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ORIGINAL ARTICLE

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase deficiency: urinary organic acid profiles and expanded spectrum of mutations

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Abstract Mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (HMCS2) deficiency results in episodes of hypoglycemia and increases in fatty acid metabolites. Metabolite abnormalities described to date in HMCS2 deficiency are nonspecific and overlap with other inborn errors of metabolism, making the biochemical diagnosis of HMCS2 deficiency difficult. Urinary organic acid profiles from periods of metabolic decompensation were studied in detail in HMCS2-deficient patients from four families. An additional six unrelated patients were identified from clinical presentation and/or qualitative identification of abnormal organic acids. The diagnosis was confirmed by sequencing and deletion/duplication analysis of the *HMGCS2* gene. Seven related novel organic acids were identified in urine profiles. Five of them (3,5-dihydroxyhexanoic 1,5 lactone; *trans*-5-hydroxyhex-2-enoate; 4-hydroxy-6-methyl-2-pyrone; 5-hydroxy-3-ketohexanoate; 3,5-dihydroxyhexanoate) were identified by comparison with synthesized or commercial authentic compounds. We provisionally identified *trans*-3-hydroxyhex-4-enoate and 3-hydroxy-5-ketohexanoate by their mass spectral characteristics. These metabolites were found in samples taken during periods of decompensation and normalized when patients recovered. When cutoffs of adipic >200 and 4-hydroxy-6-methyl-2-pyrone >20 μmol/mmol creatinine were applied, all eight samples taken from five HMCS2-deficient patients during episodes of decompensation were flagged with a positive predictive value of 80 % (95 % confidence interval 35–100 %). Some ketotic patients had increased 4-hydroxy-6-methyl-2-pyrone. Molecular studies identified a total of 12 novel mutations, including a large deletion of *HMGCS2* exon 1 in two families, highlighting the need to perform quantitative gene analyses. There are now 26 known *HMGCS2* mutations, which are reviewed in the text. 4-Hydroxy-6-methyl-2-pyrone and related metabolites are markers for HMCS2 deficiency. Detection of these metabolites will streamline the biochemical diagnosis of this disorder.

Abbreviations

4HMP	4-hydroxy-6-methyl-2-pyrone
HMCS2	Mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase protein
MLPA	Multiplex ligation-dependent probe amplification

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Introduction

Mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (HMCS2) catalyzes the first step of ketone biosynthesis from fatty acids and is critical for providing energy to the brain during periods of fasting. Human HMCS2 deficiency (OMIM 605991) was first described in 1997 (Thompson et al. 1997), and a small number of patients have subsequently been described with mutations identified in the *HMGCS2* gene that encodes HMCS2 (Aledo et al. 2001, 2006; Bouchard et al. 2001; Ramos et al. 2013; Shafqat et al. 2010; Wolf et al. 2003). HMCS2 deficiency is characterized by episodes of severe hypoglycemia and metabolic acidosis that occur during fasting and can progress rapidly to life-threatening coma. During these episodes, blood levels of free fatty acids are increased with inappropriately low levels of ketones (Fukao et al. 2014; Zschocke et al. 2002). Levels of urine dicarboxylic acids, which are secondary metabolites of incompletely oxidized fatty acids, are also increased. However, these biochemical features also occur in several inborn errors of fatty acid metabolism or secondary to other causes such as liver failure and are therefore not specific for HMCS2 deficiency. Typically, no clinical or biochemical abnormalities are apparent between clinical episodes. The lack of specific biochemical markers and the need to obtain samples during a metabolic crisis make the diagnosis of HMCS2 deficiency challenging. Consequently, only a few individuals with HMCS2 deficiency have been reported.

The HMCS2 enzyme is poorly expressed in readily available tissues such as skin fibroblasts; thus, enzymatic diagnosis requires an invasive liver biopsy. This is further complicated by the presence of the cytosolic form of the enzyme encoded by the *HMGCS1* gene. Diagnosis can be confirmed by DNA analysis, but the mutations described to date have generally been private, and pathogenicity is not always definitive. It would therefore be useful to have more specific biomarkers available to improve the diagnosis of HMCS2 deficiency and as a means of prioritizing patients for *HMGCS2* sequencing.

In this report, we describe the identification of seven novel organic acids in urine that may serve as biomarkers for HMCS2 deficiency during periods of metabolic decompensation. Combining data from other families with HMCS2 deficiency, we report 12 novel disease-causing mutations, including a deletion of exon 1 that requires quantitative gene analyses for its detection, and provide an update on *HMGCS2* mutations identified so far.

