAemia.

Conclusions: Calculating the MA/MMA ratio in plasma allows differentiation between CMAMMA and classic MMAemia. The full clinical spectrum of CMAMMA remains to be delineated.

Introduction

Combined malonic and methylmalonic aciduria (CMAMMA) has thus far been described in two disorders, malonyl-CoA decarboxylase (MCD) deficiency (OMIM #248360) and, recently, acyl-CoA synthetase family member 3 enzyme (ACSF3) deficiency (OMIM #614265). Malonyl-CoA decarboxylase catalyzes the conversion of malonyl-CoA to acetyl-CoA and carbon dioxide, and patients excrete significantly higher malonic acid (MA) than methylmalonic acid (MMA) levels (de Wit et al. 2006). ACSF3 adds a coenzyme A moiety to both MA and MMA, and patients with mutations in ACSF3 excrete more MMA than MA (Alfares et al. 2011). The incidence of CMAMMA due to mutations in the ACSF3 gene has been estimated at approximately 1 in 30,000 (Sloan et al. 2011). However, at present only 11 patients with mutations (missense, in frame deletion, and nonsense mutations) in the ACSF3 gene have been reported in literature (Alfares et al. 2011; Sloan et al. 2011). Patients with mutations in the ACSF3 gene exhibit a highly heterogeneous clinical phenotype, ranging from asymptomatic (Alfares et al. 2011) to severe (Sloan et al. 2011). Symptoms and signs reported thus far in children include coma, ketoacidosis, hypoglycemia, failure to thrive, elevated transaminases, microcephaly, dystonia, axial hypotonia, and/or developmental delay (Sloan et al. 2011). In those who were identified as adults,

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symptoms include (mild) memory problems, seizures, and encephalopathy (Sloan et al. 2011).

Here, we describe two sisters who both exhibited an encephalopathic event upon an influenza infection in whom urinary analysis initially revealed repetitive “isolated” methylmalonic aciduria (MMAemia). Diagnostic follow-up for MMAemia (genetic complementation analysis, enzymatic and/or mutation analysis) did not yield a primary defect. Diagnostic exome sequencing – to our surprise – identified compound heterozygosity for mutations in the ACSF3 gene. MA excretion was missed in the routinely performed organic acid analysis since it co-elutes with MMA and was negligible in comparison with the elevated MMA excretion.

Therefore, we developed a simple, fast, sensitive, and robust assay to determine MA in plasma using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and applied it to samples of the patients. Clinical data of all of the formerly reported patients were compared.

Material and Methods

Patients

Patient A is the first child of unrelated parents. She had a normal development until the age of 6 years when she was hospitalized because of dehydration and drowsiness thought to be caused by vomiting. Despite rehydration her neurological condition deteriorated. Within a few hours, she exhibited opisthotonus, as well as oculogyric crises. She developed a paresis of both arms. In addition, a Babinski sign was present bilaterally. On suspicion of an encephalitis, she was treated with acyclovir, methylprednisolone, and haloperidol without clear clinical benefits. Catatonia was diagnosed and treated with lorazepam. Viral PCR analysis (nose/throat specimen) was positive for influenza A virus and treatment with oseltamivir was initiated. The initial diagnostic work-up included broad metabolic screening. Urinary organic acid analysis revealed an increased MMA without other overt abnormalities. Metabolic investigations in plasma revealed increased MMA concentrations, while free carnitine, acylcarnitines (including C3DC-carnitine and C4DC-carnitine), amino acids, and total homocysteine were normal. MMA was also quantified in cerebrospinal fluid (CSF) and was found to be increased (13 $\mu\text{mol/L}$; controls: not detectable). Since some forms of MMAemia respond to cobalamin, vitamin B12 injections were administered, however without a clear clinical or biochemical effect. Initially, brain MRI did not show abnormalities, but a second MRI (performed 3 weeks later) showed increased signal on T2 of the genu of the corpus callosum compatible with demyelination or infection (Fig. 1). In addition, an

increased signal was found in the cervical myelum (c2–c7) suggesting edema. She gradually recovered, but upon recovery mutism became evident, which cleared only partially and necessitated revalidation. During follow up she has shown continued improvements. At the age of 10 years, she is visiting a regular elementary school, with higher than average scores.

Patient B, the sister of patient A, developed normally until the age of 5.5 years. She became encephalopathic at the age of 5.5 years after a period of vomiting and fever. Viral PCR (nose/throat specimen) revealed an influenza B infection. She exhibited an opisthotonus and developed oculogyric crises identical to her sister. Likewise there was notable pyramidal tract involvement with bilateral Babinski signs. On examination she had catatonia manifested by stupor, mutism, and *flexibilitas cerea*. She improved clinically after treatment with lorazepam. MRI investigation showed a diffusely increased T2 signal in the white matter, of note corpus callosum appeared normal. The liver size was slightly increased. At follow-up (age 7.5 years), her development is in the normal range.

