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RESEARCH ARTICLE

RARS1-related hypomyelinating leukodystrophy: Expanding the spectrum

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Abstract

Objective: Biallelic variants in *RARS1*, encoding the cytoplasmic tRNA synthetase for arginine (ArgRS), cause a hypomyelinating leukodystrophy. This study aimed to investigate clinical, neuroradiological and genetic features of patients with *RARS1*-related disease, and to identify possible genotype-phenotype relationships. **Methods**: We performed a multinational cross-sectional survey among 20 patients with biallelic *RARS1* variants identified by next-generation sequencing techniques. Clinical data, brain MRI findings and genetic results were analyzed. Additionally, ArgRS activity was measured in fibroblasts of four patients, and translation of long and short ArgRS isoforms was

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quantified by western blot. **Results**: Clinical presentation ranged from severe (onset in the first 3 months, usually with refractory epilepsy and early brain atrophy), to intermediate (onset in the first year with nystagmus and spasticity), and mild (onset around or after 12 months with minimal cognitive impairment and preserved independent walking). The most frequent *RARS1* variant, c.5A>G, led to mild or intermediate phenotypes, whereas truncating variants and variants affecting amino acids close to the ArgRS active centre led to severe phenotypes. ArgRS activity was significantly reduced in three patients with intermediate and severe phenotypes; in a fourth patient with intermediate to severe presentation, we measured normal ArgRS activity, but found translation mainly of the short instead of the long ArgRS isoform. **Interpretation**: Variants in *RARS1* impair ArgRS activity and do not only lead to a classic hypomyelination presentation with nystagmus and spasticity, but to a wide spectrum, ranging from severe, early-onset epileptic encephalopathy with brain atrophy to mild disease with relatively preserved myelination.

Introduction

Hypomyelinating leukodystrophies are a heterogeneous group of genetic white matter disorders resulting from a significant and permanent deficit in myelin deposition within the central nervous system.[1] Since the description of the first hypomyelinating leukodystrophy, Pelizaeus-Merzbacher disease (PMD) in 1885[2] and its pathology in 1910,[3] numerous disorders characterized by hypomyelination have been identified through MRI pattern recognition analysis,[4] genetic linkage and more recently next generation sequencing techniques.[5,6] This combined approach has resulted in the identification of a number of genetic variants associated with hypomyelination, many of which are individually so rare that the resultant phenotypes are yet to be fully defined.[1,5,7]

Variants in the *RARS1* gene have been previously reported in 10 patients[5–7] with a hypomyelinating leukodystrophy (MIM 616140),[7–9] each presenting with nystagmus, ataxia and spasticity resembling PMD. *RARS1* encodes cytoplasmic arginyl-tRNA synthetase (ArgRS), a monomeric enzyme in class 1 of the aminoacyl-tRNA synthetase (aaRS) family, essential for protein synthesis.[8] ArgRS exists in a short and a long isoform, both translated from the same transcript, with the short isoform being translated from an alternative start codon causing the absence of the N-terminal 72 amino acids in the short isoform. This isoform is found free in the cytosol,[9] whereas the long isoform is found in a subcomplex together with aaRS complex-interacting multifunctional protein 1 (AIMP1) and glutaminyl-tRNA synthetase (GlnRS), within a larger multisynthetase complex of nine tRNA synthetases and three accessory proteins in total.[8]

Although the exact mechanism(s) underlying pathogenicity of *RARS1* variants remain(s) unknown, there is increasing evidence in other tRNA synthetase disorders that aminoacylation errors contribute to cellular dysfunction.[10,11] However, whether aminoacylation is impaired in *RARS1*-related hypomyelination, has not yet been demonstrated yet. In this paper we report 20 patients with hypomyelination and *RARS1* variants, 16 new and four reported previously,[12] expanding the clinical and neuroradiological presentation. In addition, ArgRS activity was analyzed for four patients, confirming the impact of *RARS1* variants on aminoacylation.

Patients and Methods

Patients and data collection

We included 20 patients from 15 unrelated families and multinational institutes. Four patients (P1–4) were published previously.[12] *RARS1* variants were identified locally by clinical next generation sequencing techniques (either WES or WES with a filter for leukodystrophy genes,

which included *RARS1*, a known disease gene) following local procedures. After the identification of biallelic *RARS1* mutations by the referring centers, the Centre for Childhood White Matter Diseases, Amsterdam was contacted by the treating clinician, and clinical and radiological data were retrospectively collected there. These data were evaluated by LG and NW at the Centre for Childhood White Matter Diseases, Amsterdam. The study was approved by the Institutional Review Board of VU University Medical Centre and the participating institutes. All patients/parents gave appropriate informed consent.

