

Supplementary Appendix

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Supplementary Appendix to:

Monocarboxylate transporter type 1 deficiency and ketone utilization

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Targeted whole exome sequencing

Original genomic DNA was isolated from peripheral lymphocytes of the index patient and family members according to standard procedures. Snp-array assays were performed on all five family members using a high resolution snp array (HumanCytoSNP-12 BeadChip, Illumina). Homozygous stretches larger than 1 MB were identified, using an in house developed algorithm (available upon request). 36 homozygous stretches larger than 1 MB were identified. A custom capture array was designed targeting the exonic parts plus 50 bp flanks within the homozygous stretches (1). The designed targeted area of 3.1 Mb, probes could be designed for 92% of the requested genomic regions. Sequencing libraries were prepared as described previously (1) and sequenced on the Solid 5500 platform with an average coverage ranging between 160 to 239 fold. Mapping and variant analysis was performed as described previously (2), with the exception that a recessive inheritance model was applied. Initial filtering did

not yield good candidate variants, therefore the filtering criteria were relaxed to allow for improved detection of small insertions and deletions. The used settings for filtering were: allele frequency <0.01, coverage >10 reads, percentage variant >50% for the patient, >10% for parents and <80% for unaffected siblings and exclusion of missense variants annotated as benign by PolyPhen2.

Sanger sequencing, cDNA and deletion analysis

The complete coding regions of MCT1/SLC16A1, MCT2/SLC16A7, MCT3/SLC16A8, MCT4 /SLC16A3 and CD147/BSG were sequenced in 96 patients suffering from episodes of severe ketoacidosis. The primer sequences are available upon request. Homozygous mutations were confirmed by verification of the heterozygous nature of the mutation in both parents.

For the analysis of the mutation state in mRNA, cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Life technologies) on RNA isolated from fibroblasts using Trizol reagent (Life technologies). Subsequently a PCR was performed using cDNA specific primers (sequence available upon request), followed by Sanger sequencing.

To test for potential copy number variation in heterozygous patients, we performed an MLPA-assay in our ISO15189 accredited diagnostic lab according to the manufacturers protocols. 6 multiplex amplification probes (Multiplicon) were designed covering the 5 exons of SLC16A1, with the largest exon 4 covered twice. Primer sequences are available upon request.

Expression and functional analysis

Erythrocyte lactate transport assay

Erythrocyte lactate transport was measured essentially as described by Fishbein (3). Within 26 hours after drawing (EDTA) anticoagulated blood samples were incubated at 37°C for 2 h without stirring to load the erythrocytes with lactate. Erythrocytes were then packed, washed twice with buffer (134 mM NaCl, 4.8 mM KCl, 0.96 mM MgCl₂, 10 mM sucrose, 10 mM MES pH 6) and subsequently diluted tenfold in incubation buffer (134 mM NaCl, 4.8 mM KCl, 0.96 mM MgCl₂, 10 mM sucrose, 10 mM Tricine pH 7.5). At six 1-min intervals 0.5 ml aliquots were pipetted into iced buffer (134 mM NaCl, 4.8 mM KCl, 0.96 mM MgCl₂, 10 mM sucrose, 10 mM MES pH 5) to stop the reaction. The samples were then spun (1 min, 20,000 x *g* at 4°C), washed with buffer (134 mM NaCl, 4.8 mM KCl, 0.96 mM MgCl₂, 10 mM sucrose, 10 mM MES pH 6), spun again (1 min, 20,000 x *g* at 4°C) and lysed in 200 µl 0.59 M perchloric acid. Samples were thoroughly mixed, spun (5 min, 20,000 x *g* at 4°C) and the supernatant was used for measurement of lactic acid. Lactic acid concentration was determined by following the production of NADH spectrophotometrically at 340 nm for 30 min at 37°C in the presence of L-glutamate (50 mM in 400 mM NaCO₃, pH10), NAD⁺ (2 mM), Glutamate-pyruvate transaminase (3.5 U/ml, Roche) and Lactate dehydrogenase (105 U/ml, Sigma). Specific activity was expressed as lactate efflux from the erythrocyte in time per g Hb. For each patient sample, at least one control sample was included within the same experiment, which was treated exactly the same from blood drawing until analysis. All analyses were also performed in the presence of 20 µM p-chloromercuribenzenesulphonate

(pCMBS), a potent inhibitor of MCT1, to verify that the measured lactate efflux was transporter dependent.

