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### **Expanded phenotype of AARS1-related white matter disease**

Guy Helman, BS<sup>1,2</sup>, Marisa Mendes, PhD<sup>3</sup>, Francesco Nicita MD PhD<sup>4</sup>, Lama Darbelli, PhD<sup>5,6,7,8</sup>, Omar Sherbini, MPH<sup>9</sup>, Travis Moore, MSc<sup>5,6</sup>, Amy Pizzino, MGC<sup>9</sup>, Rosalba Carrozzo, PhD<sup>4</sup>, Alessandra Torracco, PhD<sup>4</sup>, Michela Catteruccia, MD<sup>4</sup>, Chiara Aiello, PhD<sup>4</sup>, Paola Goffrini, MS PhD<sup>10</sup>, Sonia Figuccia, MS<sup>10</sup>, Desiree Smith<sup>3</sup>, Kinga Hadzsiev, MD PhD<sup>11</sup>, Andreas Hahn, MD<sup>12</sup>, Saskia Biskup, MD PhD<sup>13</sup>, Ines Brösse, MD<sup>14</sup>, Urania Kotzaeridou, MD<sup>14</sup>, Darja Gauck, MSc<sup>15</sup>, Theresa A. Grebe, MD<sup>16</sup>, Francis Elmslie, MD FRCP<sup>17</sup>, Karen Stals, PhD<sup>18</sup>, Rajat Gupta, MSc, FRCPC<sup>19</sup>, Enrico Bertini, MD<sup>4</sup>, Isabelle Thiffault PhD FABMGG<sup>20,21,22</sup>, Ryan J. Taft, PhD<sup>23</sup>, Raphael Schiffmann, MD<sup>24</sup>, Ulrich Brandl, MD<sup>25</sup>, Tobias B. Haack, MD<sup>14</sup>, Gajja Salomons, PhD<sup>3</sup>, Cas Simons, PhD<sup>1,2</sup>, Geneviève Bernard, MD, MSc, FRCPC<sup>5,6,7,8</sup>, Marjo S. van der Knaap, MD PhD<sup>26,27</sup>, Adeline Vanderver, MD<sup>9,28,\*</sup>, Ralf A. Husain, MD<sup>25,\*</sup>

1. Murdoch Children's Research Institute, The Royal Children's Hospital, Victoria, Australia 3042
2. Institute for Molecular Bioscience, The University of Queensland, Queensland, Australia 4072
3. Metabolic Unit, Department of Clinical Chemistry, VU University Medical Center, 1081 HV Amsterdam, The Netherlands
4. Department of Neurosciences, Unit of Muscular and Neurodegenerative Disorders, Laboratory of Molecular Medicine, Bambino Gesù Children's Hospital, IRCCS, 00146 Rome, Italy
5. Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada H3G 1A4
6. Child Health and Human Development Program, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada H3H 2R9
7. Department of Pediatrics, McGill University, Montreal, Quebec, Canada H4A 3J1
8. Department of Human Genetics, McGill University, Montreal, Quebec, Canada H3A 0C7
9. Department of Neurology, Children's Hospital of Philadelphia, Philadelphia, PA USA 19104
10. Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, 43124 Parma, Italy
11. Department of Medical Genetics, University of Pécs, 7621 Pécs, Hungary
12. Department of Child Neurology, Justus-Liebig-University, 35389 Giessen, Germany
13. Praxis fuer Humangenetik and CeGaT GmbH, 72076 Tuebingen, Germany
14. Division of Child Neurology and Inherited Metabolic Diseases, Centre for Paediatrics and Adolescent Medicine, University Hospital Heidelberg, 69120 Heidelberg, Germany
15. Institute of Medical Genetics and Applied Genomics, University of Tuebingen, 72076 Tuebingen, Germany
16. Division of Genetics and Metabolism, Department of Child Health, Phoenix Children's Hospital, University of Arizona College of Medicine, Phoenix, Arizona 85016
17. South West Thames Regional Genetics Service, St George's University Hospital, London, UK SW17 0RE
18. Molecular Genetics Department, Royal Devon and Exeter NHS Foundation Trust, Exeter, UK EX2 5DW
19. Department of Neurology, Birmingham Children's Hospital, Birmingham, UK B4 6NH
20. Children's Mercy Kansas City, Center for Pediatric Genomic Medicine, Kansas City, MO USA 64108
21. Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals, Kansas City, MO, USA 64108