Enzyme assay

Aminoacylation was assessed by measuring ArgRS activity in cultured fibroblasts of 4 patients. Fibroblast 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 $MgCl_2$, 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 $[^{15}N_2]$-arginine, $[^{15}N]$-valine and $[D_2]$-glycine. The reaction was terminated using trichloroacetic acid. After sample washing with trichloroacetic acid, ammonia was added to release the labeled amino acids from the tRNAs. $[^{13}C_6]$-arginine, $[^{13}C]$-valine and $[^{13}C_2, {}^{15}N]$-glycine were added as internal standards and the labeled amino acids were quantified by LC-MS/MS. Intra-assay variation was <15%. Valyl-tRNA synthetase and Glycyl-tRNA synthetase activity were simultaneously detected as control enzymes.

Western blot

To confirm the presence of ArgRS protein in fibroblasts, a western blot was performed. Cell pellets were resuspended in urea lysis buffer (10 mmol/L Tris HCl, 8 mol/L urea, 100 mmol/L NaCl, pH 8.0). After DNA shearing using a 29-gauge needle, protein concentration was determined and 30 µg of total protein were separated in a 12% stain-free SDS gel (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred onto a polyvinylidenfluoride membrane (Bio-Rad, Hercules, CA) using a Trans-Blot Turbo Transfer System (Bio-Rad). Immunodetection was performed using a primary antibody (rabbit) directed at ArgRS (PAS-30145, Thermo Fisher Scientific, Waltham, MA) and a secondary anti-rabbit antibody (PO448, Dako, Glostrup, Denmark). Immune complexes were detected by enhanced chemiluminescence (Lumilight Plus), according to the manufacturer's specifications (Roche, Indianapolis, IN). Images were acquired in a charge-coupled device imager ChemiDoc XRS (Bio-Rad) using the Image Lab software (Bio-Rad).

Results

Clinical characteristics

Detailed clinical characteristics are provided in Table S1. Eighteen of the 20 patients presented in the first year of life, 11/18 under the age of 3 months. Seven patients presented with delayed motor development, five with seizures and one with nystagmus. The other five presented with microcephaly ($n = 2$), irritability ($n = 2$) or failure to thrive ($n = 1$). The two patients who had their first signs after the age of 12 months presented with nystagmus and frequent falls at age 3 years and with delayed language and social development at age 2 years. Over time, 12 patients developed nystagmus, 13 spasticity, seven ataxia and 10 epilepsy. Seizures were refractory to treatment in eight of these 10 cases, the clinical picture suggesting infantile epileptic encephalopathy. At the time of reporting five patients, all with disease onset before the age of 3 months, were deceased (aged 21–42 months).

Of the surviving 15 patients, 13 had intellectual disability, ranging from mild to moderate ($n = 6$) to severe ($n = 7$). Of the six patients who achieved walking, two continued to require support at their latest follow-up (P1, aged 11 years, and P3, aged 26 years), four were independently mobile, and one became wheelchair dependent later in life. The most mildly affected patient (P10) initially presented aged 3 years with transient nystagmus and clumsiness, and did not develop concerns regarding cognition and progressive lower limb spasticity until the fourth and fifth decades.

Based on these findings, we classified clinical presentation as:

1 Severe (presenting in the first 3 months of life, and usually with refractory epilepsy),
2 Intermediate (the classic hypomyelinating phenotype resembling PMD and related disorders, with onset within the first year of life, nystagmus and spasticity, but sometimes with the ability to walk with support), and
3 Mild (with an onset around age 12 months and the ability to walk without support).

This spectrum is a continuum: for example, P4 is an example of a borderline patient between the severe and intermediate form.

