FARS1-related disorders caused by bi-allelic mutations in cytosolic phenylalanyl-tRNA synthetase genes: Look beyond the lungs!

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Abstract
Aminoacyl-tRNA synthetases (ARSs) catalyze the first step of protein biosynthesis (canonical function) and have additional (non-canonical) functions outside of translation. Bi-allelic pathogenic variants in genes encoding ARSs are associated with various recessive mitochondrial and multisystem disorders. We describe here a multisystem clinical phenotype based on bi-allelic mutations in the two genes (FARSA, FARB) encoding distinct subunits for tetrameric...
1 | INTRODUCTION

Aminoacyl-tRNA synthetases (ARSs) are ubiquitous enzymes that fulfill an essential role in protein biosynthesis by covalently linking a tRNA molecule to its cognate amino acid. In addition, ARSs have key functions in various cellular pathways outside of translation.\(^1\) In humans, two subsets of ARSs can be distinguished based on their cytoplasmic or mitochondrial localization. With rare exception, separate genes, unique for each amino acid, encode for both cytoplasmic and mitochondrial ARSs. More than 60 disorders are associated with mutations in ARS genes and, depending on their individual genotype, follow both dominant or recessive inheritance patterns.\(^6\)\(^\text{10}\)

Cytosolic phenylalanyl-tRNA synthetase (FARS1) ranks among the most complex of the ARSs with a hetero-tetrameric structure, consisting of two FARSA alpha (FARSA) and two FARSB beta (FARSB) subunits.\(^11\) The hypothesis that both genes may be associated with a similar multisystemic human disease, including features of interstitial lung disease, liver disease, neurological manifestations, and growth restriction (MIM: 619013, 613 658), needs further support. Whereas 11 individuals presenting with bi-allelic mutations in FARSA are described,\(^9\)\(^\text{12}\)\(^\text{13}\) information on FARSA-related disease is rare and comes from a single case report describing a patient with mutations in FARSA and features of interstitial lung disease, cerebral and skeletal abnormalities, hypotonia, and liver dysfunction.\(^10\)

Here, we report three unrelated individuals with identified bi-allelic variants in FARSA (MIM: 602918), and two unrelated individuals presenting bi-allelic variants in the gene encoding the beta subunit, FARSB (MIM: 609690). We provide biochemical and functional proof of novel genetic variants, spotlight the value of rare but indicative interstitial lung disease as a clue for diagnosis, give details on the long-term clinical course including treatment and outcome, and review the spectrum of FARS1-associated recessive disease.

2 | MATERIAL AND METHODS

Patients were recruited from the European Management Platform for Interstitial Lung Disease (chILD-EU) registry by searching the database for children with chronic interstitial lung disease of unknown cause, and in-depth analysis of ARS genes by whole exome sequencing (WES) was performed. Six subjects with predicted disease-causing variants in FARSA and FARSB were retrieved, one was published independently.\(^10\) Patients and families gave their written informed consent to participate in the chILD-EU consultation and diagnosis program. All human subject research was in accordance with current ethical standards and approved by the Ethics Committee of the University of Munich, Germany (EK 111-13, EK 20-329).

2.1 | Whole exome sequencing and variant prioritization

Genomic DNA was isolated from whole blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Exonic regions were enriched with SureSelectXT Human All Exon V6 + UTR kit (Agilent, Santa Clara, CA) and sequenced as 150 bp paired-end runs on an Illumina NextSeq500 (ILLUMINA, San Diego, CA). Reads were aligned to the human reference genome GRCh37 by Burrows-Wheeler Aligner (v.0.7.15).\(^14\) Genome Analysis Tool Kit (GATK v3.8)\(^15\) software was used to analyze the data. Functional annotation was added using Variant Effect Predictor (VEP), Ensembl release 95.\(^16\) Rare variants (MAF < 1%) were filtered using an in-house SQL database incorporating frequency information from the 1000 Genomes Project\(^17\) and gnomAD.\(^18\) Filtered variants were evaluated based on predicted pathogenicity scores and an autosomal recessive inheritance pattern including homozygous and compound heterozygous variant pairs identified by SmartPhase.\(^19\) All patients were analyzed as trios; variants that remained after filtering are presented in Table S2. Sanger sequencing was used to confirm the identified mutations in FARSA and FARSB and to determine the carrier status of parents.
and the unaffected sibling in family 1 (for primers, see supplementary information S1).