22. School of Medicine, University of Missouri-Kansas City, Kansas City, MO, USA 64108
23. Illumina, Inc., San Diego, CA, USA 92121
24. Baylor Scott & White Research Institute, Dallas, TX, USA 75204
25. Department of Neuropediatrics, Jena University Hospital, 07747 Jena, Germany
26. Department of Child Neurology, Emma Children's Hospital, Amsterdam University Medical Centers, Vrije Universiteit Amsterdam and Amsterdam Neuroscience, 1081 HV Amsterdam, The Netherlands
27. Department of Functional Genomics, Center for Neurogenomics and Cognitive Research, VU University, 1081 HV Amsterdam, The Netherlands
28. Department of Neurology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA 19104

# Denotes Shared Senior Author

Correspondence to:

Adeline Vanderver | vandervera@email.chop.edu

Ralf A. Husain | Ralf.Husain@med.uni-jena.de

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## **Abstract**

**Background:** Variants in genes *ARS1* and *ARS2*, encoding cytoplasmic and mitochondrial aminoacyl-transfer RNA synthetases (aaRSs) have been increasingly implicated in human disease. Biallelic *AARS1* variants were initially described as causing a discrete phenotype. As recent reports of individuals with other *ARS1* related diseases have identified cases with phenotypic variability from the index presentations, we sought to assess phenotypic variability in individuals with *AARS1*-related disease.

**Methods:** A cross-sectional survey was performed on individuals with biallelic variants in *AARS1*. Clinical data, neuroimaging, and genetic testing results were reviewed. AlaRS activity was measured in available fibroblasts of affected individuals.

**Results:** We identified 11 individuals with biallelic *AARS1* variants. Two phenotypic presentations emerged, one with early infantile-onset disease resembling the index cases of *AARS1*-related epileptic encephalopathy with deficient myelination (n=7). The second with a later onset disorder (n=4), where disease onset occurred after the first year of life and was characterized on neuroimaging by a progressive posterior predominant leukoencephalopathy evolving over time to include the frontal white matter. AlaRS activity was significantly reduced in five affected individuals tested with both early infantile-onset and late-onset phenotypes.

**Conclusions:** We suggest that variants in *AARS1* result in a broader clinical spectrum than previously appreciated. The predominant form observed results in early infantile-onset disease with epileptic encephalopathy and deficient myelination. However, a subgroup of affected individuals manifests with late-onset disease with a similarly rapid progressive clinical decline. Further studies including longitudinal imaging and clinical follow up will be valuable in understanding disease progression and outcome.

**Keywords:** AARS1, Magnetic Resonance Imaging, Phenotype, Epileptic encephalopathy, Deficient myelination

## Introduction

Variants in genes *ARS1* and *ARS2*, encoding cytoplasmic and mitochondrial aminoacyl-transfer RNA synthetases (aaRSs) have been implicated in a series of heritable neurologic conditions. AaRSs make up a group of ubiquitously expressed, essential enzymes that play a role in the first step of protein translation through the charging of tRNA molecules with their cognate amino acids. In general, each amino acid is coupled to its own charging enzyme and these enzymes are encoded by 37 unique genes with function either primarily in the cytoplasm (designated -*ARS1*) or in mitochondria (designated -*ARS2*).<sup>1,2</sup> Recently, these genes have been associated with dominant and/or recessive forms of diseases that are thought to arise due to dysfunction in tRNA charging activity and the inability to meet high translational demand.<sup>1,3</sup>

A recent deep phenotyping study of recessive *ARS1* defects demonstrated that all identified disorders were associated with central nervous system (CNS) signs or impairment of sensory functions such as hearing and/or vision. In most cases, individuals presented with failure to thrive, feeding, and gastrointestinal problems.<sup>3</sup> Liver disease, facial dysmorphisms, various endocrine abnormalities, and mitochondrial dysfunction were reported for >30% of the different *ARS1* defects.<sup>3</sup> Often, this pattern of severe multi-system disease occurred in the first months of life and was concurrent with periods of stress, particularly infection.<sup>3</sup>

For many of the recessive *ARS1*-related disorders, the earliest reports revealed unique phenotypes that permitted the grouping of patients and identification of disease.<sup>4-6</sup> However, increased identification of affected individuals with variants in these genes has revealed significant phenotypic diversity amongst individuals with variants in *ARS1* genes. As genomic evaluation continues to grow in the diagnostic workup of patients, knowledge of the phenotypic spectrum of a given disorder can help to corroborate genetic findings,<sup>7</sup> in particular in the absence of absolute evidence regarding the pathogenicity of an identified variant.