Radiological findings

MRI scans were available for 17 of the 20 patients: for one patient (P6) no MRI was available, and for two patients (P13 and P19), we had only selected images. Detailed MRI findings are provided in Table S2 and Figures 1, 2. Throughout the cohort, T2-weighted images demonstrated supratentorial white matter hyperintensity with corresponding T1 hypointensity, in keeping with

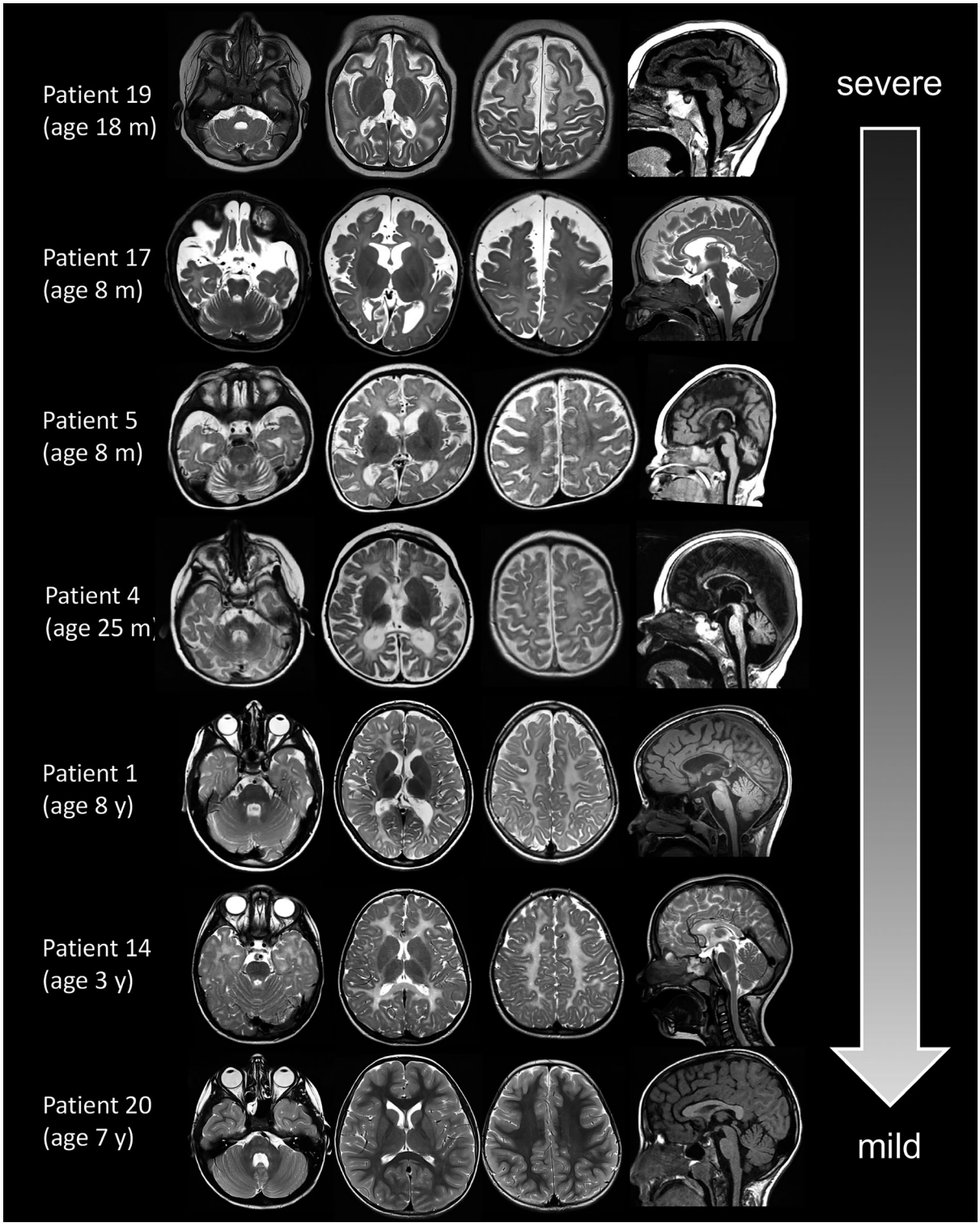

Figure 1. Demonstrating the spectrum of MRI findings in selected patients with *RARS1*. Axial T2-weighted and sagittal T1-weighted (P19, P5, P4, P1, P20) and T2-weighted images (P17, P14), from the most to the least severely affected patient. The severely affected patients have early-onset cerebral atrophy and, in patient 5, also cerebellar atrophy, in addition to abnormal T2 hyperintense signal of the cerebral white matter, indicating myelin deficit. P19, the most severely affected patient, also has a simplified gyral pattern and a round, small cerebellum.

