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Biochemical signatures mimicking multiple carboxylase deficiency in children with mutations in *MT-ATP6*

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Abstract

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AAL and JWA: study design, primary drafting of manuscript, data analysis; SB, JC, LCB, RM BHG, GAD, EG, NH, BH, GH, AM, SLR, JVH, AW, LE: data acquisition and analysis; editing and critical appraisal of manuscript. The authors have no competing interests to declare or disclose. The content of the article has not been influenced by sponsors.

Elevations of specific acylcarnitines in blood reflect carboxylase deficiencies, and have utility in newborn screening for life-threatening organic acidemias and other inherited metabolic diseases. In this report, we describe a newly-identified association of biochemical features of multiple carboxylase deficiency in individuals harboring mitochondrial DNA (mtDNA) mutations in *MT-ATP6* and in whom organic acidemias and multiple carboxylase deficiencies were excluded. Using retrospective chart review, we identified eleven individuals with abnormally elevated propionylcarnitine (C3) or hydroxyisovalerylcarnitine (C5OH) with mutations in *MT-ATP6*, most commonly m.8993T > G in high heteroplasmy or homoplasmy. Most patients were ascertained on newborn screening; most had normal enzymatic or molecular genetic testing to exclude biotinidase and holocarboxylase synthetase deficiencies. *MT-ATP6* is associated with some cases of Leigh disease; clinical outcomes in our cohort ranged from death from neurodegenerative disease in early childhood to clinically and developmentally normal after several years of follow-up. These cases expand the biochemical phenotype associated with *MT-ATP6* mutations, especially m.8993T > G, to include acylcarnitine abnormalities mimicking carboxylase deficiency states. Clinicians should be aware of this association and its implications for newborn screening, and consider mtDNA sequencing in patients exhibiting similar acylcarnitine abnormalities that are biotin-unresponsive and in whom other enzymatic deficiencies have been excluded.

Keywords

Mitochondrial disease; Organic acidemias; Newborn screening; Inherited metabolic diseases; Leigh disease; mtDNA

1. Introduction

The objective of newborn screening is to identify treatable conditions during a presymptomatic period and inform intervention to alter the natural history of the condition. Many newborn screening programs utilize tandem mass spectrometry to analyze the acylcarnitine profile. Propionylcarnitine (C3) elevations may be suggestive of propionic acidemia, methylmalonic acidemia, or other inherited disorders of intracellular cobalamin metabolism. Elevated hydroxyisovalerylcarnitine (C5OH) can be seen in 3-methylcrotonyl-CoA carboxylase (3-MCC) deficiency (OMIM 210200; Schulze et al., 2003). Concurrent elevation of C3 and C5OH indicates multiple carboxylase deficiency, usually due to biotinidase or holocarboxylase synthetase deficiency (Burri et al., 1981; Wolf et al. 1985). Newborn screening results may be suggestive (or diagnostic) of conditions other than those specifically targeted. In this paper, we report twelve patients with acylcarnitine signatures of multiple carboxylase deficiency or organic acidemia who were ultimately diagnosed with mutations in *MT-ATP6*.

Mutations in *MT-ATP6*, and in particular m.8993T > G at high levels of heteroplasmy (typically > 90% heteroplasmy in blood), account for approximately 10% of cases of Leigh disease (OMIM 256000), an early-onset, progressive, neurodegenerative and genetically heterogeneous mitochondrial disease. Of 106 individuals with Leigh or Leigh-like syndrome evaluated in Japan, ten of the 30 individuals with mtDNA pathogenic variants had variants in *MT-ATP6* (Ogawa et al., 2017). At lower levels of heteroplasmy (typically

75–90% in blood), mutations in *MT-ATP6* can cause Leigh disease phenotypes or the neuropathy, ataxia, and retinitis pigmentosa (NARP) syndrome (Rahman et al., 1996; Sofou et al., 2014). Besides *MT-ATP6*, > 75 other nuclear and mitochondrial genes are implicated as causal for Leigh/NARP spectrum disease (Lake et al., 2016). Characteristic imaging findings in Leigh disease are bilateral T2 hyperintense lesions of the basal ganglia, brainstem and midbrain. Neurological manifestations include hypotonia, dystonia, chorea, ataxia and central apnea (Sofou et al., 2014), and neurodegenerative episodes with stepwise developmental regression, often in the setting of acute viral illness. Consensus recommendations support specific management precautions for patients with known or suspected mitochondrial disease, particularly at times of acute illness, although data that definitively demonstrate that such precautions prevent or attenuate clinical worsening are lacking at present.