*AARS1* encodes the cytoplasmic aminoacyl tRNA synthetase for alanine (AlaRS) and has been previously associated with dominant and recessive disease. Heterozygous variants in *AARS1* are associated with a dominant form of Charcot-Marie-Tooth disease, type 2N (CMT2N [OMIM: 613287]). In the case of recessive *AARS1*-related disease (OMIM: 616339), the index cases had a

distinct clinical presentation, with early infantile epileptic encephalopathy, deficient cerebral myelination and peripheral neuropathy.<sup>5</sup> Subsequent reports of 4 individuals from 3 families with *AARS1*-related disease matched this phenotype<sup>8-10</sup> and a more recent report revealed recurrent acute liver failure in a single case.<sup>11</sup> Here we present data on 11 additional individuals from ten families with biallelic variants in *AARS1*. We provide insight into a divergent presentation of recessive *AARS1*-related disease through clinical and neuroimaging evidence in cases with atypical presentations. Further, we provide functional assessment of AlaRS aminoacylation activity in five individuals.

## **Materials and Methods**

### *Patient recruitment*

Affected individuals and their families were recruited under studies with approval from the institutional review board at their respective institutions. Written informed consent for collection of clinical information, neuroimaging, and genetic information was obtained for each study participant.

### *Abstraction of clinical data*

Clinical and demographic data was abstracted from available medical records of affected individuals. Neuroimaging was reviewed for all cases where available. Genetic testing reports were reviewed or variants were provided by the referring provider and classified using the American College of Medical Genetics (ACMG) criteria for variant curation.<sup>12</sup>

### *Aminoacylation studies*

Cytosolic AlaRS activity was measured in available patient fibroblast lysates, which were incubated at 37°C for 10 min in reaction buffer (50 mM Tris buffer [pH 7.5], 12 mM MgCl<sub>2</sub>, 25 mM KCl, 1 mg/mL bovine serum albumin, 0.5 mM spermine, 1 mM ATP, 0.2 mM E. coli total tRNA, 1 mM dithiothreitol, 0.3 mM [D<sub>3</sub>] alanine and 0.3 mM [<sup>15</sup>N<sub>2</sub>] arginine). Aminoacyl-tRNA was precipitated with trichloroacetic acid (TCA). After washing free amino acids with TCA, [D<sub>3</sub>] alanine and [<sup>15</sup>N<sub>2</sub>] arginine were detached from the tRNA by addition of ammonia. [D<sub>7</sub>] alanine and [<sup>13</sup>C<sub>6</sub>] arginine were added as internal standards. Labelled amino acids were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

## Results

### *Clinical Summaries of Divergent Presentations*

Eleven individuals from ten families were seen at 8 independent institutions and were evaluated for the possibility of an encephalopathy. In each of these cases, biallelic variants in *AARS1* were identified (Table 1).

Across this series, age at disease onset ranged from birth to 28 years of life. Six previously unreported individuals presented within the first four months of life, while the remaining four presented after the first year (12 months – 27 years). All individuals developed a spastic tetraparesis over time. In most affected individuals, there was developmental delay (9/11), seizures (9/11), truncal hypotonia (8/11), and microcephaly (primary or secondary, 7/11). Four individuals had optic atrophy and vision loss over the course of their disease (Supp Fig. S1).

All seven individuals with early infantile-onset disease had epileptic encephalopathy, developmental delay, microcephaly, and hypotonia. Microcephaly was primary in four of six cases and secondary in the remaining. These individuals did not achieve early developmental milestones (7/7) and were profoundly neurologically disabled. One individual (I-7) had congenital vertical talus, similar to the index cases.<sup>5</sup> There was no remarkable family history in any affected individual.

