

Defective hepatic bicarbonate production due to carbonic anhydrase VA deficiency leads to early-onset life-threatening metabolic crisis

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Purpose: Four mitochondrial metabolic liver enzymes require bicarbonate, which is provided by the carbonic anhydrase isoforms VA (CAVA) and VB (CAVB). Defective hepatic bicarbonate production leads to a unique combination of biochemical findings: hyperammonemia, elevated lactate and ketone bodies, metabolic acidosis, hypoglycemia, and excretion of carboxylase substrates. This study aimed to test for CAVA or CAVB deficiencies in a group of 96 patients with early-onset hyperammonemia and to prove the disease-causing role of the CAVA variants found.

Methods: We performed CA5A and CA5B sequencing in the described cohort and developed an expression system using insect cells, which enabled the characterization of wild-type CAVA, clinical mutations, and three variants that affect functional residues.

Results: In 10 of 96 patients, mutations in CA5A were identified on both alleles but none in CA5B. Exhibiting decreased enzyme activity or thermal stability, all CAVA mutations were proven to cause disease, whereas the three variants showed no relevant effect.

Conclusion: CAVA deficiency is a differential diagnosis of early-onset and life-threatening metabolic crisis, with hyperammonemia, hyperlactatemia, and ketonuria as apparently obligate signs. It seems to be more common than other rare metabolic diseases, and early identification may allow specific treatment of hyperammonemia and ultimately prevent neurologic sequelae.

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Key Words: baculovirus expression system; hyperammonemia; metabolic acidosis; neonatal onset; Sf9 cells

INTRODUCTION

Bicarbonate (HCO_3^-) is the predominant extracellular buffer and is essential for maintaining pH homeostasis throughout the human body. Carbonic anhydrases (CAs), a family of zinc metalloenzymes, catalyze the reversible hydration of CO_2 ($\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$).¹ These enzymes are involved in processes such as respiration, renal acidification, bone resorption, formation of aqueous humor and cerebral fluid, gastric secretion, gluconeogenesis, lipogenesis, and ureagenesis in humans.² The 14 isoenzymes known in humans differ in tissue-specific expression, subcellular localization, kinetic parameters, and susceptibility to various inhibitors; for example, CAs I, II, III, VII, and XIII are located in the cytoplasm; CAs IV, IX, XII, and XIV are membrane bound; CAs VI, X, and XI are secreted; and CAVA and VB are located in the mitochondria (<http://www.proteinatlas.org>).

Human CAVA is a 267-amino acid mitochondrial enzyme expressed exclusively in the liver, kidneys, and skeletal muscle.³

It provides bicarbonate to four hepatic mitochondrial enzymes involved in essential metabolic pathways: carbamoyl phosphate synthetase 1 (CPS1; the first step of the urea cycle), pyruvate carboxylase (gluconeogenesis), propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase (branched-chain amino acid catabolism) (**Figure 1a**). The encoding gene CA5A (OMIM 114761) spans ~50 kb, contains 7 exons and 6 introns, and maps to 16q24.2 (refs. 4 and 5). Of all human CAs, CAVB is the only other isoform expressed in mitochondria, but it has a wider tissue distribution, including the liver.³ CA5B (OMIM 300230) spans ~50 kb as well, but it maps to Xp22.2 (ref. 6).

The liver disease CAVA (CAVA) deficiency (OMIM 615751) is a rare autosomal-recessive inborn error of metabolism (IEM)⁷ that results in defective hepatic bicarbonate production. This novel disorder was recently described in four patients⁸ exhibiting lethargy caused by neonatal or infantile hyperammonemia, combined with elevated blood lactate and urine ketone bodies,

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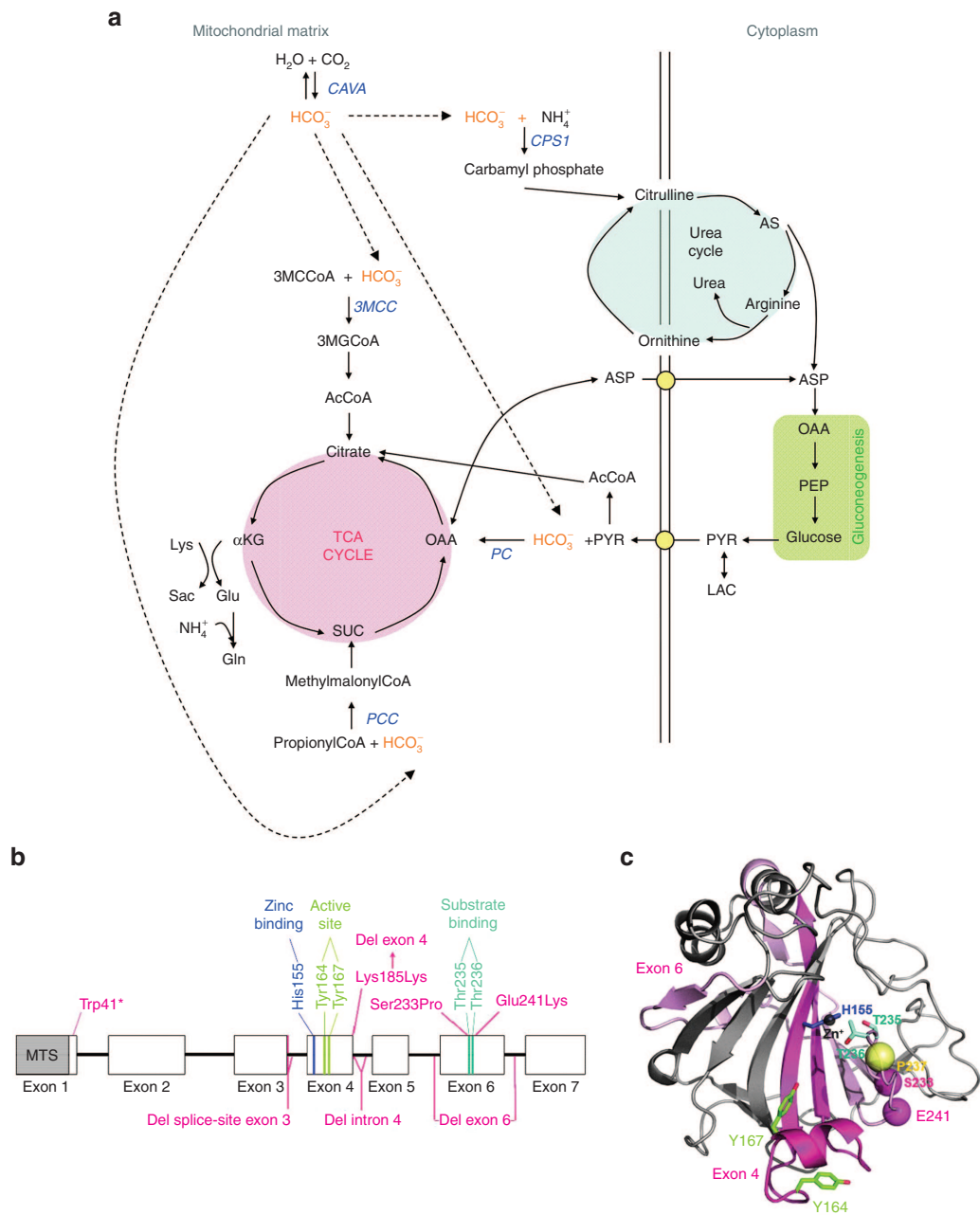


