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De-novo and biallelic pathogenic variants in *NARS1* cause neurodevelopmental delay due to dominant negative and partial loss-of-function effect

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Abstract

Aminoacyl-tRNA-synthetases (ARSs) are ubiquitous, ancient enzymes that charge amino-acids to cognate tRNA molecules, the essential first-step of protein translation. Here, we describe 20 families consisting of 31 individuals with *de-novo* heterozygous and biallelic mutations in the asparaginyl-tRNA-synthetase gene (*NARS1*), with a phenotype characterised by microcephaly, neurodevelopmental delay, seizures, peripheral neuropathy and ataxia. *NARS1* mRNA and protein expression from both patient fibroblasts and induced neural progenitor cells (iNPCs) were reduced; *NARS1* enzyme levels in these tissues were also significantly reduced. Yeast complementation assay on the recessive p.Arg545Cys mutation was not detrimental, but molecular modelling of this mutation showed weaker spatial positioning and tRNA selectivity. Zebrafish modelling of the prevalent p.Arg534* *de-novo* mutation gave a severely abnormal locomotor phenotype. Here, we show that *de-novo* and biallelic mutations in *NARS1* are a prevalent cause of abnormal neurodevelopment, where the mechanism for *de-novo* mutations is dominant negative and for recessive mutations partial loss-of-function.

Introduction

The attachment of tRNA to cognate amino acids is essential for the translation of genes into proteins. This conjugation is performed by a group of enzymes, aminoacyl-tRNA synthetases (ARSs), which are ubiquitously expressed, ancient and highly evolutionarily conserved genes¹⁻³. There are 21 proteinogenic amino acids, 20 of them are esterified to cognate tRNA by a designated ARS enzyme. There are 37 genes encoding members of the ARS gene family; 20 of them encode an enzyme that functions in the cytoplasm (17 encode a monofunctional either monomeric or homodimeric ARS, one encodes the bifunctional EPRS, and two encode the heterotetrameric $\alpha_2\beta_2$ FARS1) and 17 encode an enzyme that exclusively functions in the mitochondria. Alternative splicing or translation initiation of the transcript encoding respectively cytosolic *KARS1* and *GARS1* allows synthesis of the mitochondrial isoforms of these enzymes. The missing mitochondrial *QARS* is compensated by the so-called tRNA-dependent transamidation pathway involving mitochondrial *EARS* (*EARS2*) and the GatCAB amidotransferase encoded by the *gat* genes⁴⁻⁶. The most recent ARSs genes to be linked with disease were *FARSA*, *VARS1*, *CARS1* and *TARS1*, where a number of families presented with recessive mutations, associated with neurodevelopmental phenotype and partial loss of these ARS protein function⁷⁻¹¹.

Despite the essential canonical function and ubiquitous expression of ARS enzymes, mutations in ARS genes have been implicated in a variety of human diseases with both recessive and dominant inheritance patterns^{2, 5, 6, 12}. ARS genes have received increased scientific attention recently due to their association with a range of neurological disorders, with phenotypes ranging from mild late-onset peripheral neuropathy to severe, multisystemic developmental syndromes^{5, 6, 13-15}. Mutations in the majority of ARSs (36 out of 37) have been associated with a variety of human disease (**Table S1**).^{2, 5, 6, 12}

Mutations in cytoplasmic ARS-encoding genes mainly lead to neurodegeneration of the peripheral nervous system or brain stem and spinal cord, such as in Charcot-Marie-Tooth neuropathies (*GARS1* [MIM 600287]), *AARS1* [MIM 601065] and brain stem and spinal cord hypomyelination (*DARS1* [MIM 603084]). ARSs and ARSs interacting proteins have been implicated in neurodevelopmental disorders and epilepsies such as *DARS1* [MIM 615281], *RARS1* [MIM 616140], *AIMP1* [MIM 260600], *AARS1* [MIM 601065], and mitochondrial *ARS2* mutations often associated with leukoencephalopathy [*AARS2* [MIM 615889], *DARS2* [MIM 611105] or pontocerebellar hypoplasia [*RARS2*, MIM 611524].

Patterns of inheritance are either dominant, recessive, *de-novo* or in the cases of *AARS1*, *YARS1*, *MARS1*, *HARS1* and *GARS1* both recessive and dominant patterns occur¹². Mutations in *QARS1* [MIM 603727], were identified in two unrelated families characterized by progressive microcephaly, developmental delay, intractable seizures in infancy, diffuse atrophy of the cerebral cortex and cerebellar vermis¹⁶. Functional studies have mainly revealed loss of function due to mutations in ARSs, with decreased aminoacylation efficiency or misfolding, although ARS mutations

have been difficult to functionally interpret. According to the available literature, the vast majority of ARS mutations surveyed lead to at least some degree of loss of primary function, and many appear to lead to increased instability resulting in lower steady state levels. There are a limited number of outliers (in *GARS1*, *YARS1*, and *AARS1*) where it is difficult to ascribe the phenotype to a loss of primary aminoacylation function. Genetic proof of pathogenicity has been the most important in understanding the pathogenicity of ARSs in the past and given the key function of these proteins it is likely that defects in all ARSs will be associated with disease¹². Here, we report on *de-novo* dominant and biallelic, autosomal recessive mutations in the *asparaginyl-tRNA synthetase* gene (*NARS1*) in 20 families and 31 affected individuals. Asparaginyl-tRNA^{Asn} is generated by *NARS1* in a reaction that proceeds in two steps. *NARS1* first catalyses the ATP-dependent activation of asparagine (Asn) into Asn~AMP with the release of pyrophosphate, and then transfers the activated Asn onto tRNA^{Asn} with the release of AMP (**Figure 1A**). We assess herein the genetic proof of these mutations and analyse their impact on patients, using different models (patient cell lines, neural progenitor cell, yeast, zebrafish and molecular modelling).

Results

Clinical characteristics

Table 1 summarizes the core clinical features of the families and individuals with *NARS1* defects and a detailed table is given in **Supplementary section, Tables S2-4 (detailing *de-novo* and recessive mutations)**. Essentially, all affected individuals had GDD and intellectual disability, which varied from moderate to profound in severity. They had marked delays in language development. Motor development was also severely impaired, with one patient never acquiring autonomous ambulation. Microcephaly was observed in the vast majority of cases, and whereas some individuals had primary microcephaly, secondary microcephaly was noted in a proportion. Epilepsy was highly associated with the phenotype, affecting approximately two thirds of cases, with six patients experiencing seizures before 1 year of age. The semiology of these attacks was varied, with a mixture of partial, myoclonic and generalised tonic-clonic seizures described. An ataxic gait, poor balance and dysarthria were frequently detected on examination suggesting an additional neurodegenerative process, however no structural abnormality of the cerebellum was observed on imaging. A demyelinating peripheral neuropathy occurred in several patients presenting with distal leg muscle atrophy, predominantly affecting those with the p.Arg545Cys or *de-novo* mutations. Several dysmorphic features were consistent across cases. These included abnormal hands (e.g., clinodactyly, foetal fingers pad, 2-3 toe syndactyly, slender fingers) and/or feet (e.g., small feet, toe syndactyly, slender feet). Upslanting palpebral fissures were the most common facial dysmorphism reported. Others included a broad forehead, wide mouth, wide set teeth and low set ears with overfolded helices. Skeletal abnormalities including scoliosis, pronounced thoracic kyphosis and *pes-cavus* were also noted. Behavioural traits associated with the phenotype included impulsivity, stereotypies with repetitive speech / hand movements and selective feeding rituals.

Genetic analysis

We identified 20 families and 31 affected individuals with mutations in *NARS1* (See **Figure 1B** for *NARS1* mutation schematic, **Figure 2** for family trees).

Heterozygous *de-novo* variants

Eight families had *de-novo* heterozygous mutations, six had c.1600C>T, p.Arg534* (F1-6; P1-6), one c.1525C>T, p.Gly509Ser (F7; P7) and one with c.965G>T, p.Arg322Leu (F8; P8). These variants were not present in our 652 neuropathologically normal brain series or in GnomAD databases.

Biallelic variants

Biallelic mutations were found in 12 families, one with homozygous c.50C>T, p.Thr17Met (family 17; P26), two siblings with compound heterozygous c.1067A>C, p.Asp356Ala; c.203dupA, p.Met69Aspfs*4

(family 19; P29 and P30), two siblings with homozygous c.32G>C, p.Arg11Pro (family 16; P24 and P25), two siblings with compound heterozygous c.1049T>C, p.Leu350Pro; c.1264G>A;p.Ala422Thr mutations (family 18; P27 and P28), one with compound heterozygous c.268C>T, p.Arg90*; c.394G>T, p.Gly132Cys (family 20, P31) and seven families (families 9-15; P9-23) had homozygous c.1633C>T, Arg545Cys mutations. In ExAC p.Arg545Cys and p.T17M were present as heterozygotes, p.Ala422Thr in six heterozygote individuals. In ExAC p.Met34Leu, p.Met69Aspfs*4 and p.Leu350Pro were absent, p.Arg11Pro was present in one individual. The c.1067A>C, p.Asp356Ala variant in family 19 was present in 122 heterozygotes, suggesting this mutation may modify the phenotype and only be pathogenic when associated with a severe mutation, such as c.203dupA, p.Met69Aspfs*4 mutation.

Genotype-phenotype correlations

Individuals with *de-novo* mutations have a more severe phenotype. The majority of cases presented with an earlier onset and severe microcephaly, (head circumference more than 3 SDs below the mean). Epilepsy was observed in all but one case, and all individuals exhibited evidence of ataxia on clinical examination. Peripheral neuropathy was also common in this sub-group of patients and all patients with *de-novo* mutations had a high degree of intellectual disability which varied from severe to profound. Family 16 with the homozygous mutation p.Arg11Pro also produced a particularly severe clinical picture with severe developmental delay, seizures, microcephaly and atrophy on MRI (**Supplementary data, S2-4 and Figure 3**).

Patients homozygous for p.Arg545Cys demonstrated hypotonia and, predominantly distal, weakness. Spasticity was more frequently observed in patients with the p.Arg11Pro or *de-novo* mutations. A demyelinating polyneuropathy was documented in a number of cases and, in one case (family 9, P9 and 10), this was confirmed with a sural nerve biopsy.

MRI was unremarkable for a common structural change across all cases. On the whole, imaging was normal apart from microcephaly. In family 6, with *de-novo* p.Arg534* variant, mild atrophy was seen and severe atrophy and delayed myelination was seen in family 16, (P24 and P25) that had a particularly severe phenotype (**Supplementary data, S2-4 and Figure 3**).

Pathogenicity of *NARS1* mutations

The *NARS1* gene is constrained (intolerant to loss of function), which is not surprising given the important function, pLI=0, o/e for missense variants Z=0.87 with Z=0.83, to missense variation in *NARS1*. In the gnomAD database of >100,000 sequenced individuals only two homozygous missense variants were seen, one at p.Ile397Val and one at p.Phe131Val. We identified *de-novo* *NARS1* mutations in eight families, with similar phenotypes. Six families presented the same mutation at codon 534. The two other *de-novo* variants were located at codon 322 and 509. The p.Arg534* hotspot mutation is located 15 AAs from the end of the 548 AAs protein, the location of a number of *NARS1* likely pathogenic mutations. The amino acids Arg534 and Arg545 are universally conserved in *NARS1* genes from all three major taxonomic, suggesting that they play a significant structural or functional role.

The homozygous p.Arg545Cys mutation was found recurrently in seven recessive families and affects the same C-terminal catalytic stretch as p.Arg534*. Given its proximity to residue 534, p.Arg545Cys may have a comparable mechanistic effect to p.Arg534*.

The p.Asp356Ala variant was found in *trans* with the only recessive truncating allele observed thus far at c.203dupA; p.Met69Aspfs*4 (P29 and P30). Two missense mutations (p.Arg322Leu and p.Asn218Ser) were found in a compound heterozygous state in P8, but p.Asn218Ser appears to have allelic frequencies that would be unlikely to be associated with a severe phenotype. The p.Arg322 residue is essential for enzymatic activity and it would thus be predicted to have loss of normal enzyme activity (Pr. H. Becker, personal communication). Both the p.Thr17Met and p.Arg11Pro mutations are in the N-terminal UNE-N appended domain of *NARS1*, specific to eukaryotes, and that was only very recently shown to have a chemokine¹⁷. Western blot analysis revealed reduced protein levels for these cases (**Figure 4B**).

Functional characterisation

Yeast complementation assays

These experiments showed that a yeast strain carrying an *nrs2* deletion was well complemented by the p.Arg545Cys mutation. Given the increased growth, it would therefore fit with a conformational effect present in higher species that would only change the code here likely by tRNA interaction (**Figure 4**).

Induced Neural progenitor cells

Trans-differentiation was performed for fibroblasts cultured from patient lines using Sendai virus treatment followed by culturing in neural progenitor cell (NPC) induction media, to produce induced NPCs (iNPCs) (**Figure 5**). iNPC colonies were produced and isolated from fibroblasts from P26 (p.Thr17Met) and P29/30 (p.Asn356Ala; p.Met69Aspfs*4). From the isolated colonies gene expression was determined by qPCR to select iNPC populations which presented decreased expression of fibroblast markers *col1a1*, *col3a1*, *twist2* and *DKK3*, as well as increased NPC markers *nestin*, *sox1* and *MSI-1*, and the iNPC population was expanded to be subsequently used for RNA sequencing (RNAseq). RNAseq showed normal *NARS1* expression in iNPCs from patients carrying the p.Thr17Met and p.Asp356Ala mutations, and decreased expression of the p.Met69Aspfs*4 *NARS1* allele in the P11 cells. Interestingly, iNPCs from patients show increased expression of several other ARSs (*DARS1*, *GARS1*, *RARS1*, *SARS1*, *TARS1*, *WARS1*, *YARS1*). This could be explained by the fact that the *NARS1* mutant(s) are inducing the Integrated Stress Response (ISR), which activated a number of ARS genes. Analysis of the cellular pathways (using ConsensusPath database) associated with genes with significantly altered mRNA levels showed that upregulated genes were involved in pathways heavily associated with protein translation and processing such as ER and Golgi protein processing and ribosomal homeostasis, which is consistent with other studies showing reduced aminoacylation activity resulting from disease-associated mutations in cytosolic ARSs ⁶. In addition, increased action of VEGFR1/2 (upregulated by ATF4, which is one of the key transcription factors in the ISR) was suggested, which may be of interest considering the reported actions of other ARSs, as in GARS and EPRS via GAIT complex, SARS1, TARS1 and mini WARS1 on VEGF-related signalling ¹⁸. Pathways associated with downregulated genes typically were associated with cell cycle progression and DNA repair and replication such as G2/M checkpoint, homology directed repair and telomere maintenance pathways, may be a reflection of the alteration of cellular proliferation resulting from decreased protein synthesis (**Figures S5-10**).

Blue-native polyacrylamide gel electrophoresis (BN-PAGE)

This experiment identified the amount of the *NARS1* dimer complex, and showed that P26 (p.Thr17Met) and P29 (p.Asn356Ala and p.Met69Aspfs*4) had severely reduced dimer formation compared to controls (**Figure 5E**). Interestingly, the patient's unaffected parents carrying one heterozygous mutation each also appeared to show decreased level of the *NARS1* dimer. P9 (p.Arg545Cys) showed comparable *NARS1* dimer amount to healthy controls. Similar to most other disease-associated ARSs, *NARS1* functions as a class II homodimer. ¹⁹ The decreased *NARS1* dimer expression observed in fibroblasts from P26 and P29 shown by BN-PAGE accounts for the apparent deficit in aminoacylation capacity, despite finding no consistent decrease in *NARS1* monomer expression. This is further supported by our molecular simulation (**Figures 7**) that predicts an unstable dimer for p.Asn356Ala mutant, since this substitution is located at the interface between the two *NARS1* monomers.

Western blotting and RT-PCR

We show by semi-quantitative RT-PCR and Western blotting of *NARS1* from lymphoblasts and fibroblasts from families with p.Arg545Cys, p.Thr17Met, p.Asp356Ala; p.Met69Aspfs*4, p.Arg11Pro, that *NARS1* expression is reduced (**Figure 4A-E**).

Zebrafish experiments

Microinjection of 225pg of capped *in vitro* transcribed RNA encoding WT human *NARS1* rarely resulted in morphological abnormalities (3.2%) the same dose of Arg534* RNA generated animals with severe truncation of the body axis and a single cyclopic eye with a much greater frequency (31.0%) than WT ($p < 0.0001$, Fisher exact test) (**Figure 6A**). At a dose of 450pg, microinjection of WT or Arg534* RNA induced these defects in 25.0% or 60.9% of larvae, respectively ($p < 0.0189$, Fisher exact test). Notably, these same phenotypes have previously been associated with mutations in other microcephaly genes, *ORC1* and *PLK4*, when modelled in zebrafish by RNA microinjection²⁰. While we noted a trend toward body length reduction when mutant RNA was injected, this was not statistically significant. We therefore conclude that expression of Arg534* mutant *NARS1* in zebrafish causes dose-dependent cyclopia and body axis reduction, acting through a dominant-negative effect²¹.