Clinical features of the two sisters were compared with formerly reported patients (Table 1).

Samples

Six plasma samples and sixteen urine samples from the two CMAMMA patients were available and were compared with 20 stored plasma samples from a total of six patients with both nonresponsive and responsive MMAs (classic MMAemia) (MMAA or MMAB). Urine samples from 40 females with a similar age were selected as control group.

Reagents

MA, stable isotopically labeled MA ($^{13}\text{C}_3\text{-MA}$) and dithiothreitol (DTT) were purchased from Sigma (Steinheim, Germany). Formic acid was purchased from Merck (Darmstadt, Germany) and acetonitrile was obtained from Biosolve (Valkenswaard, the Netherlands).

MMA and MA in Urine

Quantification of MMA excretion was performed by GC/MS in standard organic acid analysis. Quantification of MA excretion was performed by GC/MS using selected ion monitoring (SIM) of m/z 233 in the same assay. Prior to analysis the samples were trimethylsilylated.

MMA and MA in Plasma

Sample preparation and chromatographic and mass spectrometric conditions on an UPLC/MS/MS system were used as

Fig. 1 Sagittal T2 flair showing hyperintense lesion in the genu of corpus callosum

described before (de Sain-van der Velden et al. 2015) with some slight modifications. In short, apart from other internal standards, $^{13}\text{C}_3\text{-MA}$ ($12\text{ }\mu\text{mol/L}$) was added to plasma.

The MS operated in negative electrospray ionization (ESI) mode for MA. Mass transitions m/z 102.8 to m/z 58.8 (MA) and m/z 105.8 to m/z 60.8 ($^{13}\text{C}_3\text{-MA}$) were measured. For quantification a calibration curve of eight standards with spiked concentrations of 0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and $5.0\text{ }\mu\text{mol/L}$ MA was prepared in plasma. For quality control (QC), heparinized blood from a volunteer was used to prepare three QC samples: QC-B (blank, without spiking), QC-L (low, spiked with $1.2\text{ }\mu\text{mol/L}$ MA), and QC-H (high, spiked with $3.7\text{ }\mu\text{mol/L}$ MA). The method was validated for accuracy, process efficiency, carryover, LOD, LOQ, linearity, intra-variation, inter-variation, cross talk, and stability (after preparation of the samples, freeze/thaw cycle) according to recently published guidelines (Honour 2011).

MA in plasma was quantified in control subjects within a wide age range to determine age dependency of this

compound. These data were used to set reference ranges for MA.

Results

Genetic Analysis

Diagnostic exome sequencing in both patients identified compound heterozygous missense mutations in ACSF3, a recognized cause of CMAMMA (Alfares et al. 2011). The first mutation c.1075G>A p.(Glu359Lys) has been described before (Alfares et al. 2011). The second mutation c.311A>T p.(Asn104Ile) was not described before in controls ($>60,000$ controls; Exome Aggregation Consortium (ExAC), Cambridge, MA (<http://exac.broadinstitute.org> [03–2015]) or patients and concerns a change of an evolutionary highly conserved amino acid. In silico predictions suggest a pathogenic effect for this mutation.

Table 1 Symptoms and signs of patients with known mutation in the ACSF3 gene

Symptoms and signs	Frequency
T2 hyperintensities ^a	4
Increased signal on T2 of the genu of the corpus callosum ^b	1
Diffusely increased T2 signal in the white matter ^b	1
Extrapyramidal tract involvement ^a	3
Oculogyric crises ^b	2
Encephalopathy ^a	3
Loss of speech/mutism ^a	3
Mutism ^b	2
Psychiatric symptoms ^a	3
Catatonias ^b	2
Memory problems	3
Neurodegeneration of the brain and spinal cord ^a	2
Increased T2 signal in the cervical myelum (c2–c7) ^b	1
Pyramidal tract involvement ^b	2
Opisthotonus ^b	2
Acidosis/ketoacidosis	2
Axial hypotonia	2
Psychomotor delay	2
Poor weight gain/failure to thrive	2
Seizure	1
Complex partial seizures	1
Speech delay	1
Elevated transaminases	1
Failure to thrive	1
Hypoglycemia	1
Incontinence	1
Microcephaly	1
Ocular migraine	1

^a Present in either of the two sisters^b Not previously reported

Nomenclature is according to HGVS guidelines and is based upon transcript NM_174917.4.

Quantification of MMA and MA

MMA in plasma ranged from 15.3 to 1,327.4 $\mu\text{mol/L}$ in the group with MMAemia. Urine excretion ranged from 196 to 14,500 $\mu\text{mol/mmol creatinine}$ in this group (Fig. 2).