2. Materials and methods

Patients were ascertained as a result of discussion of the initial index cases on the metab-L email listserv, an online, email based resource and discussion platform for biochemical geneticists and metabolic physicians. A protocol was approved as an exempt study by the Colorado Multiple Institution Review Board (COMIRB #15-2296). Providers gathered clinical, biochemical, radiological and molecular genetic data, which were aggregated by the primary authors (AAL and JDWA). Biochemical and genetic investigations were performed at clinical laboratories using standard methods. All individuals initially presented for care based on abnormal blood spot NBS performed and analyzed according to local protocols apart from Patient 2, Patient 8, and Patient 11. Patient 2 was previously reported, and Patient 7 was recently published as a single case (Hauser, 2014; Balasubramaniam et al. 2016). For whole exome sequencing (WES) of Patient 1, the University of British Columbia (H12–00067) approved the study protocol and parents provided informed consent for publication. Parents of Patient 9 and Patient 10 provided informed consent as part of an IRB-approved research study of mitochondrial disorders at Baylor College of Medicine.

3. Results

Eight of the twelve individuals (67%) presented as newborns due to abnormal C5OH on dried blood spot acylcarnitine analysis undertaken as part of a state or nationally-mandated NBS program. Fig. 1a outlines abnormal values in patient newborn screening (NBS) samples compared to cutoffs in respective laboratories. Patient 2 and Patient 8 did not have NBS and were identified after presenting with neurological symptoms. Patient 11 had elevated C3 and not C5OH on NBS, but was not flagged as abnormal due to absence of secondary markers. Patient 3 and Patient 6 had elevated C3 on NBS in addition to elevated C5OH; the remaining seven patients had normal C3 on NBS.

All patients underwent urine organic acid analysis; eight of the twelve had elevations of metabolites consistent with multiple carboxylase deficiency (3-hydroxyisovaleric acid, 3-hydroxypropionic acid, methylcitric acid). All patients had enzymatic testing of biotinidase performed (either on NBS or as a stand-alone assay) with normal results. Five patients underwent sequencing of *BTD* (associated with biotinidase deficiency, OMIM 253260)

with normal results. Eight patients underwent sequencing of *HLCS* (associated with holocarboxylase synthetase deficiency, OMIM 253270) with normal results, and one patient (Patient 2, for whom *HLCS* sequencing was not performed) had normal holocarboxylase synthetase enzyme activity. For both *BTD* and *HLCS*, sequencing was either undertaken as Sanger sequencing or as WES with adequate coverage of the loci of interest. Patient 9 had biotin-dependent carboxylase enzyme assay (propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, and pyruvate carboxylase activities), which showed no deficiencies of carboxylase activity in that assay. mtDNA sequencing results, together with heteroplasmy levels in blood for identified pathogenic variants, are presented in Table 1, together with details of the clinical, biochemical, and molecular genetic characteristics of each case. Eleven patients had known pathogenic mutations in *MT-ATP6* in a homoplasmic or high heteroplasmic state in peripheral blood; the most prevalent *MT-ATP6* mutation was m.8993T > G, present in a ten patients and m.9176T > G present in one patient. In eight of the ten harboring m.8993T > G, the mutation was homoplasmic in blood; the two remaining m.8993T > G patients had heteroplasmy levels of 87% and 82% in blood. In four of the eight homoplasmic m.8993T > G patients, maternal testing revealed that the mutation had appeared *de novo*, although maternal testing was not completed in some patients' mothers. No patients in this series harbored the m.8993T > C mutation at any level of heteroplasmy. One remaining patient had two rare variants in *MT-ATP6*, one of which was absent from > 30,000 population controls and both of which are predicted to be pathogenic with *in silico* tools (Benson et al., 2013). Both variants were present at lower levels of heteroplasmy in the patient's mother's blood. For most patients, clinicians performed multiple follow-up acylcarnitine profiles on serum or whole blood. Concentrations of C3 and C5OH on follow-up acylcarnitine profile are shown in Fig. 1b–d. Nine patients had plasma citrulline (Cit) concentration measured as part of the NBS or on follow-up testing; all had low Cit concentrations on at least one time point. Data on plasma arginine concentrations on NBS or at later times were not collated for this series of patients.

