Supplementary Materials and Methods

Linkage analysis

Genotyping was performed using Illumina Human Omni Express Bead Chip (Illumina, San Diego, California, USA), with >750K SNPs per sample. Linkage analysis as well as haplotyping and multipoint logarithm of odds (LOD) score calculation were done using SUPERLINK ONLINE SNP 1.1 (http://cbl-hap.cs.technion.ac.il/superlink-snp), assuming an autosomal recessive mode of inheritance. Homozygosity mapping was done using the Homozygosity-Mapper software (1).

Genetic analysis

DNA was extracted from peripheral blood. Whole-exome sequencing (HiSeq2000, Illumina, San Diego, CA, USA) was done as previously described (2). Whole-genome sequencing was done as previously described (3). Data were analyzed using QIAGEN's Ingenuity® Variant Analysis™ software (www.qiagen.com/ingenuity) from QIAGEN Redwood City. Our filtering cascade excluded common variants which demonstrated an allele frequency greater than or equal to 0.1% in the Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/) or Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/), unless established as Pathogenic common variant. In addition, we excluded homozygous variants that had an allele frequency greater than 2% in our in-house whole exome sequencing database of 180 ethnically matched Bedouin control samples. Furthermore, we kept variants which were predicted to have a deleterious effect on protein coding sequences (e.g. Frameshift, in-frame indel, stop codon change, missense or predicted to disrupt splicing by MaxEnt Scan) and variants that were experimentally observed to be associated with a phenotype: pathogenic, possibly pathogenic or disease-associated, according to the Human Gene Mutation Database (HGMD). Following filtering, we selected only homozygous variants that were shared between both affected individuals sequenced. All physical positions are according to the GRCh37/hg19 genome assembly.

The authors will make all processed and raw high-throughput sequencing data available for individual researchers upon request.

Multiple sequence alignment

Nine orthologues of *LDHD* were selected to represent protein conservation using multiple sequence alignment. Protein sequences were acquired from the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov). RefSeq sequence accession numbers used for the analysis were: NP_705690.2 (H.sapiens), XP_511108.2 (P.troglodytes), XP_001103000.1 (M.mulatta), XP_852976.3 (C.lupus), NP_001068911.1 (B.taurus), NP_081846.3 (M.musculus), NP_001008893.1 (R.norvegicus), XP_003641953.1 (G.gallus), NP_956167.1 (D.rerio). Percentage of identity and similarity were acquired from the Basic Local Alignment Search Tool (Blast) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein sequence alignment was carried out using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Structure prediction

Complete protein sequence of human D-lactate dehydrogenase (NP_705690) was deposited into Swiss-model server (https://swissmodel.expasy.org/) and XtalPred (http://ffas.burnham.org/XtalPred-cgi/xtal.pl) to identify closest homologs based on sequence and predicted structure. Both resources identified homology between D-lactate dehydrogenase and other members of a large family of FAD-binding proteins. This is in line with data obtained from the previously solved crystal structure of D-lactate dehydrogenase in *E.coli*, demonstrating that the enzyme contains an FAD-binding domain (4). A human D-lactate dehydrogenase model was created using Swiss-Model based on the structure of the closest structural homolog found, a putative dehydrogenase from *Rhodopseudomonas palustris* (PDB ID 3PM9), with 30% protein sequence identity and an E (expect) score $<10^{-99}$. QMEAN and GMQE scores were -2.31 and 0.65, respectively. The structural model predicted a homo-dimer similar to the template, in line with solved structures of D-lactate dehydrogenase in other organisms (5). The dimerization surfaces were apparently preserved, although there are some differences in amino acid residues. All hydrophobic pockets and the FAD-binding pocket in particular, were very similar and

apparently preserved in the model. The residues composing the catalytic pocket are identical between the model and the 3PM9 protein. Figures 3C,D were created using PyMOL (6).

LDHD constructs

Full mRNA of human *LDHD* was amplified from a liver cDNA library (Clontech Laboratories, Palo Alto, CA) and cloned into 2 separate pCDNA^{TM} 3.1 (-) expression vectors (Invitrogen), containing a 3' FLAG epitope sequence or a 3' red fluorescent protein (tdTomato) sequence. The c.1108C>T mutation was then inserted using DpnI-mediated site-directed mutagenesis. PCR primers used for mutagenesis: Forward 5'- GACAGCATGGCACAATGCCTGGTACG-3'; Reverse 5'- CATTGTGCCATGCTGTCCAAAGCCGG-3'.