ARS enzymatic assays

These experiments showed a reduction of *NARS1* enzymatic activity in patient-derived fibroblasts and lymphoblasts with *NARS1* variants. The most dramatic decrease was observed for P2 (carrying the *de-novo* p.Arg534* variant) and the mildest decrease was observed for P24 (homozygous for the p.Arg11Pro) (80% of the controls). Amino acid residue Arg11 is located in the 5' end of the non-canonical UNE-N domain (**Figures 8, S11**).

Discussion

Here, we report the identification of *de-novo* heterozygous and biallelic mutations in the cytoplasmic asparaginyl-tRNA-synthetase (*NARS1*) gene which are associated with a neurodevelopmental phenotype in 31 patients derived from 20 families. Within the last 40AA of *NARS1* several mutations were identified: a *de-novo* hotspot and homozygous recessive mutations at amino acid residues p.Arg534*, p.Gly509 and p.Arg545 respectively. In the first few amino acids of the gene two homozygous mutations were identified at p.Arg11 and p.Thr17, with both having severe clinical phenotype. Other mutations in *NARS1* were spread throughout and not clustered in any particular region of the gene. Interestingly the clinical phenotype correlates with the reduced expression where tissue was available (such as the p.Arg11Pro and p.Thr17Met), and might correlate with reduced protein stability, as suggested by structural modelling of the p.Arg545Cys. This is also the case for the MRI imaging which show atrophy and white matter abnormalities in the patients carrying the variants p.Arg11Pro and p.Thr17Met but normal MRI in the p.Arg545Cys patients. The N- and C-terminus region mutation numbers, cluster and phenotypes suggests these are functionally important regions of the gene that likely disrupt the protein homodimer and ATP-binding/catalytic domain in *NARS1*. These two mutations gave elevated *NARS1* enzyme activity, which attributed to their location in the N-terminal extension domain. This domain has additional non-translational functions which enables enzymatic activity of the modified protein. By examining protein expression, protein synthesis and the aminoacylation activity, it is clear that the non-translational functions of such ARS proteins, regulated by the newly-evolved appended domains such as UNE-N, seem not specifically necessary for ARS activity (**Figures 8, S11**).

The functional data on *NARS1*, based on fibroblasts and iNPCs examined, suggests that mutations cause loss of the enzymatic protein by reduced expression and disruption of dimer formation in the majority of mutations, with increased expression of other ARSs. Modelling the *de-novo* p.Arg534* in zebrafish phenotype gave an abnormal phenotype associated with the clinical picture (**Figure 6**). We reasoned that this mutation may compromise *NARS1* activities dependent on a critical C-terminus, or it may cause a gain of function or dominant negative (*i.e.* interfering with the function of the normal allele/protein). Given that it is located in the last exon, transcripts encoding the recurrent Arg534* mutation are predicted to escape nonsense-mediated mRNA decay, and the encoded protein is only truncated by 15 AAs. This mutation is present in the ATP-binding/catalytic domain that would seemingly be critical for *NARS1* enzymatic function. We therefore predicted that this mutation may act through either a dominant-negative mechanism, and to this end we hypothesised that overexpression of this

mutation in zebrafish embryos may cause abnormal phenotypes with a higher incidence than the matched wild-type (WT) allele.

The common homozygous p.Arg545Cys mutation in the yeast models was difficult to interpret as this showed an increased growth that would suggest a gain in function mechanism, but the protein modelling with the p.Arg545Cys mutation with loss of the helix linker suggests reduced tRNA interaction and catalytic activity (**Figure 7**). Potentially, the effect could be much more harmful for cells of the nervous system than a unicellular organism. One of the possibilities is that the *NARS1* mutant mischarges a tRNA in the human cells that might be less conserved in fungi and thus the mischarging would be of a much lesser extent in yeast, hence the better growth without side effects. Molecular modelling of the p.Arg545Cys mutation lies within a region that probably interacts with the sugar-phosphate backbone of the tRNA (at positions 68-69) close to the active site of the enzyme. Replacing the bulky arginine with a cysteine does not seem to perturb the enzyme overall structure as assessed by molecular modelling. However, by affecting the tRNA-enzyme contact, this mutation may alter the enzyme selectivity towards its tRNA and therefore could reduce the overall affinity for tRNA. Furthermore, *NARS1* enzyme activity for patients homozygous for this variant (P9 and P20) showed a decreased *NARS1* activity.

We thus propose that genotypes with biallelic loss of function alleles would be embryonic lethal, given the essential function of *NARS1*, genotypes with single *de-novo* mutations cause a dominant negative effect and biallelic missense or missense and nonsense/out of frame deletion/insertion mutations cause partial loss of function. Similar effects are seen in Aicardi-Goutières syndrome for example, caused by ADAR mutations. In each case there are relatively frequent alleles that are pathogenic when *in trans* to a null, but never found in patients at the homozygous state²². The only exception is for the distal C-terminus mutations such as the homozygous p.Arg545Cys mutation that structurally has a milder phenotype where the mechanism is likely due to abnormal protein structure and catalytic activity (**Figures 1C, 7, S1, S2**).

The spectrum of disease symptoms and signs is widespread in the central nervous system and peripheral nervous system as affected individuals had a broad neurodevelopmental phenotype characterized by motor and intellectual developmental delay, microcephaly, ataxia, neuropathy and seizures. This is likely due to a broad tissue expression throughout the body but high expression in the cortex, cerebellum and brainstem in mouse brain may reflect the more severe neurological picture^{23, 24} (**Figure S3**). Mutations have been reported for the majority of ARSs (35 out of 37). In addition, aminoacyl-tRNA synthetase interacting multifunctional proteins 1-3 (AIMP1-3) that participate together with nine cytosolic ARSs are constituent of the so-called multi-synthetase complex, and have also been associated with a variety of human diseases²⁵. Almost certainly, with time all ARSs are likely to have a disease association, given their critical role¹³⁻¹⁵. The *NARS1* data brings the number to 35 out of 37 and places *NARS1* in close proximity on a modified Taylor's Venn diagram of amino acid (AA) properties with other AA with similar properties (*IARS1, LARS1, DARS1, EPRS1, NARS1, RARS1, QARS1*) and interestingly these also have more severe phenotypes²⁶ (**Figure S4**). The functional work that we have carried out has helped to understand the role of *NARS1* mutations in disease but developing CRISPR/Cas9 animal models will be the next important step in understanding the molecular rationale of these *NARS1* mutations. In addition, the mutations identified here and the numbers of patients, could add *NARS1* to genetic testing panels for children and young adults presenting with NDD, epilepsy and/or a demyelinating neuropathy.

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Subjects and Methods

Study participants

Samples were taken with informed consent from each institution using local IRB ethics for functional analysis of human DNA and biomaterial. Individuals with likely pathogenic *NARS1* variants were ascertained via an international collaborative network of research and diagnostic sequencing laboratories. Samples were collected from 18 families and 27 affected individuals. Phenotypic data were collected from a detailed review of medical records, phone interviews, clinical re-evaluation and photographs. All participants provided informed consent to local Research Ethics Committees from all the participating institutes approved the study. See **Supplementary Table 2** for clinical details and countries that referred cases.

Sequencing

Exome sequencing was carried out using a number of methods in different centres with different analysis platforms and pipelines used. The majority of cases were sequenced based on the Nextera Rapid Capture Exome kit (Illumina) and sequenced on the HiSeq 2500 platform (Illumina). The resulting 100 bp paired-end sequence reads were mapped against the human reference genome assembly 19 (GRCh37) with the Burrows-Wheeler Aligner package²⁷ and read duplicates were removed with Picard (<http://broadinstitute.github.io/picard/>). Variant calling and indel realignments were performed with the Genome Analysis Toolkit (GATK)²⁸ and variants were submitted to ANNOVAR for annotation²⁹. For Families 17 and 19, Agilent sequence capture was used, as described elsewhere³⁰. For families sequenced at GeneDx (families 5, 6, 8, 10), genomic DNA was extracted from the proband and parents (when available). The exonic regions and flanking splice junctions of the genome were captured using the Clinical Research Exome kit (Agilent Technologies, Santa Clara, CA) or the IDT xGen Exome Research Panel v1.0. Massively parallel (NextGen) sequencing was done on an Illumina system with 100bp or greater paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19, and analyzed for sequence variants using a custom-developed analysis tool. Additional sequencing technology and variant interpretation protocol has been previously described³¹. The general assertion criteria for variant classification are publicly available on the GeneDx ClinVar submission page (<http://www.ncbi.nlm.nih.gov/clinvar/submitters/26957/>)

Bioinformatic analysis

cDNA and protein sequence variants are described in accordance with the recommendations of the Human Genome Variation Society (<http://www.hgvs.org>) using Ensembl ENSG00000134440; ENST00000256854.10 as the reference sequences (<http://www.ensembl.org/>). Evolutionary conservation of nucleotides was assessed using PhyloP (46 vertebrate species) and GERP scores, which were accessed through the UCSC Genome Browser

(<https://genome.ucsc.edu/>) using genomic coordinates from GRCh37/hg19. Grantham scores were used to assess the physicochemical nature of the amino acid substitutions. *In silico* analyses of sequence variants were performed using the following pathogenicity prediction tools: SIFT (<http://sift.icvi.org/>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and Mutation Taster version 2 (<http://www.mutationtaster.org/>). All the candidate variants were further verified by Sanger sequencing.

Generation of the *nrs1* vector

pJR1-41XU-*nrs1*. Vectors to express the *Schizosaccharomyces pombe nrs1* gene were generated by amplifying the coding sequence of *nrs1* from *S. pombe* DNA with the Nrs1-PJR-F and Nrs1-PJR-R primers using Phusion HF polymerase from NEB. The PCR product was cloned into XhoI digested pJR1-41XU³² using CloneEZ from Genscript. Plasmids were sequenced to confirm the correct insertion of the fragment.

Deletion of *nrs1* gene in *S. pombe* cells

Since *nrs1* is an essential gene, we first transformed cells with the plasmid expressing *nrs1* in order to delete *nrs1* in the genome. JB775 (*h- ade6-M216 ura4-D18 leu1-32*) cells were synchronized, made competent, and transformed as described³³. Cells were transformed with the plasmid containing the *nrs1* gene, pJR1-41XU-*nrs1*, and transformants were selected by growth in EMM + ade + leu, generating the strain MR397. The *nrs1* gene was deleted in MR397 cells with the standard method by homologous recombination with the NatMx6 cassette^{34, 35}, using the primers Nrs1DelFw and Nrs1DelRv. Transformants were selected in EMM + Nat with no thiamine to promote the expression of the *nrs1* gene from the plasmid. Deletions were checked by PCR using primers Nrs1ck-L and kanR and Nrs1ck-R and kanF. The strain generated was named MR409. MR409 cells were synchronized, made competent, and transformed as described³³, with the plasmids of the pJR-41XL series containing either the empty vector, the *NARS1* wild-type gene, or the mutations described. Transformants were selected in EMM + ade. Strains and primers are provided in Supplementary data.

Yeast complementation assay of *NARS1* gene

The experiments were performed in an analogous way as described³⁶; in brief: cells were grown on EMM + ade+ ura to saturation (for 24 hours), to allow for the loss of the pJR-41XU-*nrs1* plasmid. After this time, cell density was adjusted to 4×10^6 cells /ml, and five-fold dilutions were spotted onto EMM + ade + ura plates or EMM + ade + $\frac{1}{4}$ ura + FOA plates to select for cells that have lost the plasmid.

Zebrafish experiments

Zebrafish (*Danio rerio*) embryos were obtained from a wild-type strain and were raised at 28.5 °C. Microinjections (~1 nl) were performed at the one-cell stage. The expression vector used was *NARS1* (NM_004539) Human cDNA Clone (OriGene) cloned into pCS2+ (addgene) using CloneEZ from Genscript as described above for the yeast studies. Plasmids were sequenced to confirm the correct insertion of the fragment. Mutant versions of this construct were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The sequences for all primers are available upon request. Capped RNA was synthesized using the mMACHINE kit (Ambion). Live zebrafish were imaged on a Leica MZFL III stereo microscope, and body surface area was calculated using IPLab software (Biovision). All experiments with zebrafish were performed in compliance with local ethical and Home Office (UK) regulations, project license 70/6875.

Cell culture

Fibroblasts of affected individuals carrying the homozygous p.Thr17Met, p.Arg11Pro, p.R545C, compound heterozygous, p.Asp356Ala, p.Met69Aspfs*, and corresponding controls were grown in high-glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin.

Semi-quantitative RT-PCR for patient lymphoblasts

Total RNA was extracted from immortalized lymphoblasts available from P2 and parents using TRIzol, as per manufacturer's instructions. The concentration and purity of RNA was determined spectrophotometrically. 1 µg of RNA was reverse transcribed to first strand cDNA by using random primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Primers used for *NARS1* were Forward– 5' GCGTTAGAAGGATATAGAGGCCA 3' Reverse– 5' ACCATCTCGCAACACCAGAAA 3'. Primers used for GAPDH were: Forward– 5' TGTGGGCATCAATGGATTTGG 3', Reverse – 5' ACACCATGTATTCCGGGTCAAT 3'. GoTaq® Green Master Mix was used and PCR reactions were performed with the following protocol: 95°C-2 min, (95°C-30 s, 60°C-30 s, 73°C-1 min) for 35 cycles, 73°C-5 min, and 4°C hold. Two exponential curves representing the product formation was made for both primer pairs and cycles 28 and 29 were chosen for *NARS1* and GAPDH genes respectively so that amplification rates were in the linear range for semi-quantitative comparisons.

Western blotting

For western blotting analysis, protein lysates were obtained from cultured fibroblasts and total protein concentration was measured by means of Bradford assay. Aliquots of total protein (15 µg) were loaded on 4%–12% SDS-polyacrylamide gels (NuPAGE 4%–12% Bis-Tris Protein Gels, ThermoFisher Scientific), transferred to polyvinylidene fluoride membranes, blocked and incubated overnight with a polyclonal antibody recognizing *NARS1* (anti-rabbit 1:1000; Proteintech). Secondary antibody was added for 1h and signal was detected using ECL reagents (Amersham Biosciences). Anti-beta-actin antibody (Sigma Aldrich, A3853; 1 in 5000) was used as a loading control.

Blue native polyacrylamide gel electrophoresis (BN-PAGE)

Fibroblast pellets were lysed using 10mM Tris (pH 8), 150mM NaCl, 0.1% NP40 with physical agitation for 30 minutes, centrifuged at 8,000 xg to remove debris. The supernatant was removed and total protein was quantified with the Bradford assay. Protein concentrations were equalised and prepared to 20ul at 1µg/µl with NativePAGE™ sample buffer (Thermo) and 1ul of NuPAGE 5% G-250 Sample Additive (Thermo) and loaded to a NativePAGE 3-12% Bis-Tris Protein Gel (Thermo). Proteins were transferred to PVDF membrane using an iBlot2 PVDF Mini transfer stack (ThermoFisher Scientific) and probed with anti-*NARS1* monoclonal antibody (Abcam ab129162, 1:5000) and GAPDH (Santa Cruz).

iNPC conversion

iNPCs were generated based on the protocol published by Meyer *et al* (*J Vis Exp*, 2014) from primary fibroblasts by transduction with *Oct4*-, *Klf4* and *c-Myc*-Sendai virus, followed by culturing in NPC induction media (1:1 DMEM/F-12: Neurobasal, 2x N2, 2x B27, 1% GlutaMAX, 10ng/ml hLIF, 3µM CHIR99021, 2 µM SB431542). Following 3-4 weeks of culturing, neuroepithelial colonies form and these were isolated and expanded before total RNA was extracted from patient and control fibroblasts and iNPCs cells using the mirVana™ miRNA Isolation Kit (Ambion) for gene expression analysis by qPCR and RNAseq.

qPCR

Cell pellets from patient fibroblasts and iNPCs were lysed by Trizol reagent and, following addition of chloroform, the aqueous phase was transferred to RNeasy spin column (QIAGEN) for RNA isolation and resuspension. cDNA was generated using the reverse transcriptase kit (Applied Biosystems) and qPCR (Applied Biosystems 7900HT) was performed in triplicates using SYBR Green PCR Master Mix (Invitrogen, 4309155). Samples were normalised to expression of GAPDH and β-actin.