Immunoblot analysis

Cultured skin fibroblasts were homogenized in PBS containing protease inhibitors (Complete mini, Roche) and aliquots of 20 ug of total protein were separated a 4-12% gradient NuPAGE Bis-Tris gel (Invitrogen) in a MES buffer (pH 7.3) and transferred onto PVDF by semidry blotting. Membranes were blocked with normal goat serum (NGS) for 1 h at room temperature and then incubated overnight at 4°C with affinity purified rabbit polyclonal antibody against MCT1 (Millipore, AB3538P) or MCT4 (Millipore AB3316P) diluted 1:100 and 1:500 respectively in 4% NGS/PBS. As a loading control, the membranes were reprobed with a monoclonal antibody against α -tubulin (Molecular probes), using a 1:5000 dilution. Antigen-antibody complexes were visualized with IRDye 800CW goat anti-rabbit secondary antibody for MCT1 and IRDye 680RD donkey anti-mouse secondary antibody for tubulin using the Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA). Quantification of the signal intensities of the MCT1 and tubulin bands was done using AIDA Image analyzer software (Version 4.26, Raytest, Straubenhardt, Germany).

Calculations

The expected CO₂ excess was calculated according to Winter's formula, where $P(\text{CO}_2) = (1.5 * \text{HCO}_3^-) + 8 \pm 2$ where HCO_3^- is given in mEq / L and $P(\text{CO}_2)$ in mmHg.

Case Report

The proband, a girl, was the second child of consanguineous parents. At the age of 3.5 months she was admitted with respiratory distress due to a viral upper respiratory-tract infection and a wheeze. Unexpectedly, blood gas analysis revealed profound metabolic acidosis, pH 6.88, Base Excess (BE) -28 mmol / L, and an elevated anion gap (32 mmol / L, normal < 15 mmol /L). Glucose was borderline normal at 3.4 mmol / L. Urinary ketostick showed strongly elevated ketone levels. She was transferred to an ICU and recovered rapidly after receiving sodium bicarbonate and IV glucose on this and subsequent occasions. Cardiac ultrasound showed a hemodynamically insignificant type II atrial septum defect, persistent ductus botalli and hypoplastic left pulmonary artery. In addition, subsequently, hypoplasia and malacia of the main left bronchus was observed on bronchoscopy. Abdominal ultrasound confirmed normal sized liver and spleen. On cerebral ultrasound increased echogenicity of caudate nucleus and lentiform nucleus was noted. Borderline microcephaly was observed on follow up (SD -2.7). MRI cerebrum was unremarkable, but subsequently a developmental delay became evident.

At an age of seven months she was admitted because of vomiting and refusal to eat. Metabolic acidosis (pH 7.30, BE -10.6 mmol / L) cleared rapidly after a single correction with sodium bicarbonate. Nevertheless, strongly elevated urinary ketones were repeatedly observed, possibly due to insufficient breastfeeding. A similar episode at an age of nine months was triggered by a respiratory syncytial virus infection. At 12 months of age she was admitted for percutaneous closure of persistent ductus arteriosus. Despite regular fasting measures around this procedure, poor feeding resulted in vomiting, drowsiness and severe metabolic

acidosis within a day, pH 6.98; BE -23 mmol/L. The last significant episode occurred at 2.5 years and was triggered by poor feeding and otitis media acuta. Further episodes could be prevented with advice to avoid fasting and early admissions in case of poor feeding and vomiting. This included uneventful percutaneous closure of atrial septal defect, requiring general anesthesia at 7 years.

Metabolic investigations during bouts of ketoacidosis revealed massive urinary excretion of 3-hydroxybutyric acid (range 2100-12500 mmol /mol creatinine, normal < 8 mmol /mol creatinine) and 3-ketobutyric acid (range 3900-26500 mmol/mol creatinine, normal below 5 mmol /mol creatinine), without consistent other abnormalities. Plasma acylcarnitine profile occasionally showed elevated hydroxybutyrylcarnitine (range 0.05-1.51 μ mol / L, normal <0.2 μ mol / L).