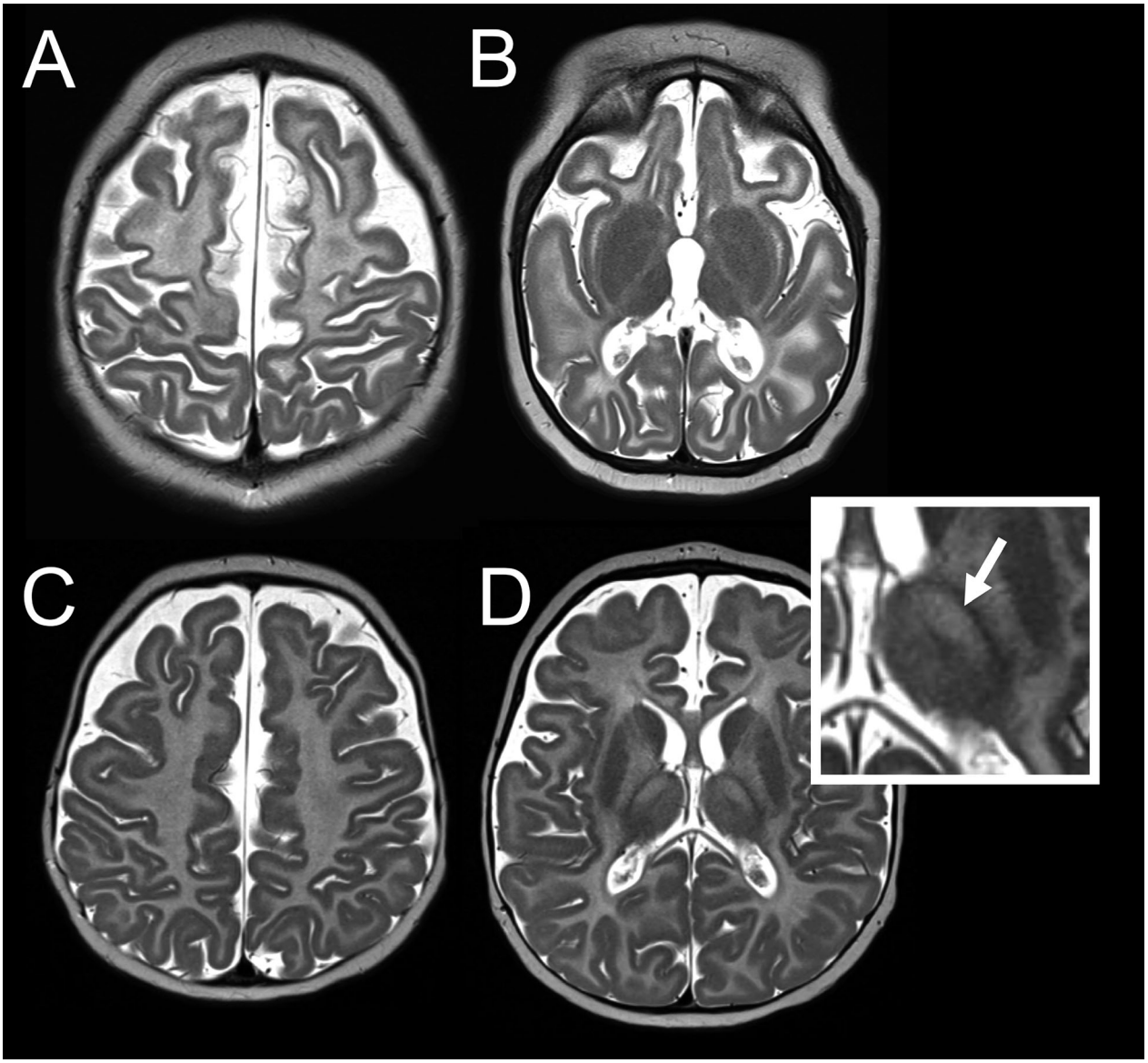

Figure 2. Additional findings beyond hypomyelination in *RARS1* variants. (A and B) T2-weighted axial images of patient 1 in the neonatal period, with simplified gyral pattern and thick posterior cortex. (C and D) Patient 15, at age 6 months, shows hyperintense T2-signal of the ventrolateral thalamus (arrow).

myelin deficit. Some patients were too young to establish a radiological diagnosis of hypomyelination, but even the youngest patients showed deficient myelination.

Nine patients demonstrated some degree of early brain atrophy; this corresponded with severe clinical presentation. P10 with a mild presentation also showed atrophy at the age of 50 years; it is unclear over how much time this had developed.

The most severely affected patient, P19, had a simplified gyral pattern in addition to hypomyelination and brain atrophy (Figs. 1, 2A and B). There was no evidence of cortical malformations in the remaining patients. One of the patients, P15, had bilateral T2-hyperintense signal in the ventrolateral part of the thalamus (Fig. 2C and D). We did not find spinal cord abnormalities in the patients we could assess this question.