RNAseq

Libraries were prepared using Illumina TruSeq Stranded Total RNA with Ribo-Zero Human kit and were sequenced on an Illumina HiSeq 2500 using paired-end protocol. Quality of sequencing reads was

ensured using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were aligned using STAR aligner and variants were called using the 2-pass protocol outlined in the GATK documentation (<https://software.broadinstitute.org/gatk/>). The numbers of reads were counted using HTSeq-count (Anders, 2015). Differentially expressed genes were identified using the DESeq2 Bioconductor package (Love, 2014). Differentially expressed genes with false discovery rate of ≤ 0.1 and a $\log_2(\text{fold change}) \geq 1$ were considered as significant. Gene set enrichment analysis for pathway analysis was performed using the CPDB web tool (<http://cpdb.molgen.mpg.de/>)

NARS1 enzyme assay

Aminoacylation was assessed by measuring *NARS1* activity in cultured fibroblasts and lymphoblasts. Cell lysates (cytosolic fraction) were incubated in triplicate at 37°C for 10 minutes in a reaction buffer containing 50 mmol/L Tris buffer pH 7.5, 12 mmol/L MgCl₂, 25 mmol/L KCl, 1 mg/mL bovine serum albumin, 0.5 mmol/L spermine, 1 mmol/L ATP, 0.2 mmol/L yeast total tRNA, 1 mmol/L dithiothreitol, 0.3 mmol/L [¹⁵N₂]-asparagine, [¹³C₄,¹⁵N]-threonine, [D₂]-glycine, [¹⁵N₂]-arginine and [D₄]-lysine. The reaction was terminated using trichloroacetic acid. After sample washing with trichloroacetic acid, ammonia was added to release the labelled amino acids from the tRNAs. [¹³C₂,¹⁵N]-glycine and [¹³C₆]-arginine were added as internal standards and the labelled amino acids were quantified by LC-MS/MS. Intra-assay variation was <15%. Threonyl-tRNA synthetase (*TARS1*), glycyl-tRNA synthetase (*GARS1*), arginyl-tRNA synthetase (*RARS1*) and lysyl-tRNA synthetase (*KARS1*) activity were simultaneously detected as control enzymes.

Molecular modelling analysis

The crystal structure of the *Brugia malayi* *NARS1* protein with 65% of identity to human *NARS1* sequence stored under the 2XGT code in the Protein Data Bank (<http://www.rcsb.org/>) was used for molecular modeling studies. Then the homology model of dimeric human *NARS1* was overlapped with *S. cerevisiae* DARS-tRNA^{Asp} ligase co-crystallized with tRNA molecule (PDB 4WJ4), that has similar domain organization and shares 27.5% of sequence identity with the human *NARS1*. The protein was visualized with the University of California–San Francisco Chimera software.³⁷

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Disclosures

None except Lindsay B. Henderson, Richard E. Person, Yue Si, Aida Telegrafi, are employees of GeneDx, Inc.

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Variant; Nucleotide, Protein	c.1600C>T, p.Arg534*	c.1525C>T, p.Gly509Ser	c.965G>T, p.Arg322Leu	c.1633C>T, p.Arg545Cys	c.32G>C, p.Arg11Pro	c.50C>T p.Thr17Met	c.1049T>C c.1264G>A, p.Leu350Pro p.Ala422Thr	c.1067A>C c.203dupA, p.Asp356Ala p.Met69Aspfs*	c.268 C>T c.394G>T, p.Arg90* p.Gly132Cys
Variant type	<i>De-novo</i> Heterozygous	<i>De-novo</i> Heterozygous	<i>De-novo</i> Heterozygous	Homozygous	Homozygous	Homozygous	Compound Heterozygous	Compound Heterozygous	Compound Heterozygous
Inheritance	AD <i>De-novo</i>	AD <i>De-novo</i>	AD <i>De-novo</i>	AR	AR	AR	AR	AR	AR
Family	1-6	7	8	9-15	16	17	18	19	20
Affected Individual(s)	1-6	7	8	9-23	24-25	26	27-28	29-30	32
Ethnicity	European	European/ Native- American	European	Pakistan / North India	Kosovo	Libya	German	Turkey	Canada
Age at onset	Birth	Birth	Birth	Childhood	Childhood	Birth	Birth	Birth	Birth
Consanguinity	No	No	No	Yes	Probable	Yes	No	No	No
Presentation	Severe NDD	Severe NDD	Severe NDD	Severe NDD	Seizures	Seizures	Mod NDD	Mod NDD	Severe NDD
ID	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Microcephaly	Yes	No	NA	Yes	Yes	Yes	Yes	Yes	Yes
Dysmorphic	Yes	Yes	Yes	Yes	Yes	NA	No	No	Yes
Seizures Affected Individuals	Yes All patients	Yes	Yes	Yes 9,14,15,18,19,21,22,23	Yes All patients	Yes	Yes 23	Yes All patients	Yes
Spasticity Affected Individuals	Yes 3,4,6	No	Yes	No Hypotonia	Yes All patients	NA	Yes	NA	No Hypotonia
Neuropathy Affected Individuals	Yes 1, 2, 5	NA	No	Yes 9,10,20	NA	NA	Yes	NA	NA
Ataxia Affected Individuals	Yes All patients	NA	Yes	Yes 9,10,11,12,21	NA	NA	Yes	NA	

Table 1. Summary of *NARS1* variants and clinical features of affected individuals. NDD = neurodevelopment delay, ID = intellectual disability AD = autosomal dominant, AR = autosomal recessive, Mod = moderate, NA = not available.

Figure legends

Figure 1. *NARS1* protein structure and function. **a.** Amino acid asparagine (Asn) is ligated to tRNA^{Asn}, catalysed by *NARS1* and ATP to produce Asn-tRNA (Asn), AMP and pyrophosphate. **b.** Schematic representation of human *NARS1* primary structure. Three main domains are depicted, the unique domain of *NARS1* (UNE-N), the anticodon binding domain (ABD) and the catalytic domain (ABD). The nature and position of the mutants are shown above the primary structure, de-novo boxed in red, while the position of the domains are indicated below including motif 1 (involved in *NARS1* dimerization) and motifs 2&3 that form the active site. **c.** *NARS1* mutations and their predicted functional effect.

Figure 2. Family trees of the 20 families and 31 affected individuals identified in this study with *de-novo* and biallelic mutations in *NARS1*. Filled symbols represent affected individuals and double bars represent consanguinity in the family.

Figure 3. Radiological findings of patients in our cohort. **Set 1:** Case of homozygous p.R11P mutation. Upper row images [coronal T2-WI (1a) and axial T1-WI (1b)] at the age of 10 months show severely delayed myelination and fronto-temporal atrophy. Lower row images [axial T2-WI (1c) and axial T1-WI (1d)] repeated at the age of 18 months shows progressive and global brain atrophy with an emerging pattern of severe hypomyelination. **Set 2:** A further case of homozygous p.R11P mutation. Upper row images [axial T2-WI (2a) and axial T1-WI (2b)] at the age of 8 months show mild fronto-temporal underdevelopment and severely delayed myelination. Lower row images [axial T2-WI (2c) and axial T1-WI (2d)] repeated at the age of 2 years shows progressive and global brain atrophy along with severe hypomyelination. **Set 3:** Case of homozygous p.Thr17Met mutation. Axial FLAIR images at the age of 9 months shows global atrophy involving the cerebral and cerebellar hemispheres along with severe hypomyelination. **Set 4:** MRI images of an individual with the homozygous p.Arg545Cys mutation. Coronal T1-WI (4a), axial T2-WI (4b) and sagittal T1-WI (4c) at the age of 4 years, coronal T1-WI (4d), axial T2-WI (4e) and sagittal T1-WI (4f) at the age of 11 years as well as coronal T2-WI (4g), axial FLAIR (4h) and sagittal T2-WI (4i) at the age of 20 years demonstrating normal intracranial appearances across the 3 different ages. This patient had an upper thoracic scoliosis which was operatively corrected at the age of 4 demonstrated on the sagittal T2-WI of the spine (4j) and frontal projection radiograph of the chest/thoracic spine (4k).

Figure 4. Protein levels of *NARS1* are clearly reduced in patient-derived cells. **a.** RT-PCR, **b.** Western blotting and **c.** quantification graph of *NARS1* patients with mutations compared with controls. Ctrl = control, P7 = homozygous p.Arg545Cys, P9 = homozygous p.Thr17Met, P11 = p.Asp356Ala; p.Met69Aspfs*4, P17 = homozygous p.Arg11Pro. **d.** Immunoblot analysis in three independent controls as well as one fibroblast cell line with homozygous p.Arg11Pro. Western-blotting was performed using a rabbit anti *NARS1* antibody (1:1000; Proteintech). As loading control we used a mouse anti GAPDH antibody (1:4000; Invitrogen). **e.** Quantitative data of immunoblot analyses confirming the results depicted above. **F-h. Human *NARS1* gene is able to complement fission yeast *nrs1*.** Schematic of the yeast complementation assay. Fission yeast cells containing a plasmid expressing *nrs1* gene with uracil selectable marker whose genomic copy of the *nrs1* is deleted are transformed with plasmids containing the different variants of the human *NARS1* gene. These cells are promoted to lose the *nrs1* gene plasmids by incubating them in media with uracil for 24 hours. Five-fold serial dilutions of 2 different colonies of strains containing the different variants of *NARS1* gene wt, mutA-G (p.Arg545Cys), the empty vector and wt cells were plated in media containing uracil, or media containing FOA, which allows the growth of only those cells that have lost the yeast *nrs1* plasmid and whose *NARS1* variant is able to complement *nrs1D*.

Figure 5. BN-PAGE and iNPC RNA-seq. **a.** iNPC populations from P26 (p.Thr17Met) and P29 (c.203dupA, p.Met69Aspfs*4; c.1067A>C, p.Asp356Ala) exhibit increased expression of most iNPC markers (sox1, sox2, nestin, snail1, pax6, DKK3, twist2, Musashi-1) compared to fibroblast (fbb) as measured by qPCR, shown with hierarchical clustering. **b.** Heatmap with hierarchical clustering generated using all gene counts from RNAseq distinction of control (Ctrl1 a-c, Ctrl2 a-c) and patient-derived (P26 a-b, P29 a-c) iNPCs. **c.** Volcano plot showing log₂ of fold change in NARS mutant iNPCs compared to controls and -log₁₀ (adjusted p value). **d.** BN-PAGE-western blot showing reduced expression of the NARS1 dimer in patients P26, P29 and fathers compared to control, but not for patient P10. **e.** Quantification of BN-PAGE-western blot NARS1 dimer expression, showing significantly reduced expression of the NARS1 in patients P26, P29 and fathers compared to control but not change for P10. P26=homozygous c.50C>T, p.Thr17Met, P29=c.203dupA, p.Met69Aspfs*4; c.1067A>C, p.Asp356Ala, P10= c.1633C>T, p.R545C, Father of P26=heterozygous c.50C>T, p.Thr17Met, Father of P29= c.1067A>C, p.Asp356Ala.

Figure 6. Microinjection of human NARS1 RNA into zebrafish embryos. **a.** Microinjection of the indicated doses of wild-type (WT) or p.Arg534* mutant capped RNA encoding human NARS1 into zebrafish embryos resulted in complete cyclopia and severe truncation of the body axis, a dwarf-like appearance. **b.** Low-power representative images of each experimental group at 5 days post-fertilisation. Dwarves are indicated by the red arrows. **c.** Quantification of animal body length and the proportion of animals exhibiting the dwarfic appearance for each experimental group, as indicated. Error bars are standard deviations.

Figure 7. Molecular modelling of the NARS1 Arg545Cys homozygous mutation. The crystal structure is based on *B.malayi* NARS1. NARS1 is a homodimer; one NARS1 monomer given in yellow and one in orange. Analog of the transition state presented in the surface representation, C-terminus in dark blue, Asp230, Asp226 in cyan, Arg545 and Arg545Cys in magenta. **a.** Interaction between NARS1 and tRNA with residues on the helical linker. **b-e.** Zoom in on the C-terminus helical linker region, **b and e.** show loss of molecular interaction and folding of the NARS1 protein with the Arg545Cys mutation (*).

Figure 8. a. Reduced asparaginyl-tRNA synthetase activity in patients cell lines specifically in p.Arg534* (P2), p.Arg545Cys (P9, P20), p.Arg11Pro (P24), p.Thr17Met (P26) and p.Asp356Ala / p.M69Aspfs*4 (P29) NARS1 variants in comparison to the average of 3 unrelated fibroblast cell lines (all cell lines are fibroblast except P2 which is a lymphoblast cell line, control values for lymphoblast are similar to fibroblasts); n=9, p value FDR<0.01 **b.** Other tRNA synthetases, TARS: Threonyl-tRNA synthetase; KARS: lysyl-tRNA synthetase; GARS: Glycyl-tRNA synthetase and RARS: arginyl-tRNA synthetase were measured as control for NARS1 activity.

Figure. 1

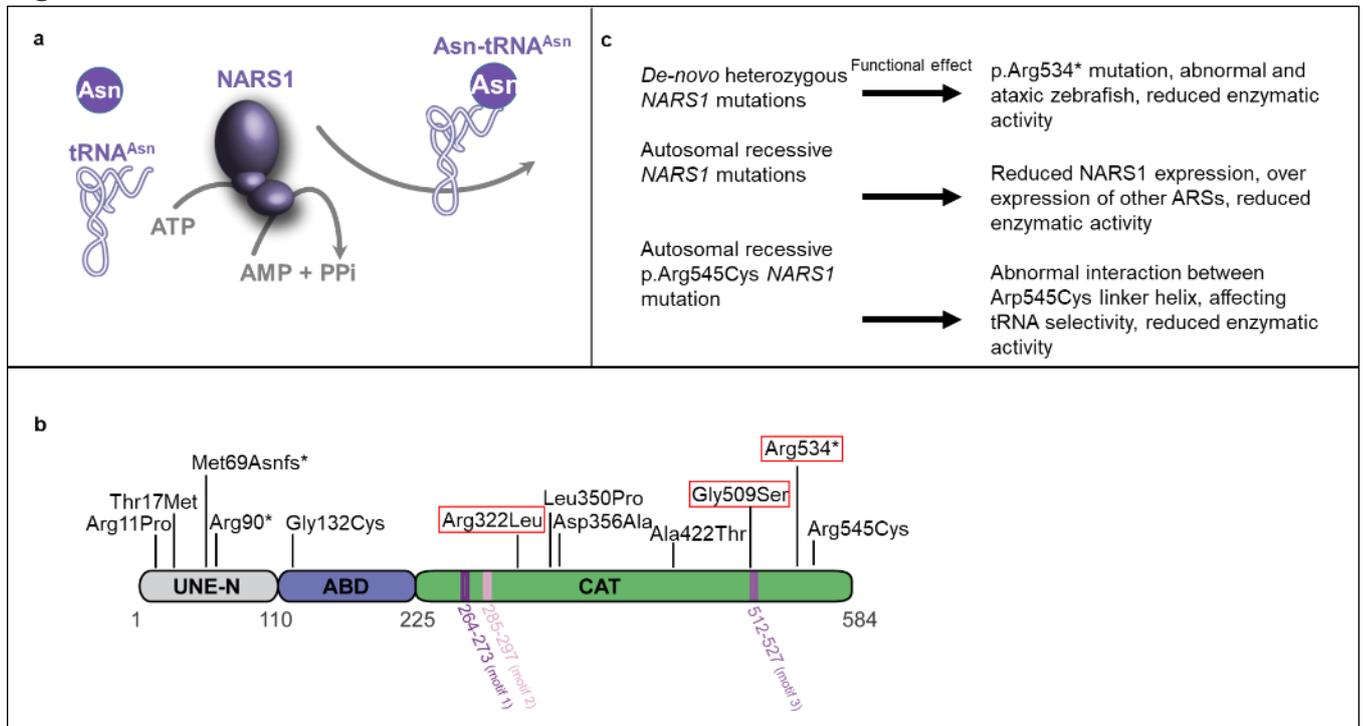


Figure 2.