This group included responsive as well as nonresponsive ($n = 7$ samples) patients for vitamin B12. One sample (from the responsive patients) before starting B12 was included. The other samples from the responsive ones were from the time after B12 application. In both patients, MMA concentrations were consistently elevated in urine as well as plasma (see Fig. 3a and b, respectively). In all urine

Fig. 2 MMA concentration in plasma (filled circle; responders $N = 13$, open circle; nonresponders $N = 7$, left Y-axis) and in urine (filled square; responders $N = 13$, open square; nonresponders $N = 7$, right Y-axis) from patients with classic MMAemia

samples from the CMAMMA patients, excretion of MA was increased. The range of urine MA excretion was 15–201 and 13–226 $\mu\text{mol/mmol creatinine}$ in patient A and patient B, respectively (Fig. 3c). Notably, in some urine samples, this was only just above the upper value (range 0.23–10.0 $\mu\text{mol/mmol creatinine}$) observed in 40 female control subjects with a mean age of 7.5 years (range 5–11 years). The urinary MA/MMA ratio (ranging from 1.7 to 9.1 in patient A and from 1.1 to 8.2 in patient B) overlapped with those calculated in control subjects (range 0.06–2.4).

General aspects of assay performance for MA in plasma are shown in Table 2. The 95 percentile of MA in plasma was 0.31 (range 0.08–0.79 $\mu\text{mol/L}$) in 87 control subjects (median age: 13.9 (range 2 months–75 years) and was not age dependent. The range of MA/MMA in plasma was 0.4–5.3 in control subjects. The range of MA was 0.9–3.4 $\mu\text{mol/L}$ in CMAMMA (Fig. 3d) and overlap with those measured in classic MMAemia (0.17–2.12 $\mu\text{mol/L}$; Fig. 4a). The range of MA was 0.17–0.43 $\mu\text{mol/L}$ in the responders and 0.41–2.12 $\mu\text{mol/L}$ in the nonresponders. In contrast, the MA/MMA ratio in plasma fully discriminates CMAMMA from classic MMAemia (Fig. 4b).

Discussion

Here, we describe two patients with compound heterozygous missense mutations in the ACSF3 gene identified by diagnostic exome sequencing. Both patients presented with a severe psychiatric presentation after a viral infection. Until now 11 patients have been reported in literature with mutations in the ACSF3 gene with various clinical presentations (Table 1). The clinical presentation of the patients described in the present paper was strikingly different from other reports (Table 1). While psychiatric

Fig. 3 (a) MMA excretion ($\mu\text{mol}/\text{mmol creatinine}$), (b) MMA in plasma ($\mu\text{mol}/\text{L}$), (c) MA excretion ($\mu\text{mol}/\text{mmol creatinine}$), and (d) MA in plasma ($\mu\text{mol}/\text{L}$) in both patients. Dotted lines represent 95th percentile of normal

Table 2 General aspects of assay performance and influence of stability for MA in plasma

	MA
Accuracy (%)	91
Process efficiency (%)	57
LOD (μM)	0.02
LOQ (μM)	0.06
Carryover (%)	<0.2
Linearity (μM)	62
Intra-variation (%) ($n = 10$)	6.7
Inter-variation (%) ($n = 10$)	13.4
Cross talk (%)	<0.02
Stability (after sample preparation) (h)	24
Stability (freeze/thaw cycles)	10 ^a

^a Maximum tested

features and neurological problems have been described in adult patients, this was not observed in the younger group (Sloan et al. 2011). Striking features in our patients included mutism (in both) and corpus callosum lesions (in one patient). Interestingly, corpus callosum lesions have

been associated with mutism, suggesting a pathophysiological link. However, no clear corpus callosum abnormalities were observed on MRI imaging in the other patient.

CMAMMA is biochemically characterized by elevated concentration of the diagnostic compounds MMA and MA in both urine and plasma. While MMA excretion in patients with mutations in the ACSF3 gene has been reported to be 8–194 (Alfares et al. 2011; Sloan et al. 2011) times normal, MA excretion is typically present at relatively lower levels 3–91 times normal excretion (Alfares et al. 2011; Sloan et al. 2011).

In our laboratory, elevated urinary MA values were initially missed using routinely performed organic acid analysis. Although we successfully increased the sensitivity of our assay for MA – by using selected ion monitoring (SIM) at m/z 233 – it should be noted that even then, the elevation of urinary MA concentration may be so subtle that it could easily be missed. Moreover, there was an overlap in urine MA/MMA ratio between control subjects and patients with CMAMMA.