In this group of individuals with early infantile-onset disease, reported symptoms were primarily neurological although extra-neurological features were also seen. Feeding problems were prominent (6/7) with failure to thrive noted in six individuals and gastrostomy tube (g-tube) dependence in most cases (5/7). Gastroesophageal reflux (6/7) was also a common gastrointestinal complication of their neurologic disease. I-9 had hepatomegaly with elevated GGT on laboratory studies and periods of supraventricular paroxysmal tachycardia. Several patients had musculoskeletal complications from their neurologic disease. I-6a had congenital joint contractures and a congenital pulmonary airway malformation. I-8 was reported to have severe osteoporosis with at least one pathological fracture. Two individuals (I-6a and I-8) died from complications of their neurological disease at 8 months and 15 years of age; in I-8, this was

noted to be due to complications from central respiratory failure. There were no other cardiac, respiratory, or gastrointestinal disturbances reported in these individuals.

In the individuals with late-onset disease, there was a significant range in age of disease-onset. The eldest patient, I-1, presented at 27 years of age with severe headaches, dizziness, loss of balance and blurred vision. She had a history of headaches early in adolescence. She had peripheral neuropathy and examination also revealed a wide-based gait with progressive left lower extremity spasticity and hyperreflexia. Last evaluated at 30 years of age, she had progressive cognitive decline, gait imbalance, peripheral vision loss, facial weakness, and dysarthria.

I-2 is a female with intrauterine growth retardation but normal growth and development in the first year of life. Best acquired motor development was supported walking. In the second year of life, she had a regression in speech and motor skills and had progressive spasticity of the upper and lower extremities. As the disease progressed, she developed severe spastic tetraparesis by 38 months of age which required an intrathecal baclofen pump placed over time. She developed neurogenic bladder and optic atrophy with loss of vision at 42 months and focal epilepsy at 4 years of age. She also suffered from chronic constipation, hip dislocation, scoliosis, pes equinovarus and severe osteoporosis with pathologic fracture. Respiratory chain analysis in muscle tissue showed diminished activities for complexes I, II/III and IV. Electrophysiological studies showed pathologic evoked visual and somato-sensory potentials. Laboratory studies showed chronic hypoalbuminemia. She was last evaluated at 12 years of age and had severe neurological impairment but a stable disease course.

I-3 presented at 36 months with regression in mobility, motor skills and speech after a normal pregnancy, neonatal period and early psychomotor development. She developed a severe spastic tetraparesis with dystonia and had hip dislocations. She developed intractable focal epilepsy. On examination at 19 years of age she had microcephaly and remained alert and interactive. She had recurrent lower respiratory tract infections thought to be related to aspiration in her late teenage years and was transitioned to g-tube feedings. She died at 19 years of age as a result of central respiratory failure during a respiratory tract infection.



I-4 developed normally in the first year of life, but mild development delays were noted in the second year of life after a suspected episode of encephalopathic episode with no determined cause. During his initial episode of encephalopathic episode he was noted to be hypoalbuminemic which has since resolved. He started to walk autonomously at the age of 16 months and language delay resolved with speech therapy **at age XX**. He developed normally and remained healthy until 12 years of age when he had an abrupt onset of neurological symptoms with right-sided hemiparesis, which evolved to a tetraparesis over a two-week period, and followed by significant motor and cognitive regression over the next five months. He also developed bradykinesia and dystonic movements. Communication was limited as he developed a severe spastic dysarthria but was able to understand simple commands.

**Commentato [GH1]:** Francesco please comment if you know

### *Neuroimaging*

Seventeen neuroimaging studies were available for review from 8 affected individuals while in one case only the clinical report of MRI findings was available. In three individuals, all with early infantile onset disease, brain MRI showed deficient myelination in the earliest scans, where early myelinating structures such as the brainstem and posterior limb of the internal capsule (PLIC) suggest absent myelination from at the earliest study 4 months of age. In I-6a there was immature gyration, absent myelination, and evidence of early atrophy. I-9 had a brain MRI and CT performed at 4 and 5 months of age, respectively (Figure 1A and 2B). The earliest scans show absent myelination in early myelinating structures, including the cerebellum, brainstem, and PLIC, with early ventriculomegaly and enlarged subarachnoid spaces. A follow-up CT scan at 23 months of age revealed calcifications in the cerebellar white matter and the dentate nuclei (Figure 2B). An MRI performed at 6 years and 5 months of age showed total cerebral white matter volume loss with profound ventriculomegaly and enlarged subarachnoid spaces.