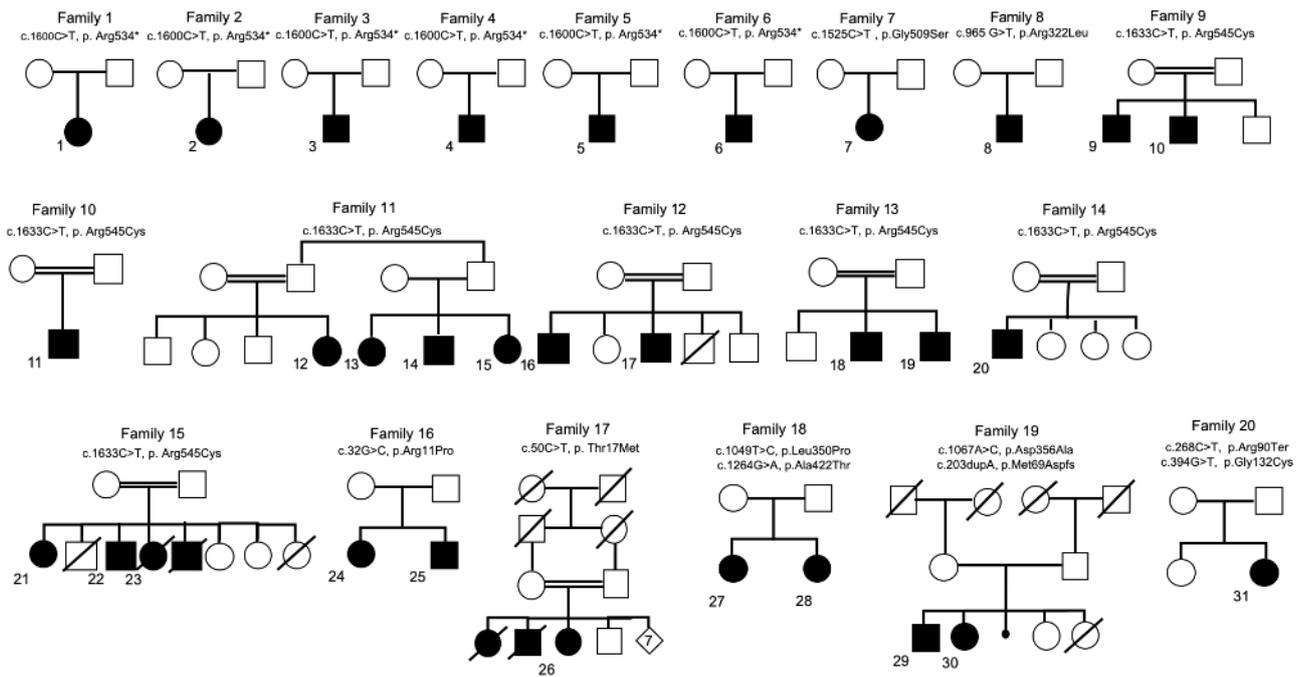


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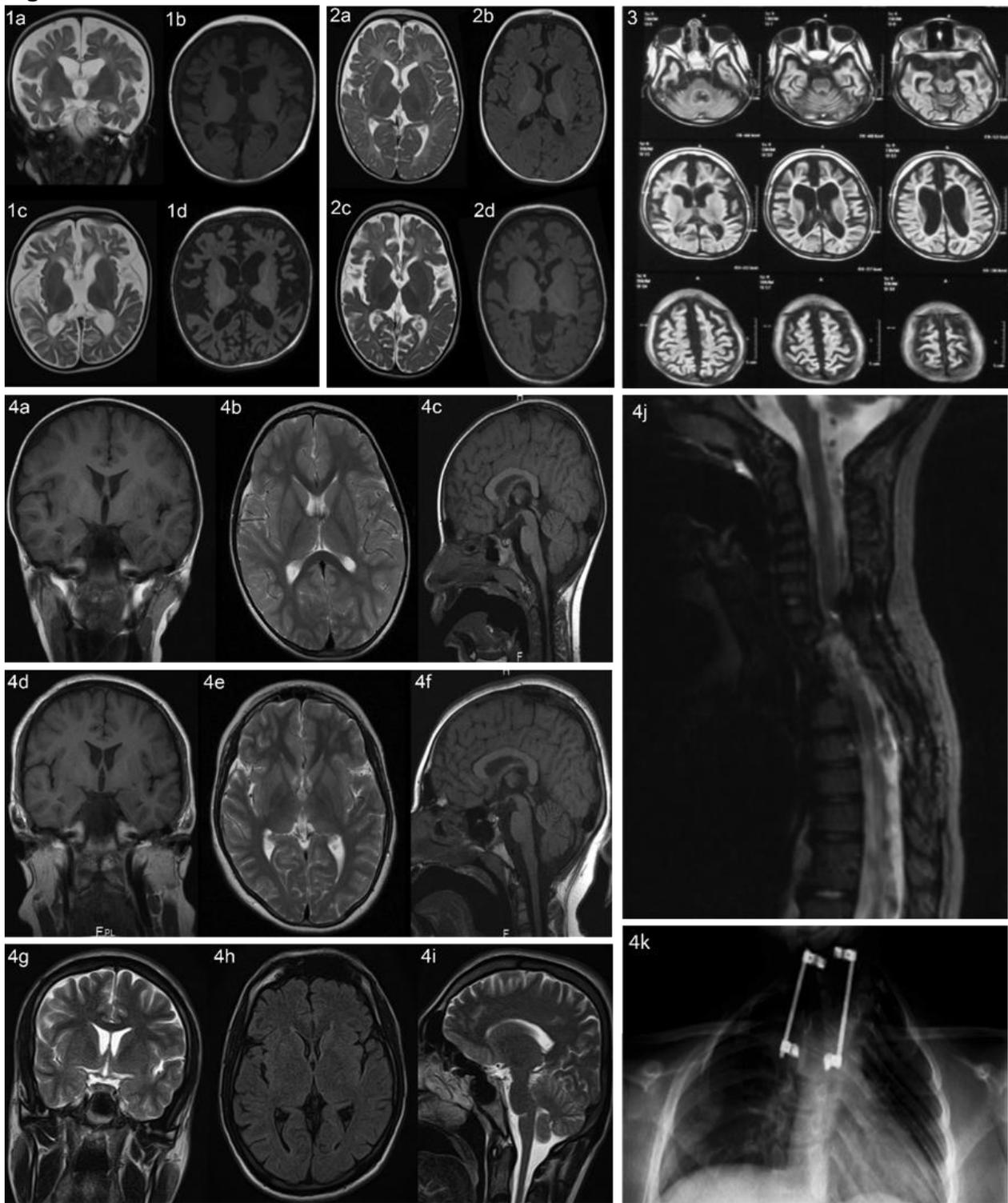


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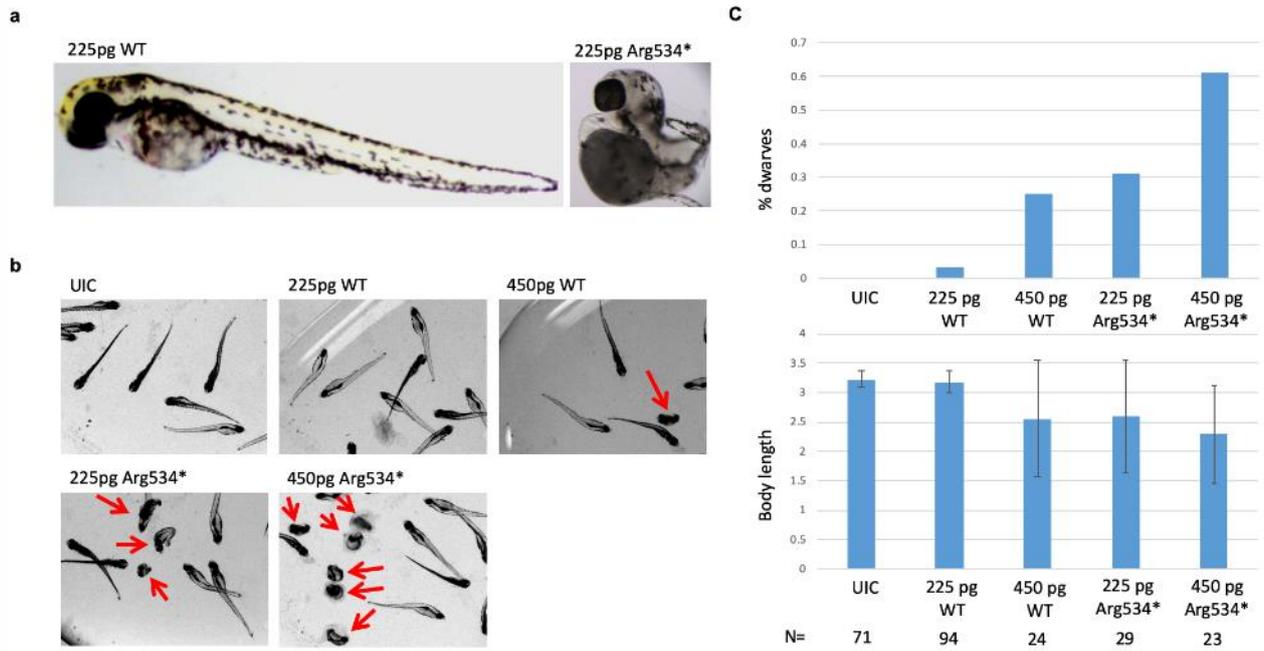


Figure 7.

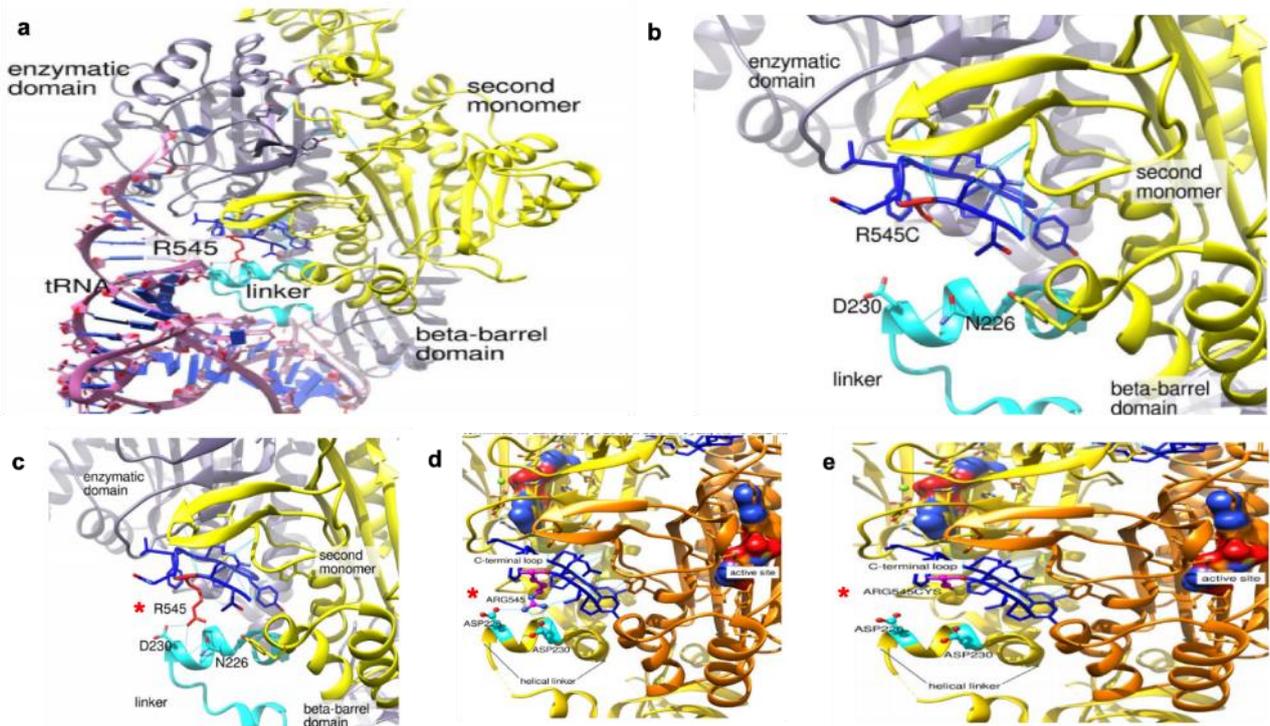
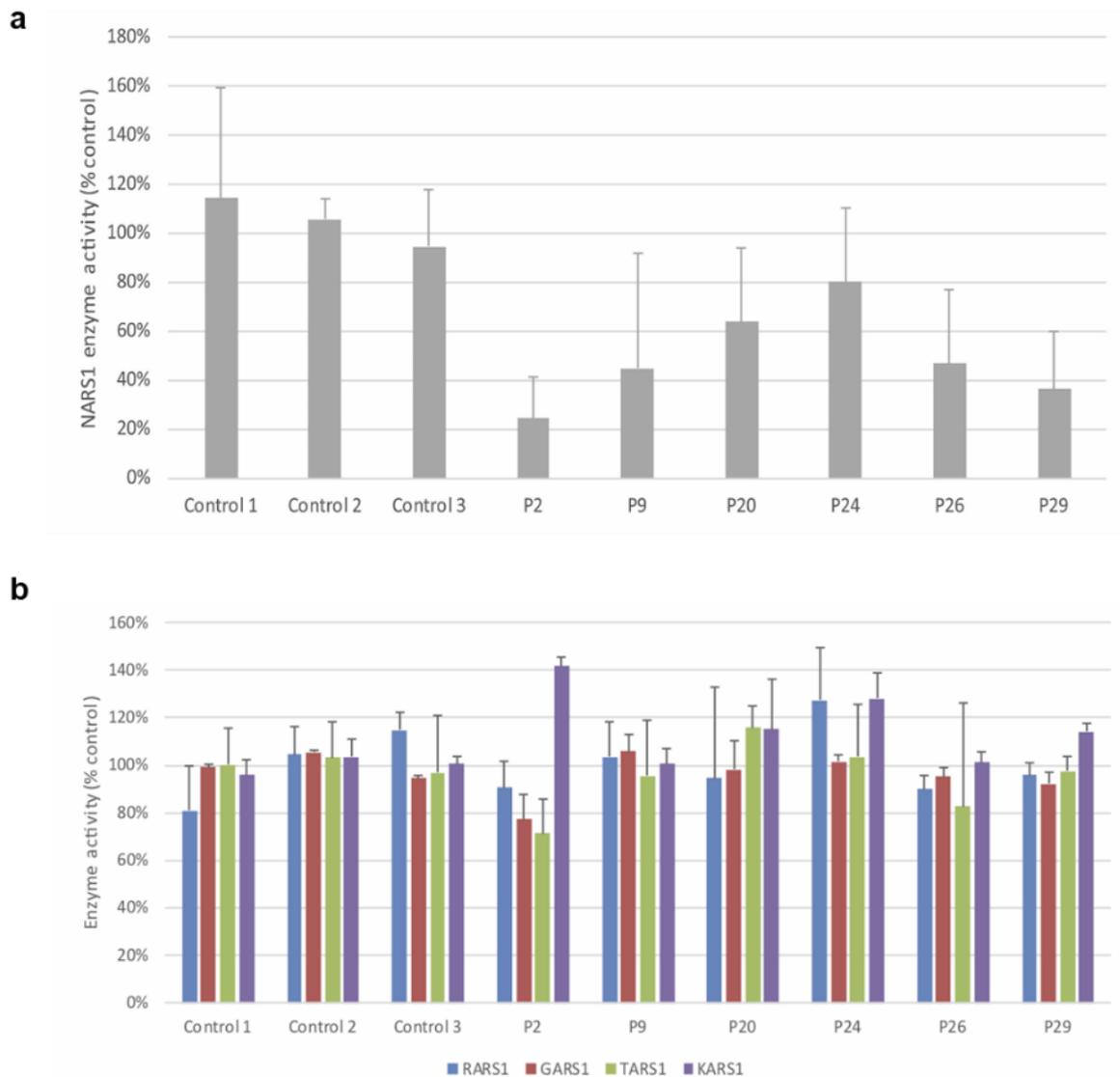


Figure 8.



Supplementary information

Clinical description

Family 1: Patient 1: NARS1 de-novo mutation, c.1600C>T, p.Arg534*

The proband was a Dutch female born to healthy non-consanguineous parents. She had an uneventful perinatal course and was born with a normal weight for gestational age. Feeding difficulties were immediately apparent, requiring tube feeding in neonatal period. As an infant, she had global development delay (GDD), failing to meet developmental milestones in multiple areas of functioning. In terms of motor milestones, sitting was achieved aged 18 months and she began walking aged 36 months. She had severe delay in language development, speaking first words aged 2 year, and at follow-up aged 17 years, her vocabulary is limited to approximately 10 words. She was also found to have progressive microcephaly with an occipital frontal circumference (OFC) of 45.8 cm (2nd percentile, -2.1 SD) at 3.5 years and 49 cm (<1st percentile, -5 SD) at 16 years. She had an MRI aged 4 years, which was otherwise unremarkable. Recurrent febrile seizure were reported aged of 11, 16, 23 and 33 months. As she grew older, she continued to exhibit signs of severe intellectual disability with limitations across all adaptive domains. Her examination was notable for a number of dysmorphic features including brachycephaly, deep-set

eyes, upslanting palpebral fissures, short philtrum, long slender fingers and persistent fetal finger pads. She had bilateral pes cavus requiring surgical correction and a unilateral foot-drop. She was unable to fully co-operate in a neurological examination, but there was evidence of wasting distally in the upper and lower limbs, she also appeared ataxic on mobilising and had absent ankle reflexes bilaterally.

Family 2: Patient 2: NARS1 de-novo mutation, c.1600C>T, p.Arg534*

A Dutch female born to non-consanguineous parents. She presented with global developmental delay with failure to meet age-appropriate milestones, first sitting aged 16 months old and walking at 26 months. She spoke her first words aged 2 years and 6 months and continued to show profound speech delay. Now, as an adult, she speaks only a few words with a nasal pitch and has severe intellectual disability. Microcephaly was confirmed on examination with an OFC measuring 46.8 cm (<1st percentile, -4.3 SD) aged 8 years and 11 months. She has epilepsy, experiencing her first GTC seizure aged 3 years, with epileptiform discharges confirmed on EEG in the posterior temporal regions. On examination, she has several dysmorphic features including mild upslanting palpebral fissures, wide set teeth, a broad jaw, clinodactyly and marked thoracic kyphosis. She has severe bilateral foot drop with atrophy of the lower legs and intrinsic foot muscles. She is hypotonic with muscle weakness, which is more pronounced distally. Vibration and sensation are impaired to the level of the ankles. Her coordination is grossly normal but she has a slight intention tremor in the right upper limb. She has had several investigations including an MRI brain, aged 9 years, which was reported as normal. Nerve conduction studies confirmed a demyelinating polyneuropathy and muscle ultrasound indicated hyperechogenicity distally in lower limbs bilaterally.