RARS1 variants

Full details of the *RARS1* variants identified within the cohort are given in Table 1 and Figure 3. Five patients were identified to have homozygous and 15 compound heterozygous variants; six variants were previously reported whilst 15 were novel. The most common variant

Table 1. Genotype-phenotype relationship.

Patient	Subtype	Genotype		Age of onset	Nystag-mus	Micro-cephaly	Highest motor milestone	Epi-lepsy	Feeding difficulties	Main MRI findings	
		Variant 1	Variant 2							Hypomyelination	Atrophy
Nafisinia et al. (patient 3)	Severe	**c.1367C>T p.(Ser456Leu)**	c.1846_1847del p.(Tyr616Leufs*6)	3 months	Y	Y	Sitting without support	Y	Unknown	Y	Y
Rezaei et al. (patient 1)	Severe	**c.2T>C p.(Met1?)**	**c.2T>C p.(Met1?)**	First months	Y	Y	Head control (lost age 7 months)	N	Unknown	Y	N
Rezaei et al. (patient 2)	Severe	**c.2T>C p.(Met1?)**	**c.2T>C p.(Met1?)**	First months	Y	Y	Head control	Y	Unknown	Y	Y
P4[1]	Severe	c.1A>G p.(Met1?)	**c.1535G>A p.(Arg512Gln)**	2 months	Y	Y	Rolls over	N	Y	Y	Y (mild)
P5	Severe	c.1316C>A p.(Ala439Asp)	c.1316C>A p.(Ala439Asp)	3 months	Y	Y	Partial head control	Y	Y	Y	Y
P6	Severe	c.1316C>A p.(Ala439Asp)	c.1316C>A p.(Ala439Asp)	Congenital	Unknown	Y	Partial head control	Y	Y	n/a	n/a
P7	Severe	**c.173T>C p.(Leu58Pro)**	c.1790T>C p.(Leu597Pro)	Congenital	Y	Y	No milestones achieved	Y	Y	Y	Y (mild)
P11	Severe	c.67_70del p.(Thr23Leufs*6)	c.67_70del p.(Thr23Leufs*6)	Congenital	Y	Y	Partial head control	Y	Y	Y	Y
P12	Severe	c.67_70del p.(Thr23Leufs*6)	c.67_70del p.(Thr23Leufs*6)	Congenital	Y	Y	Partial head control	Y	Y	Y	Y
P15	Severe	c.2T>A p.(Met1?)	c.448_456del p.(Cys150_Glu152del)	6 weeks	N	Y	Partial head control	Y	Y	Y	Y
P16	Severe	**c.2T>C p.(Met1?)**	**c.1535G>A p.(Arg512Gln)**	Congenital	Y	Y	Rolls over, partial head control	Y	Y	Y	Y
P17	Severe	c.1452+1G>A p.(?)	c.1534C>T p.(Arg512Trp)	2 months	N	Y	No milestones achieved	Y	Y	Y	Y
P18	Severe	c.1452+1G>A p.(?)	c.1534C>T p.(Arg512Trp)	Congenital	Y	Y	No milestones achieved	Y	Y	Y	Y
P19	Severe	c.3G>T p.(Met1?)	**c.96_97del p.(Cys32Trpfs*39)**	Congenital	N	Y	No milestones achieved	Y	Y	Y	Y
Ji et al. (patient 118)	Inter-mediate	**c.5A>G p.(Asp2Gly)**	c.1625+2T>G p.(?)	3 months	Y	N	Unknown (severe motor impairment)	N	N	Y	Y (mild)
P1[1]	Inter-mediate	**c.5A>G p.(Asp2Gly)**	c.45+1G>T p.(?)	1 year	Y	N	Walks with support	N	N	Y	N
P2[1]	Inter-mediate	**c.5A>G p.(Asp2Gly)**	c.45+1G>T p.(?)	5 months	Y	N	Sits without support, crawls	N	N	Y	N
P3[1]	Inter-mediate	**c.5A>G p.(Asp2Gly)**	**c.96_97del p.(Cys32Trpfs*39)**	10 months	Y	N	Walks with support	N	N	Y	N

(*Continued*)

Table 1. Continued.

