TITLE PAGE

Biallelic variants in *WARS2* encoding mitochondrial tryptophanyl-tRNA synthase in six individuals with mitochondrial encephalopathy

Saskia B. Wortmann[1,2,3,18*], Sharita Timal[4,5,18], Hanka Venselaar[6], Liesbeth T. Wintjes[4], Robert Kopajtich[2], René G. Feichtinger[1], Carla Onnekink[7], Mareike Mühlmeister[4], Ulrich Brandt[4], Jan A. Smeitink[4], Joris A. Veltman[8,9], Wolfgang Sperl[1], Dirk Lefeber[5], Ger Pruijn[10], Vesna Stojanovic[11,12], Peter Freisinger[13], Francjan v Spronsen[14], Terry GJ Derks[14], Hermine E. Veenstra-Knol[15], Johannes A Mayr[1], Agnes Rötig[16], Mark Tarnopolsky[17], Holger Prokisch[2,3,19], Richard J. Rodenburg[4,19*]

[1]Department of Pediatrics, Salzburger Landeskliniken (SALK) and Paracelsus Medical University (PMU), Salzburg, Austria

[2]Institute of Human Genetics, Helmholtz Zentrum Munich, Neuherberg, Germany

[3]Institute of Human Genetics, Technische Universität München, Munich, Germany;

[4]Radboud Center for Mitochondrial Medicine, Department of Pediatrics, Radboud University Medical Center, Nijmegen, The Netherlands

[5] Department of Neurology, Donders Center for Brain, Cognition, and Behavior, Translational Metabolic Laboratory, Radboud University Medical Center, Nijmegen, The Netherlands

[6]Center for Molecular and Biomolecular Informatics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands

[7]Department of Biomolecular Chemistry, Radboud Institute for Molecular Life Sciences and Institute for Molecules and Materials, Radboud University Medical Center, Nijmegen, The Netherlands

[8]Department of Human Genetics, Radboud Institute for Molecular Life Sciences

[9] Institute of Genetic Medicine, International Centre for Life, Newcastle University, Newcastle upon Tyne, United Kingdom

[10]Department of Biomolecular Chemistry, Radboud Institute for Molecular Life Sciences and Institute for Molecules and Materials, Radboud University Medical Center, Nijmegen, The Netherlands

[11] School of Medicine, University of Novi Sad, Novi Sad, Serbia

[12]Institute for child and youth health care of Vojvodina, Intensive Care Unit, Novi Sad, Serbia

[13]Children's Hospital, Klinikum am Steinenberg, Reutlingen, Germany

[14]University of Groningen, University Medical Center of Groningen, Beatrix Children's Hospital, Division of Metabolic Diseases, Groningen, the Netherlands.

[15]Department of Genetics, University of Groningen, University Medical Center of Groningen, Groningen, the Netherlands

[16]INSERM U1163, Université Paris Descartes - Sorbonne Paris Cité, Institut Imagine, Paris, France

[17]Department of Pediatrics, Division of Neuromuscular and Neurometabolic Diseases, McMaster University Medical Center, Hamilton, Ontario, Canada

[18,19]equally contributing

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*Correspondence:

Saskia Wortmann, MD PhD. Department of Pediatrics, Salzburger Landeskliniken (SALK) and Paracelsus Medical University (PMU), Salzburg, Austria. T +43-(0)57255-26101, F - 26399, s.wortmann-hagemann@salk.at.

Richard Rodenburg, PhD. Radboud Center for Mitochondrial Medicine, Department of Pediatrics, Radboud University Medical Center, Nijmegen, The Netherlands. T +31-(0)24-3611111, Richard.Rodenburg@radboudumc.nl.

ABSTRACT

Mitochondrial protein synthesis involves an intricate interplay between mitochondrial DNA encoded RNAs and nuclear DNA encoded proteins, such as ribosomal proteins and aminoacyl-tRNA synthases. Eukaryotic cells contain 17 mitochondria-specific aminoacyl-tRNA synthases. *WARS2* encodes mitochondrial tryptophanyl-tRNA synthase (mtTrpRS), a homodimeric class Ic enzyme (mitochondrial tryptophan-tRNA ligase; EC 6.1.1.2). Here, we report six individuals from five families presenting with either severe neonatal onset lactic acidosis, encephalomyopathy and early death or a later onset, more attenuated course of disease with predominating intellectual disability. Respiratory chain enzymes were usually normal in muscle and fibroblasts, while a severe combined respiratory chain deficiency was found in the liver of a severely affected individual. Exome sequencing revealed rare biallelic variants in *WARS2* in all affected individuals. An increase of uncharged mitochondrial $tRNA^{Trp}$ and a decrease of mtTrpRS protein content were found in fibroblasts of affected individuals. We hereby define the clinical, neuroradiological and metabolic phenotype of

WARS2 defects. This confidently implicates that mutations in *WARS2* cause mitochondrial disease with a broad spectrum of clinical presentation.

KEY WORDS

mitochondrial disorder, liver, lactic acidosis, COX deficiency

INTRODUCTION

Aminoacylation of tRNAs plays a key role in both cytosolic and mitochondrial (mt) protein synthesis, and requires aminoacyl-tRNA synthases (aaRS). Eukaryotic cells contain 37 different aaRS proteins, of which the 17 so-called mt-aaRS proteins (encoded by *aaRS2* genes) are exclusively localized in mitochondria. Biallelic variants in 16 of the genes encoding the mitochondrial aaRS2 proteins have already been reported with a clinical phenotype (Almalki, et al., 2014; Dallabona, et al., 2014; Nishri, et al., 2016; Vanlander, et al., 2015). Very recently, *WARS2* (MIM *604733) encoding tryptophanyl-tRNA synthase (mtTrpRS), a homodimeric class Ic mt-aaRS enzyme, was reported as a candidate gene in two single case reports (Musante, et al., 2017; Theisen, et al., 2017). Here, we report six individuals from five families **(Figure 1)** and define the clinical, neuroradiological, and metabolic phenotype of mtTrpRS deficiency, confidently implementing *WARS2* as a disease gene involved in mitochondrial disease.

MATERIAL AND METHODS

Case reports are summarized in **Table 1**. All procedures followed were in accordance with the Helsinki Declaration, all parents gave written informed consent (2013).

Family 1, Individual I1: Individual 1 was born as second child (female) of healthy parents from a closed Slowakian community in Serbia. Cesarean section was performed in the 38th gestational week due to intrauterine growth restriction. Birth weight was 1240 g, length 38 cm, head circumference 29 cm (all p<5), APGAR scores were 6 and 8 at 1 and 5 minutes respectively, she was transferred to the neonatal intensive care of a tertiary referral center. Lactate was 21 mmol/l (normal range (NR) <2 mmol/l), there were no laboratory signs suggestive for sepsis, TORCHES, glucose, liver function tests (LFT), coagulation tests and ammonia were within normal range. Brain, abdominal and heart ultrasound were unremarkable. Due to a distensed abdomen the abdominal cavity was explored, showing dilated loops of the small intestines and air and meconium filled colon. There were no signs of obstruction nor perforation, an ileostomy was performed.