4. Discussion

In this report, we present twelve patients with persistent biochemical (acylcarnitine) features of multiple carboxylase deficiency and known pathogenic or likely pathogenic mutations in the *MT-ATP6* loci identified in a homoplasmic or high heteroplasmic state. Primary biotinidase deficiency was formally excluded in all twelve patients (either by enzyme analysis of the NBS sample, or by enzyme analysis and/or sequencing at a later time or sequencing of *BTD* prompted by NBS results or a clinical presentation); holocarboxylase synthetase deficiency was excluded (by enzyme analysis and/or sequencing prompted by NBS or a clinical presentation) in nine patients. There was no apparent biochemical or clinical response to biotin supplementation for most patients. All but two patients that underwent NBS had abnormally elevated C5OH acylcarnitine. Interestingly, most patients who underwent NBS had isolated C5OH elevation, with C3 elevation becoming evident only on follow-up testing. Isolated elevation of C5OH is a feature of 3-MCC deficiency, an organic acidemia that is asymptomatic in the majority of affected individuals (Rips et al., 2016). The fact that the majority of patients with 3-MCC deficiency are asymptomatic has led to a discussion of removing isolated C5OH elevation from the NBS panel in

some regions (Wilcken, 2016). Patients with isolated C5OH elevation may be discharged from ongoing care with a presumed diagnosis of 3-MCC deficiency without extensive diagnostic testing, implying that there may be a degree of under-ascertainment of *MT-ATP6* mutations in this population. Our findings also introduce the possibility that some presumed or confirmed 3-MCC deficiency patients presenting with clinical disease may also have *MT-ATP6* mutations, compounding their phenotype.

In addition to the twelve patients reported here with *MT-ATP6* mutations, we are aware of one patient with a persistent biochemical profile consistent with multiple carboxylase deficiency for whom only a m.1555A > G mutation in *MT-RNR1* was detected on mtDNA sequencing (Rutledge, personal communication, unreported data). This *MT-RNR1* variant is associated with susceptibility to aminoglycoside-related ototoxicity. We are also aware of at least four additional patients known to harbor the m.8993T > **C** mutation (NOT m.8993T > **G**, as was observed in nine patients in our NBS cohort) in *MT-ATP6* and ascertained clinically, all of whom have had moderate elevations of C3 or C5OH acylcarnitines. One of these patients also had low plasma Cit, with the remaining three patients having Cit in the low normal range (range 10–20 micromol/L; Christodoulou, personal communication, unreported data). Given the biochemical appearance of multiple carboxylase deficiency noted in the patient with the *MT-RNR1* mutation, the association may not be limited to mtDNA mutations in *MT-ATP6*, though prospective collection of data from a broader population of mitochondrial disease patients is required to examine this issue in further detail. Currently, it appears that *MT-ATP6* mutations account for the majority of patients with ‘multiple carboxylase deficiency-like’ acylcarnitine profiles and mtDNA mutations. Broader biochemical or metabolomic evaluation of patients of different ages and with varying phenotypes and who are known to harbor mtDNA mutations (particularly *MT-ATP6* variants at varying levels of heteroplasmy, and with a specific focus on C3, C5OH, Cit and other urea cycle intermediates) may clarify the prevalence of this characteristic biochemical profile in that population, help to augment mechanistic understanding of these biochemical changes and inform genetic counseling for such patients, including presymptomatic infants identified *via* NBS.

Specific pathogenic mechanisms of acylcarnitine abnormalities that resemble those observed in multiple carboxylase deficiency in patients with *MT-ATP6* mutations remain unclear and determination of the underlying biochemistry merits further investigation. Biotin-protein ligase (encoded by *HLCS*), propionyl-CoA carboxylase and 3-MCC enzymes all require ATP for enzymatic activity (Murthy and Mistry, 1972) and may be sensitive to ATP depletion due to complex V dysfunction in the setting of pathogenic *MT-ATP6* mutations. Another recently described disorder resulting in a similar biochemical signature to that observed in multiple carboxylase deficiency is carbonic anhydrase-V (CA-V) deficiency (van Karnebeek et al., 2014) The mechanism of this biochemical signature in CA-V deficiency may be insufficient generation of intra-mitochondrial bicarbonate ions as substrate for carboxylases. Secondary dysfunction of CA-V resulting from *MT-ATP6* mutations could also be considered among possible etiologies for the elevations in C5OH and C3 in this population. For Patients 1, Patient 9, and Patient 10, WES excluded other genetic diagnoses potentially contributing to the clinical and biochemical phenotypes.

