



## SHORT REPORT

# Combined malonic and methylmalonic aciduria: exome sequencing reveals mutations in the *ACSF3* gene in patients with a non-classic phenotype

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## ABSTRACT

**Background** Combined Malonic and Methylmalonic Aciduria (CMAMMA) is a rare recessive inborn error of metabolism characterised by elevations of urine malonic acid (MA) and methylmalonic acid (MMA). Nearly all reported cases are caused by malonyl-CoA decarboxylase (MCD) deficiency. Most patients have metabolic acidosis, developmental delay, seizures and cardiomyopathy. CMAMMA was also described in symptomatic patients with normal MCD activity, suggesting heterogeneity in this disorder.

**Methods and results** We identified two probands with a non-classical CMAMMA variant through the Quebec newborn urine screening program. While they share the biochemical phenotype of elevated MA and MMA, the MMA excretion was higher than MA, the clinical courses were benign, *MYLCD* gene sequencing was normal and MCD activity, measured in one proband, was normal. Using exome sequencing in the single consanguineous proband, we identified a homozygous missense allele in the *ACSF3* gene, encoding an Acyl-CoA Synthetase (ACS) with unknown substrate and function. The second proband was homozygous for a different *ACSF3* missense allele. Both substitutions were in conserved residues and were identified in less than 0.5% of their respective ethnic control populations.

**Conclusion** These results suggest that *ACSF3* is a candidate gene for non-classical CMAMMA observed in our patients and document the value of exome sequencing of a limited number of patients for the identification of novel disease genes.

Several types of patients with elevated malonic acid (MA) have been described in the literature. The most well characterised is that related to malonyl-coenzyme A (CoA) decarboxylase (MCD) enzyme deficiency (OMIM #248360) in which the biochemical abnormalities include elevated MA alone, or combined elevations of MA and methylmalonic acid (CMAMMA) with MA mainly being higher than methylmalonic acid (MMA).<sup>1–11</sup> 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. Two patients have been described with similar combined elevations of MA greater than MMA, but with normal MCD enzyme activity in fibroblast cultures.<sup>12 13</sup>

suggesting that this disorder is heterogeneous. All of these patients have shown a variety of clinical features that include metabolic acidosis, developmental delay, seizures, cardiomyopathy, gastrointestinal distress and dysmorphic features, although normal development was also reported.<sup>11</sup> Through the Quebec newborn urine screening programme<sup>14</sup> we identified asymptomatic individuals with CMAMMA, but with MMA higher than MA, and sought to define the underlying molecular cause. We refer to these patients as having non-classic CMAMMA.

## METHODS

### Metabolite detection

The Quebec newborn urine screening programme, including MMA since 1975, evaluates urine from 4–6-week-old infants by thin layer chromatography. Subsequent urine organic acid analysis by gas chromatography mass spectroscopy confirms CMAMMA. MMA is considered elevated when there is more than 200 µmol/mmol creatinine in the newborn period.<sup>15</sup> Patients were followed in our medical genetics clinic and informed consent for research studies was obtained according to institutional guidelines.

### Molecular analysis

Genomic DNA was isolated from patient fibroblast cell lines. All exons and intronic flanking sequences of *MYLCD* were amplified and sequenced by Sanger methods; primers and PCR conditions were modified from Wightman *et al.*<sup>11</sup> *ACSF3* exons 5 and 8 were amplified from gDNA in 315 controls using primers and conditions reported here (see supplementary material, available online only). GenBank ref seq nos: *MYLCD*, NM\_012213; *ACSF3*, NM\_174917; *TAS2R43*, NM\_176884.

### Exome sequencing

A total of 3 µg of DNA was subjected to the Sure-Select Human All Exon v2 Kit from Agilent Technologies (Santa Clara, CA, USA), according to the manufacturer's protocols. This kit targets approximately 44 Mb of sequence including the human exome defined by the NCBI consensus coding sequence database (version 20090902) and additional ref seq sequences. The captured DNA was sequenced by Illumina GAIIx (San Diego, CA, USA) on one lane, generating approximately 32 million

76 bp quality-controlled reads. Reads were aligned to the human reference genome (University of California, Santa Cruz (UCSC) hg19 assembly) using Burrows-Wheeler Aligner's Smith-Waterman Alignment (BWA).<sup>16</sup> Reads that mapped non-uniquely or to unfinished chromosomes were discarded from further analysis.

