A recurring *NFS1* pathogenic variant causes a mitochondrial disorder with variable intra-familial patient outcomes

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ABSTRACT

Iron-sulfur clusters (FeSCs) are vital components of a variety of essential proteins, most prominently within mitochondrial respiratory chain complexes I-III; Fe—S assembly and distribution is performed *via* multi-step pathways. Variants affecting several proteins in these pathways have been described in genetic disorders, including severe mitochondrial disease. Here we describe a Christian Arab kindred with two infants that died due to mitochondrial disorder involving Fe—S containing respiratory chain complexes and a third sibling who survived the initial crisis. A homozygous missense variant in *NFS1*: c.215G>A; p.Arg72Gln was detected by whole exome sequencing. The *NFS1* gene encodes a cysteine desulfurase, which, in complex with ISD11 and ACP, initiates the first step of Fe—S formation. Arginine at position 72 plays a role in NFS1-ISD11 complex formation; therefore, its substitution with glutamine is expected to affect complex stability and function. Interestingly, this is the only pathogenic variant ever reported in the *NFS1* gene, previously described once in an Old Order Mennonite family presenting a similar phenotype with intra-familial variability in patient outcomes. Analysis of datasets from both populations did not show a common haplotype, suggesting this variant is a recurrent *de novo* variant.

Our report of the second case of NFS1-related mitochondrial disease corroborates the pathogenicity of this recurring variant and implicates it as a hot-spot variant. While the genetic resolution allows for prenatal diagnosis for the family, it also raises critical clinical questions regarding follow-up and possible treatment options of severely affected and healthy homozygous individuals with mitochondrial co-factor therapy or cysteine supplementation.

1. Introduction

Iron-sulfur clusters (FeSCs) are an important component of a variety of proteins found throughout the cell. FeSCs play a vital role in many essential biochemical processes, most notably energy production in the mitochondria [1,2], as well as various other crucial activities, such as DNA repair, iron homeostasis, and heme biosynthesis [3,4]. FeSC biogenesis occurs *via* a multi-step process beginning with the transfer of sulfur from cysteine, catalyzed by the cysteine desulfurase NFS1, in complex with ISD11, ACP and ISCU along mitochondrial scaffold proteins (Fig. 1A). Additional molecular machinery is involved in iron incorporation and in trafficking and delivery of FeSCs to target proteins

[1]. Pathogenic variants in genes encoding components of this process have been reported in several disorders, such as combined oxidative phosphorylation deficiency 19 *(LYRM4/ISD11)* [5], mitochondrial myopathy *(FDX1L)* [6], Friedreich ataxia *(FXN)*, and sideroblastic anemia *(GLRX5)* [2,7].

Although cysteine desulfurase is essential in the first step of FeSC assembly, to date, disease-causing variation in the *NFS1* gene has only been reported in a single pedigree from an Old Order Mennonite community, resulting in a mitochondrial disorder [8]. Here we report a second family of Christian Arab descent with recurrence of the same reported variant. We discuss intra-familial variability and propose possible treatment options for severe mitochondrial disorders caused by disruption of this pathway.