Family 3: Patient 3: NARS1 de-novo mutation, c.1600C>T, p.Arg534*

The proband was a Dutch male born to healthy non-consanguineous parents. His pre-natal course was remarkable for inter-uterine growth retardation and oligohydramnios. At birth, he was found to have a low weight and OFC for gestational age. GDD was evident from early infancy as he failed to reach several milestones including gross motor parameters, walking for the first time aged 30 months. In particular, his speech was severely delayed. Now, aged 10 years his language is limited to short sentences with a notably nasal quality. Dysmorphic features were evident on follow-up examination including medial eyebrow flare, a short upturned nose, retrognathia and clinodactyly of his fifth fingers. He was notably dysarthric and had a bilateral tremor with occasional myoclonus. He was hypertonic with clonus at the knee and ankle. Power was reduced with weakness in a pyramidal distribution in the upper and lower limbs. He was hyperreflexic with upgoing plantar reflexes bilaterally.

Family 4: Patient 4: NARS1 de-novo mutation c.1600C>T, p.Arg534*

Caucasian male born to healthy non-consanguineous parents. He presented with his first seizure aged 4 months old, suffering from both partial and myoclonic seizures with associated EEG abnormalities. In addition, he has a dilated aortic root, which was diagnosed in infancy. He suffered from GDD. Gross motor skills were severely delayed, first sitting aged 16 months and never acquiring the ability to walk unaided. His speech was also profoundly delayed and is now limited to 1-2 words at the age of 13 years. On examination, he had a dysmorphic appearance with arachnodactyly, pectus excavatum, dolichostenomelia, long palpebral fissures and hypertelorism. He exhibited stereotypies with hand and mouth repetitive movements. He had microcephaly with an OFC of 49cm aged 13 years old (<1st percentile, -4.7 SD). His neurological examination was notable for severe spasticity in upper and lower limbs and impaired co-ordination.

Family 5: Patient 5: NARS1 de-novo mutation c.1600C>T, p.Arg534*

Female born to healthy non-consanguineous parents. Characterised by severe global developmental delay, with profound speech delay. Now aged 16 using she has only 1-2 word phrases. She first walked age 3 years. She had feeding difficulties in infancy requiring G-tube feeding.

In terms of epilepsy, she had an initial seizure at 3 months of age continued to have both partial and generalized seizures throughout her childhood, which was well managed with levetiracetam. An EEG at 15 years was consistent with chronic static encephalopathy. Her examination was notable for microcephaly with a sloping forehead, slanting eyes, low set ears with fleshy helices, widely spaced teeth and small hands with tapered fingers. She walked with a broad based ataxic gait. There was wasting distally especially involving the extensor digitorum brevis. She was hypotonic in upper and lower limbs with distal weakness and hyporeflexia. She had impaired sensation to pinprick in the upper and lower limbs. Nerve conduction studies showed findings consistent with a demyelinating neuropathy.

Family 6: Patient 6: NARS1 de-novo mutation c.1600C>T, p.Arg534*

Male proband born to healthy non-consanguineous parents. He presented in infancy with neurodevelopmental delay (NDD), walking for the first time aged 23 months. His speech development was severely delayed and, now aged 8 years, he has a vocabulary limited to approximately 20 words. He began having seizures at 11 months, which were initially considered febrile seizures; however, these occurred recurrently throughout childhood and were classified as atypical febrile seizure. At this time, he underwent an EEG which failed to capture epileptiform activity. On examination he had microcephaly (<1st percentile, -3.25SD) and several dysmorphic features including a long philtrum, a thin upper lip, an everted lower lip, a wide mouth, midface hypoplasia and low set ears with overfolded helices. He also had syndactyly of his toes and short first toe bilaterally. His physical examination was notable for a broad-based ataxic gait and increased tone in the lower limbs bilaterally with hyperreflexia and clonus. MRI brain showed mild cortical atrophy with enlargement of the CSF space at 8 months however subsequent imaging aged 3 years was unremarkable for structural abnormalities.

Family 7: Patient 7: NARS1 de-novo mutation c.1525C>T, p.Gly509Ser

The proband was a British female born to non-consanguineous parents. Of note, exome sequencing also revealed a complex X chromosome rearrangement. She first presented at 9 months with focal seizures with secondary generalisation. She subsequently underwent EEG, which failed to capture epileptiform discharges. She had GDD, failing to reach a number of developmental milestones, resitting for the first time aged 12 months and walking aged 27 months with severe delays in speech. She persisted to have learning difficulties throughout her childhood and suffered with chronic constipation. On examination, she was normocephalic. Dysmorphic features included broad forehead, large ears, tented upper lip, long and slender fingers, slender feet, hypermetropia and a unilateral convergent squint. She was hypotonic with brisk DTR and down-going plantars. She unexpectedly died while sleeping aged 10 years 8 months. Autopsy showed a normally formed brain with focal calcification of basal ganglia and dentate nucleus.

Family 8: Patient 8: NARS1 de-novo mutation c.965 G>T, p.Arg322Leu

The proband is a 15 year old male of mixed race (German-Irish / English-Native American / Russian-Polish heritage) born to healthy parents. In infancy, he had feeding difficulties and was slow to reach motor milestones, sitting aged 8 months and walking at 35 months. His speech was profoundly delayed speaking first words aged 8-9 years old. He has intractable epilepsy characterised by both absence and myoclonic seizures, confirmed on EEG with slow wave bursts with spike & poly-spike bursts. On examination, dysmorphic features included a prominent nose and broad forehead. He is dysarthric with limited speech, which mostly consists of repetitive phrases. He had increased tone in the lower limbs bilaterally and hyperreflexia throughout.

Vibration and sensation were intact. Co-ordination was impaired with both appendicular and axial ataxia. MRI was unremarkable for structural abnormalities.

Family 9: Patient 9 and 10: NARS1 Homozygous mutation c.1633C>T, p.Arg545Cys

The proband, patient 9, is a 33 year old male of Indian descent, born to consanguineous parents. He had a normal prenatal course and uncomplicated birth. He failed to meet developmental milestones in infancy, with severe delays in speech and fine motor skills. He began having generalised tonic-clonic seizures aged one year, which were poorly controlled with anti-epileptic agents and persisted into adulthood. Contractures in his lower limbs required him to undergo a tendon lengthening procedure aged 14 years. Additionally, he has severe sensory and motor neuropathy with NCV showing complete absence of sensory action potentials in the upper limbs, and absent motor action potentials in the upper and lower limbs. On examination, he is microcephalic and has bilateral foot drop with a left sided foot deformity consistent with a Charcot joint. He wears AFOs to enable ambulation and has a broad-based ataxic gait. There are contractures of his fingers bilaterally and evidence of muscle atrophy distally. He is hypotonic with reduced power proximally (3/5) with a pronounced weakness distally (2/5). He is areflexic and has impaired sensation to pinprick to the level of elbows and ankles in the upper and lower limbs respectively. His younger brother, patient 10, is similarly affected. He also had an unremarkable prenatal course and birth but failed to meet expected developmental milestones. As an older child, he had learning difficulties requiring special schooling. He has no history of seizures. He has scoliosis, a broad based gait and difficulty walking. Nerve conduction velocities revealed a demyelinating neuropathy in upper and lower limbs.

Family 10: Patient 11: NARS1 Homozygous mutation c.1633C>T, p. Arg545Cys

The proband, is an 8 year old male born to consanguineous parents from Pakistan. As an infant, he initially had feeding difficulties, choking and regurgitating with feeds. He failed to meet developmental milestones with severe delays in gross motor skills, sitting at 12 months and walking at 24 months. Speech was also severely delayed and now, aged eight, his vocabulary is limited to a few sentences. On examination, he had severe microcephaly with an OFC of 46.5cm (<1p, -4.2 SD). He had fifth finger clinodactyly bilaterally and toe syndactyly of the right foot. Neurological examination was limited in a setting of intellectual disability. There was no evidence of wasting. He had a broad based gait and poor balance with difficulty running. Patellar reflexes were 3+ bilaterally; otherwise reflexes were normal with down-going plantars. His MRI was unremarkable for structural abnormalities.

Family 11: Patients 12-15: NARS1 Homozygous mutation c.1633C>T, p.Arg545Cys

The proband is 17-year-old female born to Pakistani parents. She has severe intellectual disability across all adaptive domains. As a child, she was slow to meet several developmental milestones, sitting for the first time at 12 months and walking at 20 months. She also had difficulty feeding, choking regularly. She has microcephaly with and OFC of 49.5 cm (<1p,-4.5 SD). She has no history of seizures. Her physical examination is notable for reduced power (3/5) in all muscle groups with and an ataxic gait. She has no evidence of wasting or impaired sensation. She has two siblings, an older brother and sister and a female cousin who are similarly affected with GDD in childhood and severe intellectual disability. Additionally, her older brother and sister also have epilepsy characterised by generalised tonic clonic seizures and her eldest sibling is the most severely affected with profound intellectual disability.

Family 12: Patients 16-17: NARS1 Homozygous mutation c.1633C>T, p.Arg545Cys

The proband is a male born to consanguineous parents was reviewed aged 8 years. He has severe intellectual disability with a history of GDD, including severe delay in meeting motor milestones, walking for the first time age 6.5 years. He also had severe speech delay and at the

time of follow-up had a limited vocabulary and difficulty forming sentences. His neurological examination was notable for hypotonia and lower limb weakness. His brother, now aged 21 years, is also affected with severe intellectual disability, wasting, and weakness in lower limbs.

Family 13: Patients 18-19: NARS1 Homozygous mutation c.1633C>T, p.Arg545Cys

Two siblings born to consanguineous parents from Pakistan. The proband is 6 months old male who presented in the neonatal period with microcephaly, seizures and failure to thrive. His older brother also has epilepsy, microcephaly and global developmental delay. He walked age 3 years. His speech is severely delayed and, at follow-up aged 6.5 years, he only speaks a few words and is unable to form sentences.

Family 14: Patients 20: NARS1 Homozygous mutation c.1633C>T, p.Arg545Cys

The proband is a 16 year old boy born to consanguineous parents from Pakistan. He was born following a normal pregnancy and perinatal course, but began to exhibit signs of moderate global developmental delay in infancy. He sat at 7 months, began walking at 2 years of age and had moderate delay in speech development. He had learning disabilities in school and persisted to have moderate intellectual disability. His examination was notable for bilateral pes cavus, weakness and wasting distally in upper and lower limbs, absent deep tendon reflexes and impaired sensation. Nerve conduction velocities revealed severe sensorimotor polyneuropathy with primary axonal degeneration. His MRI Brain was unremarkable.

Family 15: Patients 21-23: NARS1 Homozygous mutation c.1633C>T, p.Arg545Cys

This family consists of eight siblings born to consanguineous Pakistani parents. Four siblings were affected with GDD, microcephaly and seizures. The eldest, a female, was reviewed aged 30 years. She presented with severe GDD in childhood, sitting for the first time at 4 years of age and walking first steps at 10 years. She did not speak at all until she was 4.5 years old and now has limited speech with difficulties communicating. At 4.5 years of age she fell downstairs, after which, she began having left sided focal seizures, which were controlled with sodium valproate. Her seizures stopped after 2 years, however she had persistent left sided weakness which was attributed to the fall. On examination, she had bilateral foot drop and several dysmorphic characteristics including; large dysplastic ears, a large nose with broad nares, a large mouth with widely spaced teeth, and syndactyly of the second and third toes bilaterally. She had normal tone with reduced power in lower limbs bilaterally particularly on dorsiflexion (1/5). She had an ataxic gait with absent ankle reflexes. She was unable to participate in sensory examination. Her younger brother, who is now 16, is similarly affected but has less severe intellectual disability. He also has microcephaly and had delayed developmental milestones as child, sitting at 1 year of age, walking aged 2 years and speaking first words at 3 years of age. His speech remains limited, he is unable to speak in full sentences, and he has a tendency towards aggressive behaviour. He also had focal seizures in infancy, however they have now resolved and his last witnessed seizure occurred aged 2 years. Two other siblings, also affected, died in childhood. A girl, who was reviewed aged 13 years, also presented with GDD and microcephaly in infancy. She had severe intellectual disability and was unable to feed herself independently. She also had a history of delayed milestones in childhood, sitting aged two and walking aged three, and had poor language abilities and spoke only a few words. She began having generalised tonic clonic seizures aged 4 years which were treated with sodium valproate but poorly controlled. She died at the age of 16 years due to sepsis secondary to burns which she obtained following a fall into a stove. Her brother also died aged 6 years of age. He was unable to sit, stand or speak and had severe and seizures which commenced when he was 2 months old. His death was attributed to respiratory distress secondary to unrelated respiratory complications. Besides these siblings, these patients also had six first degree relatives (not shown) that had GDD and died in early childhood.

Family 16: Patients 24-25. NARS1 siblings with homozygous mutation c.32G>C, p.Arg11Pro

Two affected siblings born to parents from Kosovo, who were not known to be related. The couple's first child (a girl) was unaffected. Their second child, a boy, was born at full term following an uneventful pregnancy. He had a normal postnatal course and birth parameters. No abnormalities were detected until he was 5 months old when he presented with generalised tonic-clonic seizures. Moreover, his motor development deteriorated with progressive muscle spasticity. CT imaging of his brain revealed widening of bifrontal subarachnoid spaces as well as small subdural hygroma. Laboratory work-up including CSF analysis was unhelpful. Brain MRI at the age of 10 months revealed prominent cerebral atrophy and delayed myelination. During the following year, he was treated with phenobarbital, levetiracetam and baclofen to control seizures and spasticity. Progressive microcephaly was noted OFC 43 cm (<1st percentile, -4 SD). Motor and cognitive milestones were not reached (e.g. no crawling, no sitting without support, no development of language, etc). Follow-up brain MRI at the age of 1 ½ years showed severe atrophy with no progression of myelination. At the age of 2 ½ year, the boy deteriorated with poor feeding, vomiting and dehydration. An MRI brain demonstrated basilar thrombosis and associated infarction in the cerebellum, pons and midbrain. His neurological function deteriorated further and he died aged 4 years in a palliative care setting with severe aspiration pneumonia. The third born, a girl, had normal pre- and postnatal development. She presented aged 8 months with status epilepticus. Physical examination revealed microcephaly, developmental delay and moderate spasticity of the extremities. Brain MRI showed comparable findings to her brother with severely delayed myelination. Follow-up brain MRI at the age of 2 years revealed progressive brain atrophy and arrest of myelination.

Family 17: Patient 26. NARS1 siblings with homozygous c.50C>T, p.Thr17Met

The proband is a female born to Libyan parents who were first cousins. She was reviewed aged 7 years, at which point was found to have severe microcephaly 42cm (<1st percentile, -7.7 SD). She has severe intellectual disability. She presented with generalised tonic clonic seizure at 6 months and was diagnosed with GDD in infancy failing to meet age appropriate milestones. She has 2 additional siblings who were similarly affected but passed away in childhood.

Family 18: Patients 27 and 28. NARS1 siblings with compound heterozygous c.1049T>C, c.1264G>A, p.Leu350Pro, p.Ala422Thr

The proband is a 15-year-old female born to healthy non-consanguineous German parents. She presented in childhood with neurodevelopmental delay and progressive microcephaly. She failed to reach appropriate motor milestones sitting for the first time age 3 years. Speech developmental was also severely delayed and now aged 15 years her vocabulary is limited to a few words and she is unable to communicate effectively. She persisted to have severe intellectual disability and experiences frequent episodes of inappropriate laughter. She had her first generalised tonic clonic seizures at approximately 4 years of age, at which point she was documented as having an abnormal EEG. Her neurological examination was notable for an ataxic gait. She had muscle atrophy which is more pronounced in her lower limbs. She is hypotonic with reduced power distally and hyporeflexic in the lower limbs with upgoing plantars bilaterally. Sensation to sharp touch was impaired. A MRI brain ruled out any structural abnormalities and nerve conduction velocities confirmed demyelinating peripheral neuropathy. Her sister who is now 21 years of age and is clinically similar to her sister with severe intellectual disability, microcephaly and demyelinating peripheral neuropathy. However, she has never had seizures.

Family 19: Patient 29-30. NARS1 siblings with compound heterozygous .1067A>C, c.203dupA, p.Asp356Ala, p.Met69Aspfs*

The proband is a male born to non-consanguineous Turkish parents. He was born with a low weight (-2.38 SD) and height (-3.76 SD) for gestational age. He failed to meet age appropriate

developmental milestones in terms of motor skills and did not walk until 3 years of age. His speech was also severely delayed speaking his first words aged 4 years. He began having generalised tonic clonic seizures aged 6 years at which point he underwent an MRI brain, which revealed thickening of gyri. Examination at follow up at 14 years of age revealed severe microcephaly 49.2 cm (<1st percentile, -3.4 SD). His sister, also affected, is clinically identical with GDD and epilepsy.