Approximately 28.9 million reads were uniquely mapped to the reference genome, providing an average NCBI consensus coding sequence coverage of 25X. We imposed similar variant identification filtering criteria as described.<sup>17</sup> Variants were identified using SAMtools.<sup>16</sup> In addition, we compared our variants against a pool of 34 exomes previously sequenced by us, of which three were reported<sup>17</sup> while the remainder are unpublished. Variants seen in two or more individuals were discarded. Functional annotation of the predicted variants was carried out using ANNOVAR,<sup>19</sup> which cross-references variants against public databases, including dbSNP131 and the 1000 Genomes project. Novel variants were defined as those absent from dbSNP and had a 1000 Genomes allele frequency of less than 0.005. This allowed us to consider variants observed in 1000 Genomes at very rare frequencies. Potentially damaging variants included non-synonymous substitutions caused by missense and nonsense single-nucleotide polymorphisms (SNP), splice-site SNP, and frameshift changes due to indels.

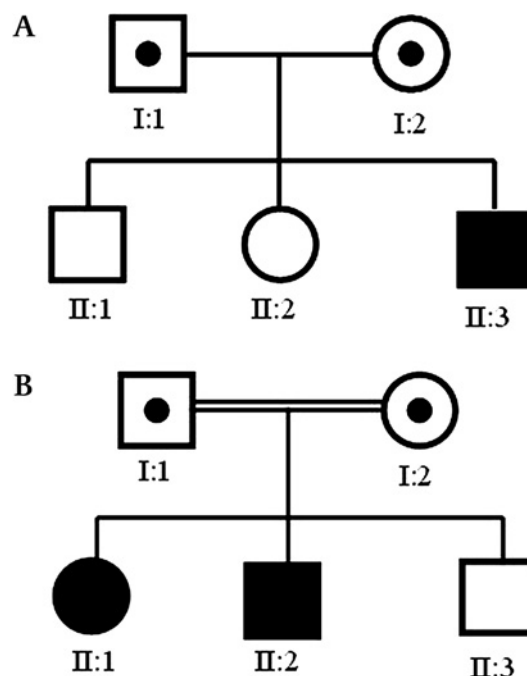
## RESULTS

The clinical and biochemical features of the two non-classic CMAMMA probands in this report are described below.

Patient 1, a 14-year-old boy, was born to non-consanguineous Ashkenazi Jewish parents (figure 1A, II:3) and was briefly reported previously.<sup>15</sup> His initial urine MMA level was 891  $\mu\text{mol}/\text{mmol}$  creatinine. Confirmatory testing by gas chromatography mass spectroscopy showed a urine MMA of 745  $\mu\text{mol}/\text{mmol}$  creatinine (normal <25) and MA of 60  $\mu\text{mol}/\text{mmol}$  creatinine (normal <6). His plasma MMA level was 18  $\mu\text{mol}/\text{l}$  (normal <0.5). On follow-up the ranges of urine MA and MMA were 20–68 and 214–470  $\mu\text{mol}/\text{mmol}$  creatinine, respectively; the plasma MMA range was 20–32  $\mu\text{M}$ .

Patient 2, a 4-year-old girl, was born to first cousin French Canadian parents (figure 1B, II:1). Her initial MMA level was 800  $\mu\text{mol}/\text{mmol}$  creatinine, and confirmatory testing showed urine MMA and MA levels of 737 and 67  $\mu\text{mol}/\text{mmol}$  creatinine, respectively; the plasma MMA level was 39.2  $\mu\text{mol}/\text{l}$ . On follow-up the ranges of urine MA and MMA were 43–260 and 690–1830  $\mu\text{mol}/\text{mmol}$  creatinine, respectively. The range of plasma MMA was 28–40  $\mu\text{M}$ . She has a 2-year-old similarly affected sibling (figure 1B, II:2) with urine MMA 843 and MA 94  $\mu\text{mol}/\text{mmol}$  creatinine.