In addition to multiple carboxylase deficiency, plasma citrulline deficiency was observed in nine out of ten of our patients who have documented quantitative plasma amino acids; the tenth patient (Patient 12) has borderline low Cit (10 micromol/L). Hypocitrullinemia is a well-reported finding in the setting of *MT-ATP6* mutations. It is less prevalent (although also seen) in other etiologies of mitochondrial disease (Ribes et al., 1993; Naini et al., 2005). Plasma Cit is included on many NBS panels as a mechanism for early detection of citrullinemia type I (OMIM 215700) and citrin deficiency (citrullinemia type II; OMIM 605814), both of which are characterized by hypercitrullinemia. The likely mechanism of hypocitrullinemia in mitochondrial disease is inhibition of the proximal urea cycle due to functional deficiency of carbamoyl phosphate synthase (CPS1) and/or Δ^1-pyrroline-5-carboxylate synthetase, both ATP-dependent enzymes. The fact that borderline or frank hypocitrullinemia is seen in all of the patients for whom citrulline concentrations were analyzed in this case series suggests that a similar mechanism could account for both acylcarnitine abnormalities resembling multiple carboxylase deficiency and for hypocitrullinemia. Other areas meriting further study include whether either the observed acylcarnitine abnormalities or hypocitrullinemia have any prognostic significance or additional implications for management for patients harboring *MT-ATP6* or other mtDNA mutations. This includes the question of whether secondary perturbations of the urea cycle are relevant to the clinical phenotype in these patients, and whether supplementation of citrulline and/or arginine may be of benefit to them. None of the patients in this series have any documented history of hyperammonemic episodes, a feature that characterizes proximal urea cycle disorders (UCD). Supplementation of citrulline and/or arginine are recommended as standard of care for patients with primary UCD (Häberle et al., 2012).

Clinicians responsible for clinical follow-up of NBS results should be aware that pursuit of mtDNA sequencing of patients with C5OH elevation or other biochemical features of multiple carboxylase deficiency may identify pre-symptomatic individuals with future risk for serious degenerative phenotypes including Leigh disease or NARP. Patients' families should be offered appropriate genetic and prognostic counseling prior to mtDNA testing by experienced counselors with knowledge and experience of the complexities of counseling patients with mtDNA-related mitochondrial disease. For the m.8993 T > G mutation, a predictive model exists to correlate future risk of symptoms with measured level of heteroplasmy in blood (White et al., 1999a). Based on this model, patients with a high level of heteroplasmy for m.8993T > G are at very high risk of developing Leigh disease/NARP. Previous studies of patients with m.8993T > G and m.8993T > C suggest that level of heteroplasmy in blood of these mutations correlates to a fair extent with levels in other tissues and with clinical phenotype (age of clinical onset exhibiting inverse correlation with level of heteroplasmy), a characteristic that is not a feature of other mtDNA mutations (White et al., 1999b). Many patients in this study developed neurological symptoms during intercurrent viral illnesses. It is hoped that when additional patients are identified pre-symptomatically, interventions can then be instituted that might decrease risk of progression or delay symptomatic onset. As previously stated, the benefits of this approach are currendy theoretical and, as yet, unsubstantiated with supportive data. At this time, there are no prospective data to guide care or counseling in this circumstance, though we suggest that clinicians refer patients found to harbor pathogenic *MT-ATP6* mutations and

identified in this manner to centers of expertise with mitochondrial disease. With central coordination from a clinician with expertise with mitochondrial disease, multidisciplinary consensus-based precautions during acute intercurrent illness (as recommended in recently published guidelines on care for patients with mitochondrial disease, Parikh et al., 2015) can be implemented, and appropriate clinical surveillance for known manifestations of Leigh disease/NARP spectrum clinical phenotypes employed. Specifically, regular developmental assessments, electrocardiogram, and echocardiogram may be indicated. MRI of the brain should be considered in the setting of developmental delay, regression, or new neurological symptoms. Avoidance of medications that impair mitochondrial function (especially sodium valproate, which has been associated with fatalities in patients with known or retrospectively diagnosed mitochondrial disease) together with maintenance of anabolism (provision of calories and avoidance of fasting) during illness may reduce the likelihood of sudden devastating illness and decompensation. Precautionary care of this nature lacks clear supportive data, may be anxiety-inducing for parents and caregivers, and difficult to implement in families where the child is entirely asymptomatic at the time the mtDNA mutation is identified.