Amino acids in plasma were within the normal range values with exception of clearly elevated alanine 720 (NR 116-376) umol/l, and mildly elevated valine 203 (NR 59-199), tyrosine 209.7 (NR 40-160), lysine 301 umol/l (NR 42-242), histidine 153.3 (NR 33-121) and arginine 11.3 (NR 12-104) umol/l. Urinary organic acids showed elevated lactate 480 mmol/mol (NR 13-46).

Lactic acidosis remained (max 25 mmol/l) despite corrections with continous i.v. bicarbonate. The child was clinically stable until she deteriorated at day 21 and subsequently died of multi organ failure at day 23 despite maximal intensive care treatment

Family 2, Individual 2 and I3: Individual 2 (male) was born at term as first child to healthy, unrelated Dutch parents after an uneventful pregnancy (birth weight 2680 gram (p5-10), APGAR scores 3 and 9 after 1 and 5 minutes). From the second day of life progressive tachypnoea was noted, he displayed a lactic acidosis (max. 23.2 mmol/l, NR < 2.2 mmol/l) and a hypoglycemia (1.5 mmol/l (NR>2.6)). The next day he was transferred to a tertiary

center and lactate levels decreased to 2.7 – 6 mmol/l under high amounts of i.v. glucose (up to 10 mg/kg/min). He was discharged at day 30.

The repetitive lactate measurements in the next months were higher in fasted (max. 5.6 mmol/l) than in fed state (max. 4.2 mmol/l). At the age of nine months he was diagnosed with West syndrome. His development was globally delayed. He had frequent periods where he cried, was hypertonic and presented dystonic movements. Due to the need of frequent feeds and his impaired swallowing he received tube feeding. MRI at age 19 months showed subcortical hypointensive white matter lesions in the pre- and post central gyrus and in the center semi-ovale going along with absent myelinisation. No evident petechial cortical bleedings (as seen in hypoglycaemia) were present. MRS of the cranial regions of the lateral ventricles showed (aspecifically) elevated choline with normal N-acetylaspartate and absent lactate, arguing against hypoxia. At age 3.5 years he died from respiratory insufficiency probably following a viral infection.

Individual 3 (male) was the younger brother of I2. The pregnancy was complicated by intrauterine growth retardation. A caesarean section was performed at 34^{+1} weeks of gestation due to fetal distress. Birth weight was on p10, APGAR scores were 9 after both 1 and 5 minutes. A few hours after birth, blood lactate levels reached 11.2 mmol/l and blood glucose was in the lower normal range (2.4 mmol/l) without clinical signs. He was first treated with high i.v. glucose (10 mg/kg/min) on which lactate concentrations normalized. Cerebral ultrasound in the first week was normal and cerebral MRI in the third week of life showed edema of the white matter and elevated lactate in all voxels measured on MRS. He was discharged after one month. At age three months his suckling reflex had disappeared and he was started on drip feeding. He seemed to have stopped with any development, neurological examinations showed generalized muscular hypotonia and brisk tendon reflexes. A follow-up

MRI at age 18 months showed marked frontal cortical and subcortical atrophy, the MRS was unchanged. Shortly after, he died after an epileptic seizure.

Plasma aminoacids, urinary organic acids and repetitive LFT, as well as ammonia and coagulation parameters were unremarkable in both children.

Family 3, Individual I4: Individual I4 (female) was born as first child at term after an uneventful pregnancy to healthy, unrelated French parents. Delivery, anthropometrics at birth and neonatal course were unremarkable. At age four months she presented muscular hypotonia and strabismus, at age six months she was diagnosed with West syndrome. At that time also global developmental delay and retinitis pigmentosa were noted. At the age of one year she developed cardiomyopathy. At the age of three years she died in an epileptic seizure. Lactate levels and LFT were not reported. An MRI was not available.

Family 4, Individual I5: Individual I5 was born as third child to healthy first cousins of Iraqi origin. No details concerning the pregnancy, delivery and neonatal course are reported. The parents report jaundice at the age of two months necessitating several hospitalizations and periods with altered consciousness, muscular hypertonia and tong bite necessitating artificial ventilation, but deny epilepsy. The individual came to attention at age 13 months after fleeing to Germany by foot. He was in a very dystrophic nutritional state (weight 6 kg, 3.8 kg under p5) and showed dystonic movements of the rump and arms, axial hypotonia and head lag. An MRI showed bilateral hypoxic ischemic basal ganglia lesions. He could not swallow nor suck efficiently, but the family refused further investigations or treatment. More than a year later he was admitted to another hospital with fever (> 40 °C) and dehydration. The clinical examination was unchanged. The fever did not respond to antipyretics and lasted with exception of some fever free days for four weeks. No focus of the infection could be detected despite intensive diagnostics. Suddenly a hepatosplenomegaly occurred and the individual deteriorated presenting clinical features of sepsis and an

intermittent diabetes insipidus. A CMV infection was proven. After intensive care treatment including artificial ventilation, and inotropic drugs, and in improved nutritional state he showed lesser unintended movements, became more alert and started to babble. He is currently aged three years, tube fed, globally delayed and shows a muscular hypotonia mainly of the rump with dystonia of the legs. Ophthalmological investigations, echocardiogram and EEG were unremarkable. LFT, ammonia, coagulatin tests and serum lactate levels were unremarkable (0.9-1.5 mmol/l), as were urinary and CSF lactate. Urinary organic acids and plasma amino acids were unremarkable, CK was intermittently elevated up to 1000 U/L (NR < 250 U/l).

Family 5, Individual I6: Individual I6 (female) was born at 37 weeks of gestation as first child to healthy unrelated Canadian parents by cesarean section due to fetal distress. Pregnancy, anthropometrics at birth and neonatal course were unremarkable. At age 18 months she presented with delayed speech and fine motor skills. A mild ataxia and weakness of the left arm and leg with mild spasticity were noted. She did not walk independently until the age of 7.5 years and currently, at the age of 10 years, she can only walk very short distances using a walker due to severe ataxia. She has mild to moderate intellectual disability, mild optic atrophy (but no apparent visual impairment) and nystagmus, severe ataxia, spastic legs (left more than right) and rigidity of her arms and legs. Lactate was first measured at age 18 months and was 2.9 mmol/L (NR < 2.0), and has varied between mildly elevated and normal over the years. LFT, bilirubin, glucose and coagulation studies were normal. MRI examinations performed at the age of 18 months and four years showed mild cerebral volume loss and mild cerebellar volume loss, respectively.

Mitochondrial oxidative phosphorylation (OXPHOS) enzymes measurements were performed as described previously (Feichtinger, et al., 2014; Janssen, et al., 2003; Rodenburg, 2011; Rustin, et al., 1994).

Whole exome sequencing (WES) was initiated independently at three different centers on leukocyte derived DNA of I1, I2, I4, I5, I6 following previously described procedures (Kremer, et al., 2016; Neveling, et al., 2013; Wortmann, et al., 2015). Based on the suspected autosomal recessive pattern of inheritance, we prioritized genes carrying biallelic variants. For each individual, common variants were filtered out based on a minor allele frequency of <1% in dbSNP (v.137), the Genome Aggregation Database (gnomAD) (Lek, et al., 2016), and the respective in-house databases.

