**E-appendix**

***ACO2* homozygous missense mutation associated**

**with complicated hereditary spastic paraplegia**

Christian G. Bouwkamp, Zaid Afawi, Aviva Fattal-Valevski, Inge E. Krabbendam, Stefano Rivetti, Rafik Masalha, Marialuisa Quadri, Guido J. Breedveld, Hanna Mandel, Muhammad Abu Tailakh, H. Berna Beverloo, Giovanni Stevanin, Alexis Brice, Wilfred F.J. van IJcken, Meike W. Vernooij, Amalia M. Dolga, Femke M.S. de Vrij, Vincenzo Bonifati, Steven A. Kushner

**Aconitase enzyme assay**

Activity of the aconitase enzyme was measured using the colorimetric Aconitase Enzyme Activity Microplate Assay Kit (Abcam) according to the manufacturer’s instructions. LCLs were lysed by homogenization in lysis buffer (2x106 cells/sample), followed by centrifugation for 15 minutes at 10.000g and 4°C. The activity was measured both in supernatant of whole cell lysates, and in isolated mitochondrial and cytoplasmic fractions using a complementary cellular fractionation kit (Abcam). The conversion of isocitrate to cis-aconitate was quantified by fluorometric absorbance at 240 nm in a microplate reader. All experiments were performed at least twice with similar results. Absorbance data were corrected for blank measurements and normalized to control levels.

**Western blotting**

LCL lysates were diluted 1:1 with 25% XT Sample Buffer (Biorad) and 10% 100mM dithiothreitol (DTT) followed by heating to 65°C for 15 minutes. Samples were electrophoresed through 4%-12% Criterion™ XT Bis-Tris gradient gels (Biorad) in XT-Mops buffer (Biorad). Proteins were transferred overnight on 0.45µm pore nitrocellulose membrane (Biorad) in a Tris-Glycine buffer consisting of 10% Tris/Glycine buffer (Biorad) and 20% anhydrous methanol in distilled water. Membranes were blocked for 1 hour while shaking at room temperature in TBS buffer with 0.1% Tween-20 (TBST) containing 4% blotting grade blocker (Biorad). Primary antibody labelling of the membranes for -actin (Millipore) and aconitase 2 (Abcam) were performed in TBST buffer containing 2% BSA for 24 hours at 4°C, followed by washing with TBST buffer. Subsequently, blots were incubated with IRDye® conjugated secondary antibodies (LI-COR Biosciences) in TBST with 2% non-fat dry milk for one hour at room temperature and washed again. Antibody fluorescence was visualized using an ODYSSEY® CLx scanner (LI-COR Biosciences).

**High-resolution cellular respirometry**

A semi-automated method was used to isolate crude mitochondria from lymphocytes [11]. LCLs were cultured in T75 flasks and collected in isolation medium (pH 7.5) containing sucrose (250 mM), EDTA (3 mM), and HEPES (20 mM). High-precision cell breakage was achieved with a Balch homogenizer and a 4µm wall-to-ball bearing clearance. Cell homogenates were pumped 3 times through the homogenizer (0.71 mL/minute) and centrifuged at 800 rpm for 10 minutes. The supernatant was collected and centrifuged at 9000 g for 10 minutes. The mitochondrial pellet was collected in 150 µL of isolation buffer. Protein content was measured using the Pierce BCA assay.

Mitochondrial respiration of LCLs was analyzed by high-resolution respirometry using the Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria). Mitochondrial respiration was monitored during continuous stirring at 750 rpm in 1ml MiR05 buffer (0.5mM EGTA, 3mM MgCl2, 60mM lactobionic acid, 20mM taurine, 10mM KH2PO4, 20mM HEPES, 110mM D-Sucrose, BSA, 1g/L essentially fatty acid free, at pH7.4). Oxygen polarography was performed at 37°C. Oxygen flux per tissue mass (pmol O2.s−1\*mg−1) was recorded using DatLab software. Non-phosphorylating respiration or mitochondrial respiration state 2 was induced by adding the complex I-linked substrates pyruvate (5mM) and malate (2mM). Subsequently, the oxidative phosphorylation capacity of complex I-linked activity (state 3) was measured after addition of a saturating concentration of ADP (2.5 mM). Oligomycin (2μg/ml) was added to inhibit the ATP synthase and measure proton leak. The maximum respiration (state 4u) was induced by stepwise titration of the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) in 1 μM steps until the maximum oxygen consumption was reached. The oxygen concentration and the first derivative of the oxygen concentration, reported as oxygen consumption [O2 slope, pmol\*(mL/s)] of crude isolated mitochondria were recorded in 2 sec intervals following instrumental background correction and calibration of the polarographic oxygen sensors.