Family 20: Patient 31. NARS1 mutation compound heterozygous c.268C>T, c.394G>T, p.Arg90*, p.Gly132Cys

The proband is an 8 year old Canadian female born to healthy non-consanguineous parents. She was born at 37 weeks and had feeding difficulties from the beginning with associated failure to thrive. Developmental milestones were profoundly delayed. She did not walk until 6 years and 10 months and did not speak until she was 5 years old. At follow-up aged 8 years, her speech was limited to 3 words and she was unable to form sentences. From 1 to 3 years of age, she experienced five generalised tonic-clonic seizures. She is now seizure free and does not require anti-epileptic drugs. On examination, she was small for age with a weight of 11kg (-5.6 SD) and height of 95.3 cm (-7.6 SD). She has severe microcephaly (-3SD) and several dysmorphic features including hypotelorism, deep set eyes, a prominent nasal bridge and thin upper lip with smooth philtrum. Skeletal abnormalities include a right sided hip dysplasia and bilateral varus deformities requiring de-rotational osteotomies. Due to severe intellectual disability, she struggled to cooperate with the neurological examination, however she appeared to be hypotonic and have impaired co-ordination. An MRI brain showed microcephaly with a thin corpus callosum and decreased white matter volume throughout.

Gene	Locus	Location of Protein	Mode	Disease Phenotype(s)	Clinical severity	References
AARS1	16q22	Cytoplasm	AD	Charcot Marie Tooth disease type 2N	Mild	38-41
			AR	Epileptic encephalopathy, early infantile, 29	Moderate	
AARS2	6p21.1	Mitochondria	AR	Mitochondrial Infantile Cardiomyopathy	Moderate	36, 42, 43
				Leukoencephalopathy with ovarian failure	Moderate	
CARS1	11p15.4	Cytoplasm	AR	Microcephaly, DD, brittle hair and nails	Moderate	44
CARS2	13q34	Mitochondria	AR	Epilepsy encephalopathy, myoclonic epilepsy	Moderate	45-48
				Combined oxidative phosphorylation deficiency 27		
DARS1	2q21.3	Cytoplasm	AR	Hypomyelination, brainstem, spinal cord and leg spasticity	Moderate	49-51
DARS2	1q25.1	Mitochondria	AR	Hypomyelination, brainstem, spinal cord, elevated lactate	Severe	52-61
EPRS1	1q41	Cytoplasm	AR	Leukodystrophy, hypomyelinating, 15	Moderate	62
EARS2	16p12.2	Mitochondria	AR	Leukoencephalopathy and high lactate	Severe	63-68
				Combined oxidative phosphorylation deficiency 12		
FARSA	19p13.2	Cytoplasm	AR	Rajab interstitial lung disease with brain calcifications	Moderate	11
FARSB	2q36.1	Cytoplasm	AR	Rajab interstitial lung disease with brain calcifications	Moderate	69, 70
FARS2	6p25.1	Mitochondria	AR	Combined oxidative phosphorylation deficiency 14	Moderate	71-76
				Spastic paraplegia 77		
GARS1	7p15	Cytoplasm & Mitochondria	AD	Charcot Marie Tooth disease type 2D	Mild	39, 77-92
			AR	Distal SMA type V, myalgia, cardiomyopathy		
HARS1	5q31.3	Cytoplasm	AD	Charcot Marie Tooth disease type 2W	Mild	93, 94
			AR	Usher Syndrome 3B	Moderate	
HARS2	5q31.3	Mitochondria	AR	Perrault Syndrome 2	Mild	95
IARS1	9q22.31	Cytoplasm	AR	ID, GR, muscular hypotonia, hepatopathy, cholestasis	Severe	96
IARS2	1q41	Mitochondria	AR	Cataracts, GH deficiency, deaf, neuropathy, bone dysplasia	Severe	97-99

				Leigh syndrome		
KARS1	16q23.1	Cytoplasm & Mitochondria	AR	Intermediate Charcot Marie Tooth disease type B	Mild	100-102
				Autosomal recessive deafness-89		
				Visual impairment, microcephaly, DD, seizures		
LARS1	5q32	Cytoplasm	AR	Infantile hepatopathy	Severe	103
LARS2	3p21.31	Mitochondria	AR	Perrault syndrome 4	Mild	104
				Hydrops, lactic acidosis and sideroblastic anaemia		
MARS1	12q13.3	Cytoplasm	AD	Charcot Marie Tooth disease type 2U	Mild	105-108
			AR	Interstitial lung and liver disease	Moderate	
MARS2	2q33.1	Mitochondria	AR	DD, sensorineural hearing loss, Spastic ataxia 3	Mild	109, 110
				Combined oxidative phosphorylation deficiency 25	Moderate	
NARS1 2	11q14.1	Mitochondria	AR	Alpers, Leigh syndrome, DD, ID, epilepsy, myopathy	Severe	111-116
				Combined oxidative phosphorylation deficiency 24		
PARS2	3p21.31	Mitochondria	AR	Infantile-onset developmental delay and epilepsy	Moderate	116
				Alpers syndrome		
QARS1	3p21.31	Cytoplasm	AR	Microcephaly, seizures, DD, cerebral cerebellar atrophy	Severe	16
RARS1	5q34	Cytoplasm	AR	hypomyelinating leukodystrophy 9	Severe	117
RARS2	6q16.1	Mitochondria	AR	Epileptic encephalopathy, lactic acidosis neurological symptoms, pontocerebellar hypoplasia 6	Severe	118-120
SARS1	1p13.3	Cytoplasm	AR	Ataxia, weakness, ID, microcephaly, speech impaired	Moderate	121
SARS2	19q13.2	Mitochondria	AR	Hyperuricemia, pulmonary HT, renal failure, alkalosis	Moderate	122
TARS1	5p13.3	Cytoplasm	AR	Trichothiodystrophy, ichthyosis, ID, decreased fertility	Moderate	123
TARS2	1q21.2	Mitochondria	AR	Axial hypotonia and severe psychomotor delay	Mild	124, 125
				Combined oxidative phosphorylation deficiency 21		
VARS1	6p21.33	Cytoplasm	AR	Severe DD, microcephaly, seizures, cortical atrophy	Moderate	7-10
VARS2	6p21.33	Mitochondria	AR	Microcephaly, epilepsy, encephalocardiomyopathy	Moderate	124, 126-129
				Combined oxidative phosphorylation deficiency 20		
WARS 1	14q32.2	Cytoplasm	AD	Distal hereditary motor neuropathy	Mild	130
WARS 2	1p12	Mitochondria	AR	Ataxia, weakness, microcephaly, speech impaired, ID	Moderate	121, 131, 132
YARS1	1p35.1	Cytoplasm	AD	Dominant-intermediate Charcot Marie Tooth disease	Mild	127, 133
			AR	Multisystem disease, DD, failure to thrive	Moderate	
YARS2	12p11.2	Mitochondria	AR	myopathy, lactic acidosis, and sideroblastic anaemia	Moderate	134-136

Table S1. ARS gene implicated in 36 human diseases from a total of 37 genes. The first letter of the ARSs' gene name corresponds to the amino acid specificity of the corresponding ARS (based on the amino acid one-letter code) and 2 indicates that the gene encodes a mitochondrial-restricted isoform¹⁻³. Clinical phenotype is based upon publications. Key: ID = intellectual disability, GH = growth hormone, GR = growth retardation, DD = developmental delay, AR = autosomal recessive, AD = autosomal dominant, HT = hypertension, SMA = spinal muscular atrophy.

Table S2 : Detailed clinical features of patients with NARS1 de-novo heterozygous mutations. Families 1-8.

Family	1	2	3	4	5	6	7	8
Patient	1	2	3	4	5	6	7	8
Inheritance	<i>de-novo</i> Heterozygous	<i>de-novo</i> Heterozygous	<i>de-novo</i> Heterozygous	<i>de-novo</i> Heterozygous	<i>de-novo</i> Heterozygous	<i>de-novo</i> Heterozygous	<i>de-novo</i> Heterozygous	<i>de-novo</i> Heterozygous
Variant	c.1600C>T p.Arg534*	c.1600C>T p.Arg534*	c.1600C>T p.Arg534*	c.1600C>T p.Arg534*	c.1600C>T p.Arg534*	c.1600C>T p.Arg534*	c.1525C>T p.Gly509Ser	c.965 G>T p.Arg322Leu
Origin	Dutch	Dutch	Dutch	Caucasian	Hispanic, European	German Irish Belgian	UK	German-Irish English-American Russian- Polish
Consanguinity	N	N	N	N	N	N	N	N
Gender / Age at follow-up	F / 17	F / 22	M / 10	M / 13	F / 16	M / 5y 8m	F / 2y 10m	M / 15
Occipital Frontal Circumference (OFC)								
Birth	45.8cm <2p (-2.1SD)	NA	NA	33cm (10p)	NA	NA	NA	NA
Follow -up Percentile / SD	49cm <1p (-5SD)	46.8cm <1p(-4.3 SD)	50cm <1p (-2.2 SD)	49cm <1p (-4.7SD)	49.3cm at 13 y <1p (-4.5 SD)	47cm <1p (-3.2SD)	50 cm 86p	54cm 27p
Microcephaly	Y	Y	Y	Y	Y	Y	N	N
Developmental Delay								
GDD	Y	Y	Y	Y	Y	Y	Y	Y
Sitting (Months)	18	16	NA	16	11	NA	12	8
Walking (Years)	3	2y 2 m	2y 6m	N	3	23	2y 3m	2y 11m
Language	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed
Neurological Findings								
Intellectual Disability	Severe	Severe	Severe	Profound	Profound	Severe	Severe	Severe
Seizures	GTC	GTC	NA	Myoclonic / Partial	GTC / Partial	GTC	GTC	Partial/Myoclonic
Peripheral Neuropathy	Y	Y	NA	N	Y	N	NA	NA
Ataxia	Y	Y	Y	Y	Y	Y	NA	Y
Imaging	NAD	NAD	NAD	NA	NA	Mild atrophy and enlargement of CSF space	NA	NAD
Clinical Features								
Dysmorphic Features	Upslanting palpebral fissures Pes-cavus	Clinodactyly Upslanting palpebral fissures Thoracic Kyphosis Wide spaced teeth	Clinodactyly Retrognathia	Upslanting palpebral fissures Hypertelorism Arachnodactyly, Pectus Excavatum	Upslanting palpebral fissures Wide spaced teeth Low set ears Fleshy Helices	Low set ears Overfolded Helices Syndactyly	Large ears Long slender fingers	Broad Forehead
Tone	NAD	Reduced	Increased	Increased	Reduced	Increased	Reduced	Increased
Power	Reduced	Reduced	Reduced	NAD	Reduced	Reduced	NA	NAD
Sensation	NA	Reduced	NA	NAD	Reduced	NAD	NA	NA
Co-ordination	Ataxic gait	Unilateral Intention Tremor	Dysarthria	Ataxic gait	Ataxic gait	Ataxic gait	NA	Ataxia
Reflexes	Reduced	Reduced	Increased	Increased	Reduced	NAD	Increased	Increased
Other	N	N	Tremor /Myoclonus	Stereotypies	N	N	N	Stereotypies

Y = Yes, N= No, M= Male, F= Female, NA= Not Available, p = percentile, SD = standard deviation, NAD = No abnormalities detected, GTC = Generalised Tonic Clonic Seizures

Table S3: Detailed clinical features of patients with NARS1 Homozygous mutation c.1633C>T, p.R545C including families 9-15.

Family	9	9	10	11	11	11	11	12	12	13	13	14	15	15	15
Patient	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Origin	North India	North India	North India	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan
Consanguinity	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Gender / Age at follow-up	M / 33	M / 17	M / 8	F / 17	F / 17	M / 19	F / 23	M / 8	M / 21	M / 6m	M / 6.5	M / 16	F / 30	M / 16	F / 13
Occipital Frontal Circumference (OFC)															
Birth	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Follow-up Percentile / SD	NA	NA	46.5cm <1p (-4.2 SD)	52cm 2p (-2.2 SD)	49.5cm <1p (-4.5 SD)	52cm 2p (-2.1 SD)	50.3cm <1p (-3.8 SD)	46cm <1p (4.6SD)	51cm <1p (2.8SD)	NA	49cm 2p (-2.0SD)		50.8cm <1p (-3.3SD)	48.2cm <1p (-4.5SD)	48cm <1p (-4.4SD)
Microcephaly	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y
Developmental Milestones & Intellectual Disabilities															
GDD	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Sitting (Months)	12m	12m	12m	12m	14m	12m	12m	NA	NA	N	NA	7m	4y	1y	2y
Walking (Years)	NA	NA	2y	1y 8m	2y 8m	2y 8m	2y 8m	6y 6m	3y		3y	2y	10y	2y	3y
Language	Delayed	Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed		Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed
Neurological Findings															
Intellectual Disability	Severe	Moderate	Severe	Severe	Severe	Severe	Profound	Severe	Severe	Severe	Severe	Moderate	Severe	Moderate	Moderate
Seizures	GTC	N	N	N	N	GTC	GTC	N	N	GTC	GTC	N	Partial	Partial	GTC
Ataxia	Y	Y	Y	Y	N	N	N	NA	NA	NA	NA	NA	Y	NA	NA
Peripheral Neuropathy	Y	Y	N	N	N	N	N	NA	NA	NA	NA	Y	NA	NA	NA
Imaging	NAD	NAD	NAD	NA	NA	NA	NA	NA	NA	NA	NA	NAD	NA	NA	NA
Clinical Findings															
Dysmorphic Features	Contractures	Scoliosis	Clinodactyly Syndactyly	Clinodactyly	N	Clinodactyly Short Limbs	N	N	N	N	N	NAD	Dysplastic ears Syndactyly	NA	NA
Tone	Reduced	Reduced	NAD	NAD	NAD	NAD	NAD	Reduced	Reduced	NA	NA	N	N	NA	NA
Power	Reduced	Reduced	NAD	NAD	Reduced	Reduced	Reduced	Reduced	Reduced	Reduced	Reduced	Reduced	Reduced	Reduced	NA
Sensation	Reduced	Reduced	NAD	NAD	NAD	NAD	NAD	NA	NA	NA	NA	Reduced	NA	NA	NA
Co-ordination	Ataxic	Ataxic	Ataxic	Ataxic	NAD	NAD	NAD	NA	NA	NA	NA	NA	Ataxic	NA	NA
Reflexes	NAD	NAD	NAD	Reduced	Reduced	NAD	Reduced	NA	NA	Reduced	Reduced	Reduced	Reduced	NA	NA

Y = Yes, N= No, M= Male, F= Female, NA= Not Available, p = percentile, SD = standard deviation, NAD = No abnormalities detected, GTC = Generalised Tonic Clonic Seizures

Table S4: Detailed clinical features of patients with NARS1 mutations. Autosomal recessive inheritance. Families 16-20.								
Family	16	16	17	18	18	19	19	20
Patient	24	25	26	27	28	29	30	31
Inheritance	Homozygous	Homozygous	Homozygous	Compound Heterozygous	Compound Heterozygous	Compound Heterozygous	Compound Heterozygous	Compound Heterozygous
Variant	c.32G>C p. Arg11Pro	c.32G>C p. Arg11Pro	c.50C>T p. Thr17Met	c.1049T>C, p. Leu350Pro c.1264G>A, p. Ala422Thr	c.1049T>C, p. Leu350Pro c.1264G>A, p. Ala422Thr	c.1067A>C, p. Asp356Ala c.203dupA, p. Met69Aspfs*	c.1067A>C, p. Asp356Ala c.203dupA, p. Met69Aspfs*	c.268C>T, p. Arg90* c.394G>T, p. Gly132Cys
Origin	Kosovo	Kosovo	Libya	German	German	Turkey	Turkey	Canada
Consanguinity	N	N	Y	N	N	N	N	N
Gender / Age at follow-up	M / 2	F / 2y 6m	F / 7	F / 15	F / 21	M / 14y 2m	F / 7y 10m	F / 8
Occipital Frontal Circumference (OFC)								
Birth	34cm 20p	NA	NA	33cm 11p	NA	NA	NA	NA
Follow-up Percentile / SD	43cm <1p (-4SD)	40.5cm <1p (-5 SD)	42cm <1p (-7.7SD)	NA	NA	49.2Cm <1p (-3.4SD)	47.5cm <1p (-3.4SD)	41 cm <1p (-8.7SD)
Microcephaly	Y	Y	Y	Y	Y	Y	Y	Y
Developmental Milestones & Intellectual Disabilities								
GDD	Y	Y	Y	Y	Y	Y	Y	Y
Sitting (Months)	N	N	NA	NA	NA	NA	NA	NA
Walking (Years)	N	N	NA	3	3	3	2	6y 10m
Language	N	N	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed	Severely Delayed
Neurological Findings								
ID	Profound	Severe	Severe	Severe	Severe	Severe	Severe	Severe
Seizures	Myoclonic / GTC	GTC	GTC	GTC	N	GTC	GTC	GTC
Peripheral Neuropathy	NA	NA	NA	Y	Y	NA	NA	NA
Ataxia	NA	NA	NA	Y	Y	NA	NA	Y
Imaging	Delayed Myelination	Delayed Myelination	NA	NAD	NA	Thickening of gyri	NA	Thin corpus callosum Decreased white matter
Clinical Features								
Dysmorphic Features	NAD	NAD	NA	NAD	NAD	NAD	NAD	Hypotelorism
Tone	Increased	NAD	NA	Reduced	Reduced	NA	NA	Reduced
Power	Reduced	Reduced	NA	Reduced	Reduced	NA	NA	N
Sensation	NA	NA	NA	Reduced	Reduced	NA	NA	N
Co-ordination	NA	NA	NA	Ataxic gait	Ataxic gait	NA	NA	Ataxic
Reflexes	Reduced	Reduced	NA	Reduced	NA	NA	NA	N
Other	N	N	N	N	N	N	N	Hip dysplasia

Y = Yes, N= No, M= Male, F= Female, NA= Not Available, p = percentile, SD = standard deviation, NAD = No abnormalities detected, ID = Intellectual Disability, GTC = Generalised Tonic Clonic Seizures, ASD = Autistic Spectrum Disorder

Residue	Predicted effect of the mutation on <i>NARS11</i> protein
Arg11Pro	Unique domain of <i>NARS11</i> (UNE-N),
Thr17Met	Unique domain of <i>NARS11</i> (UNE-N),
Arg322Leu	Disturbance of the stabilization of aa-adenylate
Leu350Pro	Probably little effect on enzymatic activity and tRNA recognition, predictable effect on <i>NAR1</i> dimer formation.
Asp356Ala	Mutant at the interface between the two enzymes, loss of the side chain of Asp356 and therefore probably an interaction with the dimmer becoming more unstable
Ala422Thr	Affects the 3' end of the acceptor arm
Gly509Ser	Disrupts the end of the interface between the two subunits of <i>NARS11</i>
Arg545Cys	Potential disruption of the interaction with the acceptor arm of the tRNA

Table S5. Missense mutations in *NARS11* with the description of protein affects. The substrate in the figures is the aminoacyl-adenylate of *Brugia malayi* Asparaginyl-tRNA synthetase complexed with ATP: Mg and L-Asp-beta-NOH adenylate: PPI: Mg. The models of wild, mutant and *Brugia malayi* structures were superimposed with very good agreement. Apart from the Unique domain of *NARS1*, which cannot be modelled. The strongest effect in terms of catalysis would be expected for Arg322Leu and Asp356Ala.