The effect of the *missense variants identified in mtTrpRS* was evaluated by using a mtTrpRS homology model which was constructed using PDB file 1i6l as a template. This PDB file contains the structure of tryptophanyl-tRNA synthase from *Geobacillus stearothermophilus* complexed with tryptophanyl-5'AMP. This sequence shares 44% sequence identity with human mtTrpRS. The YASARA (Krieger, et al., 2002) and WHAT IF Twinset (Vriend, 1990) were employed (with default parameters) for model building and subsequent analyses.

For *western blot analysis a* total of 20 μg of mitochondrial protein extract prepared from fibroblasts of two controls (C1, C2) and two affected individuals (I1, I5) were loaded on a 10% SDS gel. After separation, proteins were blotted on a nitrocellulose membrane (Feichtinger, et al., 2014). The western blot was incubated with antibodies in the following order: polyclonal rabbit anti-WARS2 (ThermoFisher, PA5-43808, 1:1000 o/n 4 °C); monoclonal rabbit anti-MT-CO2 (Abcam, ab79393, 1:1000 1h RT); monoclonal mouse anti-

NDUFS4 (Abcam, ab55540, 1:1000 1 h RT); monoclonal mouse anti-VDAC1 (Abcam, ab 14734, o/n 4 °C); monoclonal mouse anti-UQCRC2 (Abcam, ab14745, 1:1000 1 h RT); monoclonal mouse anti-ATP5A1 (Abcam, ab14748, 1:1000 1 h RT); monoclonal mouse anti-SDHA (Abcam, ab14715, 1:30000 1h RT). After incubation with the MT-CO2 antibody the WB membrane was stripped.

Immunohistochemical Staining was performed on formalin-fixed and paraffin-embedded tissues of liver and muscle autopsy samples of I1. Antibodies against complex I subunit NDUFS4 (1:1000), complex II subunit SDHA (1:2000), complex III subunit UQCRC2 (1:1500), complex IV subunit I subunit I (mouse monoclonal,1:1000; Abcam ab14705), complex V subunit ATP5A1 (1:2000), VDAC1 (1:2000). See details for antibodies in western blot section above. All antibodies were diluted in Dako antibody diluent with background reducing components (Dako, Glostrup, Denmark). For antigen retrieval, the sections were immersed for 45 min in 1 mM EDTA, 0.05% Tween-20, pH 8, at 95 °C. Tissue sections were incubated for 60 min with the above-mentioned primary antibodies (Feichtinger, et al., 2017).

For *WARS2 mass spectrometry m*itochondria were isolated in the presence of protease inhibitor (Sigma) and mixed with reducing sample buffer. 50 µg of protein were separated on a 12.5 % Tricine-SDS PAGE at 4 °C (Schagger, 2006). The gel was fixed in 50 % methanol, 10 % acetic acid and 100 mM ammonium acetate, washed briefly with water and stained overnight in Coomassie solution (0.025 % Coomassie dye G250 in 10 % acetic acid). After destaining in 10 % acetic the region of interest of each lane (12-65 kDa) was cut into seven even slices, guided by a standard protein marker mix in separate lane to allow an estimation of the molecular mass range of each slice for later data analysis. The slices were cut into

smaller pieces and transferred separately into a 96-well filter plate (Millipore). After further

destaining in 50 % methanol, 50 mM ammonium hydrogen carbonate (AHC), in-gel tryptic

digest was performed as follows: The gel pieces were incubated for 60 minutes in 120 µl 5

mM dithiothreitol in 50 mM AHC followed by an incubation in 120 µl 15 mM 2-

chloroacetamide in 50 mM AHC for 45 minutes. After washing in 50 % methanol, 50 mM

AHC for 15 minutes and air-dehydration for 45 minutes, rehydration of the gel pieces was

carried out in 20 µl 5 ng/µl trypsin (Promega), 50 mM AHC, 1 mM $CaCl_2$ for 30 minutes at 4

°C. After addition of 50 µl 50 mM AHC, the sealed microplate was incubated for tryptic

digest over night at 37 °C. The peptides were eluted afterwards by centrifugation into a 96-

well PCR microplate (Axygen). Remaining peptides in the gel were further extracted by 50 µl

30 % acetonitrile, 3 % formic acid and the supernatants subsequently dried in a SpeedVac

concentrator. The tryptic peptides were resuspended in 20 µl 5 % acetonitrile, 0.5 % formic

acid and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in a

Thermo Scientific Q Exactive 2.0 Orbitrap Mass Spectrometry System with a front end nano-

flow high performance liquid chromatography system (Easy nLC-1000) using the Thermo

Scientific Xcalibur 2.2 SP1 Software Package. Peptides were separated in 40 minutes HPLC

runs on a PicoTip emitter column (8±1 µm Silica Tip; New Objectives) filled with 3 µm

reprosil-pur C18 beads (Dr Maisch GmbH) using a 30 minutes gradient of 5 % to 35 %

acetonitrile with 0.1 % formic acid, followed by a column wash with 80 % acetonitrile and

re-equilibration of the column with 5 % acetonitrile for 5 minutes each. The peptides were

analyzed on-line in positive mode by a mass spectrometry method programmed to fragment

the top 20 most abundant peptides. Full scan MS mode (400 to 1400 m/z) was operated at a

resolution of 70 000 with automatic gain control (AGC) target of 1x106 ions and a maximum

ion transfer of 20 ms. Selected ions for MS/MS were analyzed using the following

parameters: resolution 17500; AGC target of 1x105; maximum ion transfer of 50 ms; 4.0 m/z

isolation window; normalized collision energy 30 % for CID and dynamic exclusion of 30s.

A lock mass ion (m/z=445.12) was used for internal calibration (Olsen, et al., 2005). The

analysis of the RAW files was performed using the MaxQuant software package (version

1.5.0.25, www.maxquant.org)(Cox and Mann, 2008). Extracted spectra were matched against

the reviewed H. sapiens NCBI RefSeq database release 71. Sequences of known

contaminants were added to this database and the reverse decoy was strictly set to FDR of

0.01. Database searches were done with 20 ppm and 0.5 Da mass tolerances for precursor

ions and fragmented ions. Trypsin with the allowance of two missed cleavages was selected

as the protease. Modifications were set as N-terminal acetylation and oxidation of methionine

for dynamic modifications and cystein carbamidomethylation as a fixed modification. Protein

abundancies were determined by label-free quantitation using the composite iBAQ intensity

values determined by MaxQuant (Jia, et al., 2002). The abundance of mtTrpRS 37.9 kDa

isoform 1 (NP_056651.1) as iBAQ intensity was normalized to total iBAQ intensity of the

voltage-dependent anion-selective channel protein 1 (VDAC1; NP_003365.1) to adjust for

protein differences in the analyzed mitochondrial samples.

tRNA aminoacylation assay was performed using total RNA isolated from patient

fibroblast cells grown to subconfluent monolayer using Trizol (Life Technologies) extraction

following the manufacturer's procedures. After washing three times with 75% ethanol, the

RNA pellet was dissolved in 0.1 mM EDTA, 10 mM sodium acetate, pH 5.0. Ten

micrograms of RNA was separated by denaturing gel electrophoresis for 24 hours at 4 °C,

using a 10% polyacrylcrylamide gel containing 8 M urea and 0.1 M sodium acetate pH 5.0.