The four individuals with late-onset disease all had a distinctly different white matter disease, characterized by a posterior predominant leukoencephalopathy (Figure 1C-E). In all cases, the splenium and the posterior aspect of the body of the corpus callosum (4/4) were significantly affected while the genu is spared in I-1 and I-4. In I-3, there was isolated involvement of the inner blade of the genu which progressed to total involvement on follow-up scans. The frontal

white matter was spared in I-1 and I-4 (2/4) and the subcortical U-fibers were spared in all 4 individuals (4/4). There was extension of the white matter signal hyperintensity through the corticospinal tracts into the brainstem. In I-2 there was total involvement of the ventral midbrain structures, and progressive atrophy of these structures on follow-up imaging. There were areas of restricted diffusion in the affected periventricular white matter (2/3) and suspected tissue necrosis with white matter rarefaction seen on FLAIR (1/3). Follow-up imaging in I-2 and I-3 suggested a progressive degenerative disease with marked atrophy affecting the cerebrum and cerebellum on repeat imaging scans. CT scan in I-2 revealed the presence of cerebral and cerebellar calcifications (Figure 2C). No follow-up scans were available for I-1 and I-4 from their initial presentation which showed evidence of active demyelination posteriorly and mild white matter volume loss. MRS in I-2 revealed elevated lactate and in I-4 showed peaks of choline and lactate and mild NAA reduction.

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Commentato [GH3]: Ask Marjo r.e. her case

#### *AARS1 variants identified*

Across these 11 individuals from ten unrelated families, 15 unique variants in *AARS1* were identified through next-generation sequencing (Table 1; *AARS1* NM\_001605.2), of which most were not previously reported in affected individuals. One individual was compound heterozygous for a variant observed in the initial description of this condition, p.(Arg751Gly, found *in trans* with a unique missense variant (p.(Asn604Lys)). I-7 and I-10 each were compound heterozygous for p.(Gly913Asp) and a truncating variant (p.(Arg330Ter) and p.(Arg333Ter), respectively). The p.(Gly913Asp) variant was previously seen in the early infantile-onset form of *AARS1*-related disease.<sup>8,9</sup> All other variants had not been previously identified in the literature or the ClinVar variant database. Two individuals, one with late and the other with early onset disease (I-3 and I-9, respectively), shared the same variant, p.(Gly581Ser). However, in I-3 the variant was found in the homozygous state, while in I-9 it was compound heterozygous with a frameshift variant (p.(Gln154HisfsTer9)). All identified variants were absent or found at very low allele frequencies in the gnomAD population allele frequency database (range 0.0 - 0.00002), are predicted damaging across a suite of *in silico* tools and affect highly evolutionarily conserved residues (Table 1).

Human *AARS1* consists of four functional domains derived from sequence alignment and structural modelling: the aminoacyl-tRNA synthetase domain (residues 1-254), the tRNA recognition domain (residues 255-451), the editing domain (residues 468-757), and the oligomerization (C-terminal) domain (residues 758-968). The identified variants fell across all domains of the protein (Figure 3A) and no discernible genotype-phenotype relationship emerged from this analysis.

Missense variants identified in I-1 were associated with increased transcription and mRNA levels on reverse-transcription quantitative polymerase chain reaction (RT-qPCR, [Supp Fig. S2]). Despite increased mRNA levels, western blot showed reduced AlaRS protein levels (Supp Fig. S3), suggesting protein instability. In I-4, the p.(Val666Ala) variant and multi-exonic deletion were found to cause decreased mRNA and protein level (Supp Fig. S4 - S5). Moreover, the p.(Val666Ala) expressed in a yeast *AARS1* knockout model demonstrated reduced ability to rescue yeast growth relative to wt *AARS1* (Supp Fig. S6), supporting pathogenicity of this missense variant.