Figure 1 Biochemical pathways using bicarbonate produced by carbonic anhydrase VA (CAVA) and mapping of CAVA clinical mutations. (a) Dotted lines link CAVA-produced bicarbonate (orange) with the bicarbonate-dependent carboxylases (in italics and blue). Enzymes: CPS1, carbamoyl phosphate synthetase 1; PC, pyruvate carboxylase; PCC, propionyl CoA carboxylase; 3MCC, 3-methylcrotonyl CoA carboxylase. Metabolites: AS, argininosuccinate; 3MCCoA, 3-methylcrotonyl CoA; 3MGCoA, 3-methylglutaconyl CoA; AcCoA, acetyl CoA; α KG, α -ketoglutarate; OAA, oxaloacetate; SUC, succinyl CoA; Glu, glutamate; Gln, glutamine; Sac, saccharopine; Lys, lysine; ASP, aspartate; PEP, phosphoenolpyruvate; PYR, pyruvate; LAC, lactate. **(b)** Human wild-type CA5A gene representation with predicted mitochondrial targeting sequence (MTS) and predicted functional residues for zinc binding (blue), active site (green), and substrate binding (cyan). Clinical mutations are shown in pink. p.Ser233Pro, p.Lys185Lys, leading to the deletion of exon 4 and the deletion of exon 6, were published in ref. 8. **(c)** Mouse CAVA structure (Protein Data Bank code 1DMX) in the cartoon representation is gray. Functional residues are in the stick representation (C atoms are colored as in (b), and O atoms are red). Residues harboring the missense mutations studied here are shown as pink spheres, and the regions corresponding to exons 4 (residues 154–186) and 6 (residues 207–258) are colored in dark and light pink, respectively. Polymorphism p.Pro273Leu is represented as a yellow sphere and labeled. Polymorphisms p.Asn45Lys and p.Asn46Lys are not represented since the most N-terminal part of the protein is not included in the crystal structure. Zinc is represented as a small, dark gray sphere and labeled.

metabolic acidosis, hypoglycemia, and urinary excretion of carboxylase substrates and related metabolites.⁸ On the other hand, no human disease has been attributed to a defect in CAVB.

Until 2014, the only known human disease attributable to the deficiency of a CA was CAII deficiency. CAII is found in the cytosol of essentially every tissue or organ,² and its deficiency is

associated with osteopetrosis, renal tubular acidosis, and cerebral calcifications.^{9,10} Bone marrow transplantation has been used to attenuate osteopetrosis and cerebral calcification,¹¹ and sodium bicarbonate has been used to treat renal tubular acidosis¹² in these patients. By contrast, deficiency of CAI, a cytosolic isoform highly expressed in erythrocytes, does not produce a clinical phenotype¹³ in either humans or in pigtail macaques.^{14,15}

There exist few animal models with defective CAs. One is the CAII-deficient mouse, which only partly recapitulates the human disease: it exhibits only renal tubular acidosis and cerebral calcifications, not the prominent osteopetrosis found in humans with this disease.¹⁶ Promising results have been obtained using gene therapy in CAII-deficient mice, which was able to restore the ability to acidify urine after oral administration of ammonium chloride.¹⁷ The mouse model lacking Car5A (murine homologue to human CAVA), like humans, exhibited life-threatening hyperammonemia, whereas the Car5B (murine homologue to human CAVB) knockout mice had no clinical phenotype.¹⁸ Interestingly, double-knockout (Car5A^{-/-}, Car5B^{-/-}) mice developed even more severe hyperammonemia than the Car5A knockout mice, suggesting that even though both Car5A and Car5B seem to contribute to ureagenesis in mice, Car5A has a predominant role.¹⁸

Given the amenability to treatment and therefore the prevention of irreversible brain damage, our group was motivated to investigate further the effect of impaired hepatic bicarbonate production, at both the clinical and biochemical levels, as a cause of hyperammonemic encephalopathy. Our aims were, first, to test for CAVA deficiency in patients with “isolated” neonatal-onset hyperammonemia, in whom the biochemical profile thus was not indicative of any other urea cycle disorder (UCD) or organic aciduria. To do so, we sequenced CA5A in a group of 96 patients who were initially considered to suffer from a proximal UCD but in whom no mutations in NAGS and CPS1 were found. In addition, to rule out a CAVB defect, we sequenced CA5B. Third, to prove the disease-causing role of all detected CA5A mutations, we developed an expression system based on insect cells, already proven valid by other groups to express other CA isoforms^{19,20} and by our group to study another liver mitochondrial enzyme, CPS1.^{21–23} We expressed the recombinant wild-type CAVA, all published and newly identified mutations, and three non-disease-associated variants, and we tested their enzyme activity and thermal stability. Finally, we described the clinical and biochemical features of the patients with CAVA deficiency.