Subsequently, the RNA was transferred to Hybond N+ membranes (Amersham RPN303B)

by electroblotting, followed by UV-crosslinking. The blots were blocked for 1 hour at 55 °C

in hybridization solution (6x SSC, 0.1 mg/ml sheared herring sperm DNA, 0.2% SDS in 10x

Denhardt's solution) and were subsequently incubated overnight at 55 °C with ^{32}P-labeled antisense RNA probes **(Supp. Table 1)** in hybridization solution. After hybridization, the blots were washed with 1x SSC, 0.1% SDS for 15 min. at 55 °C and subsequently with 0.1x SSC at room temperature. Bound probes were detected by phosphor imaging (Bio-Rad Molecular Imager FX). Hybridization probes were generated by in vitro transcription by T7 RNA polymerase in the presence of ^{32}P-labeled alpha-UTP, using linearized templates. The respective constructs were generated by PCR using mitochondrial DNA as starting material and forward and reverse primers for the mitochondrial tRNA^{Trp} and tRNA^{Arg} genes (**Supp. Table 1**; T7 RNA polymerase promoter sequence in the reverse primer).

RESULTS

Based on the multisystem involvement in combination with an elevated lactate a mitochondrial disorder was suspected. The individuals showed a varying degree of abnormalities in the mitochondrial oxidative phosphorylation (OXPHOS) enzymes (**Table S1**). Mild enzyme deficiencies were observed in muscle tissue of I6 and in both muscle tissue and fibroblasts of I4, while the clinically most severely affected patient I1 showed severe combined complex I and IV deficiency in liver tissue but normal amount in muscle tissue (**Figure. 2**). In muscle tissue of I3 and fibroblasts of I1 - I3 no significant abnormalities of OXPHOS enzymes were observed **(Supp. Table S1).**

With WES *WARS2* (NM_015836.3, MIM 604733) biallelic variants were prioritized in all five index cases. A total of six different missense mutations, one frameshift mutation and one insertion/deletion were detected (**Figure 1, Supp. Table 2).** Sanger sequencing confirmed variants in all individuals, as well as an additional affected family member (I3), and carrier status in available parents. In our collective, the variants were spread throughout the entire gene. One of the variants was found in two independent families but there were no

hotspot regions. All missense variants affected evolutionary conserved residues (**Figure 1**).

No other rare variants were detected in known disease associated genes or in genes encoding

proteins with a mitochondrial location. All variants have been reported to the LOVD database

(http://www.lovd.nl/WARS2).

Figure 3 provides an overview of the missense variants described in this report in the

three dimensional structure of the mtTrpRS dimeric protein. All variants are predicted to alter

the protein structure e.g. with regard to the stability of the tertiary structure or ligand binding.

The detailed descriptions of the predicted effects for all individual mutations are given in the

supplementary data **(Supp. Figure S1A-G).**

The western blot analysis showed the absence of mtTrpRS protein in fibroblasts of I1 and the

diminished content in I4 (**Figure 4A**). Quantification of the western blot results **(Figure 4 B-

G)** showed very little differences in the expression levels of several subunits of OXPHOS

enzyme complexes, with exception of complex IV subunit COX2 encoded by *MT-CO2* that

appeared moderately reduced (**Figure 4 C**).

Next, we investigated the effect of the *WARS2* genetic variants on the expression of

the mtTrpRS protein by SDS-PAGE and mass spectrometry in fibroblasts of individuals I2

and I3 and a muscle biopsy of individual I2 using the same protocol. Despite the presence of

protease inhibitor during sample preparation, the mtTrpRS peptides were hardly detectable

around the expected size of 38 kDa, but rather spread over a wide mass rage. We concluded

that the *WARS2* mutations present in individuals I2 and I3 rendered the mtTrpRS protein

more prone to proteolysis **(Figure 4H)**. On the basis of the total amount of mtTrpRS peptides

detected in the different gel slices, the residual mtTrpRS expression levels in individuals I2

and I3 were calculated to be around 7% in fibroblasts (I2 and I3) and 6% in muscle (I2),

compared to the levels in healthy control samples **(Supp. Figure 2, Figure 4H)**. The

expression levels of the four respiratory chain complexes were unchanged in fibroblasts of

individuals I2 and I3, as determined by averaging the abundance of several subunits of each complex that were well detectable by mass spectrometry (**Figure 4H**).

Investigation of aminoacylation by northern blot analysis showed a clear decrease in charged mt-tRNA^{Trp} in the fibroblasts of individuals I2 and I3, while total mt-tRNA^{Trp} levels appeared normal **(Figure 5)**. This indicates that the defect in *WARS2* causes improper aminoacylation of tRNA^{Trp}, leading to abnormalities in OXPHOS protein biosynthesis.

DISCUSSION

Since the discovery of (*DARS2*) in 2007 (Scheper, et al., 2007), disease causing variants have been discovered in all 17 genes encoding mitochondrial tRNA synthases. For some of these genes, initial reports suggested an association with a distinct clinical phenotype. However, with the identification of an increasing number of individuals with mutations in these genes, it has become apparent that the clinical spectrum associated with a number of these genes (e.g. *AARS2* (Dallabona, et al., 2014), *NARS2* (Vanlander, et al., 2015), *RARS2* (Nishri, et al., 2016)*, FARS2* (Almalki, et al., 2014)) is broader than initially thought. The same holds for *WARS2*-related mitochondrial disorder as shown here in the six individuals presenting with a disease spectrum ranging from fatal neonatal lactic acidosis to a less severe phenotype dominated by intellectual disability and movement disorder. The clinical spectrum associated with defects in *WARS2* appears to be quite broad, both with respect to the clinical features (cardiomyopathy, movement disorder, retinitis pigmentosa, optic atrophy, hypoglycemia etc.) and with the age of onset and clinical course. Besides the elevated lactate, suggestive for a mitochondrial dysfunction in general, no specific metabolite was altered in the affected individuals.

The mtTrpRS protein is expected to be present in every cell and tissue that contains mitochondria. However, the effects of the *WARS2* defect on the OXPHOS enzyme activities

observed in the muscle and fibroblasts of the individuals presented here were normal with exception of mild alterations in I4 from whom samples were taken shortly before death which might have influenced the results. Also in muscle of I6 a mild enzyme deficiency was observed. By contrast, a clear combined respiratory chain enzyme deficiency was detected in the liver of I1, while investigations in other tissues were normal. I1 had a fatal clinical course, the most severe in the cohort presented, however, with no apparent liver involvement. In the same individual staining of muscle did not reveal any OXPHOS abnormalities. It can be assumed that liver is a vulnerable tissue in *WARS2* deficiency. Since no liver tissue was available from the other individuals we are unable to test this assumption at the moment. As for none of the other patients abnormal LFT or (recurrent acute) liver failure was observed, as seen in other mitochondrial disorders, we cannot correlate a clinical phenotype to the observed decrease of respiratory chain proteins. Based on the clinical and neuroradiological presentation in our patients we assume that the central nervous system (CNS) is most vulnerable in WARS2 deficiency - as in the majority of mitochondrial disorders. Again, tissue samples from the CNS for further investigations are usually unavailable on medical and ethical grounds which prevents further investigations.