#### *Defective Aminoacylation*

AlaRS activity was measured in cytoplasmic fractions isolated from fibroblasts of three of the four affected individuals with late-onset disease and performed on two individuals with early-infantile onset *AARS1*-related disease. Fibroblasts were not available from the remaining individuals. In all individuals studied, AlaRS activity was reduced. In the late-onset group, AlaRS residual activity was found to be 16%, 35%, and 11% of controls in I-1, I-2, and I-4 respectively (Figure 3B). In I-7 and I-9 from the early-infantile onset group, AlaRS activity was measured to be 9% and 15% in those individuals, respectively. Arginine tRNA synthetase (ArgRS encoded by *RARS1* [OMIM: 107820]) activity, measured as an internal control, was within control values for all affected individuals tested.

#### **Discussion**

While many disorders associated with *ARS1* variants were first described with isolated phenotypes, wider clinical variation is increasingly recognized. In this series, we present clinical and molecular data on an additional 11 cases of *AARS1*-related disease. Analysis of these

individuals identified two groups on the basis of age at disease-onset and presentation of neuroimaging findings. Early infantile-onset recessive *AARS1*-related cases presented similarly to the patients previously described in Simons et al.<sup>5</sup> and Nakayama et al.<sup>8</sup> A separate group of individuals with onset of disease beyond the first year of life and a distinctly different neuroradiologic phenotype emerged, indicating also wider neuroradiologic variation. Our findings broaden the clinical and the genetic spectrum associated with recessive *AARS1* variants beyond early infantile-onset epileptic encephalopathy with deficient myelination.

In our series, several individuals had compound heterozygous variants where one allele was distinctly classified as likely pathogenic or pathogenic based on the ACMG criteria. To the best of our knowledge, carriers of these alleles did not display any neurological signs of CMT2N and thus it is possible that in these individuals, the reduced aminoacylation activity associated with the variant allele is compensated for by the activity associated with the wild-type allele. In addition, to-date no reported pathogenic or likely pathogenic *AARS1* variant causing CMT2N as listed on ClinVar has also been associated as a disease-causing variant in recessive disease (Figure 3A). Thus, there does not appear to be a genotype-phenotype correlation predicting whether an *AARS1* variant would be causative of either dominant or recessive *AARS1*-related disease, nor does there appear to be a correlation between the residual enzyme activity and the severity of the disease or the age of onset. More recently there has been a report of a single family where a heterozygous variant in *AARS1* was suspected to be the cause of an late-onset hereditary diffuse leukoencephalopathy with spheroids of the Swedish type (HDLS-S) in an extensive family.<sup>13</sup> As there is further expansion of both dominant and recessive forms of *AARS1*-related disease, the possibility and threshold of compensation remains an intriguing clinical question.

Late-onset and rapid deterioration has previously been described in multiple individuals in another *ARS1*-related disease (*KARS1*, OMIM: 601421).<sup>14-16</sup> In these individuals, there was early development of sensorineural deafness and adult-onset cognitive decline. MRI in these individuals demonstrated a leukoencephalopathy affecting the corpus callosum, the periventricular white matter, and the anterior limb of the internal capsule. Aminoacylation studies revealed reduced capacity for LysRS in these individuals. Divergent presentations have

also been cited in disease due to *RARS1*-variants<sup>17</sup> and *QARS1*-variants (OMIM: 615760).<sup>18</sup> While our assessment is limited to the combined 15 individuals in the present study and those previously reported,<sup>5,8-11</sup> a diverging clinical presentation is evident and should be considered in the diagnostic workup of similar patients. Further it should be noted that late-onset *KARS1*-, *AARS1*- and *AARS2*-related disease share similar MRI features and may confound gene-specific diagnostic approaches. Finally, it bears mentioning that the overlap between late-onset *AARS1*- and *AARS2*-related disease is strikingly similar to that seen in disorders affecting the aspartyl-tRNA synthetase, encoded by *DARS1* (OMIM: 603084) and *DARS2* (OMIM: 610956)<sup>6</sup> and provides further impetus for broader genomic testing approaches in these individuals.