MATERIALS AND METHODS

Patients

This study was carried out in a group of 96 patients (48 male, 46 female, 2 with gender unknown to us) referred to us between 2008 and 2014 from 50 metabolic centers worldwide. We received 313 samples from index patients for NAGS and/or CPS1 mutation analysis; of these, 15 and 83 were positive for N-acetyl-glutamate synthase (NAGS) and CPS1 deficiencies, respectively. Of the negative samples, we include here

only those negative for NAGS and CPS1 genes. NAGS testing was done by single-exon amplification and DNA sequencing.²⁴ CPS1 testing was done by RNA sequencing, in most cases using stimulated lymphocytes²⁵ and in a few patients using cultured fibroblasts.²⁶ Samples of this group were received from many European countries and from Australia, Brazil, Israel, Kuwait, Saudi Arabia, and Tunisia. The majority of patients ($n = 60$) were referred from large centers that sent samples from several patients (between 2 and 9 samples), whereas 36 patients were single referrals. We thus relied on clinical and biochemical information from many different places.

All patients studied were initially suspected to suffer from a proximal UCD since they presented with symptomatic hyperammonemia in the absence of increased urinary orotic acid. Most of the patients suffered an initial hyperammonemic crisis during the first days of age, but few patients presented at a later age during infancy or childhood (Tables 1 and 2).

Molecular genetic analysis

In the entire group, standard sequence analysis of coding exons, including flanking intronic regions of CA5A and CA5B, was performed. If not provided, genomic DNA was extracted from peripheral blood leukocytes or stored skin fibroblasts. Genomic DNA was amplified by polymerase chain reaction, followed by agarose gel electrophoresis and direct sequencing according to standard protocols using the BigDye Terminator Cycle Sequencing kit version 1.1 and an ABI 3130 genetic analyzer (Applied Biosystems by Life Technologies Europe BV, Zug, Switzerland). Because of the highly homologous pseudogene sequences, the primers used were designed to be unique at their 3' end (Supplementary Table S1 online). Raw sequencing data were analyzed using the SeqPatient module of SEQUENCE PILOT software (JSI Medical Systems, Ettenheim, Germany) using the following reference sequences: CA5A Ensembl ENSG00000174990 and ENST00000309893; CA5B Ensembl ENSG00000169239 and ENST00000318636. Single-nucleotide polymorphism variants were excluded by blasting the sequencing data against the Ensemble platform. Nomenclature of mutations follows the recommendations of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>) and was checked using the online software Mutalyzer (<https://mutalyzer.nl/name-checker>). Mutation analysis was performed after written informed consent of the legal guardian was obtained. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. To confirm segregation of the mutations, parental samples were analyzed whenever available.

Amino acid conservation (Supplementary Table S2 online) was determined by ClustalW sequence alignment of CAVA from 66 species or the other 13 human CAs. The frequency of the mutations was analyzed using the Exome Aggregation Consortium browser (<http://exac.broadinstitute.org/about>) (Supplementary Table S2 online). The disease-causing probability of all variants was tested by the prediction servers PolyPhen-2 (ref. 27) and MutPred.²⁸

Table 1 Patients with carbonic anhydrase VA deficiency

Patient	Mutation C45A ^a	CAVA amino acid change ^b	Onset	Response to carbamyl glutamate	Extracorporeal detoxification	Additional crisis	Neurological outcome (long term)	Ethnic background	Parental relation	Reference to mutation
1	Exon 1 c.123G>A	p.Trp41* Truncated protein	4 Days	Yes (parallel to other action)	None	None	Normal at age 12 months	Turkish	Consanguineous	This study
2	Exon 3 c.458_459+22del24bp	p.? Predicted splicing	5 Months	Not used	None	2 Years (carbamyl glutamate not used)	Normal at age 10 years	Indian	Consanguineous (not first cousins)	This study
3	Exon 4 c.555G>A	p.His155_Leu186del Deletion of exon 4	4 Days	Yes	None	Not reported	Normal at age 6 months	Russian	Nonconsanguineous	Van Karnebeek et al. ⁸
4	Intron 4 c.555+4_555+183del180bp	p.? Predicted splicing	4 Years	Not used	None	4 Years 1 month (carbamyl glutamate not used)	Normal at age 10 years	Pakistani	Consanguineous (first cousins)	This study
5	Exon 6 c.697T>C	p.Ser233Pro	First days	Yes (single dose in crisis)	None	2.5 and 3.5 years	Normal development and behavior at age 4.5 years, below-average motor coordination	Belgian-Scottish	Nonconsanguineous	Van Karnebeek et al. ⁸
6	Exon 6 c.697T>C	p.Ser233Pro	First days	Yes	None	Not reported	Bayley Scales of infant and toddler development below average	Belgian-Scottish	Nonconsanguineous	Van Karnebeek et al. ⁸
7	Exon 6 c.721G>A	p.Glu241Lys	5 Days	Yes	None	None	Normal	Bangladesh	Consanguineous	This study
8	Exon 6 c.721G>A	p.Glu241Lys	4 Days	Yes	Hemodialysis	None	Normal No treatment at age 3 years	Pakistani	Not reported	This study
9	Exon 6 c.619-3420_c.774+502del4078bp	p.(Asp207_Gln258del) Deletion of exon 6	13 Months	Not used	None	16 Months (carbamyl glutamate not used)	Normal at 11 years	Pakistani	First-cousin consanguineous	Van Karnebeek et al. ⁸
10	Exon 6 c.619-3420_c.774+502del4078bp	p.(Asp207_Gln258del) Deletion of exon 6	2 Days	Not used	Hemodialysis (6 hours)	23 Months, with diarrhea, vomiting, and mild encephalopathy	Normal No treatment at age 6 years apart from sick-day regimen	Pakistani	First-cousin consanguineous	Van Karnebeek et al. ⁸