Material and methods

Patients

Biochemical data were available from eight individuals with HMCS2 deficiency residing in Victoria, Australia (cohort A). Their clinical features are summarized in Suppl. Table 1. The proband in each family presented with hypoglycemia and metabolic acidosis. Ammonium concentration was increased in one patient (A1-2) but was normal or only slightly increased in the other patients when measured. Lactate concentration was increased in one patient (A1-3) but was normal in the other patients when measured. Variable hepatomegaly was noted in several patients during acute presentations. Parents in family A1 are of Lebanese ancestry and are distantly related; four of eight children in this family are affected. The mode of presentation was quite similar in all affected children. The first presentation was between 5 and 9 months of age, and each child had one or more symptomatic episodes and multiple admissions to hospital for prophylactic management during intercurrent illnesses. Two siblings from this family had one or more episodes of rapid-onset, severe metabolic acidosis requiring hemofiltration during intercurrent illnesses despite preventive measures.

Parents in family A3 are first cousins of Egyptian ancestry. Patient A3-1 presented with significant hepatomegaly in addition to hypoglycemia, which initially suggested the diagnosis of glycogen storage disease type1. However, subsequent investigation and hepatomegaly resolution excluded this diagnosis. A sibling (A3-2) with the same *HMGCS2* genotype was subsequently born but has had no episodes thus far (age at this writing, 3 years).

Excluding patient A3-2, the median age for the first episode of metabolic decompensation in cohort A was 8 months (range 5–37 months). Overall, these episodes and their severity tended to diminish with patient age, and no patient had any episode after 5 years of age. The oldest patient (A1-1) is now 19 years of age. Newborn screening performed at 48–72 h of age by tandem mass spectrometry was normal for patients A1-3, A1-4, A2-1, A3-1, and A4-1. In particular, acetyl carnitine and other acyl carnitines were normal. Newborn screening acyl carnitine results were not available for the other patients.

Molecular data were available from these patients and six additional patients with HMCS2 deficiency who reside outside Victoria (cohort B). All presented in the first or second year of life with acute metabolic acidosis and hypoglycemic hypoketotic coma. Full biochemical and clinical details were not available for cohort B, and it was therefore not possible to compare their urine organic acid profiles with those of cohort A.

Biochemical analyses

The 4-hydroxy-6-methyl-2-pyrone was purchased from Sigma Aldrich. Other metabolites were synthesized as outlined in the Electronic supplementary material.

Urine organic acids were analyzed by gas chromatography-mass spectrometry (GC-MS) after ethyl acetate extraction and formation of methoxamine/trimethylsilyl derivatives. Alternative derivatives (ethoxyamine/trimethylsilyl and butyl/trimethylsilyl) were prepared to assist with identification of new metabolites. Some novel organic acids are volatile, so it was important to include precautions, such as adding ammonia during the drying stages, to minimize evaporative losses. Details of these methods are given in the Electronic supplementary material.

Mutation analysis

All nine coding exons and adjacent intron regions of the *HMGCS2* gene were amplified using polymerase chain reaction (PCR), sequenced with the Sanger method and fluorescent dye-labeled terminators using standard methods. In patients with uninformative sequencing results, quantitative gene analyses were performed by multiplex ligation-dependent probe amplification (MLPA) using a new commercial kit (P068, MRC Holland, Amsterdam, The Netherlands). Mutations were confirmed in both parents where available. Functional consequences of mutations were assessed with the Alamut prediction software suite (Interactive Biosoftware, Rouen, France). Mutations are not listed as polymorphisms in various gene-variant databases, such as the Single Nucleotide Polymorphism data base (dbSNP) (www. ncbi.nlm.nih.gov/projects/SNP/). All missense mutations were predicted to disrupt the structure of HMCS2 based on the experimental structure (Shafqat et al. 2010) and were defined as “probably damaging”, with scores >0.995 using the Polyphen 2 prediction algorithm (http://genetics.bwh.harvard.edu/pph2/).

