

Supplemental Methods:

Patient recruitment

Patients and their families were collected prospectively in the Myelin Disorders Bioregistry Project (MDBP) with approval from the institutional review board at Children's Hospital of Philadelphia, USA (IRB Approval # IRB 14-011236), "Bambino Gesù" Children's Hospital, Rome, Italy, Research Institute of the McGill University Health Center (approved by the Research Ethic Board, 11-105-PED and 2019-4972), Montreal, Canada, the Department of Neuropediatrics, Jena University Hospital, Germany and Amsterdam University Medical Centers, The Netherlands. Written informed consent for collection of clinical information, neuroimaging, and genetic information was obtained for each study participant.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for Individual 1

Total RNA from fibroblasts was isolated in appropriate amounts of TRIzol reagent according to the manufacturer's instructions (Sigma). Thirty micrograms of RNA were DNase I (Promega) treated for 30 minutes at 37°C. One microgram of RNA was reverse transcribed using random primers and MMLV reverse transcriptase according to the protocol of the manufacturer (Promega). For the quantitative PCR, primers were designed and efficiency tested according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines. Primers efficiency is between 90-110%. Real-time PCR was performed in triplicated with a 1:4 dilution of cDNA using SyBR green PCR mix on LightCycler 96 instrument (Roche). Data was normalized to *HPRT1* levels using the $\Delta\Delta C_t$ method. Primers used for *AARS1* mRNA amplification are: Forward 5'-GCCAATACCCAGAAGTGCAT-3' and reverse 5'-GCATCCTCAGCACCAACTTT-3'. Primers used for *HPRT1* mRNA amplification are: Forward 5'-TGCTGAGGATTTGGAAAGGG-3' and reverse 5'-ACAGAGGGCTACAATGTGATG-3'.

Immunoblotting for Individual 1

Protein samples were extracted from patient fibroblasts as well as from age and sex-matched control fibroblasts. Cultured cells were washed once with ice cold PBS. Cells were lysed in ice-cold RIPA buffer (Thermo Fisher Scientific) with EDTA-free protease inhibitor (Roche). The plates were incubated on ice for 20 minutes. Cell were scraped and harvested into 1.5 ml

Eppendorf tubes and spun at 14,000g for 15 minutes at 4°C. Lysates were transferred to new tubes and protein concentrations were determined using Bradford reagent (Bio-rad). 30 µg protein/well was separated by SDS-PAGE, transferred to nitrocellulose membranes using immunoblot TurboTransfer system (Bio-rad), blocked with 5% skim milk, and immunoblotted with the indicated primary antibody, AARS1 (Abcam, 1:1000) or β-tubulin (Sigma, 1:2000), overnight at 4°C or 2 hours at room temperature. After washing, membranes were incubated with secondary antibody conjugated to horseradish peroxidase one hour at room temperature and then visualized by ECL Prime reagent (GE Healthcare). Band intensity was measured and quantified by BioRad ImageLab Software.

Sanger Sequencing Analysis for Individual 4

Sanger sequencing analysis, using BigDye chemistry 3.1 and run on an ABI 3130XL automatic sequencer (Applied Biosystems, Life Technologies), was performed to confirm the presence of the missense variant in the proband and to follow the segregation within the family members.

Quantitative PCR for Individual 4

Total RNA was isolated from control and patient skin fibroblasts, using Total RNA Purification Kit (Norgen Biotek Corp., Canada). 1 µg of total RNA was reverse transcribed using Euroscript RT-PCR KIT (Euroclone, Pero (Mi), Italy). Relative quantification of *AARS1* and using *GUSB* as a housekeeping gene) was carried out with SYBR GREEN™ PCR Master Mix (ThermoFisher scientific) and run on an ABI PRISM 7500 Sequence Detection System (Life Technologies).