### 2.2 Histopathology, Isolation of patient-derived cell lines and cell culture

Tissue for diagnosis was obtained by open lung biopsy. Following formalin fixation and paraffin embedding, slides were stained with Hematoxylin–Eosin (HE), Elastica van Gieson (EvG), Prussian Blue, and Periodic Acid-Schiff (PAS). All readings were made by a pathologist specialized in pediatric interstitial lung disease.

Fibroblasts were obtained from skin punch biopsy of affected individuals and cultured in Dulbecco’s Modified Eagle Medium/F12 (DMEM) (Pan Biotech, Aidenbach, Germany) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) (vol/vol), and 1% Antibiotic-Antimycotic (Thermo Fisher, Waltham, MA) (vol/vol), and 10 mM HEPES (Thermo Fisher), and 1% Antibiotic-Antimycotic (Thermo Fisher, Waltham, MA) (vol/vol) at 37°C and 5% CO₂. Cells were regularly split after reaching confluency of 80%-90%

PBMCs were isolated from whole blood samples by density gradient centrifugation with Biocoll (Merck, Darmstadt, Germany) and subsequently incubated with B95-8 cell supernatants (Leibniz Institute, DSMZ, Germany) and 0.01% cyclosporine A (Sigma-Aldrich). Cells were then re-aggregated with patient-derived fibroblasts to give rise to Epstein-Barr Virus Immortalized Lymphoblastoid Cell Lines (EBV-LCLs). Cells were maintained in RPMI 1640 medium with Glutamax™ (Thermo Fisher), supplemented with 10% FBS (Sigma-Aldrich) (vol/vol), 10 mM HEPES (Thermo Fisher), and 1% Antibiotic-Antimycotic (Thermo Fisher) (vol/vol) at 37°C and 5% CO₂.

### 2.3 Modeling of the human FARS1-tRNAPhe complex structure

The structure of the Thermus thermophilus tRNAPhe molecule (PDB: 2IY5)²⁰ was aligned with the crystal structure of human FARS1 (PDB: 3L4G)²¹ using PyMOL²² to model the position of tRNAPhe and the phenylalanyl-adenylate.

### 2.4 Conservation analysis of FARSA amino acid residues

Conservation analysis was performed with ConSurf Server.²³ Initial sequence selection was performed on the Uniref90 database using an E-value threshold of 0.0001. Within this pool, the best 70 FARSA sequences were manually selected. Final alignment was performed with the MAFFT-L-INS-i method and scoring was calculated using the Bayesian method.

### 2.5 RNA isolation and quantitative reverse transcription PCR

Total RNA was isolated from cultured cells using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland). RNA yield and purity were measured on a NanoDrop spectrophotometer (NanoDrop Technologies, Thermo Fisher). 1 μg of RNA was subsequently reverse transcribed with the Tetro cDNA Synthesis Kit (Bioline, Meridian Bioscience, Cincinnati, OH). Real-Time PCR was performed in a thermal cycler (StepOnePlus, Applied Biosystems, Thermo Fisher) with SensiFast SYBR Hi-ROX reagent (Bioline) capturing target genes FARSA, FARSB, and the ribosomal internal control gene RPS29 (primers, see supplementary information S1). Fold change of target gene expression was calculated based on the comparative C₇ method.²⁴

### 2.6 Western blot and densitometry

Cells were lysed in radioimmunoprecipitation assay buffer (0.15 M sodium chloride, 1% Triton X100 [vol/vol], 0.5% sodium deoxycholate [Sigma-Aldrich] [wt/vol], 5 mM ethylenediaminetetraacetic acid [GE Healthcare, Chicago, IL]), 50 mM Tris [pH = 8] [Merck]). 20 μg of protein were subjected to polyacrylamide gel electrophoresis on a NuPage Mini 3%-8% Tris-Acetate gel (Thermo Fisher) and blotted to a polyvinylidene fluoride membrane (Merck). Antigen targets were detected using the rabbit polyclonal primary antibodies anti-FARSA 18121-1-AP (Proteintech, St. Leon-Rot, Germany), and anti-FARSB HPA036677 (Sigma-Aldrich), followed by incubation with HRP-conjugated goat anti-rabbit IgG (H + L) secondary antibody (Thermo Fisher). β-actin quantification (sc-47 778 HRP, Santa Cruz, Dallas, TX) served as a loading control. Proteins were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher). Relative band intensities were analyzed using the Image J software.²⁵