Although identification of *MT-ATP6* mutations is not currently the stated intent of the NBS program, it is possible that early diagnosis may benefit these pre-symptomatic children with the passage of time. Studies of the efficacy of recommended management to prevent or delay neurological disease in NBS-identified pre-symptomatic children *versus* clinically-identified children would be of great utility. It is currently unclear if the outcome for children with *MT-ATP6* mutations and acylcarnitine features of multiple carboxylase deficiency is different from that of other children with *MT-ATP6* mutations. This uncertainty should be conveyed when discussing prognostic factors and rationale for follow-up requirements with patients and families.

In this paper, we report an uncommon but clearly reproducible biochemical and metabolomic phenotype in patients with *MT-ATP6* mutations (mostly m.8993T > G) showing biochemical features of multiple carboxylase deficiency, as well as hypocitrullinemia. We are also aware of several other patients with m.8993T > C patients not included in this series but who have similar acylcarnitine signatures. Clinicians involved in evaluation of patients with isolated elevation of C5OH or dual elevations of C3 and C5OH on newborn screening, but who have had negative molecular genetic or enzymatic testing for relevant organic acidemias (especially 3-MCC deficiency), biotinidase deficiency and holocarboxylase synthetase deficiency should be aware of this association. Young patients harboring known *MT-ATP6* mutations may be at high risk for developing neurodegenerative disease, especially Leigh disease and NARP, as well as cardiomyopathy over time, and may benefit from careful follow-up and implementation of preventive measures. Close follow-up of neurological symptoms in this group will add to the current fund of knowledge on prognosis and management of patients with *MT-ATP6* mutations. We anticipate that additional studies of mechanisms underlying biochemical phenotypes in *MT-ATP6* mutations, including registry-based biochemical phenotyping of patients with known *MT-ATP6* mutations (of different ages, clinical phenotypes and levels of mtDNA heteroplasmy in blood) will lead to progress in understanding the sensitivity and specificity of these metabolites as biomarkers for Leigh/NARP spectrum mitochondrial disease, as

well as furthering insights into pathophysiology of mitochondrial diseases and informing best practices for counseling of affected families where infants or older individuals are identified in this manner. We anticipate that these data will prompt re-evaluation of historical discussions regarding best practice for NBS algorithms, especially with regard to tiered optimization of follow-up studies in the setting of elevated C5OH acylcarnitine with or without elevated C3 and hypocitrullinemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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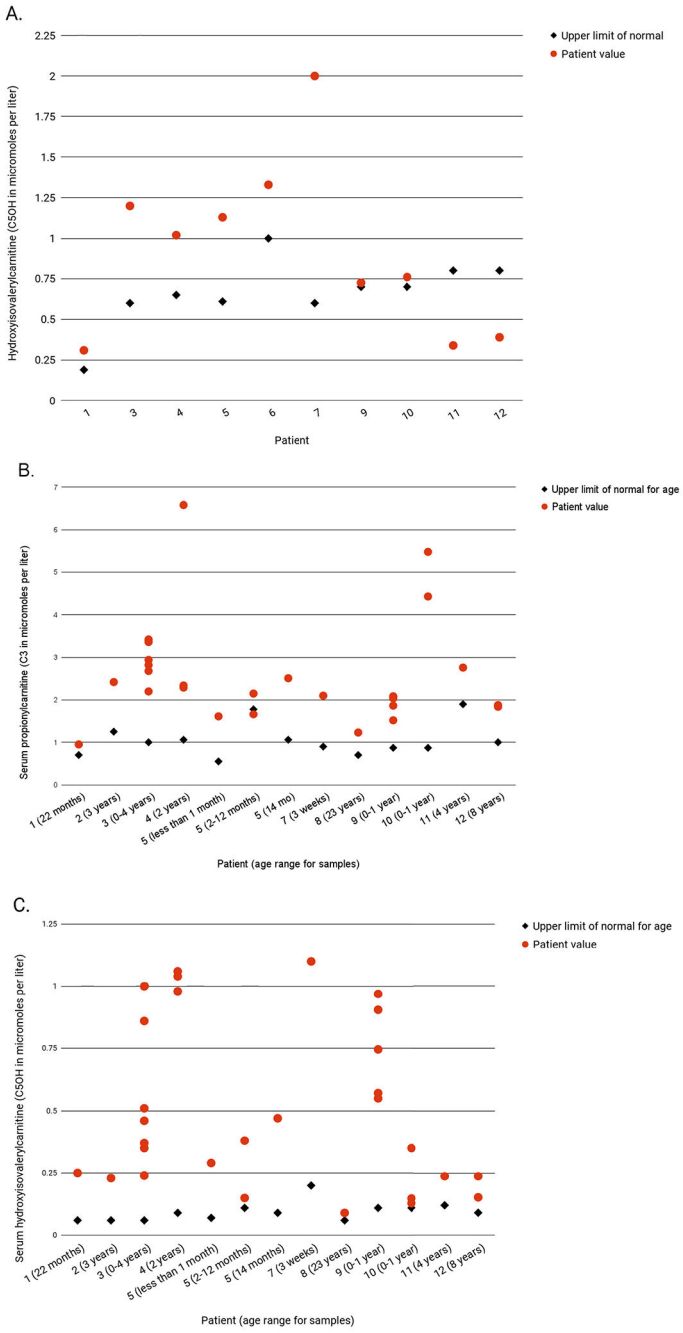