Immunoblotting for Individual 4

Immunoblotting analysis was performed to investigate the impact of *AARS1* variants on protein stability. Primary skin fibroblasts were lysed using RIPA buffer (SIGMA-ALDRICH) in the presence of protease inhibitors (Thermo scientific). 35 µg of proteins were loaded on a 3-12% Bis-Tris gel (Invitrogen-Thermo scientific). Proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane and probed with the following antibodies: anti-AARS1 (EPR11037(B)-abcam), anti-GAPDH (SIGMA-ALDRICH), anti-β-Tubulin (Cell Signaling Technology, Netherlands). Specific bands were detected using Lite Ablot Extend Long

Lasting Chemiluminescent Substrate (Euroclone, Pero (Mi), Italy). Densitometry analysis was performed using Quantity One software (BioRad, Hercules, CA, USA).

Yeast Modelling for Individual 4

The yeast strain used in this work, derived from W303-1B (*Mata ade2-1 leu2-3, ura3-1 trp1-1 his3-11, 15 can1-100*), is deleted at *ALAI* locus and expresses a wild type copy of *ALAI* gene cloned in the *URA3* bearing vector pFL38.¹ Strains were grown in synthetic complete (SC) media (0.69% yeast nitrogen base without amino acids, Formedium, UK) supplemented with 1g/l drop-out mix, except amino acids and bases necessary to keep plasmids (i.e. uracil for pFL38 and tryptophan for pFL39).² Media were supplemented with carbon sources at 2% (w/v) (Carlo Erba Reagents), in liquid phase or after solidification with 20 g/L agar (Formedium). The variant was introduced through the DpnI-mediated site directed mutagenesis³ using *ALAI* gene cloned in the pFL39 as template DNA and the modified primers (base changes in bold) as follows:

Fwd: Ala1V666A-GCAAATTAAAGAAA**ACTTACAAGCTTTTTACAAGGAAATTCC**

Rev: Ala1V666A-GGAATTCCTTGTA**AAAAGCTTGTAAGTTTTCTTTAATTGTC**

After mutagenesis, sequence of insert was verified by Sanger sequencing and the pFL39*ala1*^{V662A} mutant construct was then transformed into the *ala1Δ*/pFL38*ALAI* strain⁴ after growth in YPAD medium (1% Yeast extract, 2% Peptone, 40 mg/L adenine base, and 2% glucose). Transformants were selected for the presence of both constructs on solid SC medium lacking tryptophan and uracil. The growth on solid media containing 0.1% 5-fluororotic acid (5-FOA) was analyzed to evaluate yeast viability. Four independent clones from two independent transformation were analyzed; each colony was cultured in SC medium without uracil and tryptophan and 5 µl were then transferred in a medium lacking only tryptophan, to induce the loss of the plasmid carrying the wt*ALAI*, and grown for 24 h. 5 µl of undiluted, 1:10, 1:100 and 1:1000 dilution were spotted on 5-FOA solid medium. Plates were incubated at 28°C and growth was assessed after 48h and 72h.

Supplemental Results:

	I-1	I-2	I-3	I-4	I-5	I-6a	I-6b	I-7	I-8	I-9	I-10
Gender	F	F	F	M	F	F	M	F	M	M	F
Age at Onset	27Y	12M	36M	12Y	1M	0M	0M	3M	4M	1M	3M
Current Age	30Y	12Y	19Y*	14Y	4Y	8M*	6M	7Y	15Y*	8Y	11Y
Microcephaly											
Truncal Hypotonia											
Spastic Tetraparesis											
Extrapyramidal Symptoms											
Developmental Delay											
Developmental Regression											
Encephalopathy											
Seizures											
Ophthalmologic Dysfunction											
Dysphagia											
Failure to thrive											
Hypoalbuminemia											

Figure S1: Clinical overview of the *AARS1* cohort. Individuals I-1 to I-4 represent the late-onset disease phenotype while the remaining seven individuals (I-5 to I-10), represent the early-infantile onset *AARS1*-related disease phenotype. F: female, M: male, Y: year, M: month, *: deceased.