Patient	Subtype	Genotype		Age of onset	Nystag-mus	Micro-cephaly	Highest motor milestone	Epi-lepsy	Feeding difficulties	Main MRI findings	
		Variant 1	Variant 2							Hypomyelination	Atrophy
P8	Inter-mediate	**c.5A>G p.(Asp2Gly)**	c.1874-9_1874-5del p.(?)	10 months	Y	N	Sits without support	N	N	Y	Y (mild)
P9	Inter-mediate	**c.5A>G p.(Asp2Gly)**	c.1874-9_1874-5del p.(?)	10 months	Y	N	Sits without support	N	N	Y	Y (mild)
P13	Inter-mediate	c.668G>A p.(Arg223His)	c.1568T>A p.(Met523Lys)	9 months	N	N	Walks without support	N	Y	N	N
Nafisinia et al. (patient 1)	Mild	**c.5A>G p.(Asp2Gly)**	**c.5A>G p.(Asp2Gly)**	<12 months	Y	Unknown	Walks without support	N	N	Y	Y (late)
Nafisinia et al. (patient 2)	Mild	**c.5A>G p.(Asp2Gly)**	**c.5A>G p.(Asp2Gly)**	<12 months	Y	Unknown	Walks without support	N	N	n/a	n/a
P10	Mild	**c.5A>G p.(Asp2Gly)**	**c.5A>G p.(Asp2Gly)**	3/45 years[2]	Transient	N	Walks without support	N	N	Y	Y (late)
P14	Mild	**c.5A>G p.(Asp2Gly)**	**c.173T>C p.(Leu58Pro)**	18 months	N	Y	Walks without support	N	N	Y	N
P20	Mild	c.475C>T p.(Pro159Ser)	**c.1367C>T p.(Ser456Leu)**	2 years	N	N	Walks without support	N	N	N	N

In bold: *RARS1* variants present in more than one family. Y, yes; N, no.

[1]Previously published by Wolf et al (2014). Variants present in at least two unrelated families are depicted in bold.

[2]Transient period of clumsiness with frequent falls and rotatory nystagmus aged 3 years, followed by mild cognitive concerns not affecting daily living from early 30s, and progressive lower limb spasticity affecting walking from mid-40s.