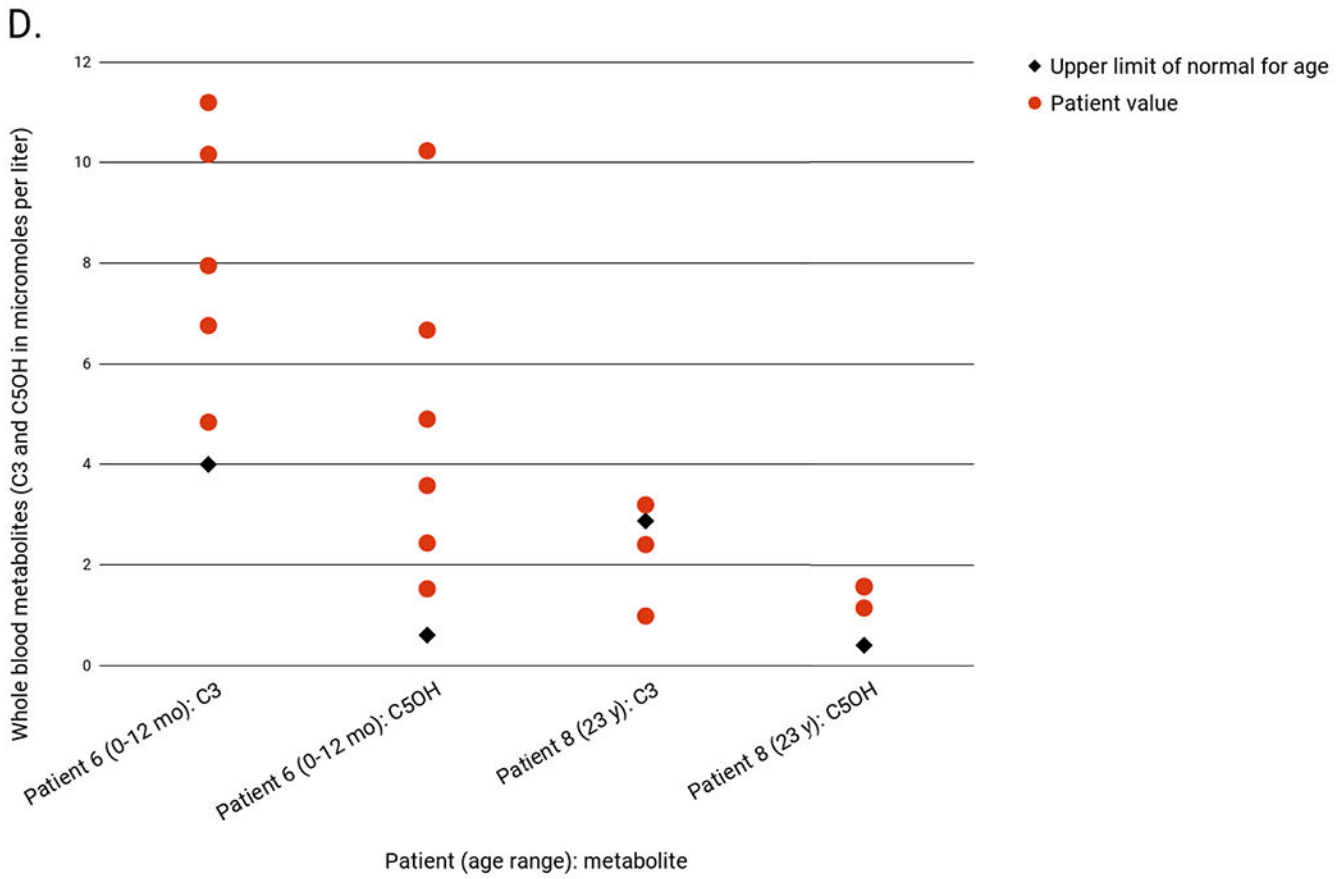

Fig. 1.

Acylcarnitine profile results for patients with mutations in *MT-ATP6*. A shows C5OH concentration from dried blood spot sample on newborn screening. Eight of ten patients that underwent newborn screening had hydroxyisovalerylcarnitine levels above the local reference range. B and C show concentrations of propionylcarnitine and hydroxyisovalerylcarnitine, respectively, on multiple serum samples. The frequency of follow-up acylcarnitine profiles was variable across the cohort, but consistent elevations outside the local reference ranges are observed for both analytes. D: Propionylcarnitine and hydroxyisovalerylcarnitine concentrations for two patients that had whole blood (rather than serum) acylcarnitine profiles. Patient 8 had both serum and whole blood acylcarnitine profiles performed.

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Table 1

Clinical, biochemical, and molecular genetic characteristics of eleven individuals with mutations in *MT-ATP6* and biochemical features of multiple carboxylase deficiency.[a]

Patient number/ gender	mtDNA variants detected in blood; degree of heteroplasmy	Abnormal NBS	Biochemical phenotype (besides ACP)	Biotin treatment initiated	*BTD* sequencing; Biotinidase enzyme assay; *HCLS* sequencing; HCLS enzyme assay	Low plasma citrulline	Clinical/neurodevelopmental course and outcome
1, M	m.8993T > G[b], 87% heteroplasmy	High C5OH: low Cit	UOA (at 15 months, during acute illness): lactic, isovaleric; propionylglycine	Yes, with resolution of biochemical abnormalities	Normal/Normal/ Normal/ND[c]	Yes	Brain MRI normal at 3.5 years; ophthalmoplegia, lethargy and encephalopathy at 5 years with MRI appearance consistent with LD; alive at age 8 years with mild speech delay
2, M	m.8993T > G, homoplasmic	ND	UOA; 3-hydroxybutyric, 3-hydroxyisovaleric	No	ND/Normal/ND/ Normal	ND	Clinically well and developmentally normal until age 5 years, then died unexpectedly with progressive encephalopathy without clear inciting event