Results

Organic acid profiles

A representative urinary organic acid profile obtained from patient A1-3 during a period of metabolic decompensation is shown in Fig. 1. The levels of dicarboxylic acids, such as adipic and suberic acids, were greatly increased, dominating the profile and reflecting increased fatty acid metabolism. A large number of other metabolites related to fatty acid metabolism, such as 3-hydroxydicarboxylic acids and 5-hydroxyhexanoic acid, were also prominent, as previously reported (Aledo et al. 2006; Bouchard et al. 2001; Morris et al. 1998; Thompson et al. 1997; Zschocke et al. 2002). In common with previous reports, the levels of metabolites rapidly normalized with treatment and were normal when the patients recovered. This was exemplified by the first episode in patient A1-3, whose organic acid profile completely normalized after 27 h.Seven unknown peaks were consistently present during periods of metabolic decompensation in affected members of family A1. Novel organic acids are labeled A–G in Fig. 1; their GC-MS characteristics are given in Table 1 and Supplementary Fig. 1. These peaks were undetectable or present at much lower levels in control urine samples.

Peak D was identified as 4-hydroxy-6-methyl-2-pyrone (4HMP) by comparison with a commercial standard. Using GC-MS data obtained from alternative derivatives (butyl esters and ethoxyamine derivatives), the other peaks were tentatively identified as saturated and unsaturated hexanoic acids containing hydroxyl and/or keto groups. Peaks B, C, E, and G were subsequently identified by synthesis of the authentic compounds with matching mass spectra and coelution with patients’ samples, whereas peaks A and F were provisionally identified from their mass spectral characteristics and comparisons with peaks C and E, respectively (see Electronic supplementary material).

A qualitative assessment of organic acids profiles from cohort A indicated that *trans*-3-hydroxyhex-4-enoic acid (peak A) and *cis*-5-hydroxyhex-2-enoic acid (peak C) were typically the most abundant peaks in the chromatograms during decompensation episodes (Fig. 1) and were present at much lower levels in control samples. However, significant levels of these two metabolites were also observed in some patients with ketosis. Preliminary observations indicated that 4HMP appeared to be the most consistently increased metabolite and offered superior discrimination between HMCS2-deficient and ketotic patients.

In order to test the diagnostic specificity of 4HMP for HMGCS2 deficiency during routine organic acid screening, we retrospectively analyzed archived data from all GC-MS urine organic acid profiles obtained in our laboratory over a 4-year period. Since it was not feasible to retrospectively analyze all archived profiles for the presence of 4HMP, a filtering process was used in which samples with significant dicarboxylic aciduria (arbitrarily defined as adipic >200 μmol/mmol of creatinine) were first identified. We also examined the relationship between urine 3-hydroxybutyric levels (an indicator of ketosis), adipic, and 4HMP. Profiles obtained from a group of patients with fatty acid oxidation disorders [seven cases of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, three of very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, two of carnitine acylcarnitine translocase (CACT) deficiency, one of carnitine palmitoyltransferase II (CPT II) deficiency, and one of multiple acyl CoA dehydrogenase deficiency] were also included to further assess specificity. These profiles were obtained from samples collected during acute episodes, frequently accompanied by dicarboxylic aciduria.

Examination of 7,369 urine organic acid profiles revealed 104 profiles with adipic >200 μmol/mmol of creatinine. Of