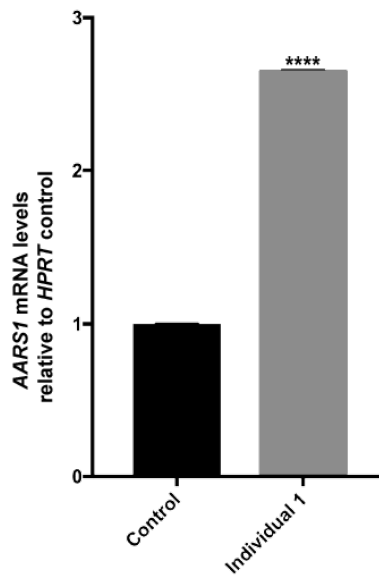


Figure S2: *AARS1* shows increased mRNA levels in fibroblasts from Individual 1. RT-qPCR analysis of *AARS1* mRNA, from patient and age-sex matched controls (n=3). The results are represented as a fold change after normalizing to *HPRT1* mRNA levels (the average of two controls). Each value represents the mean \pm standard error of mean (Student *t*-test, **** $p < 0.0001$).

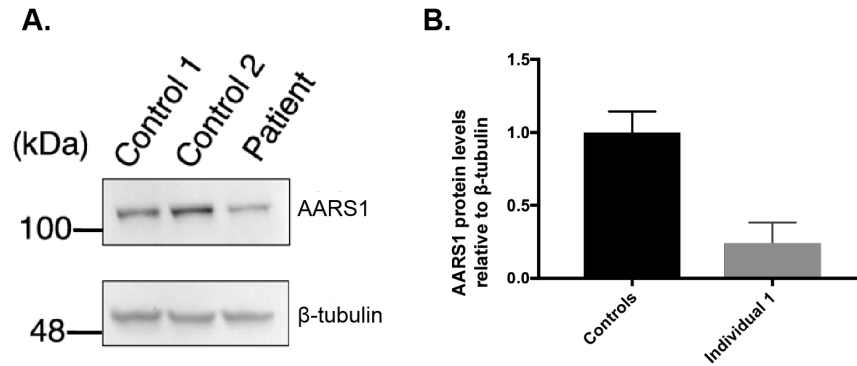


Figure S3: AARS1 protein levels are decreased in fibroblasts from Individual 1. (A) Western blot analysis of patient and age-sex matched control fibroblasts. Lysates were blotted with anti-AARS1 and anti-β-tubulin (β-TUB) antibodies. Molecular mass markers are shown on the left in kilodaltons. (B) Western blot bands from n=3 independent biological replicates were quantified using ImageJ software, normalized over control (average of both controls) and represented as mean ± standard error of mean (Student *t*-test, **p*<0.05).