Bold type represents patients previously described by van Karnebeek et al.⁸

^aThe exon affected in each patient is given in the upper line. Reference sequence C45A Ensembl ENSG00000174990. Mutations were found in all patients in a homozygous state. ^bTranslation of the complementary DNA reference sequence NM_001739.1 (GenBank). Nucleotide 57 in this sequence is considered +1 since it is the A of the translation initiation codon. The result of the mutation at the protein level is indicated in the lower line (except for missense mutations).

Table 1 (Continued)

Table 1 Continued

Patient	Mutation CA5A ^a	CAVA amino acid change ^b	Onset	Response to carbamyl glutamate	Extracorporeal detoxification	Additional crisis	Neurological outcome (long term)	Ethnic background	Parental relation	Reference to mutation
11	Exon 6 c.619-3420_c.774+502del4078bp	p.(Asp207_Gln258del) Deletion of exon 6	20 Months	Not used	None	None	Normal at age 4 years	Indian	Consanguineous (not first cousins)	Van Karnebeek et al. ⁸
12	Exon 6 c.619-3420_c.774+502del4078bp	p.(Asp207_Gln258del) Deletion of exon 6	3 Days Coma, Kussmaul breathing	Not used	Not reported	None	Normal No treatment at age 4 years	Pakistani	Not reported	Van Karnebeek et al. ⁸
13	Exon 6 c.619-3420_c.774+502del4078bp	p.(Asp207_Gln258del) Deletion of exon 6	3 Days	Not used	Hemofiltration	None	Learning difficulties and speech delay at 5 years	Pakistani	Consanguineous (first cousins)	Van Karnebeek et al. ⁸
14	Exon 6 c.619-3420_c.774+502del4078bp	p.(Asp207_Gln258del) Deletion of exon 6	2 Days	Yes, parallel to hemodialysis	Hemodialysis	None at age 9 months	Normal No treatment at age 9 months	Pakistani	Nonconsanguineous	Van Karnebeek et al. ⁸

Bold type represents patients previously described by van Karnebeek et al.⁸

^aThe exon affected in each patient is given in the upper line. Reference sequence CA5A Ensembl ENSG00000174990. Mutations were found in all patients in a homozygous state. ^bTranslation of the complementary DNA reference sequence NM_001739.1 (GenBank). Nucleotide 57 in this sequence is considered +1 since it is the A of the translation initiation codon. The result of the mutation at the protein level is indicated in the lower line (except for missense mutations).

Enzyme activity assay

CA activity assay was performed using a version adapted from the changing pH indicator method developed by Maren,²⁹ as described in the “Enzymatic Assay of Carbonic Anhydrase for Wilbur-Anderson Units” document from Sigma (<http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-carbonic-anhydrase.html>). It was based on the monitoring of pH variation resulting from the catalyzed conversion of CO₂ to bicarbonate. The assay was performed at 0–3 °C in a 24-well plate, which contained a pH-meter electrode and a magnet (for mixing), and which was placed in an ice-water bath on top of a magnetic agitator. Ice-cold CO₂-saturated water (1.6 ml) was added to 2 ml of 20 mmol/l Tris-SO₄ buffer (pH 8.3). The CO₂-saturated solution was prepared by adding dry ice chips into ice-cold water, keeping it bubbling for 15 min before starting the experiment. To ensure that the solution remained saturated with CO₂ throughout the whole experiment, dry ice chips were added regularly to maintain the bubbling. Immediately after, 15–75 µg of wild-type or mutant CA were added to the buffer–substrate solution, and a stopwatch was started simultaneously. For the blanks, an equivalent volume of buffer was added to the test tube. The time required for the solution to change from pH 8.3 to 6.3 was recorded using pH Meter 744 (Metrohm, Herisau, Switzerland). Enzyme activity was calculated in Wilbur-Anderson units according to the formula $(T_0 - T)/T$, where T_0 (blank) and T (catalyzed reaction by CA) are the time (in seconds) required for the pH to change from 8.3 to 6.3.

RESULTS

Patients with CAVA and mutations

Among the group of 96 patients, we identified CA5A mutations in 10 patients (Table 1). These patients presented with a unique combination of biochemical findings, including hyperammonemia (in all patients; range: 238–1,150 µmol/l), elevated lactate (in all patients; range: 4.8–15 mmol/l), and elevated ketone bodies in urine (in all patients) (Table 2). Glutamine and glucose concentrations varied among patients: between normal limits to 2,606 µmol/l, and between normo- and hypoglycemia (1.9 mmol/l), respectively (Table 2). Metabolic acidosis and urinary excretion of carboxylase substrates and related metabolites were also observed in variable ranges (Table 2). For a full picture of the currently known patients with CAVA deficiency, we also include the previously published⁸ patients (numbers 3, 5, 6, and 9 in Tables 1 and 2). Seven of the 10 newly described patients and 2 already described in ref. 8 presented with only one initial hyperammonemic crisis, after which they remained stable, even after tapering of treatment (Table 1). However, three newly described patients suffered a second crisis, which was milder than their first one (Tables 1 and 2); the two siblings already described in ref. 8 presented with multiple crises during illness, albeit milder the second and third times. Some patients were treated with N-carbamyl-L-glutamate (NCG) during their initial crisis, either alone or in combination with other measures, and seemed to respond positively (Table 1), with rapid reduction of ammonia.