*ARS1* variants almost invariably have a predilection for involvement of the CNS white matter. Almost all of the cytoplasmic aaRS enzymes that have been implicated in genetic disease have been associated with a prominent white matter phenotype.<sup>3,4,6,15,17</sup> In the index cases of recessive *AARS1*-related disease, there was an apparent deficiency and arrest in myelination from disease-onset, with only mild progression of myelination in early myelinating structures in one individual.<sup>5</sup> This phenotype is recapitulated in most affected individuals in our study (n=6). In individuals with late-onset disease, MRI demonstrated a prominent leukoencephalopathy, with posterior involvement affecting the splenium of the corpus callosum, PLIC, and extending through the corticospinal tracts. This appears to be progressive over time, invariably affecting the genu of the corpus callosum and extending to the frontal white matter in two individuals. Further studies including longitudinal imaging and clinical follow up will be valuable in understanding the progression of features in this disorder.

### **Supplemental Text**

A supplemental text with results regarding validation of variants identified in I-1 and I-4 is provided.

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**Table 1. AARS1 Identified Variants**

Study ID	Zygoty	Variant (GRCh37)	cDNA Change (NM_001605.2)	Protein Change	gnomAD AF <sup>a</sup>	CADD <sup>b</sup> / SIFT / Mutation Taster	ACMG Variant Classification	ACMG Criteria <sup>c</sup>	Enzyme Activity
I-1*	Mat.	16-70310906-T-C	c.296A>G	p.(Glu99Gly)	0.0	32 / D / DC	P	PS3, PM2, PM3, PP3	0.218 nmol/(min* mg protein)
	<i>De novo</i>	16-70304137-T-C	c.778A>G	p.(Thr260Ala)	0.000004	27 / D / DC	P	PS3, PS2, PM2, PM3, PP3	16% activity
I-2	Pat.	16-70305792-CT-TG	c.562_563delinsCA	p.(Ser188His)	0.0	27 / D / DC	P	PS3, PM2, PP3, PP4	0.293 nmol/(min* mg protein)
	Mat.	16-70296346-C-T	c.1574G>A	p.(Cys525Tyr)	0.0	25 / T / DC	LP	PM2, PM3, PP3, PP4	35% activity
I-3	Hom.	16-70294991-C-T	c.1741G>A	p.(Gly581Ser)	0.000007	25 / D / DC	P	PM2, PM3, PP3, PP4	NA
I-4*	Pat.	16-70292116-A-G	c.1997T>C	p.(Val666Ala)	0.000005	25 / D / DC	P	PS3, PM2, PM3, PP3, PP4	0.137 nmol/(min* mg protein)
	Mat.	Exon 1-4 Deletion			NA		P	PVS1, PM2, PM3	11% activity
I-5	Pat.	16-70289666-T-C	c.2251A>G	p.(Arg751Gly)	0.000048	27 / D / DC	P	PS1, PS3, PM2, PP3	NA
	Mat.	16-70293063-G-C	c.1812C>G	p.(Asn604Lys)	0.0	25 / D / DC	LP	PM2, PM3, PP3, PP4	NA
I-6a & I-6b	Hom.	16-70289631-C-T	c.2286G>A	p.(Lys762=) <sup>#</sup>	0.000004	23 / NA / NA	P	PVS1, PS3, PM2, PM3, PP3, pp4	NA
I-7	UNK	16-70302257-G-A	c.988C>T	p.(Arg330Ter)	0.0	37 / NA / DC	P	PVS1, PM2, PP3	0.067 nmol/(min* mg protein)
	UNK	16-70286793-C-T	c.2738G>A	p.(Gly913Asp)	0.00002	31 / D / DC	LP	PS3, PM2, PP3, PP4	9% activity
I-8	Pat.	16-70310455-AAGT-	c.410_413delACTT	p.(Tyr137LeufsTer9)	0.0	32 / NA / NA	LP	PVS1, PM2	NA
	Mat.	16-70296331-T-C	c.1589A>G	p.(Asp530Gly)	0.0	32 / D / DC	LP	PM2, PM3, PP3, PP4	NA
I-9	Pat.	16-70310405-TC-	c.462_463delGA	p.(Gln154HisfsTer9)	0.0	25 / NA / NA	P	PVS1, PS3, PM2, PM3	0.184 nmol/(min* mg protein)
	Mat.	16-70294991-C-T	c.1741G>A	p.(Gly581Ser)	0.000004	25 / D / DC	P	PS3, PM2, PM3, PP3, PP4	15% activity
I-10	Mat.	16-70302248-G-A	c.997C>T	p.(Arg333Ter)	0.000004	38 / NA / DC	P	PVS1, PM2, PM3, PP4	NA
	Pat.	16-70286793-C-T	c.2738G>A	p.(Gly913Asp)	0.00002	31 / D / DC	P	PS4, PS3, PM3, PP3, PP4, PP5	NA