S1

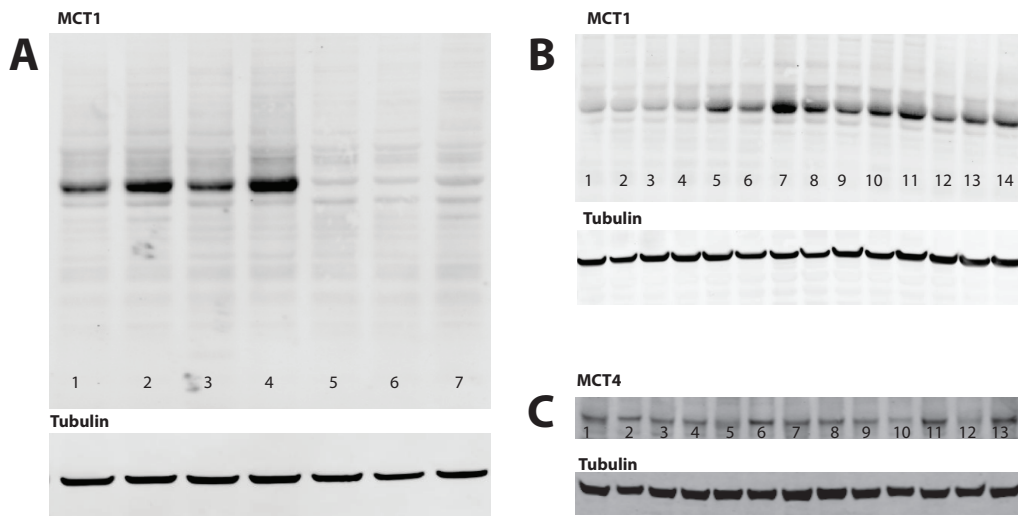


Figure S1: Immunoblot results for patients with MCT1 deficiency and controls.

Panel A and B show immunoblot results for fibroblast homogenates from MCT1 deficient patients, stained for MCT1 (45 kDa). Tubulin was used as a loading control. Panel A shows results for patients with homozygous mutations and controls. Lanes were loaded as follows: 1-4 were controls, 5: p.Asp15fs, 6: p.Arg328Ter and 7: p.Arg313Ter. Panel B shows results for patients with a heterozygous truncating mutation and controls, stained for MCT1. Lanes were loaded as follows: 1: p.Leu164ProfsTer46, 2: p.Asn250SerfsTer5, 3: , p.Val167PhefsTer13, 4: p.Leu164ProfsTer46 and 5-14 were controls. Panel C shows immunoblot results for fibroblast homogenates from MCT1 deficient patients, stained for MCT4 (50 kDa). Tubulin was used as a loading control. Lanes were loaded as follows: 1-3 homozygous mutations (1: p.Arg328Ter; 2: p.Arg313Ter; 3: p.Asp15fs); 4-7 heterozygous mutations (4: p.Arg196Ter; 5: p.Asn250SerfsTer5; 6: p.Val167PhefsTer13; 7: p.Leu164ProfsTer46); 8-13: controls.

S2

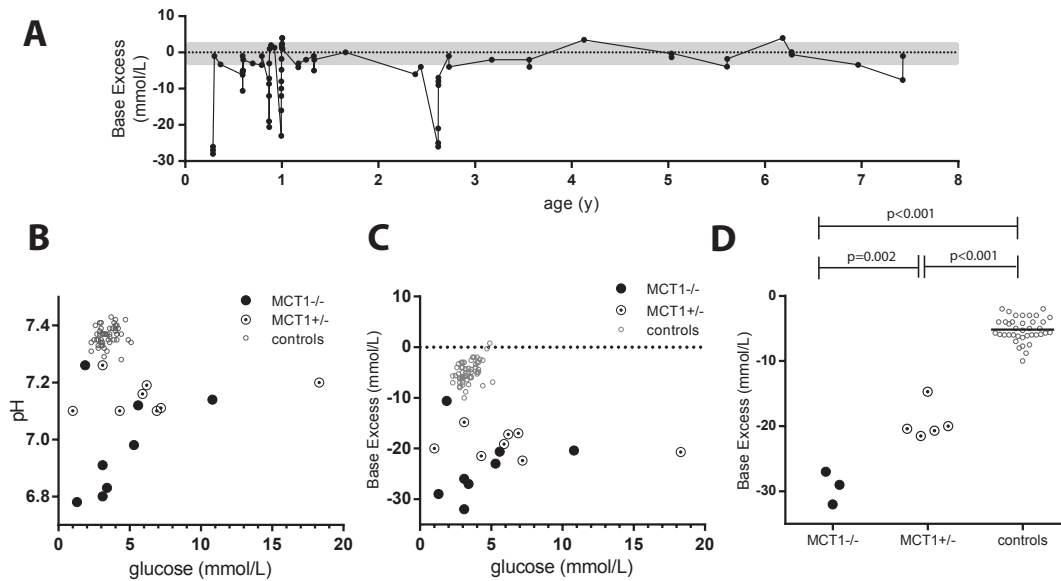


Figure S2: Base excess in patients with MCT1 deficiency.

Panel A shows the base excess over time for the index patient. Panel B and C show the blood glucose versus pH (panel B) and blood glucose versus base excess (panel C) in homozygous (-/-) and heterozygous (+/-) MCT1 deficient patients at presentation of a documented episode of catabolic stress. Panel D shows the base excess for homozygous and heterozygous patients compared to controls. The origin of the control data is described in the legend of Fig 2.

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