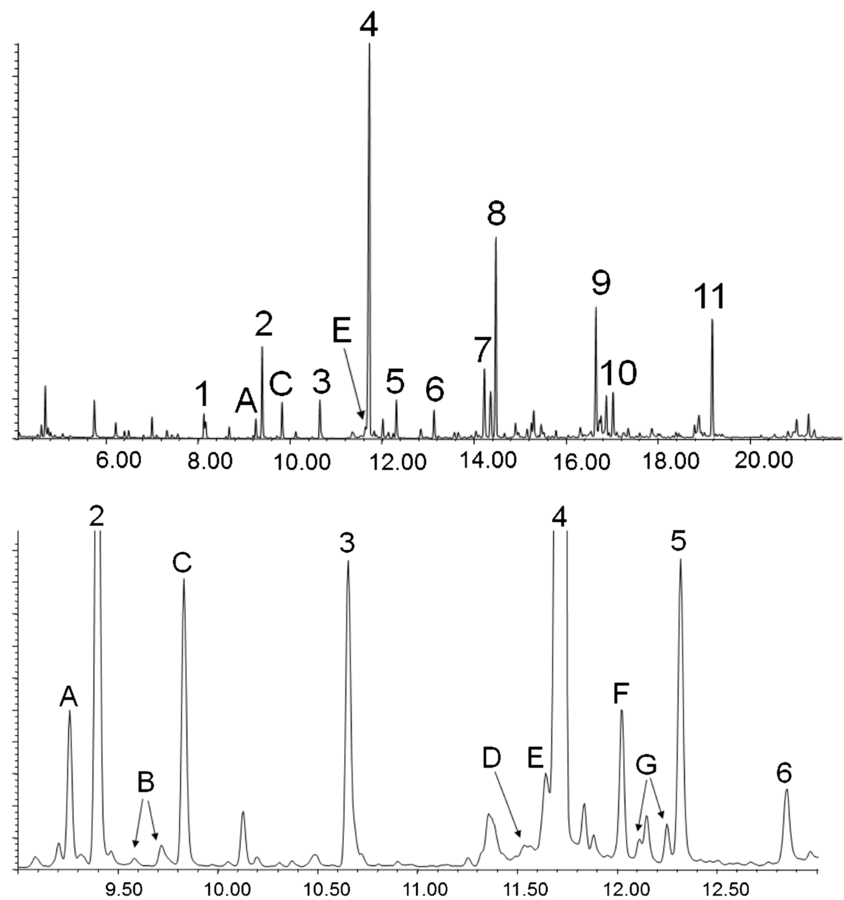

Fig. 1 Total ion chromatogram of urine organic acids from a mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (HMCS2)-deficient child during decompensation. The *lower panel* shows an expansion between 9 and 13 min. Novel organic acids (*A*–*G*) are also shown. Other metabolites: *1* urea, *2* 5-hydroxyhexanoic, *3* internal standard, *4* adipic, *5* hex-2-enedioic, *6* pimelic, *7* unsaturated suberic, *8* suberic, *9* unsaturated sebacic, *10* sebacic, *11* 3-hydroxysebacic

these profiles, eight were from patients with disorders of fatty acid oxidation (Fig. 2). Eight samples were from five patients in the HMCS2-deficiency cohort collected during episodes of metabolic decompensation. Archived electronic data were not available for two members of family A1, but hard copy spectra and chromatograms showed that the abnormal metabolites were increased in these two patients during decompensation episodes. Data from these 104 urine profiles were then reanalyzed to determine the levels of 4HMP; results are summarized in Fig. 2b. A cutoff of 20 μmol/mmol creatinine for 4HMP distinguished all eight samples of HMCS2-deficient patients at decompensation from controls, patients with fatty acid oxidation defects, and almost all patients with ketosis. Criteria of adipic acid >200 and 4HMP >20 μmol/mmol creatinine resulted in a positive

Table 1 Gas chromatography-mass spectrometry (GC-MS) characteristics of urine organic acids in mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (HMCS2) deficiency as methoxamine/trimethylsilyl derivatives

Metabolite	Structure	Retention time (min)	M+	Major ions *m/z*
A	*Trans*-3-hydroxyhex-4-enoic[c]	9.25	274	73, 143, 147, 157, 259
B	3,5-dihydroxyhexanoic 1,5 lactone[a]	9.58/9.63	202	73, 101, 145, 187
C	*Trans*-5-hydroxyhex-2-enoic	9.82	274	73, 117, 147, 230, 259
D	4-hydroxy-6-methyl-2-pyrone	11.55	198	73, 170, 183, 198
E	5-hydroxy-3-ketohexanoic[b]	11.63	319	73, 117, 147, 275, 304
F	3-hydroxy-5-ketohexanoic[b,c]	12.02	319	73, 147, 233, 304
G	3,5-dihydroxyhexanoic[a]	12.11/12.25	364	73, 117, 147, 259, 349

[a] Double peak due to two chiral centers

[b] Methoxamine derivative

[c] Provisional identification

predictive value (PPV) of 80 % for HMCS2 deficiency [95 % confidence interval (CI) 35–100 %].

Results summarized in Fig. 2 also show the expected approximate correlation between adipic acid and 3-hydroxybutyric levels due to increased fatty acid oxidation during ketosis. There was no obvious correlation between 4HMP and 3-hydroxybutyric levels. Surprisingly, we observed relatively high levels of 3-hydroxybutyric in some HMCS2-deficiency patients. For example, patient A1-3 had a 3-hydroxybutyric level comparable with ketotic patients (Fig. 2) and has a homozygous exon 1 deletion expected to completely abolish enzyme activity.