Mitochondrial extraction and western blot analysis

Constructs of wild-type and mutant *LDHD,* fused to FLAG epitope and an empty pCDNA^{TM} 3.1 (-) plasmid (Invitrogen, Carlsbad, CA, USA), were transfected into HEK293T cells (ATCC CRL-11268) seeded at ~70% confluency on 100-mm plates using Lipofectamine^{TM} 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were harvested 72 after transfection and mitochondrial and cytosolic fractions were extracted using the Mitochondria Isolation Kit for Cultured Cells (Pierce). Protein samples (20-40µg) from cytosolic and mitochondrial fractions were mixed with a standard sample buffer, loaded onto 10% polyacrylamide gel for separation using a Mini Gel Tank (Invitrogen) and transferred to a nitrocellulose membrane. Membrane was then blocked in 5% skim-milk (skim milk powder; Fluka, BioChemika) for 1 hour at room temperature, and incubated with the following primary antibodies diluted in TTBS (0.02 M Tris, pH 7.5, 0.15M NaCl, 0.9Mm Tween20) for 1-3 hours at room temperature: FLAG (Sigma–Aldrich, F1804), VDAC1 (Abcam, ab135585), α-Tubulin (T9026, Sigma-Aldrich). After each incubation with a primary antibody, membrane was incubated for 1 hour at room temperature with a secondary Goat anti-mouse HRP-conjugated antibody (sc-2005, Santa Cruz) diluted 1:10,000 in TTBS, and visualized using ChemiDoc MP imaging system (Bio-Rad).

Confocal microscopy

SH-SY5Y cells (ATCC CRL-2266) were seeded at ~50% confluency on coverslips in 12-well plates. The following day, cells were transfected with wild-type or mutant *LDHD* constructs fused to red fluorescent protein (tdTomato) and with Mito-Vector, encoding a green fluorescent mitochondrial marker protein (pAcGFP1-Mito Vector, Clontech). For transfection control and calibration of detection threshold for each fluorophore, cells were also transfected with pCDNA^{TM} 3.1 (-) plasmid containing tdTomato alone, and a Mito-Vector plasmid alone. Transfection was done using Lipofectamine^{TM} 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Forty eight hours following transfection, cells were washed twice with PBST (phosphate-buffered saline + 0.05% Tween 20), fixed in 4% paraformaldehyde for 20 min, mounted using Vectashield^{TM} containing DAPI (H-1200; Vector Laboratories) and visualized using LSM880 microscope with 40X/1.3 objectives (Carl Zeiss Microscopy GmbH, Germany). Same lenses and parameters (such as master gain, digital gain, digital offset, and pinhole size) were used in all experiments after ensuring there was no leakage of emitted light between the 3 different fluorophores used in the experiment. All pictures represent maximum intensity projection of all planes generated using ZEN 2.3 software (Carl Zeiss Microscopy GmbH, Germany).

Tissue expression

Tissue expression panel was created from total RNA derived from 21 normal human tissues (Clontech Laboratories). PCR-amplification was performed using two sets of primers designed to amplify cDNA and not gDNA of human *LDHD* and of GAPDH control. Reaction was set to 30 cycles with a Tm of 58ºC, extension time was set to 25s. Primers used for *LDHD* amplification (202bp amplicon): forward 5'-AGGAGATAGTCCAGCAGAAC-3'; reverse 5'-TTCAGATCCTCCTTGGTCTG-3'; primers used for GAPDH (452bp amplicon): forward 5'-ACCACAGTCCATGCCATCAC-3'; reverse 5'-TCCACCACCCTGTTGCTGT-3'.

Mass spectrometry

Blood and urine samples were collected from 5 affected (III:5, III:7, III:9, IV:3, IV:5) and 4 healthy (II:4, III:1, III:3, III:8) family members, early in the morning following a 12 hour fast. Blood samples were centrifuged at 2500 RPM for 15 minutes at 4ºC, plasma was then separated and frozen along with urine samples in -80ºC until downstream processing. Before freezing, samples were kept in ice (<2 hours). Metabolites were extracted from plasma and urine samples as previously described (7), and analyzed using LC-MS (Dionex Ultimate 3000 RS couples with Thermo Fisher Scientific Q-Exactive Mass Spectrometer). Separation of the two lactate isomers was achieved using Astec CHIROBIOTIC R Chiral column (Sigma-Aldrich) using a previously described protocol (8). Calibration curve was generated using known concentrations of diluted samples of D/L-lactate (71716, L7022, Sigma-Aldrich).

In-vivo experiments

Blood samples from wild-type male C57BL6/J young-adult mice (Envigo, Jerusalem, Israel) were taken to measure plasma uric acid levels before and at several time points (30, 60, 120, 180 and 300 minutes) after intraperitoneal injection of 200µl PBS or 3M D-lactate (71716, Sigma-Aldrich) diluted in PBS. At each time point, blood was drawn from the tail vein into EDTA-coated vials and immediately centrifuged at 14,000 rpm for 10 minutes at 4 ºC. Plasma was then separated and frozen at -20ºC. Plasma samples taken at all different time points were all thawed and analyzed at the same time, as follows: plasma uric acid was measured with the Amplex Red Uric Acid/Uricase Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. In brief, uricase catalyzes the conversion of uric acid to allantoin, hydrogen peroxide, and carbon dioxide. The hydrogen peroxide then reacts stoichiometrically with Amplex Red reagent in the presence of horseradish peroxidase to generate the red fluorescent oxidation product resorufin. Resorufin was measured using excitation at 530 nm and detection at 590 nm. on the Infinite 200 PRO multimode plate reader (Tecan Group Ltd., Switzerland). For each mouse, measurements were normalized to the emission detected at T=0 before injections of D-lactate / PBS were made, and average was then calculated for every time point measured.

References

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