### 2.7 Aminoacylation assay

FARS1 activity was assessed by steady-state aminoacylation assays in cells of patients and family members. Cytosolic fractions of lysates were incubated at 37°C with 0.3 mM [D₃]-phenylalanine and [D₂]-tyrosine (Cambridge Isotope Laboratories, Tewksbury, MA) in a 50 mM Tris buffer, pH 7.5, containing 1 mM ATP, 0.2 mM yeast total tRNA, 1 mg/ml bovine albumin serum, 12 mM MgCl₂, 25 mM KCl and 1 mM dithiothreitol (all Sigma-Aldrich). [¹³C]-phenylalanine and [¹³C₃]-tyrosine (Cambridge Isotope Laboratories) were added as internal standards. Labeled amino acids were quantified by liquid chromatography–tandem mass spectrometry. Intra-assay variation was <15%. For kinetic studies, Kᵅ and Vᵅₘₐₓ values for phenylalanine and tyrosine were calculated by measuring six different concentrations in the range of 0.009–0.6 mmol/L. Data were subsequently analyzed using Graph Pad Prism 8.2.1 (GraphPad Software, La Jolla, CA).

### RESULTS

#### 3.1 Clinical characteristics of patients with newly identified FARSA/FARSB variants

All five unrelated patients (P1, P2, P3, P16, P17) with novel bi-allelic variants in FARSA or FARSB (Figure 1) were identified clinically due to
their prominent and distinct pulmonary phenotype. Common to all was a peculiar chronic interstitial lung disease starting early in life and characterized by bilateral ground-glass opacification on HR-CT, and cholesterol pneumonitis in lung histology. Additional abnormalities in other organ systems were summarized, suggesting a multi-organ disorder (Figure 4, Table S1).

Patient 1 was born hypotonic with hyperflexible joints, had arachnoid fingers and delayed motor development. At the age of 5 years,
FIGURE 2  Histopathology of the lungs. (A) Prominent cholesterol pneumonia with intra-alveolar and interstitial cholesterol granulomas. Note combination with fresh and older bleeding with accumulation of iron-storing macrophages, and megakaryocyte emboli resulting from pulmonary hypertension (insert arrow) (P1, age of 8.6 years; hematoxylin–eosin (HE) staining; in upper left panel, scale bar 500 μm; insert, scale bar 50 μm, and upper right panel, scale bar 100 μm; iron (Prussian Blue, FE) staining in lower left panel and lower right panel, scale bar 500 and 200 μm). (B) Pulmonary alveolar proteinosis with dense intra-alveolar accumulation of Periodic acid–Schiff (PAS)-positive granules and cholesterol clefts (P2, age of 8 months; HE-staining in upper panels, scale bar 200 μm (left) and 100 μm (right), PAS-staining in lower panels, scale bars 100 μm) [Colour figure can be viewed at wileyonlinelibrary.com]
FIGURE 3  Legend on next page.
growth hormone resistance (Laron-type, no molecular confirmation) was diagnosed, and IGF-1 treatment was applied for 6 years (reaching 20th percentile for height). Her brain MRIs at the age of 8.8 and 9.5 years demonstrated multiple unspecific white matter lesions in a predominantly subcortical location, increased in number and size at the age of 17 years. Elongated brain arteries were noted (Figure 3(A–D)). She had hepatosplenomegaly and abdominal ultrasound revealed steatosis of the liver. At the age of 8.4 years, she presented with a protracted respiratory tract infection, was severely hypoxemic on admission, and needed intubation and ventilation for 2 weeks. Bilateral clubbing was noted. Cardiac echo (at age 8.7 years) gave slightly elevated pulmonary artery pressures, and 7 years later, there was no evidence for pulmonary hypertension. After recovery, she had a forced vital capacity of 73% of predicted, oxygen saturation during sleep between 88% and 91%, and normal pH. Her chest X-ray showed bilateral ground-glass and reticular opacities. Chest CT initially showed patchy consolidations which resolved over time, whereas fibrosing lung disease became more prominent (Figure 3(J)). Lung biopsy demonstrated intra-alveolar and interstitial cholesterol granulomas, in combination with pulmonary hemorrhage at the age of 8.6 years, consistent with cholesterol pneumonia (Figure 2(A)). The course of lung disease was progressive (Figure 3(I)) despite various treatments, including a protein-rich diet and phenylalanine substitution. Currently she is stable at low lung function and evaluated for lung transplantation.