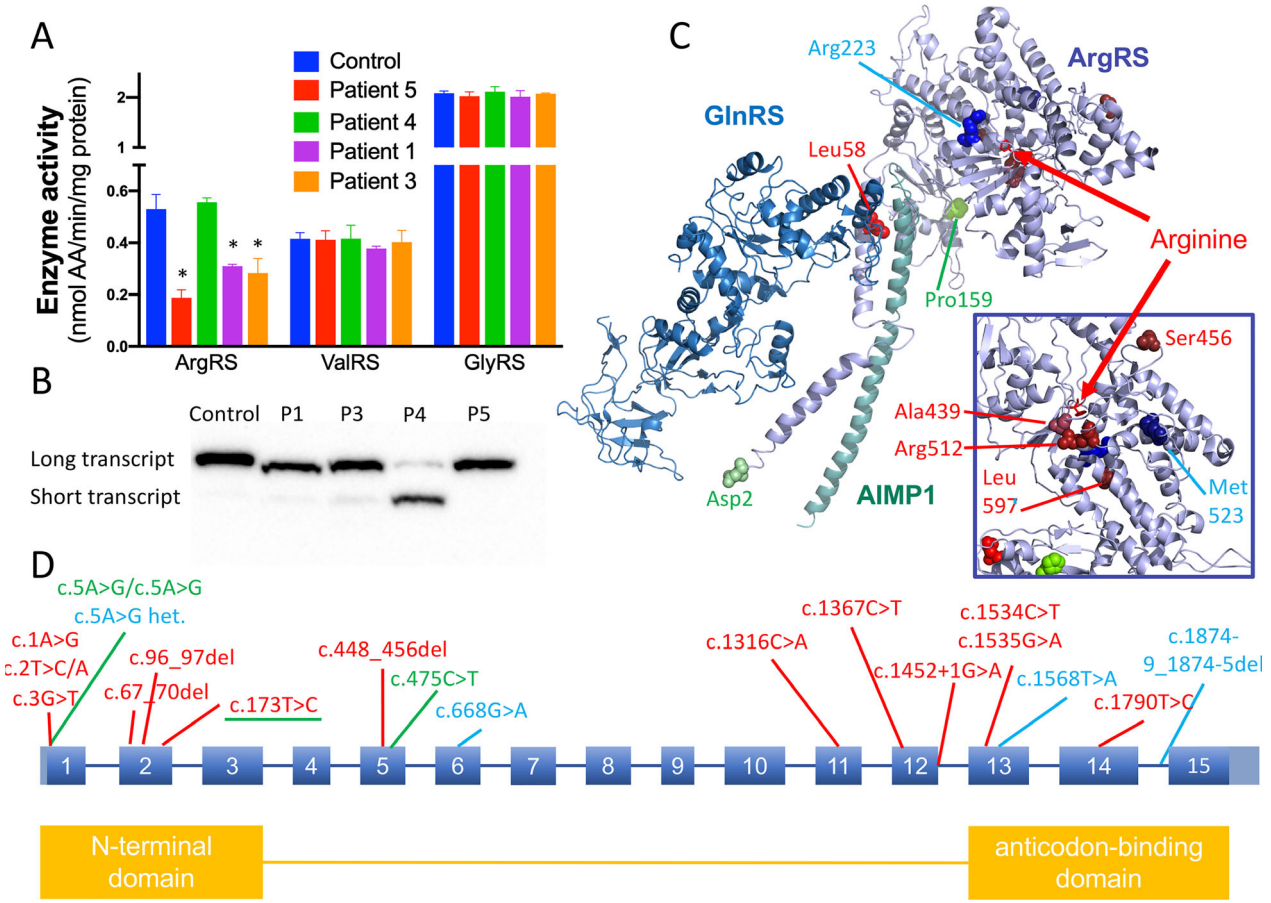

Figure 3. ArgRS activity, isoform expression and *RARS1* mutations (A) Activities of ArgRS, ValRS and GlyRS in fibroblasts of patients and controls, indicating significantly reduced ArgRS activity in patients 5, 1, and 2 (patient order according to severity with P5 most severely affected, P1 and P2 intermediately affected). (B) depicts a Western blot of ArgRS, with mainly a short transcript present in P4. (C) Is a model of ArgRS, GlyRS and AIMP1, with variants leading to a mild presentation indicated in green, variants associated with a moderate presentation in blue and variants causing a severe presentation in red. (D) Distribution of the variants (with the same color code as in (C) throughout the *RARS1* gene. ArgRS, arginyl-tRNA synthetase; GlyRS, Glycyl-tRNA synthetase; ValRS, Valyl-tRNA synthetase; AIMP1, aaRS complex-interacting multifunctional protein 1.

within our cohort was c.5A>G p.(Asp2Gly), a substitution located at the very beginning of the N-terminal domain. This variant was identified in seven of the 20 patients (one homozygous, six compound heterozygous) and was also described in two other published patients in a homozygous form.[13] All patients with this variant had an intermediate or a mild (when homozygous) phenotype.

We ascertained other recurrent variants that were present in more than one family: p.(Ser456Leu), reported previously[13] and present in P20; p.(Leu58Pro) present in P7 and P14; p.(Cys32Thrfs*39) present in P3 and P19; and several variants that affected the start codon (in four patients). Arginine at position 512, located close to the active center (Fig. 3), was affected in three unrelated families, all presenting with severe disease. Furthermore, other variants affecting amino acids in close proximity to the arginine binding site (in eight patients/six families) led to severe phenotypes (Fig. 3), with the exception of p.(Arg223His). The p.(Leu58Pro) variant, located close to the interface with GlnRS (Fig. 3), was combined in one family with p.(Asp2Gly) resulting in a mild phenotype, in another family with p.(Leu597Pro) resulting in a severe phenotype.

Several variants presumably affected translation of the full-length protein. For example, in several families the start codon was affected, resulting in (almost) absent translation of the long isoform (as demonstrated in P4, Fig. 3). These variants were also associated with a severe phenotype.

Enzyme activity and isoform translation

ArgRS activity was significantly decreased in fibroblasts of P1, P3, and P5 compared to controls (Fig. 3A). P5, with

a severe phenotype, had the lowest enzyme activity of the three patients. In contrast, P4 showed ArgRS activity in the range of the control, despite having a severe clinical phenotype. Compared to controls, P1 and P3 showed a faint band of the short ArgRS isoform; for P4, the main isoform found was the short one, although a faint band of the long ArgRS isoform was present as well (Fig. 3B).

Discussion

This study demonstrates the impact of biallelic *RARS1* variants on neurodevelopment and confirms them as a cause of hypomyelinating leukodystrophy, similar to the hypomyelination seen in patients with variants in *EPRS1* and *DARS1*, also coding for tRNA synthetases which are part of the multisynthetase complex.[14–16] Besides hypomyelination, single patients with *RARS1* variants share neuroradiological abnormalities with *EPRS1* patients (ventrolateral thalamus involvement)[14] and *DARS1* patients (spinal cord involvement)[15,16] although the latter, described so far in one *RARS1* patient[17], is not present in this cohort. We also confirm that the aminoacylating function of ArgRS is impaired, with the most pronounced reduction in a severely affected patient.