Mutations

All affected children in family A1 are homozygous for a novel, large deletion encompassing exon 1 of the *HMGCS2* gene, denoted c.1-?_104+?del. Although the exact break points of this deletion have not been determined, it is expected to completely destroy gene function and to cause complete lack of HMCS2. Patient A2-1 is compound heterozygous for c.797T > C (p.L266S) in exon 4 and c.1220T > C (p.I407T) in exon 7; p.L266 lies in a conserved alpha–helical structure, while p.I407 lies in a beta-sheet structure. Patients A3-1 and A3-2 are homozygous for mutation c.1162G > A (p.G388R) in exon 6, predicted to disrupt the thiolase fold. Since our preliminary report (Pitt et al. 2009), this mutation has also been described in a Caucasian patient (Ramos et al. 2013). Patient A4-1 is compound heterozygous for c.506G > A (p.G169D) in exon 2 and c.1514G > A (p.R505Q) in exon 9. p.G169D is predicted to disrupt the thiolase fold while p.R505Q is predicted to disrupt the dimer interface.

The homozygous exon 1 deletion c.1-?_104+?del was also identified in a child of Mediterranean origin (B1-1) who presented at the age of 1 year with postoperative hypoglycemic coma, seizures, and brain damage. One patient from England (B2-1) was found to be compound heterozygous for the missense mutation c.634G > A (p.G212R) in exon 3, previously detected in two independent German patients (Aledo et al. 2001), and the novel frameshift deletion c.431_432del (p.V144fs) in exon 2. One Australian patient (B3-1) is compound heterozygous for a novel nonsense mutation c.847C > T (p.Q283*) and a novel missense mutation c.695G > T (p.G232V), both in exon 4. Two novel missense mutations, c.553T > C (p.W185R) in exon 2 and c.1508A > G (p.Y503C) in exon 9, were detected in a patient from Northern Ireland (B4-1). One patient from an Arab country (B5-1) is homozygous for the novel missense mutation c.1078T > C (p.S360P) in exon 6. Finally, an Australian patient of mixed Chinese/Caucasian descent (B6-1) is compound heterozygous for missense mutations c.502G > A (p.G168S) and c.520T > C (p.F174L), both in exon 2. The p.G168S mutation is novel, while the p.F174L mutation has previously been described in a patient of Chinese descent (Bouchard et al. 2001). All new and previously reported *HMGCS2* mutations are listed in Table 2.

Discussion

HMCS2 is a critical enzyme in the hepatic synthesis of ketones from fatty acids. Patients with HMCS2 deficiency are therefore prone to episodic metabolic decompensation triggered by fasting or periods of catabolism that require fatty acid utilization for the provision of energy. Fatty acid oxidation is unimpaired, but ketogenesis fails as a consequence of the HMCS2 deficiency, and patients can become severely hypoglycemic and encephalopathic. Urine organic acid profiles from episodes of metabolic decompensation were consistent within our cohort A of HMCS2 deficiency patients and were dominated by secondary products of fatty acid oxidation, such as dicarboxylic acids (adipic, suberic, and sebacic acids) and 3-hydroxydicarboxylic and 5-hydroxyhexanoic acids, similar to previously published profiles. These metabolites are also frequently detected in patients with inborn errors of mitochondrial beta-oxidation (Duran 2003) and reflect the accumulation of fatty acid intermediates in enzyme steps upstream of the metabolic block. Subsequent metabolism by secondary pathways, such as microsomal omega and omega-1 oxidation and peroxisomal beta-oxidation, is proposed as a mechanism producing many metabolites detected on the organic acid profiles (see Supplementary Fig. 2).

Of greater interest were seven novel metabolites observed in HMCS2-deficient patients during decompensation (Fig. 1). These metabolites were present at lower levels than the major secondary fatty acid oxidation products mentioned above, yet they were consistent in HMCS2-deficient patients. Five of these were identified by authentic compound synthesis, and two were provisionally identified based on their mass spectral characteristics (Table 1). All contain six carbon atoms, and their structures are consistent with a buildup of fatty acid metabolites upstream from the HMCS2 enzyme, with subsequent secondary metabolism (Supplementary Fig. 2). The exact details of some of these metabolic interconversions are unclear (e.g., 4HMP formation) and require further investigation. We hypothesise that the *trans*-hex-2-enoate moiety, an intermediate in fatty acid oxidation, could be a source for some of the metabolites after additional microsomal omega-1 and omega oxidation. This is analogous to the hexanoate moiety metabolism in MCAD deficiency for producing metabolites such adipate and 5-hydroxyhexanoate. Considering the metabolic pathway, there could be additional biomarkers for HMCS2 deficiency. Complementary analytical techniques, such as nuclear magnetic resonance, may be useful for further studies in this area.