Table 2 Biochemical findings observed in patients with carbonic anhydrase VA deficiency during crisis

Patient	Maximum ammonia (μmol/l)	Maximum plasma glutamine (μmol/l)	Maximum lactate (mmol/l)	Minimum glucose (mmol/l)	Acid-base status (data from crisis) ^a	Ketone bodies in urine (spot test; data from crisis)	Organic acids in urine (μmol/mmol crea; data from crisis)
1	1,150	Not elevated	10.7	2.5	BE −10.7	Yes	Lactic acid 2,040, 3-OH-butyric acid 1,950, AcAc 730, pyruvate 415
2	490	428	10.8	4.6	pH 7.32 pCO ₂ 1.5 HCO ₃ [−] 11.0 BE −21	Grossly elevated	Not reported
	338	533	11.0	4.4	BE −17	Grossly elevated	3-OH-butyric acid 3,700
3	422	2,606	8.1	2.9	pH 7.16 pCO₂ 1.73 HCO₃[−] 5.0	Yes^b	Lactic acid 28,000, 3-OH-butyric acid 7,060, acetoacetic acid ++, 2-α-ketoglutaric acid 300, adipic acid 340, suberic acid 29
4	730	555	6.5	1.9	BE −23	Grossly elevated	3-OH-butyric acid 3700
	177	364	1.0	3.1	BE −13	Not done	Not done
5	780	1,051	9.1	2.2	pH 7.48 pCO₂ 1.44 HCO₃[−] 10.4	Yes^b	Lactic acid 3,737, 3-OH-butyric acid 7,902, acetoacetic acid 584, fumaric acid 13.8, 2-α-ketoglutaric acid 143, adipic acid 55, suberic acid 18.6, 3-OH-propionic acid 79.38, propionylglycine 1.29, methylcitrate 6.4, 3-methylcrotonylglycine 22.9, 3-OH-isovaleric acid 40.13
6	238	1,237	8.8	2.9	pH 7.46 pCO₂ 2.79 HCO₃[−] 23.0	Yes^b	Lactic acid 4,109, 3-OH-butyric acid 2,657, acetoacetic acid 927, fumaric acid 38.5, 2-α-ketoglutaric acid 254.6, adipic acid 219.8, suberic acid 48.6, sebacic acid 18.7, 3-OH-propionic acid 54.57, propionylglycine 3.28, 3-OH-isovaleric acid 55.50
7	900	1,537	4.8	No hypoglycemia	BE −22	Grossly elevated	Elevated: lactic acid, AcAc, 3-OH-butyric acid, adipic acid, suberic acid, 3-OH-suberic acid, 3-OH-sebacic acid, 3-OH-isovaleric acid
8	1,100	1,838	8.9	No hypoglycemia	pH 7.09 HCO ₃ [−] 0.5 BE −20	Grossly elevated	Lactic acid 2,330, 3-OH-butyric acid 2,690, AcAc 950, pyruvate 990, 3-OH-propionic acid 420, adipic acid 40
9	258	571	5.6	3.2	pH 7.43 pCO₂ 3.30 HCO₃[−] 14.0	Yes^b	Grossly increased: lactic acid, 3-OH-butyric acid, acetoacetic acid, 3-OH-isovaleric acid Moderately increased: fumaric acid Slightly increased: 2-α-ketoglutaric acid
10	919	1,221	13.9	3.0	pH 7.48 pCO ₂ 1.49 BE −15	Grossly elevated	Massive excretion of lactic acid and 3-OH-butyric acid, high excretion of 2-OH-butyric acid and AcAc, elevated adipic acid and 3-OH-isovaleric acid, slightly elevated suberic and sebacic acid
	119	Not reported	Not reported	Not reported	pH 7.3 pCO ₂ 3.8 BE −11.4	Not reported	Not reported
11	512	828	Yes	Not reported	Not reported	Yes	Not reported
12	700	1,880	7.0	No hypoglycemia	pH 7.20 HCO ₃ [−] 6.0	Grossly elevated	Lactic acid 7,338, 3-OH-butyric acid 15,145, AcAc: grossly elevated, 3-OH-isovaleric acid 42, 3-OH-propionic acid 22, adipic acid 54, suberic acid 14
13	890	1,504	Yes	Not reported	HCO ₃ [−] 11.0 BE −7.7	Yes	Not reported
14	700	992	15	5.8	pH 7.39 pCO ₂ 1.82 HCO ₃ [−] 8.0 BE −17	Grossly elevated	3-OH propionic acid: normal excretion Propionylglycine: traces Methylcitrate: normal 3-methyl-crotonylglycine: slightly elevated 3-OH isovaleric acid: moderately elevated Lactic acid, AcAc, 3-OH-butyric acid: Grossly elevated 3-OH-isobutyric, adipic, suberic, decandioic, 2-OH-butyric acid: elevated Pyruvate: elevated

Bold type represents patients previously reported by van Karnebeek *et al.*⁸ Italic type represents recurrent crises.

BE, base excess;

^apCO₂ in kilopascals, HCO₃[−] and BE in millimoles per liter. ^bPatients had elevated β-hydroxybutyric and acetoacetic acids.