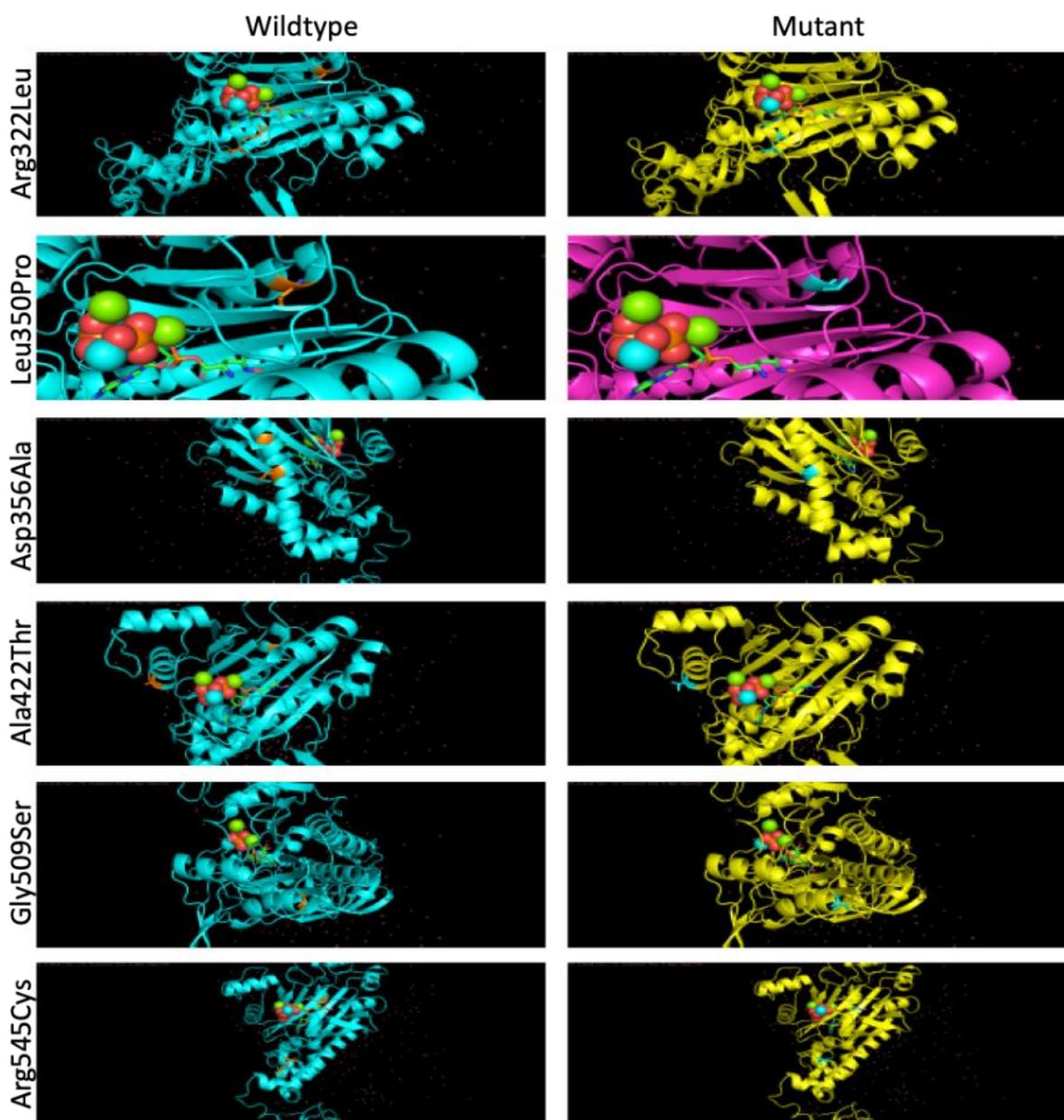


Figure S1. Molecular modelling of *NARS1* mutations. Wild type *NARS1* and each mutation was modelled using the Phyre² server¹³⁷. We observed that each model gave rise to a “wild type” *NARS1* enzyme indicating that none of the mutations completely destabilizes the protein structure (left panels). The Arg11Pro, Thr17Met and Met34Leu mutations could not be modelled because they lie in the unknown UNE-N domain. The substrate depicted with balls on the figure corresponds to the ATP:Mg and L-Asp-beta-NOHandenylate:PPi:Mg from the structure of *B. malayi* *NARS1* that was superimposed to each model we obtained. They were superimposed with a very good concordance. From this study we propose that the mutations might have the following effects: Asn218Ser (affects the interaction between *NARS1* and the anticodon arm of the tRNA), Arg322Leu (affects the stabilization of the aminoacyl-adenylate in the active site), Leu350Pro (little effect on *NARS1* activity and dimer interface), Asn356Ala (might weaken the dimer stability), Ala422Thr (little effect on 3' tRNA end binding), Gly509Ser (slight interference on dimer interface), Arg545Cys (see Figure 7).

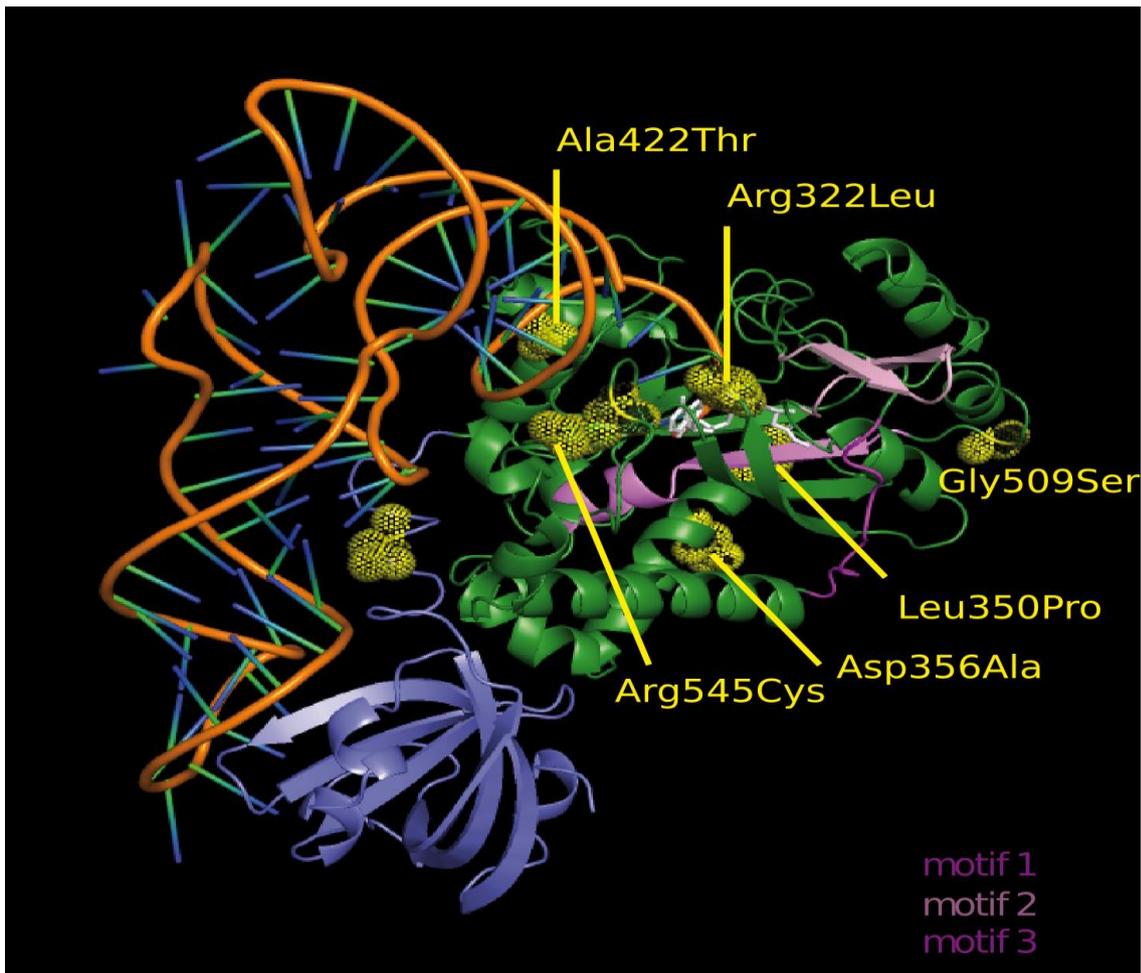


Figure S2. Model of human NARS1 obtained using the Phyre2 webserver. The latter was superposed with *Brugia malayi* NARS1 structure (2xti) complexed with ATP:Mg and L-Asp- β -NOH adenylate:PPi:Mg (L-Asp- β -NOH is shown in white color on the figure). The tRNA is tRNA^{Asp} that results from the superposition of the AspRS/tRNA^{Asp} complex from yeast with the human NARS1 model. Note that the UNE-N domain is absent from the modelled structure. The position of the mutations are indicated by yellow spheres.

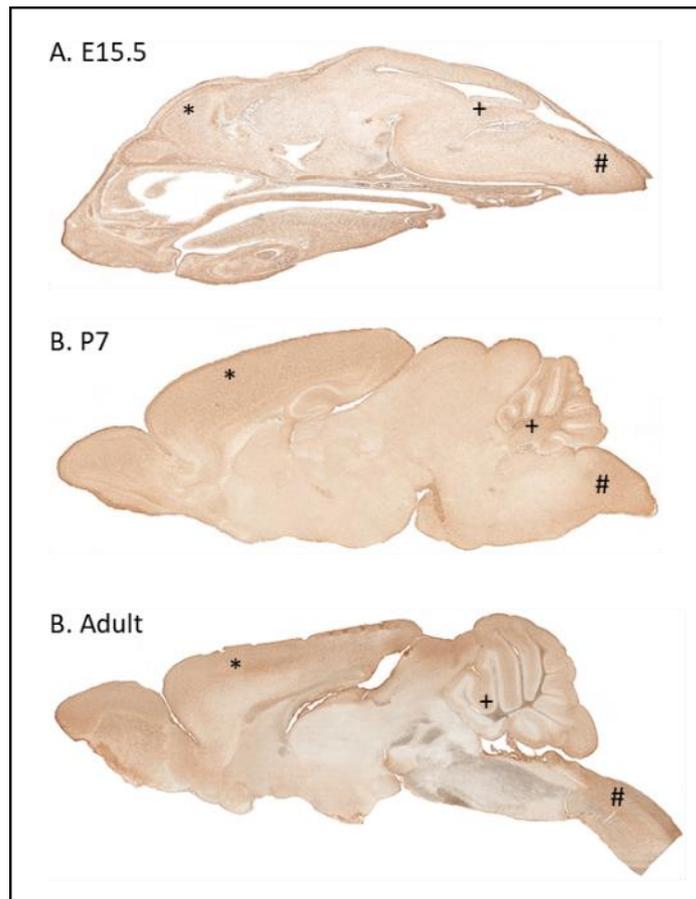


Figure S3. In-situ expression of the *NARS1* gene in mouse brain at three different ages, E15.5, P7 and adult. The *NARS1* probe used was GENSAT1-BX1431. Data from the Gene Expression Nervous System Atlas (GENSAT) Project. Expression was moderate in the brain, higher in the cortex (*), cerebellum (+) and brainstem (#).

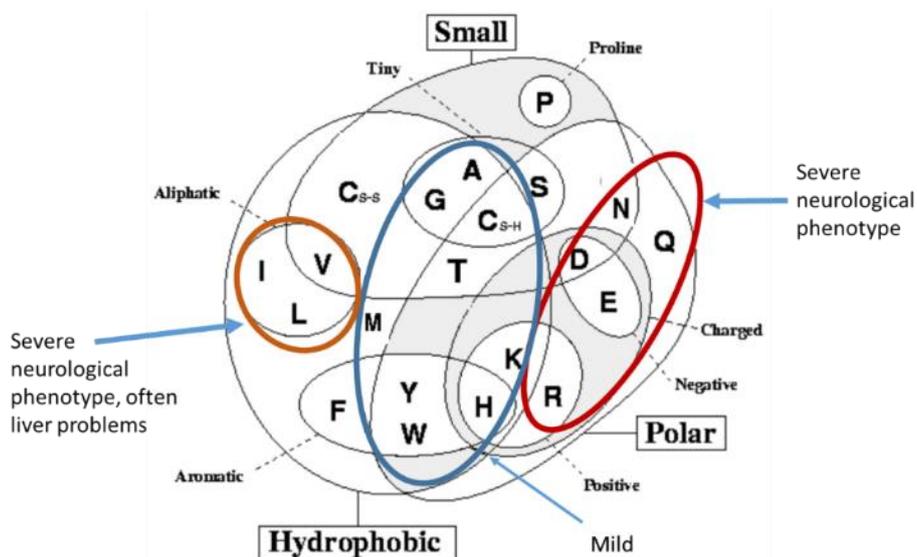


Figure S4. Modified Taylor's Venn diagram of AA properties, each amino acid is described by its physical or chemical properties (Taylor, TW, J, 1986, *Theor. Biol.* 119: 205-218). AARs clinical phenotype severity seem to cluster into two areas (red ovals) according to the amino acid that is targeted.