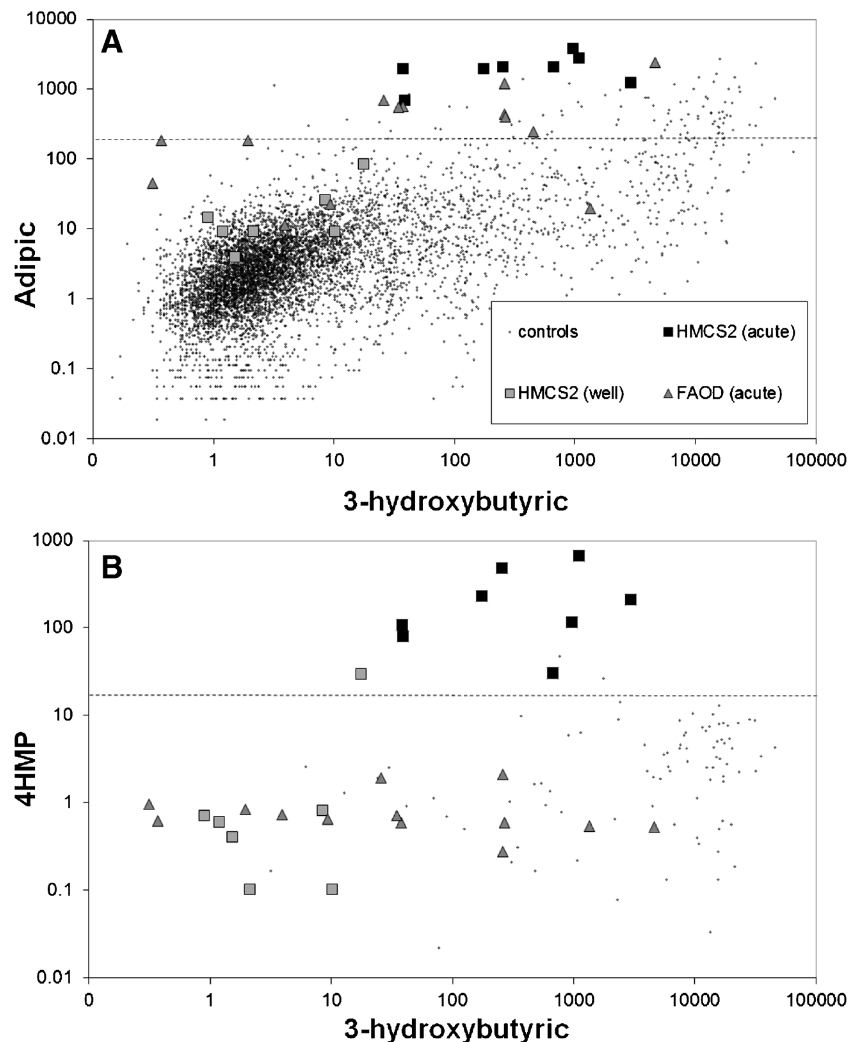

Fig. 2 Urine levels (μmol/mmol creatinine) of adipic acid (**a**) and 4-hydroxy-6-methyl-2-pyrone (4HMP) (**b**) relative to 3-hydroxybutyric levels (log–log scales). Profiles with adipic >200 μmol/mmol of creatinine (*dashed line*, panel a) comprised all eight samples from five mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (HMCS2)-deficient patients in cohort A during acute episodes. These samples also had 4HMP >20 μmol/mmol creatinine (*dashed line*, panel b). *FAOD* group of patients with fatty acid oxidation disorders

While the pattern of metabolites appeared to be quite specific for HMCS2 deficiency, we occasionally observed some of these metabolites in patients with severe ketosis: 4HMP was the most specific for HMCS2 deficiency in this regard, and we focused on the diagnostic utility of this metabolite in a wider group of controls. A retrospective examination of routine urine organic acid profiles obtained over the period in which five patients presented showed that all eight decompensation samples from these five patients had levels of 4HMP > 20 and adipic >200 μmol/mmol creatinine. Using these cutoffs, a PPV of 80 % was obtained in our study sample. Since our preliminary report of these findings (Pitt et al. 2009), two other laboratories have reported the value of these metabolites for the diagnosis of HMCS2 deficiency in abstract form (Carpenter et al. 2010, 2012; Hogg et al. 2012); a third laboratory used them to diagnose an HMCS2-deficient patient (B6-1) (personal communication, Dr Shanti Balasubramaniam).