Patient 2 was referred at 1 month of age for failure to thrive, vomiting, chronic loose stools without pancreatic insufficiency, hyperphosphatemia, and osteopenia. An initial examination revealed hepatosplenomegaly, also on CT (Figure 3(F)), with hypoaalbuminemia, increased neutral lipid storage in hepatocytes, and signs of focal fibrosis and cholestasis in liver biopsy. On physical examination, she had visible and palpable subcutaneous fat pads on her forearms and around her knees. She had hypotonic muscle tone, intermittent nystagmus, and a global neurodevelopmental delay. Brain ultrasound was normal. The infant was tachypneic since 5 months of age and suffered from recurrent respiratory infections up to the age of 1 year. Initial and consecutive chest X-rays showed bilateral homogenous ground-glass opacities, bronchial wall thickening and generalized overinflation, and chest CTs revealed interlobular septal thickening and crazy-paving pattern, increasing over time, despite therapeutic lung lavage (Figure 3(L)). Lung biopsy confirmed pulmonary alveolar proteinosis with intra-alveolar and intraseptal cholesterol crystals (Figure 2(B)). Over time respiratory distress increased. A therapeutic lavage removed 62 g of protein from the right lung, however, the infant died of respiratory failure at the age of 1.3 years.

Patient 3 was referred at the age of 5 years for recurrent lower airway infections and chronic cough for more than 3 months. He had growth retardation, pectus carinatum, digital clubbing, and a sensorineural hearing defect (Figure 3(G,H)). A CT scan at the age of 7.5 years demonstrated ground-glass opacification and reticulation, bilateral emphysema, bullae, nodules, focal consolidations, emphysema, and air trapping on expiration (Figure 3(N)). Lung biopsy showed hyperinflated and emphysematous airspaces with an increased infiltration of inflammatory cells and cholesterol pneumonia. In addition, growth hormone deficiency was detected, and cranial MRI revealed an arachnoid cyst (Figure 3(E)), but he presented neurologically normal. Despite various treatments, his clinical status deteriorated gradually with increasing hypoxemia, dyspnea, pronounced pectus carinatum, slightly altered liver enzyme values, and failure to thrive. After a several months period of increasing respiratory distress, he died of respiratory failure at the age of 12.9 years.

Patient 16 suffered from a mild bronchiolitis at the age of 2 months and a self-limiting bronchitis at the age of 7 months. At the age of 2 years, digital clubbing was observed on an emergency visit for gastroenteritis. He was asymptomatic with normal oxygen saturation on room air, however, chest X-ray and CT scan revealed diffuse ground-glass opacities, septal thickening, and subpleural cysts (Figure 3(M)). Lung tissue showed cholesterol granulomas and clefts, pneumocyte hyperplasia, and mild fibrous septal thickening. Due to chronic moderate headaches, brain MRI was performed at the age of 8 years and revealed bilateral focal and non-confluent gliotic-demeylinating lesions with dilated perivascular spaces. At the age of 10 years, he was diagnosed with sensorineural hearing loss. Otherwise, neurological examination was unremarkable. An abdominal ultrasound revealed mildly diffuse hyperemic liver parenchyma and renal asymmetry with a smaller right kidney. Currently at the age of