Both patients were born at term, were clinically asymptomatic, had normal cardiac examinations and age-appropriate development. No other organic acid abnormality was detected. Carnitine levels, acylcarnitine profiles, plasma amino acids, ammonia, blood gas values, total homocysteine, folate and vitamin B<sub>12</sub> levels were all within reference ranges, and the mothers were not vitamin B<sub>12</sub> deficient. MMA levels were not responsive to protein restriction or vitamin B<sub>12</sub> supplementation and therefore these treatments were not instituted. In cultured fibroblasts, the incorporation of [<sup>14</sup>C]-propionate, [<sup>14</sup>C]-methyltetrahydrofolate and [<sup>57</sup>C]-cyanocobalamin, and the synthesis of both adenosylcobalamin and methylcobalamin were within the reference range. These results excluded a defect in intracellular vitamin B<sub>12</sub> metabolism and methylmalonyl CoA deficiency. MCD activity in fibroblasts was measured in patient 1 and the results were within the reference range.



**Figure 1** Pedigrees of (A) family 1 and (B) family 2. Blackened boxes indicate the presence of non-classic combined malonic and methylmalonic aciduria and homozygosity for *ACSF3* mutations. Parents were heterozygote carriers of the identified mutations. Parents and unaffected siblings had normal urine malonic acid and methylmalonic acid levels; unaffected siblings were not genotyped.

## Molecular analysis

No mutations in *MYLCD* were found in either patient. Exome sequencing of patient 2, followed by the selection of novel non-synonymous variants (see Methods) that were either homozygous or potentially compound heterozygous (two mutations in the same gene) resulted in the identification of only two potential candidate genes: *TAS2R43* (taste receptor, type 2, member 43) and *ACSF3*, a novel member of the family of acyl-CoA synthetases (ACS). *TAS2R43* encodes a seven transmembrane G-protein-coupled receptor located on the surface of taste receptor cells and mediates cell responses to bitter taste.<sup>20</sup> A homozygous missense substitution in a residue conserved among mammals, c.97A→G (p.Ile33Val), was identified and predicted to be located in a potential cytoplasmic domain. We did not detect any region of homozygosity around this variant. However, based on potential biological significance, *ACSF3* was selected as the more likely candidate. Patient 2 was homozygous for c.1075G→A in exon 5, predicted to substitute a glutamate to a lysine residue, or p.Glu359Lys. The glutamate residue is highly conserved through evolution among all *ACSF3* homologues, including zebrafish, neurospora and rice, and this substitution was predicted to be damaging based on PolyPhen-2 functional analysis.<sup>21</sup> This allele was annotated in 1000 Genomes with a rare allele frequency of 0.001. There were no heterozygous SNP in the vicinity of this mutation, suggesting a region of homozygosity spanning approximately 5.4 MB at the tail of chromosome 16q. This mutation was also identified in homozygosity in the affected brother. In order to determine whether *ACSF3* play a role in the patients' phenotype, *ACSF3* was sequenced in patient 1, and a homozygous mutation, c.1411C→T (p.Arg471Trp), was identified in exon 8. The arginine residue is again highly conserved in evolution and the substitution was predicted to be damaging.<sup>21</sup> The Sanger

sequence traces for both mutations and parental genotypes are shown in figure 2.

To determine the frequency of the missense alleles in an ethnically matched population, we sequenced *ACSF3* exon 5 in 139 French Canadian controls and exon 8 in 176 Ashkenazi Jewish controls. The c.1075G→A (exon 5) mutation was found in a heterozygous state in one French Canadian control individual, predicting a minimum incidence of this phenotype to be one in 77284 in this population. The c.1411C→T (exon 8) mutation was found in a heterozygous state in a single Ashkenazi Jewish control individual, predicting a minimum incidence of this phenotype to be one in 123094.

## DISCUSSION

CMAMMA is a rare recessive phenotype characterised by elevated MA and MMA in body fluids. The non-classic patients described here differ from classic patients by having a benign clinical course. When the urine MA levels are compared they overlap; the ranges of MA are approximately 15–260 in non-classic and 19–600  $\mu\text{mol}/\text{mmol}$  creatinine in classic patients. However, the MMA levels in non-classic patients range from approximately 400 to 1000  $\mu\text{mol}/\text{mmol}$  creatinine, which is higher than the classic range of 5–300  $\mu\text{mol}/\text{mmol}$ . The detection of these patients may be unique to the Quebec newborn urine screening programme. Most newborn screening programmes analyse dried blood spots using tandem mass spectrometry in the first week of life. Infants with classic CMAMMA are suspected by elevations of C3-DC (malonyl) acylcarnitine. It is not known whether non-classic CMAMMA can be detected by this method, considering that the targeted C3-DC, C3 and C4-DC plasma acylcarnitine species were within reference ranges in our patients.