HMCS2 deficiency belongs to the hypoketotic hypoglycemia group (Aledo et al. 2006; Bouchard et al. 2001; Fukao et al. 2014; Morris et al. 1998), so the finding of moderately increased levels of ketones in urine or blood may result in this diagnosis being discounted. Importantly, increased 3-hydroxybutyrate concentration was observed in the urine of some patients during decompensation (Fig. 2), despite the fact that some of them had null mutations and were therefore expected to have negligible HMCS2 enzyme activity. It is plausible that other sources of ketones contribute a protective effect in HMCS2 deficiency (e.g., via leucine metabolism or reversal of ketone metabolism in extrahepatic tissues). Another possibility is that the 3-hydroxybutyrate is comprised of an increased proportion of the L-isomer, produced in the final steps of the fatty acid oxidation pathway, relative to the D-isomer, which is observed in fasting ketosis. Parameter measurement reflecting concentrations of circulating ketones relative to fatty acids (e.g., plasma D-3-hydroxybutyric/free fatty acids or urine D-3-hydroxybutyric/adipic acid ratios) is more appropriate for determining whether there is a functional block in

Table 2 Summary of novel and previously reported mutations identified in the mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (*HMGCS2*) gene (independent families)

Exon	Mutation	Protein effect	No. of families	Reference
1	c.1-?_104+?del	Deletion exon 1	2	This report
2	c.160G>A	p.V54M	1	(Wolf et al. 2003)
2	c.177 T>A	p.I56N	1	(Shafqat et al. 2010)
2	c.252 T>G	p.Y84*	1	(Shafqat et al. 2010)
2	c.431_432del	p.V144fs	1	This report
2	c.500A>G	p.Y167C	1	(Wolf et al. 2003)
2	c.502G>A	p.G168S	1	This report
2	c.506G>A	p.G169D	1	This report
2	c.520 T>C	p.F174L	2	(Bouchard et al. 2001)
2	c.553 T>C	p.W185R	1	This report
3	c.563G>A	p.R188H	1	(Aledo et al. 2006)
3	c.634G>A	p.G212R	3	(Aledo et al. 2001)
4	c.695G>T	p.G232V	1	This report
4	c.697A>G	p.T233A	1	(Shafqat et al. 2010)
4	c.727A>G	p.K243E	1	(Shafqat et al. 2010)
4	c.797 T>C	p.L266S	1	This report
4	c.847C>T	p.Q283*	1	This report
5	c.920 T>C	p.M307T	1	(Aledo et al. 2006)
6	c.1016+1G>A	IVS5+1 g>a	1	(Zschocke et al. 2002)
6	c.1078 T>C	p.S360P	1	This report
6	c.1162G>A	p.G388R	2	(Pitt et al. 2009)
7	c.1220 T>C	p.I407T	1	This report
7	c.1270C>T	p.R424*	2	(Bouchard et al. 2001)
9	c.1499G>A	p.R500H	1	(Aledo et al. 2001)
9	c.1508A>G	p.Y503C	1	This report
9	c.1514G>A	p.R505Q	1	This report

ketogenesis that may indicate HMCS2 deficiency (Fukao et al. 2014; Zschocke et al. 2002).

The report published here doubles the number of known disease-causing mutations to a total of 27 (Table 2). The impact of several of these mutations on the structure of the HMCS2 protein has been described (Shafqat et al. 2010), and the functional consequences of some mutations have been assessed. However, there is no obvious correlation between mutation type and clinical or biochemical characteristics. Only five mutations have been observed in more than one family, including c.634G > A (p.G212R) in three families from Germany and England, and exon 1 deletion c.1-?_104+?del in two families from Mediterranean countries. Our findings emphasize the importance of performing quantitative *HMGCS2* gene analysis by MLPA or a comparable method in individuals with suggested HMCS2 deficiency in whom standard sequence analysis yielded normal results, since mutation analysis is the only realistic method that allows the diagnosis of HMCS2 deficiency when patients are not metabolically decompensated.

Finally, the clinical observations in cohort A also support previous observations that HMCS2 deficiency is a disease of early childhood (Bouchard et al. 2001). None of the patients in cohort A had any episode of metabolic decompensation after the age of 5 years, and all are able to tolerate normal lengths of fasting. In cohort A, three patients required intensive care, including hemofiltration in two; the use of hemofiltration has been described for other patients with HMCS2 deficiency (Sass et al. 2013). All patients continue to have normal growth and cognitive and motor development. Provided metabolic decompensations are quickly recognized and treated aggressively, the prognosis for HMCS2 deficiency is very good.