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**FIGURE 3**  (A,B) Cranial MRI, axial T2-w images (P1, age 17 years) with multiple non-specific white matter lesions in a predominantly subcortical location, which may indicate cerebral small vessel disease. MR-Angio (C) and T2-w sagittal image (D) show an unusual elongation of both vertebral arteries and the basal artery as well as of all arteries of the circle of Willis (arrows). No aneurysms present. (E) T2-w cranial MRI with large arachnoid cyst (P3, age 5 years). (F) Liver enlargement in CT with hypodense liver parenchyma likely representing fatty liver (P2, age 10 months). (GH) Images of P3 highlighting pectus carinatum, failure to thrive, and digital clubbing (age 12 years). (I) Restrictive lung disease in early childhood with rapid progression. FEV<sub>1</sub> forced inspiratory volume in 1 s, FVC, forced vital capacity. (J) Chest CT (P1, 8.4 years) (first left) with bilateral diffuse ground-glass attenuation, interstitial reticular markings, and patchy consolidations. Overtime (ages 8.8, 11, 17 years) (from left to right) development of many small perisepal and subpleural cysts, diffuse ground-glass opacities, and pronounced pectus carinatum, loss of volume, architectural distortion. (K) Therapeutic bronchoalveolar lavage of the right lung (P7, age 5 years). Protein wash-out with six lavage cycles. (L) Axial HR-CT (only right side shown) before (upper panel) and 1 day after (lower panel) therapeutic lavage of the middle lobe with almost complete clearance of the diffuse ground-glass opacification and interlobular septal thickening in concert with increasing pleural effusion and dependent consolidation in the lower lobe (P2, age 10 months). (M) Chest CT with diffuse ground-glass opacities and numerous subpleural cysts (P16, age 8 years). (N) Chest CT with characteristic pectus carinatum chest deformity and differently shaped subpleural bullae and emphysematous changes, pleural thickening, multiple intraparenchymal lung cysts, and bilateral hilar lymphadenopathies (P3, age 7.5 years) [Colour figure can be viewed at wileyonlinelibrary.com]
13 years, he presents with persistent failure to thrive. He follows a protein-rich diet that was recommended to adhere to. On physical examination, lung auscultation and resting oxygen saturation are normal, but he still shows prominent digital clubbing. Lung function tests show a reduced spirometry and diffusing capacity for CO (58% of predicted) (Figure 3(i)).

Patient 17 was referred at the age of 8 years for failure to thrive and chronic cough and dyspnea, persistent for 1 year. On physical examination, she presented with pectus excavatum and digital clubbing. Chest X-ray showed bilateral interstitial appearance and CT scan identified diffuse ground-glass opacities and cysts, subpleurally and scattered throughout all lobes. Lung biopsy revealed cholesterol pneumonia associated with mild chronic interstitial inflammation and fibrosis, foam cell aggregates, mild lymphoid hyperplasia without lymphoid follicles, and mild chronic pleuritis with fat metaplasia. She has a history of chronic diarrhea from the age of 3 months, which improved over time with a specific formula of nutrition. Due to proteinuria and hypoalbuminemia, further nephrological and metabolic diagnostics were performed; however, no other abnormalities were detected. An abdominal ultrasound was normal. Currently at the age of 12 years, she has reduced resting oxygen saturation around 90% and is on long-term oxygen supplementation.

3.2  Bi-allelic variants in FARA and FARB cause FARS1-related disorders

Review of the clinical features of the five unrelated patients with novel bi-allelic variants in FARA or FARB and previously published cases described a complex phenotype, manifesting during early childhood, after an uneventful neonatal period (Figure 4). Besides the common distinct pulmonary phenotype and failure to thrive, abnormal neuromuscular features occurred in more than two thirds of the patients. About half of the subjects had abdominal and hepatic abnormalities. Less frequent findings in multiple other organ systems were present at similar prevalence, independent of the underlying FARS1 subunit affected.

Exome sequencing identified compound heterozygous FARA variants c.(1210C > T), p.(R404C) and c.(1254G > C), p.(E418D) (de novo) in patient 1 and homozygous variants (c.883 C > T), p.(R295W) and c. (829 T > G), p.(F277V) in patient 2 and 3, both with consanguineous family background. Compound heterozygous FARB variants c. (1084T > C), p.(C362R) and c.(1201C > T), p.(R401*) were identified in patient 16 and homozygous variants c.(229C > T), p.(L77=) and c. (1685G > T), p.(G562V) in patient 17 with consanguineous family background. Variants were annotated on NM_004461.3 (NP_004452.1) and NM_005687.5 (NP_005678.3), respectively (Figure 1).

3.3  FARA variant effect prediction on protein structure

With the exception of patient 2 (R295W, average conservation), the presented variants in FARA were strictly conserved (Figure S1) and absent or extremely rare in gnomAD (Table S1A). Structurally, the FARA protein consists of two main regions. The N-terminal site contains three subdomains with characteristic helix-turn-helix structures, forming a DNA/dsRNA binding domain (DBD1/2/3) and likely functioning to recognize and bind tRNA\textsuperscript{Phe}. The DBD-domain connects through a linker region to the C-terminal catalytic domain with subdomains A1/A2 which participate in tRNA binding and aminoclylation.\textsuperscript{21,26} All identified variants were located exclusively in the A1/A2 domain (Figure 5(A)).