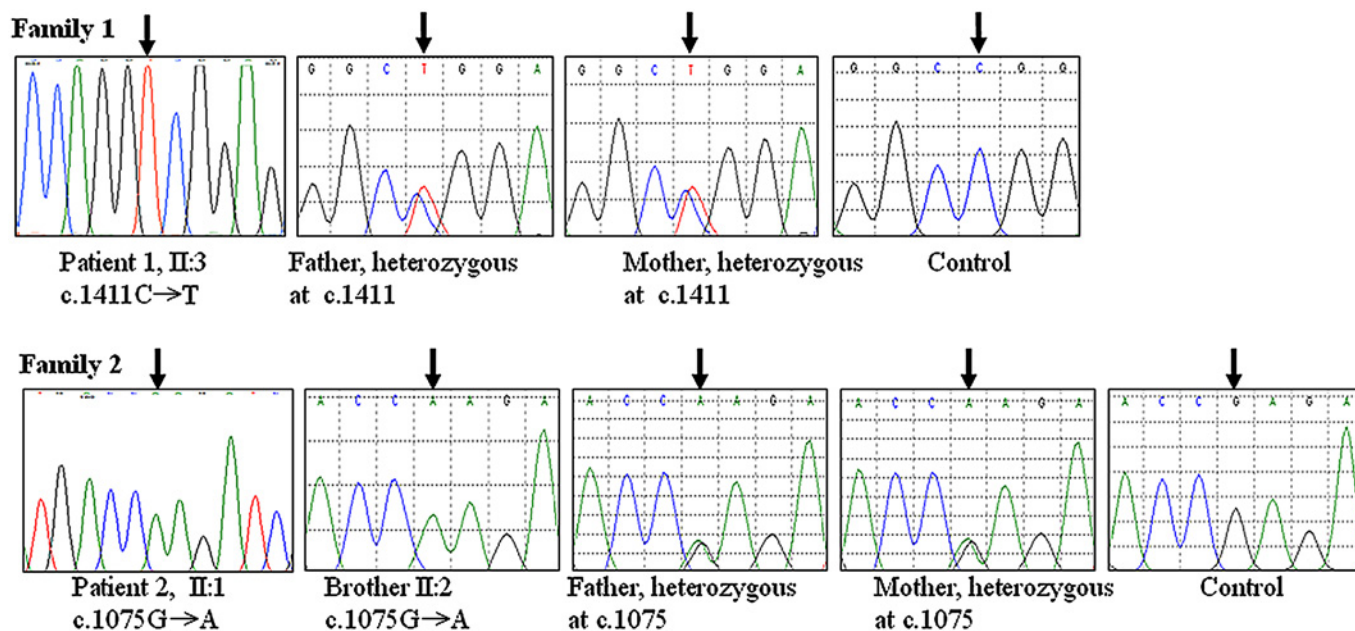
The number of newborns screened per year in Quebec is approximately 77000, and ethnicity is not determined (Dr Christiane Auray-Blais, Faculty of Medicine and Health Sciences, Université de Sherbrooke, personal communication). Therefore, approximately 2 695 000 infants were screened from 1975 to 2010 and three patients with non-classic CMAMMA were detected. This detection rate of one per 898 333 is lower than that predicted

by the heterozygote frequencies. These figures indicate that not all newborns with this biochemical phenotype are detected by the Quebec urine screening programme and further support the clinical evidence that this may be a benign phenotype.