The study also sheds new light on the disease spectrum associated with *RARS1* variants. Beyond presenting as a typical hypomyelinating leukodystrophy, a substantial number of patients with *RARS1* variants present with early epileptic encephalopathy, the most severely affected patient also displays a cortical folding abnormality. On the other end of the spectrum, patients have mild disease, even without significant myelin deficit, again reminiscent of patients with *DARS1* variants and late onset disease.[15]

Interestingly, patients with the severe phenotype resemble early-onset grey matter disorders rather than primary leukodystrophies: they have severe epilepsy and early-onset (severe) cerebral atrophy, both hallmarks of neuronal disorders. Patients with variants in *QARS1*, encoding GlnRS which closely interrelates with ArgRS within the multisynthetase complex, present with similar clinical signs.[18] One of the *QARS1* variants disturbs the interaction between GlnRS and ArgRS,[18] and we assume this is also the case for the ArgRS Leu58Pro variant, located at the ArgRS-GlnRS interface. A similar severe presentation is seen in patients with biallelic mutations in *AARS1*[19] and *VARS1*,[20,21] and also mutations in *AIMP1* and *AIMP2*, affecting two of the three scaffolding proteins of the multisynthetase complex, lead to early-onset neuronal disorders.[22,23]

Thanks to this cohort of 20 patients, we are beginning to understand the genotype-phenotype relationship.[9] The most frequent *RARS1* variant ascertained, c.5A>G p.(Asp2Gly), affects the second amino acid residue aspartate, which is part of the 72 amino-acid N-terminal domain and only present in the longer ArgRS isoform. It interacts with the long N-terminal helix of AIMP1 and also affixes the C-terminal core of GlnRS.[18] The Asp2Gly variant most likely leads to a mild or intermediate phenotype, as no patients with this variant has the severe early-infantile phenotype and all but two patients with the mildest phenotype are homozygous for this variant. In another study, this variant led to decreased levels of the longer ArgRS isoform in fibroblasts of a homozygous patient,[13] and also in our two patients compound heterozygous for this variant, we could observe a faint band for the shorter isoform in fibroblasts. One patient (P4) carries a mutation affecting the start codon c.1A>G in one allele, and shows mainly translation of the short cytosolic ArgRS isoform. We hypothesize that, due to this mutation, only the short and not the long isoform can be translated from the transcript of this allele, and that the observed faint band of the long isoform results from the transcript of the other allele. Interestingly, this patient has a severe phenotype but normal ArgRS activity in fibroblasts, in contrast to the other three patients with reduced ArgRS activity. Since the long and short ArgRS isoforms exhibit similar enzyme activities in vitro,[9] it is possible that the short isoform, the main isoform present in fibroblasts of this patient, contribute to the enzyme activity. As a consequence, normal ArgRS activity in vitro does not necessarily reflect aminoacylation in vivo, for which the long isoform is needed to form the multisynthetase complex.

The disparate clinical manifestations seen in this cohort – a severe neuronal phenotype on one hand and a typical leukodystrophy (hypomyelination) phenotype on the other – raise questions as to (1) whether the hypomyelination is a result of primary neuronal dysfunction instead of primary oligodendrocyte dysfunction; (2) whether mildly reduced ArgRS activity only affects oligodendrocytes while more severely reduced activity (also) affects neurons; and (3) whether different variants disturb different protein functions. It is postulated that the short cytosolic form of ArgRS, unaffected by some *RARS1*-variants, is involved in the ubiquitin-dependent N-end rule pathway of protein degradation by providing Arg-tRNA as a substrate for arginyl-tRNA transferase.[24] N-terminal arginylation targets certain proteins for controlled degradation, including elimination of misfolded proteins.[24] Defective protein homeostasis, due to impaired ubiquitination or ufmylation, is associated with several neurodegenerative disorders, including early-onset encephalopathies.[25–28] Therefore, defective ArgRS might hamper protein degradation in addition to affecting protein synthesis, thereby contributing to a primarily neuronal phenotype. Understanding these possible pathway(s) to disease manifestations is essential before embarking on approaches to treatment.

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Conflict of Interest

There are no conflicts of interest directly related to this work.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Clinical features of the 20 patients with RARS1 variants.
Table S2. MRI features.