To investigate the impact of both these and previously reported variants\textsuperscript{10} on FARS1 structure and function, we aligned T.\textit{thermophilus} tRNA\textsuperscript{Phe} (PDB: 2IY5) with the crystal structure of human FARS1 (PDB: 3L4G) (Figure 5(B)). In this model, three residues affected by the missense mutations potentially interfere directly with the catalytic activity, due to their proximity to the active site: E418D, N410K, and F256L. The first, E418, interacts with conserved residues K406, E431, and N434 and is located close to the 3’ end of the tRNA, where phenylalanine is loaded. The substitution by aspartate incorporates a shorter side chain that might interfere with this hydrogen network and either affects tRNA binding or the transfer of the amino acid from its binding site to the tRNA (Figure 5(Ci)). The second, N410, forms two hydrogen bonds with the conserved residue N434. These bonds have previously been predicted to influence rigidity of the phenylalanine binding site\textsuperscript{21} and the substitution by lysine at this position will very likely modify the conformation of the active site (Figure 5(Cii)). The third, F256, is an absolutely conserved residue in a very well conserved region (amino acids 253–278). Structurally, it is located at the back of the active site in a hydrophobic pocket composed of aromatic and hydrophobic residues (W257, P289, Y292, V293, A261, A319). The substitution by leucine conserves the hydrophobic nature of the residue, albeit to a lesser extent, and might slightly destabilize the pocket, thus affecting the active site (Figure 5(Ciii)).

The other variants F277V, R404C, and R295W primarily affect residues close to interfaces between subunits A/B and might interfere with the overall structure of the FARS1 complex. Residue F277 is located at a strong hydrophobic pocket at the interface between subunits and is composed of strictly conserved residues on both subunit sides. Based on the aligned homologous structures, it is also in close proximity with the tRNA. Given the strict conservation of the interface, it is likely that functional FARS/A/B is destabilized by the substitution, thus likely affecting its enzymatic activity (Figure 5(Civ)). R404 is a well conserved residue (98%) close to the active site, but it is also located near an interface with FARB and likely forms hydrogen bonds with E26 from FARB and the strictly conserved W429 from FARS. A substitution by cysteine (R404C) would impact this hydrogen bond network and might impact the interactions between subunits. Alternatively, the substitution might induce conformational changes down into the proximal active site (12.3 Å), thus directly affecting the activity (Figure 5(Cv)). The last residue, R295, is solvent exposed and located in a variable region of the protein. R295 is partially conserved (87% in our alignment) but can be substituted by other residues containing NH\textsubscript{2} groups: lysine (11%) and asparagine.
FIGURE 4  Summarized graphical clinical phenotype of patients with mutations in FARSA or FARSB described here (P1–P3, P16, P17) (marked in red) or in the literature (P4–P15) (marked in black, see Table S1 for details) (adapted from Ref. 4). Black and white squares reflect positivity and negativity for symptoms, gray squares reflect presence of symptoms “in most”\(^{12}\) (calculation with 2/3 of individuals). Unknown status is indicated by the slash symbol [Colour figure can be viewed at wileyonlinelibrary.com]
FIGURE 5  Legend on next page.
The FARS1 phenotype manifests in more than 10 organs (2%) (Figure S2). The side chains likely form two hydrogen bonds with the backbone carbonyl of the residues P445 and M446. With the substitution lysine or asparagine, one of these hydrogen bonds could be conserved. However, the substitution by a tryptophan does not allow these hydrogen bonds and would potentially weaken the structural integrity of the region (Figure 5(Cvi)).