*ACSF3* contains nine coding exons and is located on chromosome 16q24.3. There are two transcript variants that differ by the presence of an alternate exon in the 5' untranslated region. EST profiles show transcript expression in numerous tissues and organs. *ACSF3* encodes a 576 amino acid protein containing a predicted N-terminal mitochondrial targeting signal. It is a member of the family of ACS, which catalyse the activation of fatty acids by forming a thioester with CoA and a fatty acid, to form acyl-CoA. Recently, 26 distinct ACS were identified in the human genome based on the presence of five conserved amino acid motifs.<sup>22</sup> *ACSF3* could not be assigned to any of the established subfamilies (ACSS, ACSM, ACSL, ACSVL, or ACSBG and probably represented a novel ACS family member. Watkins *et al*<sup>22</sup> demonstrated that *ACSF3* did indeed have ACS activity in a heterologous expression system. p.Arg471 located in ACS motif II and mutated in patient 1, represents an ATP binding site by homology and is nearly invariant in all ACS families. Substitution of the equivalent residue in *Escherichia coli* fatty ACS to an alanine (fadD, p.Arg453Ala) resulted in the complete loss of enzymatic activity.<sup>23</sup>

We suggest that defects in *ACSF3* are good candidates for the biochemical phenotype in these patients. Future studies will need to be performed to determine whether *ACSF3* deficiency is causative of the non-classic CMAMMA described here. Given a putative role as a mitochondrial ACS enzyme, we speculate that *ACSF3* might encode a malonyl or methylmalonyl-CoA synthetase. Previous studies have suggested the possibility of a methylmalonyl-CoA synthetase providing a 'free shunt' for MMA in the mitochondria.<sup>24</sup> For malonyl-CoA synthetase, it is interesting to note that the *ACSF3* protein has 35% identity (amino acids 48–573) with bacterial malonyl-CoA synthetases using position-specific iterated BLASTp algorithms.<sup>25</sup>

Exome sequencing is a method to identify novel disease genes using one or a few affected individuals.<sup>17 26 27</sup> In this case, the



**Figure 2** Sanger sequence traces in the mutation region for patients 1, 2, parents and control.

finding of a homozygous missense allele in *ACSF3*, facilitated by consanguinity, was corroborated by finding a different homozygous missense allele in the same gene in a second proband. This highlights the growing power of this technique to find novel candidate disease genes with limited numbers of patients and families.

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**Competing interests** None.

**Ethics approval** Ethics approval was obtained from Montreal Children's Hospital and MUHC.

**Contributors** AA, NB, KAT, JM and SM identified and characterised the patients; KHH and JM performed the exome sequencing and analysis; LDN, NA and DSR performed the biochemical and molecular studies; AA and NB wrote the paper; LDN, DSR, KHH and JM contributed to the final manuscript; NB is the guarantor.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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ACSF3 intronic primers and PCR conditions for gene amplicons

Primer	Sequence	Conditions
ACSF3E02aF	GAAGGTGCAGCAGGCGTA	62°C annealing, 1min ext, 35 cycles
ACSF3E02aR	TGGGAGTCGCAGATGACATA	
ACSF3E02bF	CTACGTCGTGGCCCAGTG	62°C annealing, 1min ext, 35 cycles
ACSF3E02bR	GCAGGATGGTGACAACCTGTG	
ACSF3E03F	CAACAAAGAGCCAGCCTTCT	62°C annealing, 1min ext, 35 cycles
ACSF3E03R	CGACTGCACACCAGGAAAAG	
ACSF3E04F	CGCTGCAGACTCTTGAATGT	58°C annealing, 1sec ext, 33 cycles
ACSF3E04R	AGCACTGCTTCCAGAACTT	
ACSF3E05F	GTGAAGGAGCTGCCACTTTT	62°C annealing, 1min ext, 35 cycles
ACSF3E05R	GTCGCAGTGCATCCTGAGT	
ACSF3E06F	TCTTCTCTCTGGGTCACAGG	62°C annealing, 1min ext, 35 cycles
ACSF3E06R	AGGGGAGTCTGCCTCCAG	
ACSF3E07F	CGTTTTCCAAGCATTCCAAC	62°C annealing, 1min ext, 35 cycles
ACSF3E07R	TTCTGCCTTTGGCTGAAAAT	
ACSF3E08F	GGGTTGCCACAGGGTAGTAA	62°C annealing, 1min ext, 35 cycles
ACSF3E08R	CGACTCATCTGCAGTCGTCTA	
ACSF3E09F	AGACTGCGCTCTTCTGTGT	62°C annealing, 1sec ext, 33 cycles
ACSF3E09R	TTTCTTGATCCCTCATCTCCA	
ACSF3E10F	GGCACCTGTCCGTA CTGC	58°C annealing, 1sec ext, 33 cycles
ACSF3E10R	GATTCTACGTGACCTGATTTGG	