3, F	m.8993T > G, homoplasmic	High C3; high C5OH	UOA: 3-hydroxyisovaleric, trace methylcitric	Yes; no change	ND/Normal/ Normal/ND	Yes	Clinically well and developmentally normal age 4 years
4, F	m.8993T > G[b], homoplasmic	High C5OH; low Cit	UOA: lactic, 3-isovaleric, 3-hydroxyproprionic	Yes; no change	Normal/Normal/ Normal/ND	Yes	DD with independent ambulation from 21 months; normal brain MRI at 24 months; acute diarrheal illness at 25 months with regression and MRI showing new appearances of LD; died soon afterwards of rapidly progressive neurodegenerative disease
5, M	m.8993T > G, homoplasmic	High C5OH	UOA: lactic, isovalerylglycine, 3-hydroxypropionic, 3-hydroxyisovaleric	Yes; no change	ND/Normal/ Normal/ND	ND	Normal early milestones; walked at 11 months, mild speech delay in second year of life; alive at age 4 years with mild DD
6, F	m.9176T > G[b], homoplasmic	High C3; high C5OH, low Cit	UOA: lactic, 3-hydroxyisovaleric, 3-hydroxypropionic, methylcitric	Yes; no change	ND/Normal/ Normal/ND	Yes	Poor feeding in neonatal period with FTT over the first months of life; died at 12 months after developing neurologic symptoms in setting of acute diarrheal illness
7, M	m.8993T > G, homoplasmic	High C5OH, low Cit	UOA: normal Episodic lactic acidosis and ketoacidosis; one episode of hyperammonemia;	Yes; biochemistry unchanged but possible clinical improvement	Normal/Normal/ Normal/ND; cellular assay for biotin uptake normal[c]	Yes	Poor feeding, FTT and hypotonia in infancy; alive at 4 years with DD and DQ of 50
8, M	m.8959G > A; homoplasmic; m.9155A > T; homoplasmic[d]	ND	UOA: normal	Yes; no change	ND/ Normal/ND/ND	Yes	DD from early childhood in all domains; alive at 23 years with intellectual disability; sibling has similar phenotype but has not been investigated for mtDNA variants or for biochemical features of MCD
9, F	m.9176T > G, homoplasmic	High C5OH	UOA: elevated succinic Serum: mildly elevated lactate and alanine; normal	Yes, no change	Normal/Normal/ Normal/Normal[c]	Yes	Normal growth and meeting age-appropriate developmental milestones at 15 months of age; normal