Ninety percent of the newly described patients came from the Indian subcontinent (India, Pakistan, or Bangladesh), and one patient was Caucasian (Turkish descent). In terms of variants identified, the nonsense mutation p.W41* (**Figure 1b**), identified in a Turkish patient, is not expected to lead to nonsense-mediated decay of mRNA because it does not fulfill respective criteria,³⁰ but it will likely lead to a substantially shortened and thus inactive protein. The novel change p.Glu241Lys (**Figure 1b,c**), found in two independent patients originating in Bangladesh and Pakistan, respectively, was predicted by *in silico* analysis to cause disease (**Supplementary Table S2** online). The splice-site deletion of exon 3, found in one Indian patient, probably results in the skipping of exon 3, leading to a frame shift p.(Gly114Alafs*53) (**Figure 1b**). The remaining six patients, all from the Indian subcontinent (**Table 1**), carry an in-frame deletion of either exon 4 (patient 4) or 6 (patients 10–14) (**Figure 1b,c**); thus, because of the large protein region deleted and because of the removal of catalytic residues (**Figure 1b,c**), these mutations are expected to cause enzyme inactivation, if not lead to protein misfolding and degradation. On the other hand, prediction servers made predictions of benignity for the two most frequent polymorphisms (p.Asn45Lys, rs77325391, and p.Asn46Lys, rs74041853), included here as negative controls (**Supplementary Table S2** online). By contrast, p.Pro237Leu, the third non-disease-associated variant included in this study (**Figure 1b,c**), was predicted to be probably damaging (**Supplementary Table S2** online).

Expression, isolation, and stability of recombinant wild-type CAVA, its clinical mutants, and three known polymorphisms

To characterize all the published and newly identified CA5A variants, we introduced CA5A constructs carrying wild-type or mutant (primers are provided in **Supplementary Table S3** online) complementary DNA into Sf9 insect cells to express CAVA proteins using the baculovirus/insect cell expression system (**Supplementary Materials and Methods** online). Human CAVA is produced in the liver as a precursor, preceded by an N-terminal 38-amino acid mitochondrial signaling peptide, which is cleaved upon entry into the mitochondria. Thus, for expression of recombinant CAVA, we used the complementary DNA of mature human CAVA (the functional form *in vivo*) without the N-terminal 38 codons, which were replaced by a 28-codon N-terminal His₆ tag to facilitate purification. Since the presence of this tag drastically lowered enzyme activity (data not shown), it was removed using tobacco etch virus protease treatment (**Figure 2a**) after protein purification. The three non-disease-associated variants (p.Asn45Lys, p.Asn46Lys, and p.Pro237Leu) studied as controls and the two missense clinical mutations (p.Ser233Pro and p.Glu241Lys) did not hamper CAVA expression, solubility, or enzyme purification (**Figure 2a**). However, the mutations carrying a deletion of exon 4 or a deletion of exon 6 exhibited lower enzyme yields (**Figure 2a**) apparently as the result of poor polypeptide production and gross misfolding; only a small fraction of the expressed protein could be cleaved with the tobacco etch virus.

Mutations harboring deletions of exons 4 or 6 render CAVA inactive

In addition to the decreased enzyme production observed in the two enzyme forms carrying the deletions of exons 4 or 6, these mutants exhibited decreased thermal stability at ~3 °C and ~4 °C, respectively (**Figure 2b,c** and **Table 3**), relative to wild-type CAVA. In both cases the decreased stability might be explained by the elimination of a large fraction of the enzyme (32 residues in the case of the mutant with the deletion of exon 4, and 52 residues in the case of the mutant with the deletion of exon 6, which correspond to ~12 and ~19% of the enzymes, respectively) (**Figure 1c**). Moreover, these two mutations render CAVA inactive (**Figure 2d** and **Table 3**), confirming their disease-causing nature. Exon 4 contains the zinc binding site and the active site of the enzyme, whereas the substrate binding sites are located in exon 6 (**Figure 1b,c**). Therefore these mutations should inactivate CAVA by eliminating these key residues for the enzyme's activity.

Clinical missense mutations p.Ser233Pro and p.Glu241Lys decrease enzyme activity and thermal stability

Two clinical missense mutations, p.Ser233Pro and p.Glu241Lys, decrease enzyme-specific activity by ~50 and ~75% (**Figure 2d** and **Table 3**), respectively; this is probably explained by the fact that these two mutations affect residues mapping close to the substrate binding site (**Figure 1b,c**). In addition to the decreased specific activity (**Figure 2d** and **Table 3**), mutant p.Ser233Pro lowered the T_m (melting temperature) by ~6 °C (**Figure 2b,c** and **Table 3**), leading to the expectation of an important reduction in enzyme level in the tissue. These results corroborate those published in ref. 8, where mutant p.Ser233Pro was proven to be unstable. Similarly, the newly-described mutation p.Glu241Lys, apart from decreasing enzyme activity by ~75% (**Figure 2d** and **Table 3**), also decreased the T_m by ~2 °C (**Figure 2b** and **Table 3**). The decreased thermal stabilities may result in shorter half-lives of these two mutant forms in liver mitochondria; this fits the clinical course with metabolic decompensations triggered by febrile episodes, which is common to other IEMs as well (e.g., medium-chain acyl-CoA dehydrogenase deficiency³¹). Thus the disease-causing nature of these missense mutations would be explained by the combination of decreased residual enzyme activity as well as decreased thermal stability, in keeping with the clinical course of recurrent fever-induced decompensations during early childhood.

p.Asn45Lys, p.Asn46Lys, and p.Pro237Leu are variants without detectable functional consequences

The two most frequent polymorphisms (p.Asn45Lys and p.Asn46Lys), located in the N-terminal region of the enzyme, showed no substantial effect on CAVA activity (**Figure 2d** and **Table 3**) or thermal stability (**Figure 2b** and **Table 3**). This lack of effects can be rationalized by the fact that they map to residues located in the very N-terminal region of the enzyme, far away from the active site, and that they affect residues that vary among other CAs (**Supplementary Table S2** online). The