Aminoacylation was found to be decreased and mtTrpRS protein levels were found to be severely diminished in patient-derived cells. Similar observations have been made for defects in other mitochondrial aminoacyl-tRNA synthase (mt-aaRS) related mitochondrial disorders (Diodato, et al., 2014; Konovalova and Tyynismaa, 2013). It has been suggested that the residual mt-aaRS enzyme activity would be enough to maintain OXPHOS function in some tissues. In individuals I2 and I3, the northern blot results indeed indicate the presence of a residual amount of charged tRNA^{Trp}, which seems compatible with the residual amount of mtTrpRS protein detected by mass spectrometry. In these individual's fibroblasts a defect on aminoacylation of tRNA^{Trp} in fibroblast was detected while no clear decrease in OXPHOS

enzyme activity was observed. This tissue specificity has been reported previously for other mt-aaRS genes, such as *TARS2* (Diodato, et al., 2014), and *NARS2* (Simon, et al., 2015), and could indicate that the residual mt-aaRS activity and accompanying low levels of tRNA aminoacylation are still sufficient to generate normally functioning OXPHOS enzymes in a tissue-specific manner.

In conclusion, our study shows a broad spectrum of clinical, neuroradiological, metabolic, and biochemical phenotypes in individuals with WARS2 deficiency. It can be assumed that most of these patients will be identified by genetic screening approaches like exome sequencing.

(University of Ottawa), David Dyment (University of Ottawa) as well as Clinical Coordinator Taila Hartley and Project Manager Chandree Beaulieu at the Children's Hospital of Eastern Ontario Research Institute.

DISCLOSURE

All authors have no disclosures

References

2013. World Medical Association Declaration of Helsinki: ethical principles for medical research
involving human subjects. Jama 310(20):2191-4.

Almalki A, Alston CL, Parker A, Simonic I, Mehta SG, He L, Reza M, Oliveira JM, Lightowlers RN,
McFarland R, Taylor RW, Chrzanowska-Lightowlers ZM. 2014. Mutation of the human
mitochondrial phenylalanine-tRNA synthetase causes infantile-onset epilepsy and
cytochrome c oxidase deficiency. Biochim Biophys Acta 1842(1):56-64.

Cox J, Mann M. 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-
range mass accuracies and proteome-wide protein quantification. Nat Biotechnol
26(12):1367-72.

Dallabona C, Diodato D, Kevelam SH, Haack TB, Wong LJ, Salomons GS, Baruffini E, Melchionda L,
Mariotti C, Strom TM, Meitinger T, Prokisch H, Chapman K, Colley A, Rocha H, Ounap K,
Schiffmann R, Salsano E, Savoiardo M, Hamilton EM, Abbink TE, Wolf NI, Ferrero I, Lamperti
C, Zeviani M, Vanderver A, Ghezzi D, van der Knaap MS. 2014. Novel (ovario) leukodystrophy
related to AARS2 mutations. Neurology 82(23):2063-71.

Diodato D, Melchionda L, Haack TB, Dallabona C, Baruffini E, Donnini C, Granata T, Ragona F, Balestri
P, Margollicci M, Lamantea E, Nasca A, Powell CA, Minczuk M, Strom TM, Meitinger T,
Prokisch H, Lamperti C, Zeviani M, Ghezzi D. 2014. VARS2 and TARS2 mutations in patients
with mitochondrial encephalomyopathies. Human mutation 35(8):983-9.

Feichtinger RG, Neureiter D, Skaria T, Wessler S, Cover TL, Mayr JA, Zimmermann FA, Posselt G, Sperl
W, Kofler B. 2017. Oxidative Phosphorylation System in Gastric Carcinomas and Gastritis.
Oxid Med Cell Longev 2017:1320241.

Feichtinger RG, Weis S, Mayr JA, Zimmermann F, Geilberger R, Sperl W, Kofler B. 2014. Alterations of
oxidative phosphorylation complexes in astrocytomas. Glia 62(4):514-25.

Janssen AJ, Smeitink JA, van den Heuvel LP. 2003. Some practical aspects of providing a diagnostic
service for respiratory chain defects. Ann Clin Biochem 40(Pt 1):3-8.

Jia J, Xu F, Chen X, Chen L, Jin Y, Wang DT. 2002. Two essential regions for tRNA recognition in
Bacillus subtilis tryptophanyl-tRNA synthetase. Biochem J 365(Pt 3):749-56.

Konovalova S, Tyynismaa H. 2013. Mitochondrial aminoacyl-tRNA synthetases in human disease.
Molecular genetics and metabolism 108(4):206-11.

Kremer LS, Distelmaier F, Alhaddad B, Hempel M, Iuso A, Kupper C, Muhlhausen C, Kovacs-Nagy R,
Satanovskij R, Graf E, Berutti R, Eckstein G, Durbin R, Sauer S, Hoffmann GF, Strom TM,
Santer R, Meitinger T, Klopstock T, Prokisch H, Haack TB. 2016. Bi-allelic Truncating
Mutations in TANGO2 Cause Infancy-Onset Recurrent Metabolic Crises with
Encephalocardiomyopathy. Am J Hum Genet 98(2):358-62.

Krieger E, Koraimann G, Vriend G. 2002. Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. Proteins 47(3):393-402.

Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG, Exome Aggregation C. 2016. Analysis of protein-coding genetic variation in 60,706 humans. Nature 536(7616):285-91.

Musante L, Puttmann L, Kahrizi K, Garshasbi M, Hu H, Stehr H, Lipkowitz B, Otto S, Jensen LR, Tzschach A, Jamali P, Wienker T, Najmabadi H, Ropers HH, Kuss AW. 2017. Mutations of the aminoacyl-tRNA-synthetases SARS and WARS2 are implicated in the etiology of autosomal recessive intellectual disability. Hum Mutat.

Neveling K, Feenstra I, Gilissen C, Hoefsloot LH, Kamsteeg EJ, Mensenkamp AR, Rodenburg RJ, Yntema HG, Spruijt L, Vermeer S, Rinne T, van Gassen KL, Bodmer D, Lugtenberg D, de Reuver R, Buijsman W, Derks RC, Wieskamp N, van den Heuvel B, Ligtenberg MJ, Kremer H, Koolen DA, van de Warrenburg BP, Cremers FP, Marcelis CL, Smeitink JA, Wortmann SB, van Zelst-Stams WA, Veltman JA, Brunner HG, Scheffer H, Nelen MR. 2013. A post-hoc comparison of the utility of sanger sequencing and exome sequencing for the diagnosis of heterogeneous diseases. Hum Mutat 34(12):1721-6.

Nishri D, Goldberg-Stern H, Noyman I, Blumkin L, Kivity S, Saitsu H, Nakashima M, Matsumoto N, Leshinsky-Silver E, Lerman-Sagie T, Lev D. 2016. RARS2 mutations cause early onset epileptic encephalopathy without ponto-cerebellar hypoplasia. Eur J Paediatr Neurol.

Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, Makarov A, Lange O, Horning S, Mann M. 2005. Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol Cell Proteomics 4(12):2010-21.