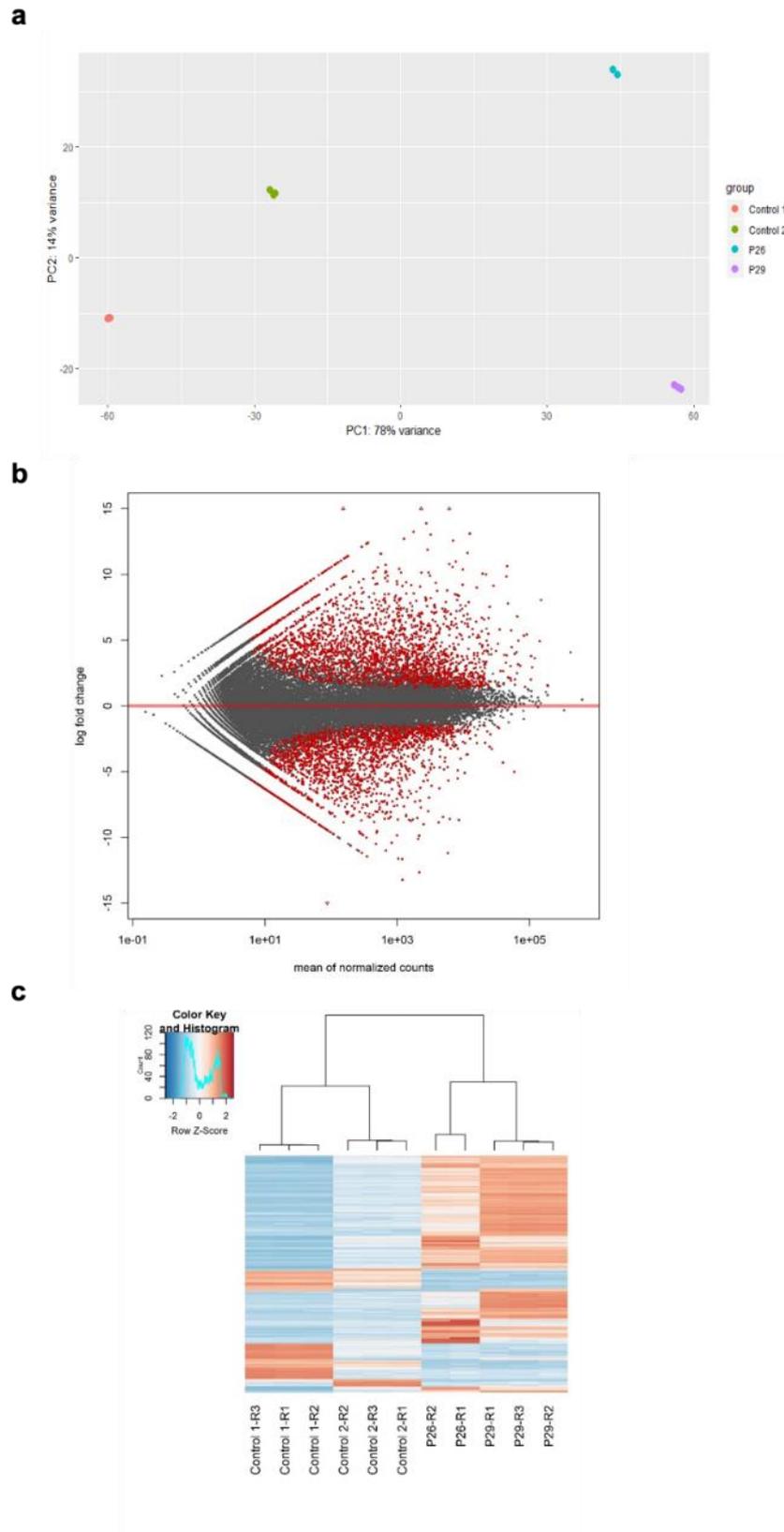


Figure S5. A. Principle component analysis of transcriptomic gene expression levels. **B.** MA plot showing normalised count number versus fold change, significantly differentially expressed gene shown in red. **C.** Cluster heatmap of top 750 most differentially expressed normalised gene counts.

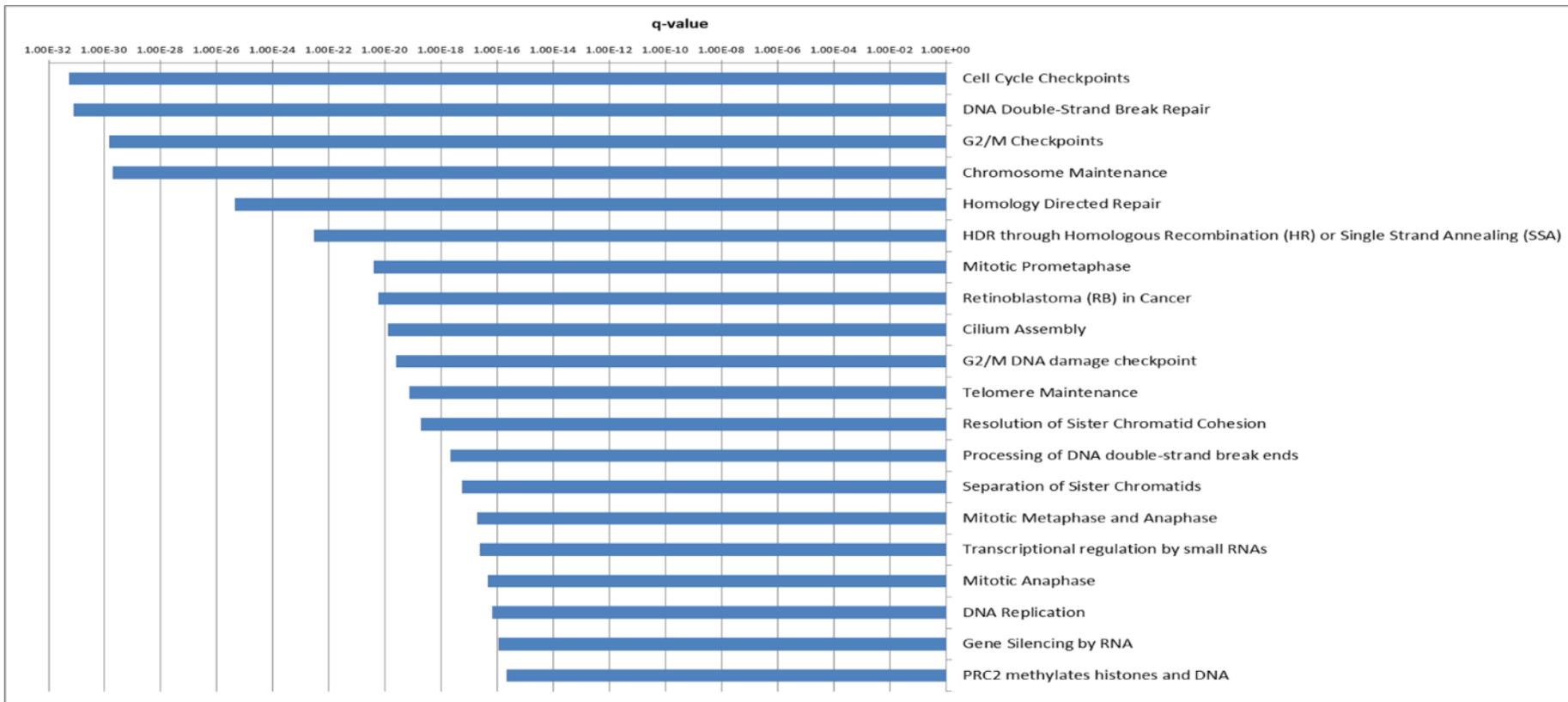


Figure S6. Supplementary iNPC data of top 20 (by significance) pathways associated with downregulated genes. (Filtered to <200 members)

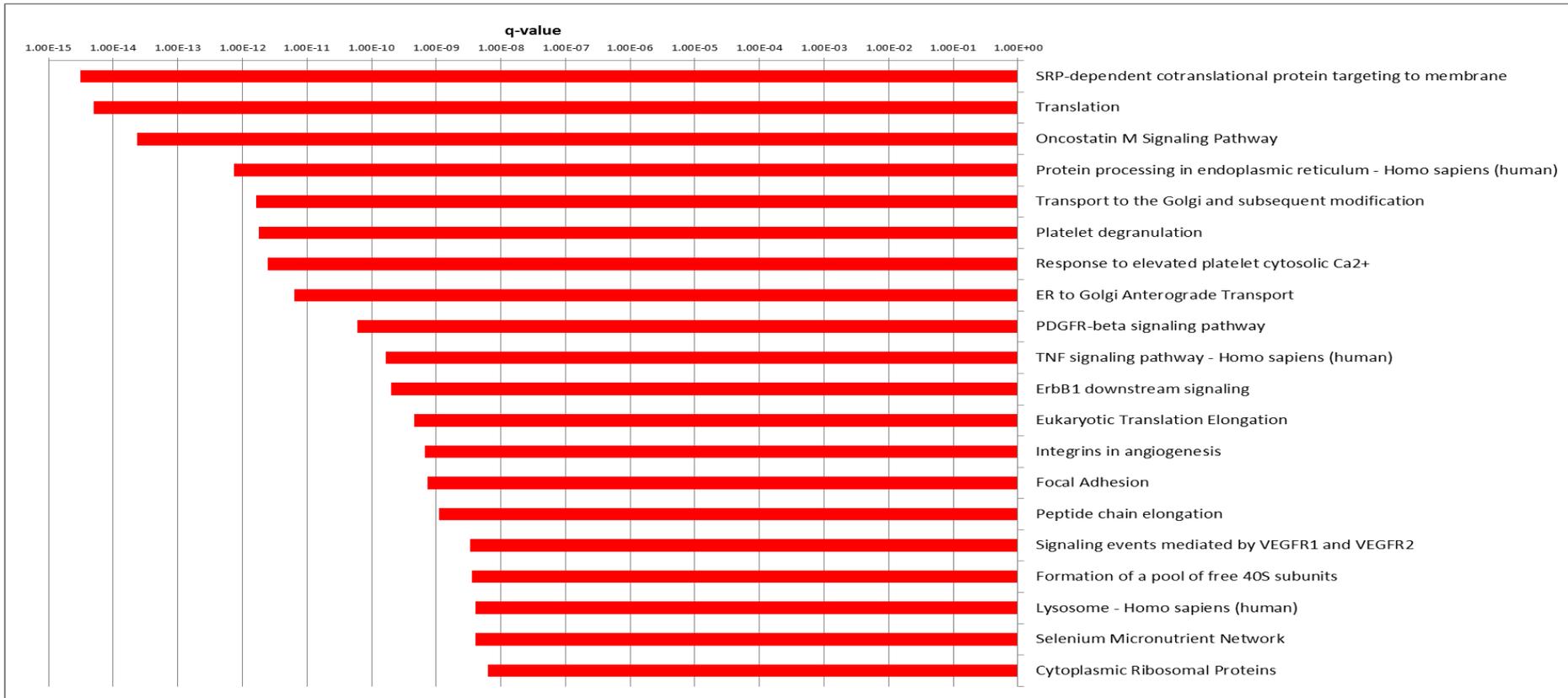


Figure S7. Supplementary iNPC data of top 20 (by significance) pathways associated with upregulated genes. (Filtered to <200 members)

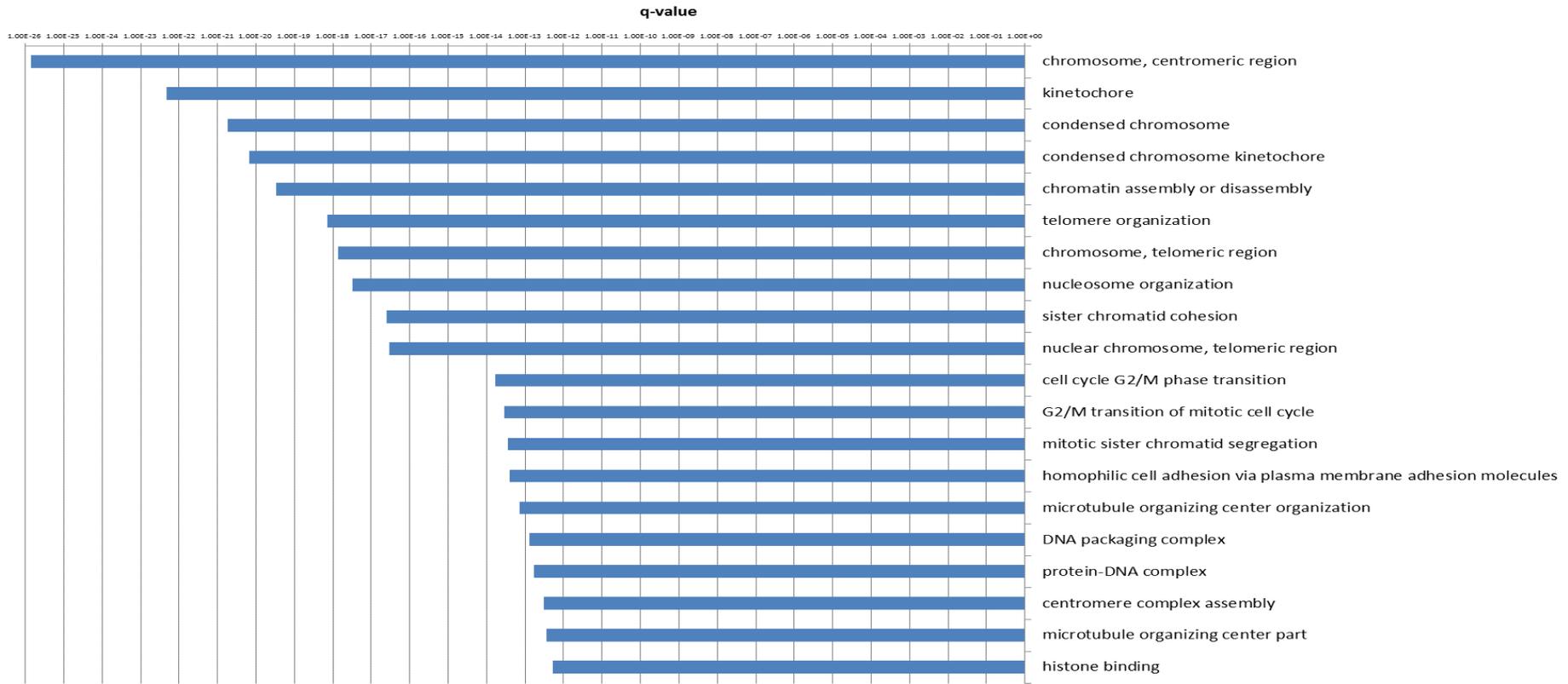


Figure S8. Supplementary iNPC data of top 20 (by significance) GO terms associated with downregulated genes. (Filtered to <200 members)



Figure S9. Supplementary iNPC data of top 20 (by significance) GO terms associated with upregulated genes. (Filtered to <200 members)

S10. Summary of Supplementary iNPC data

- Downregulated genes are especially associated with DNA replication, whereas upregulated are associated with protein synthesis and processing.
- VEGF related signalling is upregulated.
- *NARS1* gene expression was unaltered ($p=0.45$)
- Other tRNA synthetase genes were upregulated (*DARS*, *RARS*, *WARS*, *TARS*, *YARS*, *GARS*, *SARS*)
- *LARS* was downregulated & others were not altered.
- In the compound heterozygous patient, the frameshift mRNA is only expressed at a 1:9 ratio compared to the allele with a single base change (this can be visualised if desired).
- Western blots have shown decreased *NARS1* expression in patient fibroblasts in compound heterozygote only.
- Will try via qPCR to confirm NPC-associated gene expression.

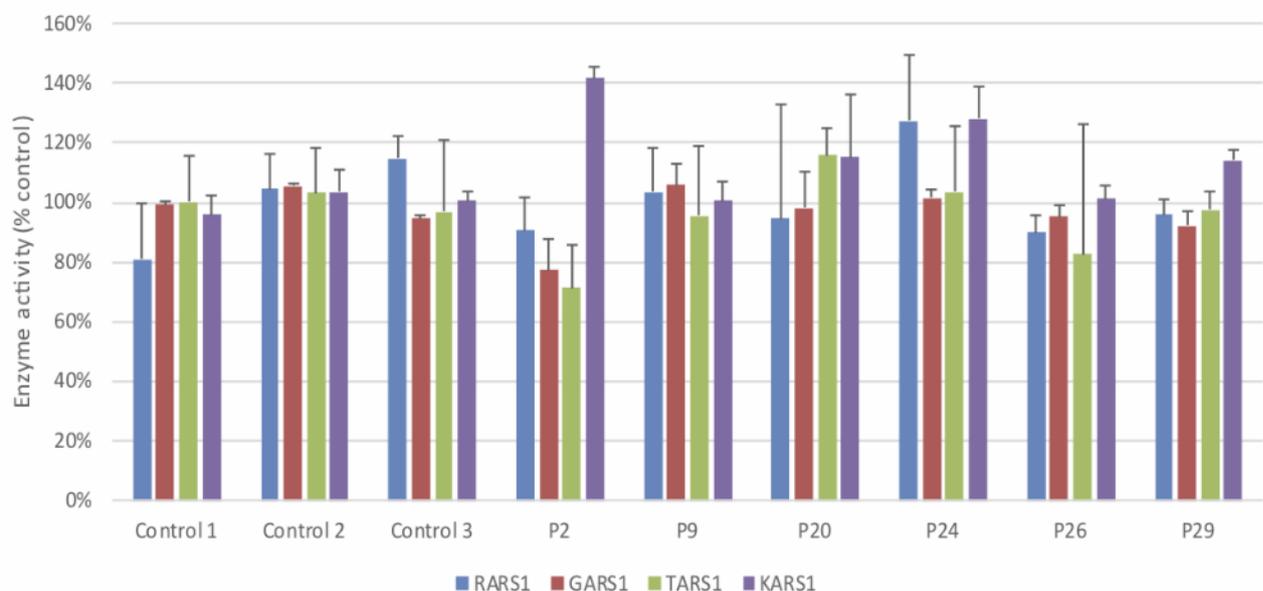


Figure S11. Patient derived fibroblast and lymphoblast cells displaying loss of *NARS1* activity compared to control. $n=9$, p value FDR <0.01 ; Asparaginyl-tRNA synthetase activity reduced significantly than other tRNA synthetase in patients' cells (carrying the p.Arg534*, p.R545C, p.Arg11Pro, p.Thr17Met and p.D356A / p.M69Dfs*4 mutations); TARS: Threonyl-tRNA synthetase; KARS: lysyl-tRNA synthetase; GARS: Glycyl-tRNA synthetase and RARS: arginyl-tRNA synthetase were measured as control for *NARS1* activity.

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