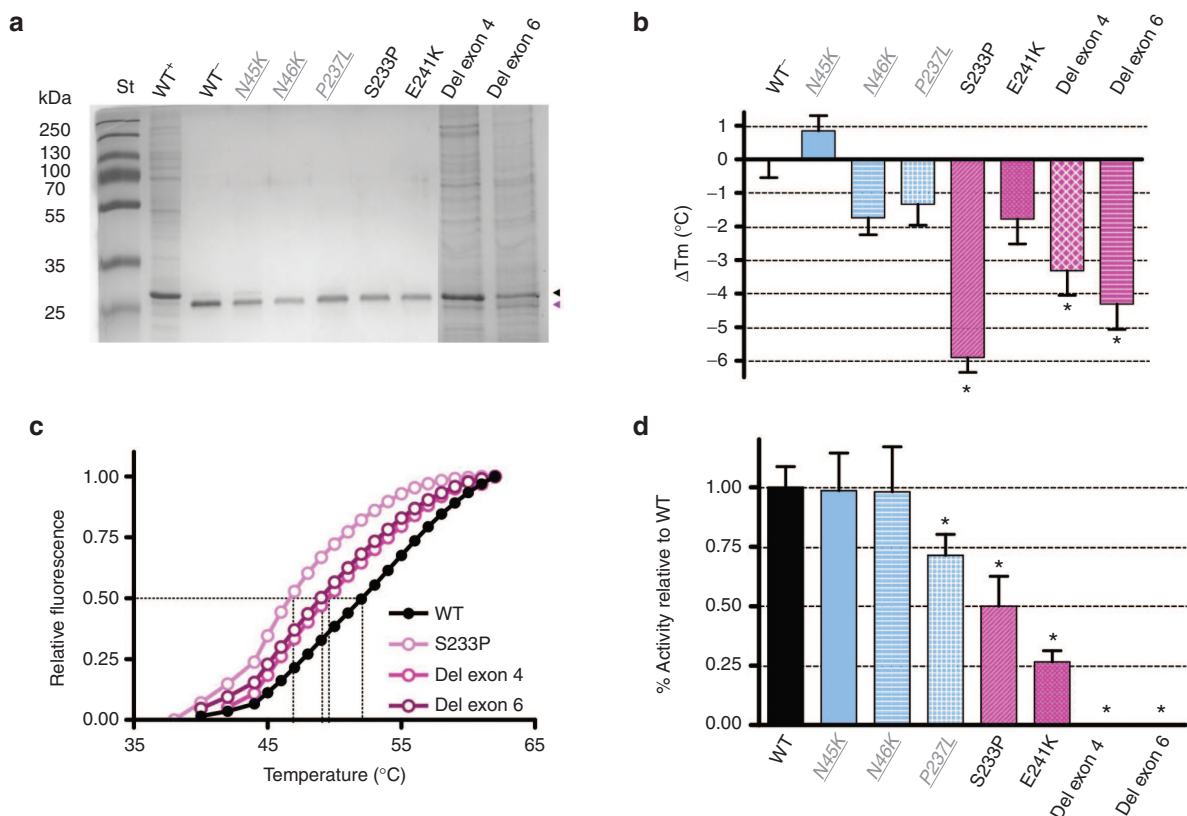


Figure 2 Production, thermal stability, and activity of wild-type (WT), polymorphisms, and clinical mutations of CAVA. WT⁺ and WT⁻ indicate the presence and absence, respectively, of the N-terminal His₆ tag in the WT enzyme. In all polymorphisms and mutant forms, the N-terminal His₆ tag has been removed by tobacco etch virus (TEV) treatment. Polymorphisms are labeled in gray and underlined. (a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (15% polyacrylamide, Coomassie blue staining) of purified human recombinant CAVA, either WT or carrying the indicated mutations. St, protein markers (PageRuler Plus prestained protein; Thermo Scientific), with masses indicated in kilodaltons. The black arrowhead indicates the position of the tag-free CAVA band of the WT form, polymorphisms, and missense mutations. The pink arrowhead indicates the position of the tag-free CAVA band of the enzyme forms carrying deletions of exon 4 or 6, respectively. (b and c) Thermostability of CAVA forms. (b) The change in T_m values (ΔT_m , compared with WT⁻) for each CAVA mutant. Error bars depict SEM from at least six measurements. *Statistically different ($P < 0.05$, Mann–Whitney test). (c) Differential scanning fluorimetry melting curves of WT CAVA and the three most unstable clinical mutations. (d) Enzyme activity per milligram of pure CAVA, expressed relative to the corresponding activity of the pure WT enzyme (438.4 U/mg). Error bars depict the SEM from at least six measurements. *Statistically different ($P < 0.05$, Mann–Whitney test).

other non-disease-associated variant p.Pro237Leu decreased CAVA-specific activity by ~30% (Figure 2d and Table 3), probably because it is involved in substrate binding (Figure 1c). However, this slight reduction in specific activity is expected to have no functional consequences in vivo.

DISCUSSION

The liver disease CAVA deficiency was described in 2014 as a human IEM⁷ in four patients.⁸ In this work we describe 10 more patients with CAVA deficiency who were identified among a group of 96 patients with unexplained hyperammonemia, suggesting that this disease may be more common than the rare forms of UCDs such as NAGS deficiency (estimated incidence: <1:2,000,000).³²

Detailed review of the phenotypes of the 10 patients identified here supports previous reports of CAVA deficiency; this IEM presents with unique biochemical findings during the neonatal period and infancy, including lethargy, poor feeding, and vomiting caused by hyperammonemia, combined with elevated blood lactate and urinary ketone

bodies, metabolic acidosis, hypoglycemia, and excretion of carboxylase substrates and related metabolites. Not all of these biochemical findings were present in all patients, but hyperammonemia, hyperlactatemia, and ketonuria seem to be obligatory (Table 2). These biochemical observations are consistent with the impairment of all four enzymes that use as a substrate the bicarbonate produced by CAVA in the liver mitochondria: CPS1, which converts waste nitrogen in the form of ammonia, bicarbonate, and adenosine triphosphate into carbamoyl phosphate in the first step of the urea cycle; pyruvate carboxylase, which links glycolysis with the Krebs cycle, by converting pyruvate to oxaloacetate, an essential substrate for the Krebs cycle; and propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase, which are involved in branched-chain amino acid catabolism (Figure 1a). In particular, the degree of hyperammonemia in CAVA-deficient patients during metabolic crisis varied between 238 and 1,150 $\mu\text{mol/L}$, similar to other UCDs, and these patients accumulated ketone bodies and organic acids such as 3-OH propionic acid and exhibited variable levels of metabolic acidosis