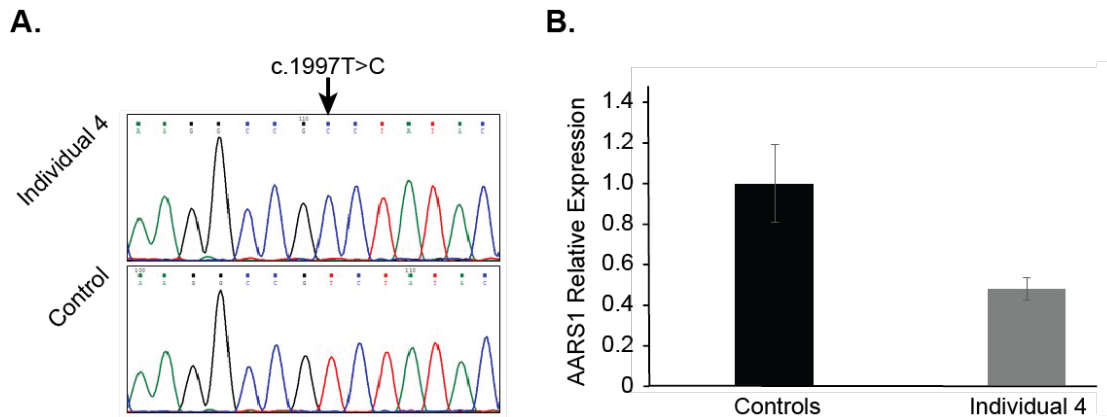


Figure S4: Effect of the deleted allele on AARS1 stability in fibroblasts from Individual 4. (A) Sanger sequencing using primers designed upstream and downstream of the c.1997C>T missense variant revealed the presence of the nucleotide change at a homozygous state confirming the loss of the allele carrying the deletion. (B) RT-qPCR showed a reduction of approximately 50% of *AARS1* transcript level in Individual 4 compared to controls (n=6). The *GUSB* transcript was used as housekeeping gene. Data are presented as a mean±SD of three independent experiments (Student *t*-test, **p*<0.05).

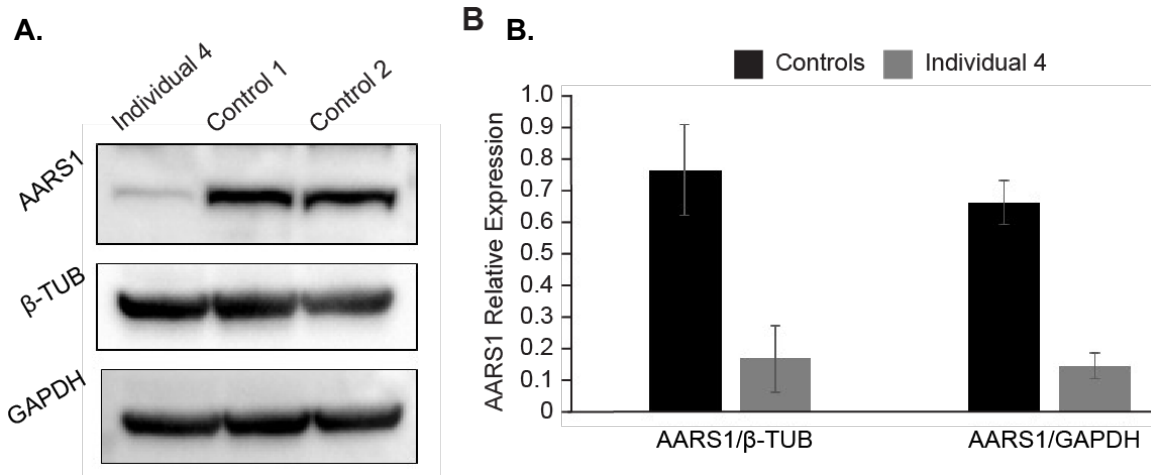


Figure S5: Expression level of AARS1 protein in Individual 4. (A) Western blotting analysis was performed on total homogenate derived from primary skin fibroblasts of the patient and control subjects. (B) AARS1 signal was normalized to either β -tubulin (β -TUB) or GAPDH levels and using a specific antibody against AARS1 showed a marked reduction of about 80% of the protein. Data are presented as a mean \pm SD of three independent experiments (Student t-test, * p <0.05; ** p <0.05).