We explored whether – in patients with an elevated MMA excretion – MA concentrations in plasma could allow differentiation. Plasma MA can be easily added to a method for quantification of MMA (and homocysteine) (de

Fig. 4 (a) Box plots showing MA in plasma ($\mu\text{mol/L}$) in control subjects, patients with MMAemia and in the two patients with CMAMMA. (b) MA/MMA ratio in plasma in both patient groups. Shown are the median and 5th–95th percentile

Sain-van der Velden et al. 2015), allowing no extra material and costs for follow-up. Our upper limit of MA ($0.79 \mu\text{mol/L}$; $n = 87$) in control subjects quantified by UPLC-MS/MS is in good agreement with the upper limit reported using GC/MS ($0.89 \mu\text{mol/L}$; $n = 19$) (Sloan et al. 2011). There was a clear increase in MA concentration in patients diagnosed with mutations in the ACSF3 gene in comparison to control subjects (Fig. 4a).

Surprisingly, MA concentrations measured in plasma of patients with CMAMMA overlap with those measured in plasma samples from patients with classic MMAemia (Fig. 4a). Plasma MA concentration in three different samples (from two different patients) with classic MMAemia was above the upper limit of control samples (Fig. 4a).

Of interest is the observation that increased excretion of both MA and MMA is observed in patients with MCD (de

Wit et al. 2006). It is speculated that MCD deficiency causes an excess of intramitochondrial malonyl-CoA, leading to the inhibition of methylmalonyl-CoA mutase and, subsequently, an increase in MMA (Alfares et al. 2011). We speculate that the opposite is true in classic MMAemia. Indeed, the plasma samples with the highest MMA concentration showed the highest MA in plasma. When calculating MA/MMA ratio in plasma, it is possible to fully discriminate between CMAMMA and classic MMAemia (Fig. 4b). The MMAemia contained both patients on vitamin B12 (responders) as well as non-responders to stress the discriminatory power of the MA/MMA ratio.

The prevalence of CMAMMA due to mutation in ACSF3 gene is not known. Given the normal concentration of propionyl (C3) carnitine in CMAMMA patients (Alfares

et al. 2011; Sloan et al. 2011), this disorder will not be detected by the current dried blood spot newborn screening. Only when MMA or MA are used as primary screening markers, detection of these patients is feasible. Screening for MCD deficiency is possible on C3DC-carnitine and other acylcarnitine ratios (McHugh et al. 2011). Given the difficulty with diagnosis, we suspect that CMAMMA is an under-recognized condition.

The high calculated incidence and the presence of asymptomatic patients (Alfares et al. 2011) leave room for the possibility that CMAMMA is a biochemical condition rather than a disease (Levtova et al. 2015), similar to SCAD deficiency (Pedersen et al. 2008) and, possibly, 3-methylcrotonylglycinuria (3MCC deficiency) (Lam et al. 2013), and screening for this disorder is then not justified. Alternatively, CMAMMA may be perceived as a risk factor rather than a disease, which could lead to clinical symptoms in combination with other genetic and/or environmental factors. The cases described here are compatible with this concept: in case of CMAMMA, certain infections may be important triggers, which provoke metabolic dysregulation. Understanding of this rare condition, e.g., by identifying factors involved in triggering the development of symptoms, will improve as more patients are identified.

In conclusion, the clinical spectrum of CMAMMA may include childhood psychiatric features. CMAMMA due to mutation in ACSF3 gene could be misdiagnosed as a classic MMAemia when urine is used as the sole matrix. Plasma MA/MMA ratio can serve as fast diagnostic tool for detection of CMAMMA. With early diagnosis of CMAMMA, additional investigation or unnecessary treatment (vitamin B12) can be avoided.

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Take-Home Message

Childhood psychiatric presentation can be added to the clinical spectrum of CMAMMA, a disorder which can easily be diagnosed by calculation the MA/MMA ratio in plasma.

Compliance with Ethics Guidelines

Conflict of Interest

Monique G.M. de Sain-van der Velden, Maria van der Ham, Judith J. Jans, Gepke Visser, Hubertus C.M.T. Prinsen, Nanda M. Verhoeven-Duif, Koen L. I. van Gassen,

and Peter M. van Hasselt declare that they have no conflict of interest.

Informed Consent

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 (5). Informed consent was obtained from all patients for being included in the study.

Animal Rights

This article does not contain any studies with animal subjects.

Details of the Contributions of Individual Authors

Monique G.M. de Sain-van der Velden and Peter M. van Hasselt were involved in conception and design, analysis and interpretation of data, and drafting the article.

Maria van der Ham was involved in conception and design, analysis and interpretation of data, and revising it critically for important intellectual content.

Koen L. I. van Gassen was involved in analysis and interpretation of data and revising it critically for important intellectual content.

Judith J. Jans, Gepke Visser, Hubertus C.M.T. Prinsen, and Nanda M. Verhoeven-Duif were involved in interpretation of data and revising it critically for important intellectual content.

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