Table 3 Influence of clinical mutations and variants on carbonic anhydrase VA activity and thermal stability

Amino acid change ^a	Specific activity (U/mg pure enzyme) ^b	Percentage of WT CAVA	T_m (°C) ^c	ΔT_m (mutant vs. WT in °C) ^d
WT	438.4 ± 37.5	100	52.47 ± 0.54	0
Variants				
p.Asn45Lys	431.8 ± 68.9	98.5	53.32 ± 0.45	+0.85
p.Asn46Lys	429.5 ± 82.3	98.0	50.73 ± 0.51	-1.74
p.Pro237Leu	312.6 ± 38.2	71.3	51.14 ± 0.63	-1.33
Mutations				
p.Ser233Pro	218.7 ± 55.2	49.9	46.57 ± 0.45	-5.90
p.Glu241Lys	116.3 ± 20.7	26.5	50.70 ± 0.75	-1.77
Deletion exon 4	—	0	49.16 ± 0.74	-3.31
Deletion exon 6	—	0	48.16 ± 0.76	-4.31

^aTranslation of the complementary DNA reference sequence NM_001739.1 (GenBank). Nucleotide 57 in this sequence is considered +1 since it is the A of the translation initiation codon. ^bEnzyme-specific activities were determined between 6 to 14 times and calculated as enzyme units per milligram of enzyme protein. ^cThermal stability was determined between 6 to 15 times. ^d ΔT_m of mutants compared to recombinant wild-type (WT) carbonic anhydrase VA (CAVA).

characteristic of secondary propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase deficiencies.

Indicating a possible but yet unproven common ancestry, 43% of the patients carried the homozygous CA5A exon 6 deletion. Indeed, all these patients come from the Indian subcontinent (either India or Pakistan), and in some cases, consanguinity is known (Table 1). In contrast to this hypothesis, patient 11 (Table 1), in spite of coming from India, had a Hindu background rather than an Islamic one; thus the exon 6 deletion probably originated genuinely in separate communities. Likewise, patient 14 (Table 1), despite being Pakistani, was born to nonconsanguineous parents. The issue of consanguinity also has to be considered as a contributing factor for the neurological symptoms in patient 13, which may be unrelated to CAVA deficiency.

NCG, although not approved as a treatment for CAVA deficiency, seemed to be beneficial for these patients (Table 1). We postulate the following explanation for its therapeutic mechanism: since propionyl-CoA carboxylase cannot perform its reaction properly because of the absence of HCO_3^- , propionyl-CoA, a potent inhibitor of NAGS,³³ accumulates. The decreased production of NAG, an essential activator of CPS1, in addition to the low concentration of its substrate bicarbonate, hampers CPS1 activity and thus ammonia detoxification by the urea cycle. The resulting secondary hyperammonemia exhibited by these patients is amenable to NCG via an increase in CPS1 activation. It must be acknowledged, however, that pyruvate carboxylase deficiency also contributes to hyperammonemia, albeit to a lesser extent given the absence of an increase in α -ketoglutarate and the relatively mild increase of Krebs cycle intermediates; thus this life-threatening symptom may strongly yet incompletely respond to NCG. In conclusion, CAVA deficiency should be considered as a treatable IEM given its amenability to NCG, in addition to extracorporeal detoxification in the case of severe hyperammonemia, as well as standard treatment of the acute metabolic crisis, which includes increased administration of calories and fluids and decreased protein intake and intravenous lipids.

A remarkable observation was that ~65% of the patients with CAVA described presented only one initial crisis, after which they remained stable, even after tapering treatment. In the remaining ~35%, the subsequent crises were triggered by catabolism and febrile episodes, and all these crises were less severe than the initial ones. A suggested explanation for this phenomenon is that the CAVB isoform, the only other CA expressed in liver mitochondria, undergoes a maturation effect,²⁷ by which the expression of functional CAVB develops during early infancy. In patients with defective CAVA, CAVB would not be sufficiently present or would be immature at the moment of birth, explaining the initial crisis, but would become functional over time, stabilizing the CAVA-deficient patient and attenuating further metabolic crisis.⁸

The expression system presented here provides evident correlations between the clinical mutations and the phenotypes observed in the patients, enabling the understanding of the degree of severity of different mutations. Indeed, the lack of functional effects of the three non-disease-associated variants studied here, and the severity of the effects (decreased enzyme activity and/or thermal stability) of the four clinical mutations analyzed, indicate that the experimental studies with recombinant CAVA mutants expressed “in vitro” validate disease-causing mutations.

In summary, our study further confirms CAVA deficiency as a novel differential diagnosis of neonatal and early-childhood hyperammonemia, and it seems more common than other rare IEMs such as NAGS deficiency. It is even possible that patients who were labeled with the exclusion diagnosis of transient hyperammonemia of the newborn could have been affected by CAVA deficiency. This novel hepatic disease exhibits a unique biochemical fingerprint including hyperammonemia, elevated blood lactate and urinary ketone bodies, metabolic acidosis, hypoglycemia, and excretion of carboxylase substrates and related metabolites. The metabolic crisis can be very severe at presentation and should be diagnosed early to start treatment quickly and to avoid unnecessary morbidity. The later course seems to be more favorable than in many other intoxication-type metabolic diseases; most patients are stable

and develop normally, with only a sick-day regimen. Research is ongoing to develop a reliable dried blood spot test so that this condition may be included in newborn screening panels.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

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DISCLOSURE

The authors declare no conflict of interest.

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