\* Support for the pathogenicity of the variants found in this individual is provided in the Supplemental Text. <sup>a</sup> Allele Frequency determined from gnomAD v2.1.1. <sup>b</sup> Max CADD score at variant site if multi-allelic variant; <sup>c</sup> ACMG scoring criteria are described in Richards et al. (2015). <sup>#</sup> Variant shown to affect splicing and is predicted by SpliceAI to cause the gain of a novel donor splice site 17 nucleotides downstream and loss of the canonical acceptor splice site at that position: DS\_DG=0.7259; DP\_DG=17; DS\_DL=0.1621; DP\_DL=0.<sup>19</sup> Abbreviations: Mat. – Maternal; Pat. – Paternal; UNK – Unknown; Hom. – Homozygous; D – Damaging; DC – Disease causing; T – Tolerated; NA – Not available; P – Pathogenic; LP – Likely pathogenic.

## Figure Legends

**Figure 1. MRI findings in individuals with recessive *AARS1*-related disease.** Evaluation of the neuroimaging findings between individuals with early infantile-onset disease in comparison to late-onset individuals. Sagittal and axial T<sub>1</sub> and T<sub>2</sub> weighted images were compared for all individuals. Diffusion-weighted and FLAIR images were compared where available. (A-B) In individuals with early infantile-onset there is early evidence of deficient myelination. At 4 months of age in I-9, there is absent myelination in early myelination structures, including the posterior limb of the internal capsule and mild ventriculomegaly suggesting a degree of cerebral volume loss. At 6 years, profound cerebral atrophy is seen. (B) Some individuals with early infantile-onset disease achieve a mild degree of myelination, but there is some cerebral atrophy and a persistent deficiency in myelination over time. (C-E) In individuals with late-onset disease, age at disease onset and neuroimaging features are more variable. In all individuals shown, there appears to be preferable involvement of the splenium of the corpus callosum seen on both sagittal (white arrow) and axial imaging (white arrowhead, D and E). (C) In the individual with late-onset disease and available follow-up imaging over time, there is progressive involvement to affect the frontal white matter and the remainder of the corpus callosum (dashed white arrow). There is also progressive cerebral atrophy. (D) There is progressive cerebral white matter involvement and areas of restricted diffusion in the affected periventricular white matter in cases with diffusion-weighted imaging obtained during the period of clinical decline.

**Figure 2. Calcium deposits in *AARS1*-related disorder.** (A) CT imaging of I-9 was performed at 5 months and 23 months of age. On early imaging studies calcifications were not evident, but (B) follow-up imaging at 23 months demonstrated calcifications in the cerebellum and thalami bilaterally. (C) CT in I-2 at 3 years of age reveals the presence of calcifications in the cerebral and cerebellar white matter.

**Figure 3. Variants identified in this cohort and aminoacylation studies of affected individuals.** (A) Schematic overview of the AlaRS functional domains and variants identified within this cohort. The amino acid changes caused by previously identified variants (Lys81Thr and Arg751Gly) are noted with a #. One individual (I-4) was found to have a structural variant (not shown). Variants associated with late-onset disease are colored in blue, while variants in black are associated with early infantile-onset disease, and variants in red seen in both phenotypes. Variants marked in grey are previously associated with autosomal dominant Charcot-Marie-Tooth disease, type 2N. (B) Aminoacylation studies performed in triplicate on fibroblast lysates from three individuals with late-onset *AARS1*-related disease and two with the more severe early infantile-onset form shown diminished AlaRS activity relative to a control sample and using ArgRS activity as an in-sample control. Activity is more severely reduced in three individuals, including two (I-7 and I-9) with early infantile-onset disease and one individual (I-4) with later disease-onset but a rapidly progressive course. Bars represent average  $\pm$  standard deviation.