In conclusion, individuals with HMCS2 deficiency exhibit characteristic urine organic acid patterns during periods of metabolic decompensation, and recognition of this pattern should facilitate the diagnosis of HMCS2 deficiency. Our results also indicate that sequencing of the coding regions and adjacent exon–intron boundaries of the *HMGCS2* gene, followed by MLPA testing for deletions, will ensure confirmation of the diagnosis at a molecular level.

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Compliance with ethics guidelines

Conflict of interest None.

Informed consent All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Declaration of Helsinki, 1975, as revised in 2000. Informed consent was obtained from all patients for study inclusion.

References

Aledo R, Zschocke J, Pie J et al (2001) Genetic basis of mitochondrial HMG-CoA synthase deficiency. Hum Genet 109(1):19–23

Aledo R, Mir C, Dalton RN et al (2006) Refining the diagnosis of mitochondrial HMG-CoA synthase deficiency. J Inherit Metab Dis 29(1):207–211

Bouchard L, Robert MF, Vinarov D et al (2001) Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase deficiency: clinical course and description of causal mutations in two patients. Pediatr Res 49(3):326–331

Carpenter K, Bhattacharya K, Ellaway C, Zschocke J, Pitt JJ (2010) Improved sensitivity for HMG CoA synthase detection using key markers on organic acid screen [Abstract]. J Inherit Metab Dis 33(Supplement 1):S62

Carpenter K, Moore F, Bhattacharya K (2012) HMG CoA synthase deficiency - how common is it and how good are our markers? [Abstract]. Twin Res Hum Genet 15(4):578

Duran M (2003) Disorder of mitochondrial fatty acid oxidation and ketone body handling. In: Blau N, Duran M, Blaskovics ME, Gibson KM (eds) Physician's guide to the laboratory diagnosis of metabolic diseases, 2nd edn. Springer, Berlin, pp 309–334

Fukao T, Mitchell G, Sass JO, Hori T, Orii K, Aoyama Y (2014) Ketone body metabolism and its defects. J Inherit Metab Dis 37(4):541–551

Hogg SL, Pierre GM, Buck J, Thalange NMPC, Calvin J (2012) 4-hydroxy-6-methyl-2-pyrone and ketonuria in HMG-CoA synthase deficiency [Abstract]. J Inherit Metab Dis 35(Suppl 1):S60

Morris AA, Lascelles CV, Olpin SE, Lake BD, Leonard JV, Quant PA (1998) Hepatic mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme a synthase deficiency. Pediatr Res 44(3):392–396

Pitt JJ, Peter H, Mishra A et al (2009) Mitochondrial 3-hydroxy-3-methyl-glutaryl CoA synthase deficiency may be recognised by increased concentrations of 4-hydroxy-6-methyl-2-pyrone in urine organic acid analysis [Abstract]. Mol Genet Metab 98(1–2):51

Ramos M, Menao S, Arnedo M et al (2013) New case of mitochondrial HMG-CoA synthase deficiency. Functional analysis of eight mutations. Eur J Med Genet 56(8):411–415

Sass JO, Kuhlwein E, Klauwer D, Rohrbach M, Baumgartner MR (2013) Hemodiafiltration in mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMG-CoA synthase) deficiency [Abstract]. J Inherit Metab Dis 36(Suppl 2):S189

Shafqat N, Turnbull A, Zschocke J, Oppermann U, Yue WW (2010) Crystal structures of human HMG-CoA synthase isoforms provide insights into inherited ketogenesis disorders and inhibitor design. J Mol Biol 398(4):497–506

Thompson GN, Hsu BY, Pitt JJ, Treacy E, Stanley CA (1997) Fasting hypoketotic coma in a child with deficiency of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase. N Engl J Med 337(17): 1203–1207

Wolf NI, Rahman S, Clayton PT, Zschocke J (2003) Mitochondrial HMG-CoA synthase deficiency: identification of two further patients carrying two novel mutations. Eur J Pediatr 162(4): 279–280

Zschocke J, Penzien JM, Bielen R et al (2002) The diagnosis of mitochondrial HMG-CoA synthase deficiency. J Pediatr 140(6): 778–780