Patient number/ gender	mtDNA variants detected in blood; degree of heteroplasmy	Abnormal NBS	Biochemical phenotype (besides ACP)	Biotin treatment initiated	*BTD* sequencing; Biotinidase enzyme assay; *HCLS* sequencing; HCLS enzyme assay	Low plasma citrulline	Clinical/neurodevelopmental course and outcome
			activity of carboxylases in leukocytes				echocardiogram; normal eye movements and fundus appearances
10, F	m.8993T > G, homoplasmic[b]	High C5OH	UOA: 3-hydroxyisovaleric, methylcitric, and 3-methylglutaconic Serum: elevated lactate and alanine with episodic metabolic acidosis	Yes, no change	Normal/ND/ Normal/ND[c]	Yes	Seizures at 2–3 months of age, hypotonia, DD; regression at 9 months with loss of head control and ability to sit; at 15 months is unable to sit or walk, has abnormal eye movements and is gastrostomy-dependent; brain MRI with delayed myelination at 3 months; normal echocardiogram; WES with *de novo* VUS in *FOXP1* (gene associated with ID/autism but not with ACP abnormalities or lactic acidosis)
11, F	m.8993T > G, homoplasmic	High C3; not flagged according to local protocol; normal C5OH	UOA: 3-hydroxyisovaleric, methylcitric, and tiglylglycine Serum: elevated lactate and alanine	No	ND/ Normal/ND/ND	Yes	Delayed gross motor development in the first year of life. Infantile spasms at 10 months. Brain MRI with T2 hyperintensity of the caudate and brainstem. Ataxia, dystonia and spasticity at age 4 years; non-verbal, ambulatory with assistance.
12, F	m.8993T > G, 82% heteroplasmy	Normal	UOA: normal Serum: lactate normal; alanine mildly elevated	No	ND/ Normal/ND/ND	borderline[e]	Clinical evaluation age 8 years for mild/moderate DD and difficulties with night vision and peripheral vision; history of amblyopia, toe-walking, social immaturity and dyspraxia without clear history of ataxia or developmental regression; normal brain MRI at 5 years; RP appearance on fundus exam at 8 years

Abbreviations: ACP = acylcarnitine profile; C3 = propionylcarnitine; C5OH = 3-hydroxyisovalerylcarnitine; Cit = plasma citrulline; DD = developmental delay(s); DQ = developmental quotient; FTT = failure to thrive; ID = intellectual disability; LD = Leigh disease; MCD = multiple carboxylase deficiency; NBS = newborn screen; ND = not done/not reported; RP = retinitis pigmentosa; UOA = urine organic acids; VUS = variant of uncertain significance; WES = whole exome sequencing.

[a] See Fig. 1.

[b] Patient's mother tested and negative for proband's mtDNA mutation.

[c] Patient also underwent WES.

[d] m.8959G > A and m.9155A > T have Polyphen2 scores of 0.992 and 0.977, respectively. m.8959G > A was present in 2/30,179 controls; m.9155A > T present in none of 30,180 controls; mother has low level of heteroplasmy for both variants.

[e] Fasting plasma citrulline was 10 micromol/L at routine clinic evaluation at 8 years of age.