Rodenburg RJ. 2011. Biochemical diagnosis of mitochondrial disorders. J Inherit Metab Dis 34(2):283-292.

Rustin P, Chretien D, Bourgeron T, Gerard B, Rotig A, Saudubray JM, Munnich A. 1994. Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 228(1):35-51.

Schagger H. 2006. Tricine-SDS-PAGE. Nat Protoc 1(1):16-22.

Scheper GC, van der Klok T, van Andel RJ, van Berkel CG, Sissler M, Smet J, Muravina TI, Serkov SV, Uziel G, Bugiani M, Schiffmann R, Krageloh-Mann I, Smeitink JA, Florentz C, Van Coster R, Pronk JC, van der Knaap MS. 2007. Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. Nat Genet 39(4):534-9.

Simon M, Richard EM, Wang X, Shahzad M, Huang VH, Qaiser TA, Potluri P, Mahl SE, Davila A, Nazli S, Hancock S, Yu M, Gargus J, Chang R, Al-Sheqaih N, Newman WG, Abdenur J, Starr A, Hegde R, Dorn T, Busch A, Park E, Wu J, Schwenzer H, Flierl A, Florentz C, Sissler M, Khan SN, Li R, Guan M-X, Friedman TB, Wu DK, Procaccio V, Riazuddin S, Wallace DC, Ahmed ZM, Huang T, Riazuddin S. 2015. Mutations of human NARS2, encoding the mitochondrial asparaginyl-tRNA synthetase, cause nonsyndromic deafness and Leigh syndrome. PLoS genetics 11(3):e1005097.

Theisen BE, Rumyantseva A, Cohen JS, Alcaraz WA, Shinde DN, Tang S, Srivastava S, Pevsner J, Trifunovic A, Fatemi A. 2017. Deficiency of WARS2, encoding mitochondrial tryptophanyl tRNA synthetase, causes severe infantile onset leukoencephalopathy. Am J Med Genet A.

Vanlander AV, Menten B, Smet J, De Meirleir L, Sante T, De Paepe B, Seneca S, Pearce SF, Powell CA, Vergult S, Michotte A, De Latter E, Vantomme L, Minczuk M, Van Coster R. 2015. Two siblings with homozygous pathogenic splice-site variant in mitochondrial asparaginyl-tRNA synthetase (NARS2). Hum Mutat 36(2):222-31.

Vriend G. 1990. WHAT IF: a molecular modeling and drug design program. J Mol Graph 8(1):52-6, 29.

Wortmann SB, Koolen DA, Smeitink JA, van den Heuvel L, Rodenburg RJ. 2015. Whole exome sequencing of suspected mitochondrial patients in clinical practice. J Inherit Metab Dis 38(3):437-43.

Figure legends

Figure 1. Rare biallelic variants in *WARS2*. (A) Pedigrees of families with autosomal-

recessive rare variants in *WARS2*. (B) Localization of the six variants in *WARS2* and

mtTrpRS (upper figure). Alignment of mtTrpRS protein sequences of various species shows

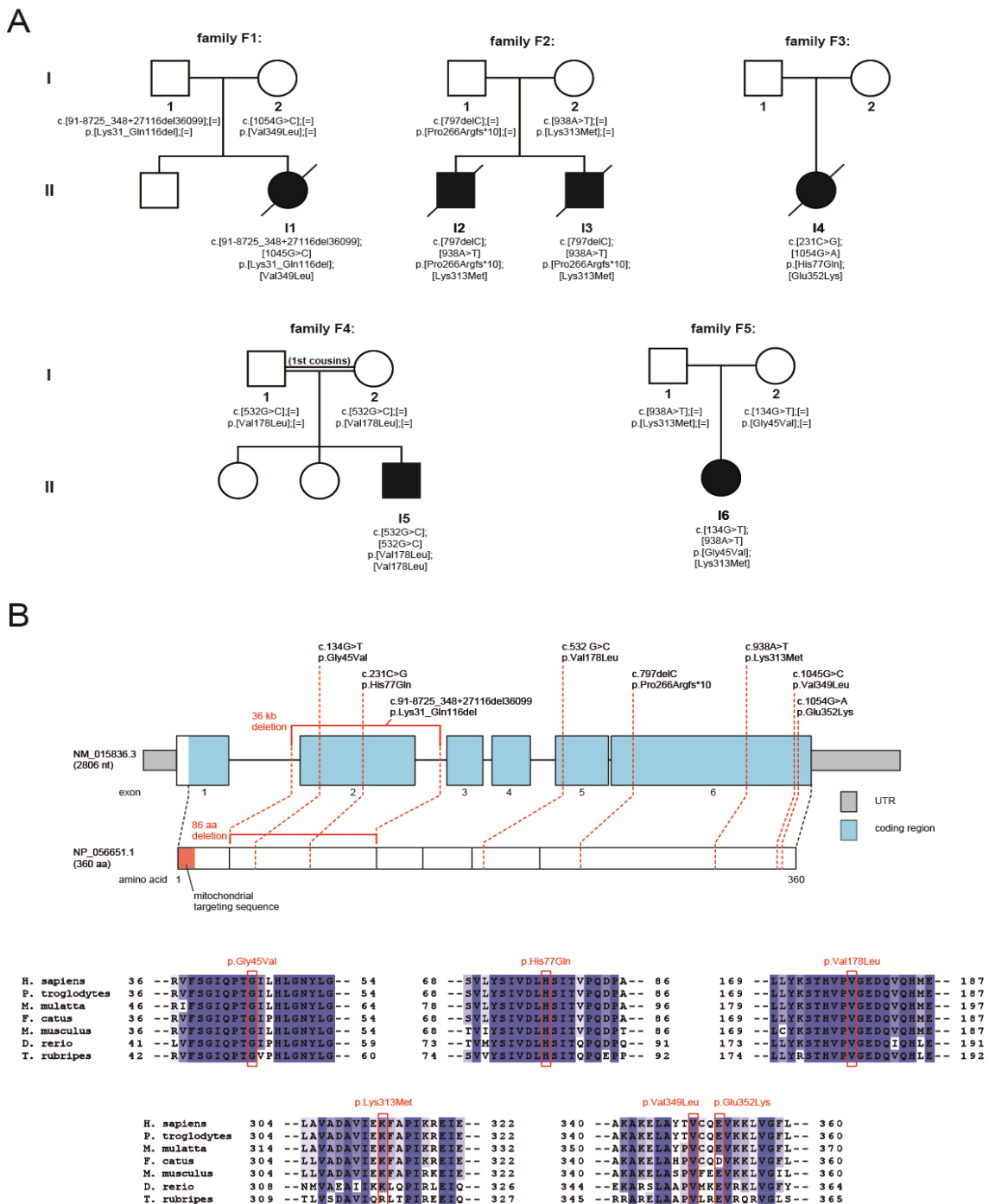that the six missense variants are evolutionary conserved.