### 3.4 Aminoacylation activity of FARS1 complex

To further characterize the impact of the mutations on aminoacylation, FARS1 enzyme activity levels were determined in fibroblasts or EBV-transformed lymphoblastoid cell lines (EBV-LCLs) of patients and family members (Figure 5(E)). Patient 1 (E418D/R404C) had FARS1 activity reduced to ~5% in fibroblasts and ~50% in the EBV-LCL. Enzymatic activity in carriers (R404C/WT), that is, the mother or the brother of patient 1, was normal indicating that E418D, consistent with its location near the tRNA end, mainly affects aminoacylation. In contrast, fibroblasts of patient 2 (R295W/R295W) had normal FARS1 activity level (see also Figure S2). In the EBV-LCLs of family 3 (F277V/F277V), we found FARS1 activity decreased to ~5% in the patient and ~30%~75% in heterozygous parents, suggesting that F277V reduces aminoacylation activity in a dose-dependent manner, but does not completely abolish it, reflective of its hypomorphic nature. All patients and carriers showed normal FARSA and FARSB transcript and protein expression (Figure 5(D), Figure S3(A,B,C)).

### 4 DISCUSSION

The data provided here support the view that FARS1-associated recessive disease represents an ample clinical phenotype involving multiple organ systems caused by a disturbance of cytosolic phenylalanyl-tRNA synthetase. Due to the heterotetrameric structure of the FARS1 complex, consisting of two alpha and two beta subunits, we expect a common underlying pathomechanism that may be rooted in either bi-allelic variants in FARSA, the gene encoding the alpha subunit of FARS1 or its beta subunit, FARSB.

The FARS1 phenotype manifests in more than 10 organs (Figure 4) and varies in expression and severity of symptoms inter-individually. It partially overlaps with the phenotype observed in other cytoplasmic ARS diseases which commonly present as a broad multisystem spectrum, typically with a neurological component. A dominant and unifying feature of FARS1 cases was its uncommon but distinctive interstitial lung disease phenotype, defined by a characteristic chest CT pattern and cholesterol pneumonitis in all individuals where histology was available. The rare pulmonary phenotype should therefore be an important red flag to start systematic search for other clinical organ involvement.

The identified variants in FARSA and FARSB were rare (MAF < 1%), predicted as pathogenic, and detected in trans compound heterozygous. The majority affect highly evolutionary conserved amino acid residues (shown for FARSA in Figure S1). Patient 16 presented a missense mutation in combination with a predicted loss of function allele. This pattern matches the typical recessive ARS disease genotype, which usually encompasses either homozygosity for missense mutations, compound heterozygosity for missense mutations or compound heterozygosity for one missense mutation and one null allele.

Considering the vital role of ARS genes for protein synthesis, mutations typically present as hypomorphic rather than loss of function alleles. Accordingly, we have detected basic FARS1 activity in all our patient-derived cell lines with cells growing normally. FARS1 aminoacylation rate was compromised in two patient cell lines, whereas it was normal in another. Normal ARS activity in vitro might not necessarily reflect its activity in vivo. Alternatively, the results suggest that aminoacylation may represent a FARS1 function not pivotal for disease pathogenesis. This concept is in line with previous mechanistic studies in FARSB mutant patient-derived cells. Bi-allelic FARS1 mutations likely express their pathogenicity through a yet undetermined molecular pathway where gene dose has fallen below a critical threshold. Concordantly, several publications link ARSs to a number of essential non-canonical functions including regulation of metabolic homeostasis, cell proliferation and differentiation, angiogenesis, tumorigenesis, and immune response.

Currently, treatment options for FARS1 patients are limited to symptomatic treatment. In some patients (P2, P7; Figure 4) pulmonary alveolar proteinosis, that is, excessive accumulation of lipoproteins in the alveolar space, characterizes their interstitial lung disease in addition to cholesterol pneumonitis. Whole lung lavage treatment achieved transient relief of respiratory insufficiency in those two subjects treated with this option (Figure 3(K)). Increasing the substrate, that is, the metabolic concentration...
of the synthetase’s respective amino acid, here phenylalanine, appears to be a safe therapeutic option which can be instituted in individual patients, but needs to be addressed in clinical trials. Anecdotal evidence supports this approach for patients with interstitial lung–liver disease due to mutations in MARS1,27

Novel therapeutic options are expected with a deeper understanding of FARSA1-linked recessive disease. Its association with a broad and variable spectrum of symptoms calls for alertness in clinical practice. Genetic screening for mutations in FARSA and FARSB may help guide diagnostics and should be considered for unclear cases of multisystem disorders in combination with chronic interstitial lung disease.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, Matthias Greise, upon reasonable request.

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SUPPORTING INFORMATION
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