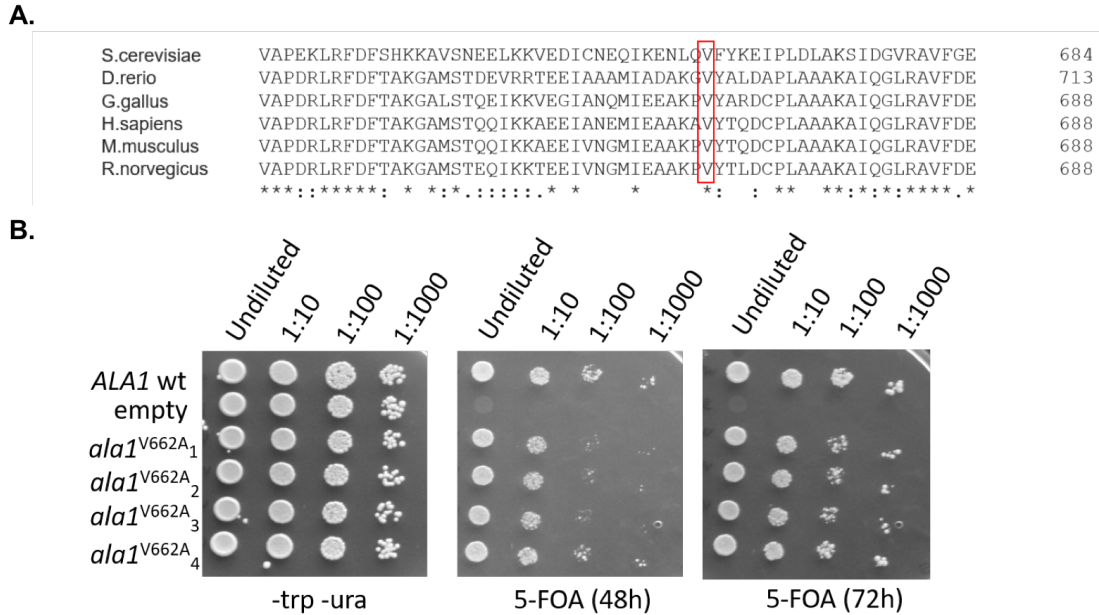


Figure S6: Yeast modelling of the p.Val666Ala variant found in Individual 4. (A) Multiple species protein alignment was generated to assess the conservation of the mutated residue. The human residue Val666 is highly conserved and corresponds to Val662 in the *AARS1* yeast (*S. cerevisiae*) orthologue, *ALAI*. (B) To evaluate the impact of the *AARS1* missense variant p.Val666Ala, we used the yeast *S. cerevisiae* as a model system. The variant was introduced into the *ALAI* gene by site-directed mutagenesis and the ability of the *ala1*^{V662A} mutant allele to complement the deletion of the endogenous *ALAI* was assessed. *ala1* Δ strain carrying a wild type *ALAI* allele on a *URA3* bearing plasmid was transformed with a *TRP1* bearing plasmid carrying wt *ALAI*, *ala1*^{V662A} or no insert. Clones resulting from the transformation were cultured in medium without uracil and tryptophan and 5 μ l were then transferred in a medium lacking only tryptophan. The survival on plates containing 0.1% 5-fluoroorotic acid (5-FOA), which select for cells that have lost the maintenance plasmid, was assessed. After 24 h of growth 5 μ l of undiluted, 1:10, 1:100, and 1:1000 dilution were spotted on SC medium without uracil and tryptophan or 5-FOA. Plates were incubated at 28°C and the growth was scored after 48 and 72 h. wt*ALAI* containing vector supported significant growth on 5-FOA, whereas the empty plasmid did not, being *ALAI* an essential gene. Regarding the Val662Ala variant, the strain expressing the *ala1*^{V662A} allele is able to complement although the growth on 5-FOA is decreased suggesting that it represents a hypomorphic allele affecting only partially *AARS1* activity.

References

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2. Bonneaud N, Ozier-Kalogeropoulos O, Li GY, Labouesse M, Minvielle-Sebastia L, Lacroute F: A family of low and high copy replicative, integrative and single-stranded S. cerevisiae/E. coli shuttle vectors. *Yeast* 1991; **7**: 609-615.
3. Fisher CL, Pei GK: Modification of a PCR-based site-directed mutagenesis method. *Biotechniques* 1997; **23**: 570-571, 574.
4. Gietz RD, Schiestl RH: Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* 2007; **2**: 35-37.