Figure 2. Immunohistochemical staining of respiratory chain enzymes and VDAC1 in autopsy liver and muscle of individual I1. A severely reduced staining of complex I and cytochrome c oxidase was observed in the liver. The nuclear encoded complex II was found in normal amount as was the mitochondrial marker protein VDAC1. In muscle tissue all enzymes were found in normal amount. Magnification: 10x objective in liver and 40x objective in muscle.
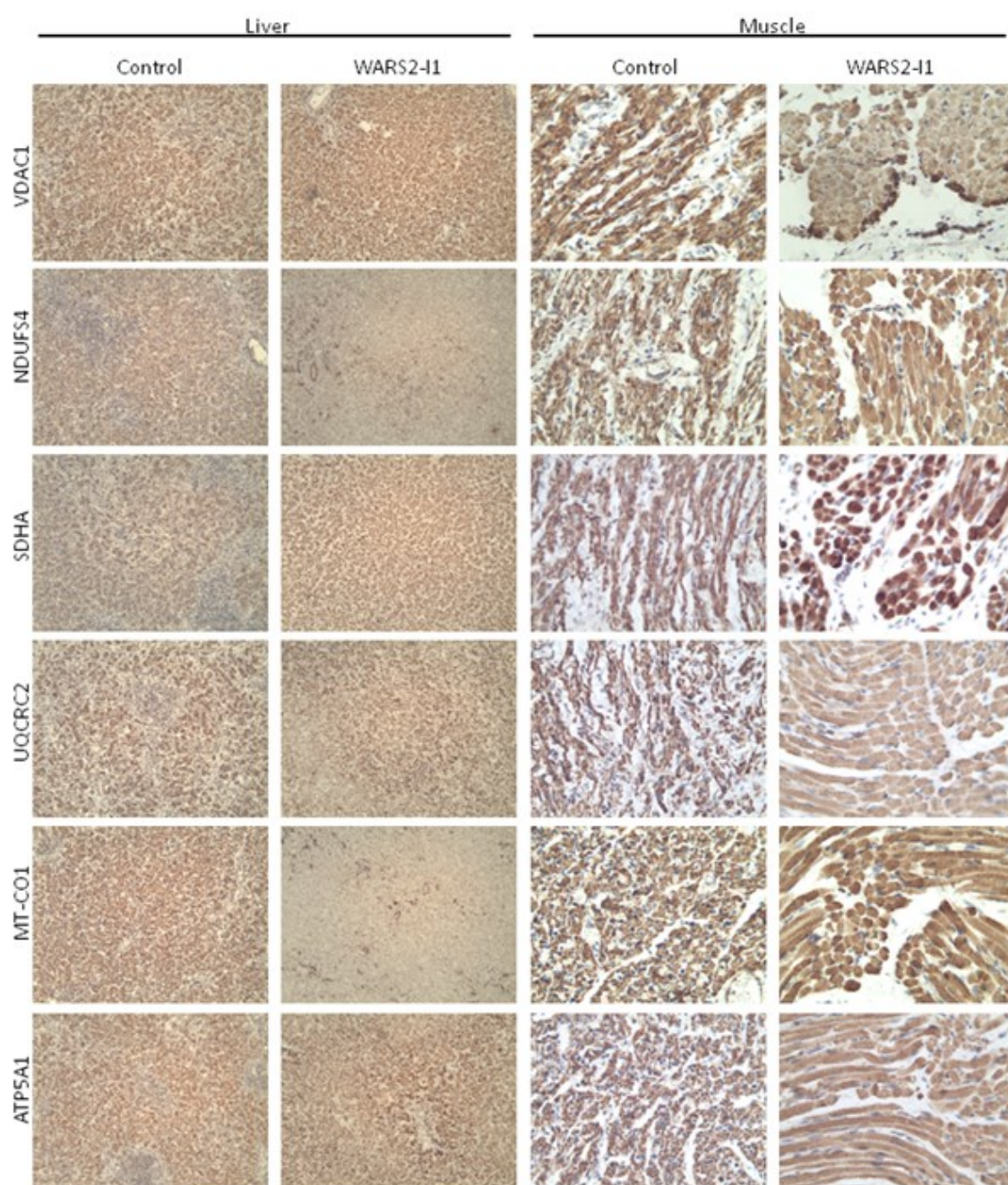
Figure 3. Modeling of the missense variants identified in mtTrpRS. Overview of the missense variants described in this report, in the three dimensional structure of the mtTrpRS dimeric protein (one subunit is indicated in grey, the other in green). The amino acid changes are indicated in red. The ligand mt-tRNA^{Trp} is indicated in yellow. Detailed images and descriptions of the predicted effects of all variants are included in the supplementary data **(Supp. Figure S1 A-G).**
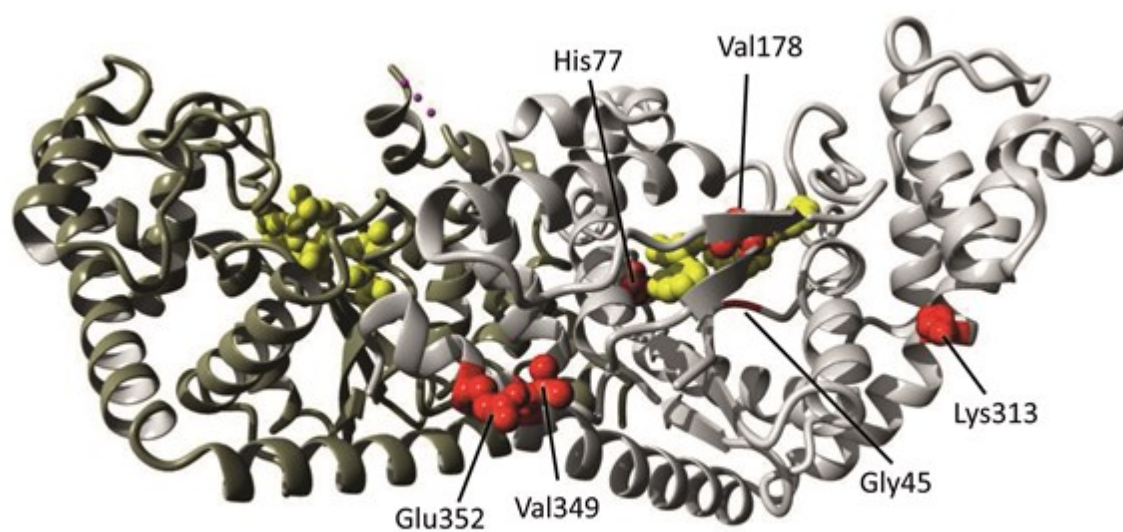
Figure 4. Western blot analysis of mtTrpRS and the OXPHOS complexes in isolated mitochondria of fibroblasts of I1 and I4. (A) Western blot of mtTrpRS and the OXPHOS complexes. Densitometric analysis of mtTrpRS protein (B), MT-CO2 subunit of complex IV (C), NDUFS4 subunit of complex I (D), SDHA subunit of complex II (E) UQCRC2 subunit of complex III (F), and ATP5A1 subunit of complex V (G). Quantification of mtTrpRS and respiratory chain complex expression amounts in muscle and fibroblasts of individuals I2 and I3 by SDS-PAGE/ESI-MS/MS as compared to controls (H). The data were normalized to the abundance level of the mitochondrial outer membrane protein VDAC1. The overall detectable amounts of mtTrpRS in individuals I2 and I3 were 7% for fibroblasts and 6% for muscle tissue compared to control expression levels.
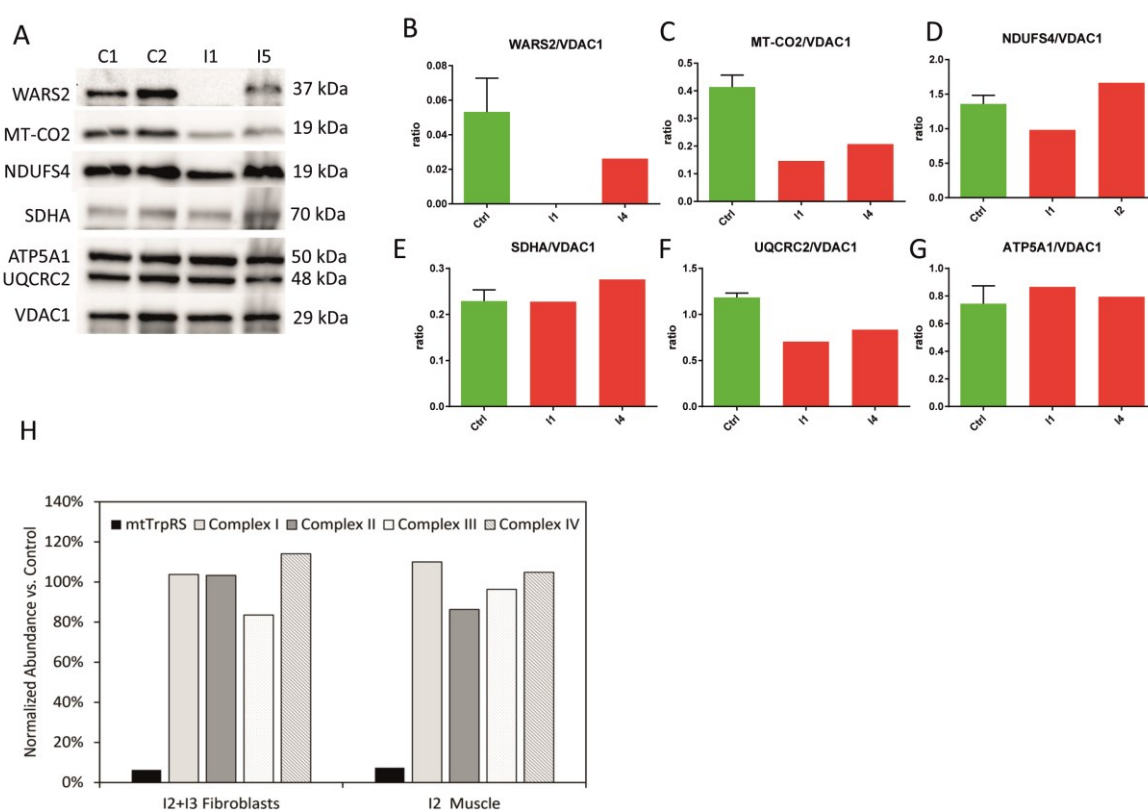
Figure 5. Charging of tRNA^{Trp} is disturbed in fibroblasts of affected individuals. Northern blot analysis of RNA extracted from cultured fibroblasts of individuals I2 and I3, two control cell lines (C1 and C2), fibroblasts of an individual with a mutation in mt-tRNA^{Arg} (P$_{Arg}$), and fibroblasts of an individual with a mutation in mt-tRNA^{Trp} (P$_{Trp}$). An aliquot of the RNA from control C1 was incubated at pH 9 to remove the amino acids from their cognate tRNAs, in order to determine the position of the uncharged tRNAs on the blots. The blots were hydridized with probes for mt-tRNA^{Trp} (upper panel) and mt-tRNA^{Arg} (lower panel). The position of the charged and uncharged tRNAs are indicated. The results show that there is a clear increase in uncharged mt-tRNA^{Trp} in the *WARS2* cell lines of individuals I2 and I3.
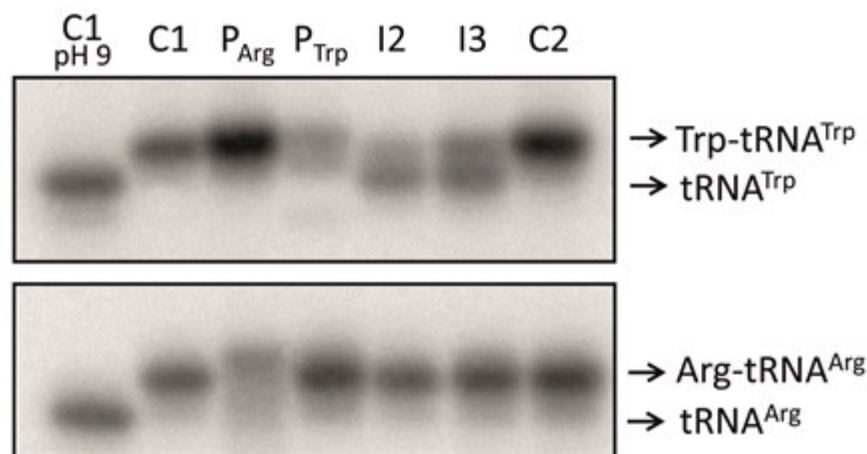
Table 1 Clinical, metabolic and radiological findings of the reported individuals

family, individual, gender, consanguinity	F1, I1, female, no	F2, I2, male, no	F2, I3, male, no	F3, I4, female, no	F4, I5, male, yes	F5, I6, female, no
neonatal course	low birth weight, lactic acidosis	low birth weight, lactic acidosis, low blood glucose	low birth weight, lactic acidosis, hypoglycaemia	unremarkable	n/a	unremarkable
presenting age (symptom)	neonatal (see above)	neonatal (see above)	neonatal (see above)	4 m (muscular hypotonia, strabismus)	13 m (DD, movement disorder)	18 m (DD)
DD/ID	n/a	yes/yes (severe)	yes/yes (severe)	yes/yes (severe)	yes/yes (severe)	yes (severe)/ yes (mild-moderate)
muscle tone	decreased	increased	increased	decreased	de-/increased	increased
movement disorder	n/a	dystonia	n/a	no	dystonia	ataxia
impaired suckling/swallowing	yes	yes	yes	n/a	yes	no
epilepsy	no	yes	yes	yes	suspected	no

other clinical findings	intestinal pseudoobstruction	no	no	cardiomyopathy, retinitis pigmentosa	no	mild nystagmus, optic atrophy, no visual impairment
neuroradiological findings (age)	ultrasound: unremarkable (neonatal)	mri: white matter defects with absent myelinisation	mri: white matter edema (neonatal), frontal atrophy (18 m)	n/a	MRI: hypoxemic-ischemic basal ganglia lesions (13 m)	MRI: cerebral and cerebellar atrophy (1.5 and 4 y)
max. serum lactate (mmol/l)	25	31	15	n/a	2.1	2.9
current age	*23 d (multi organ failure)	*3.5 years (respiratory failure)	*1.5 y (epilepsy)	*3 y (epilepsy)	alive 3 y	alive 10 y

d=days, m=months, y = years ID = intellectual disability, DD = developmental delay, n/a = not available, *

deceased