2. Methods

2.1. Participants and ethics

We studied a family consisting of healthy parents and their five children, three of whom suffered from mitochondrial disease leading to
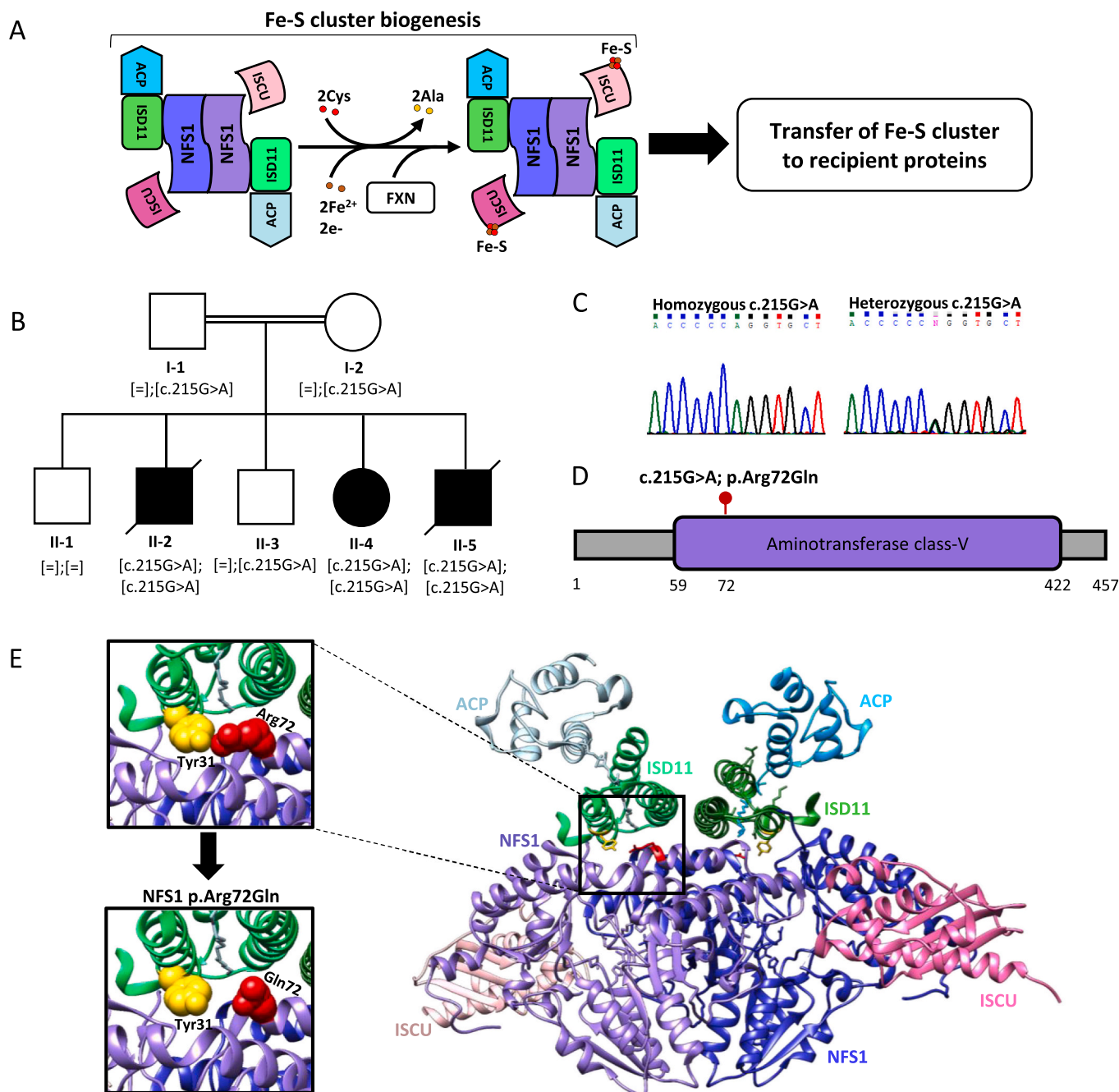

Fig. 1. The *NFS1* c.215G>A; p.Arg72Gln variant in a family with a mitochondrial disorder. (A) The cysteine desulfurase NFS1 works in complex with ISD11 and ACP and initiates the first step of iron-sulfur cluster (FeSC) formation. NFS1 catalyzes cysteine to provide the inorganic sulfur, which binds to cysteine ligands supplied by the ISCU scaffold protein. The FeSCs are then transferred to recepient proteins; (B) Family pedigree and segregation of the *NFS1* variant; (C) Sanger sequencing chromatograms displaying the c.215G>A variant in homozygous (left) and heterozygous (right) states; (D) schematic representation of the NFS1 protein. The p. Arg72Gln variant is highlighted; (E) Model of the NFS1 protein within the heterohexameric complex with ACP, ISD11 and ISCU. Arg72 of NFS1 interacts with Tyr31 of ISD11, so that the substitution of Arg with Gln at this position is predicted to hinder complex formation and proper function.

early demise of two of the affected children (Fig. 1A). A multidisciplinary medical team at Rambam Health Care Campus evaluated the family members and available medical records. The study was approved by the institutional Helsinki Committee and informed consent was obtained as customary.

2.2. Genetic analysis

Whole exome sequencing (WES) for all seven family members was performed in collaboration with the Regeneron Genetics Center (RGC), as described previously [9]. We analyzed WES data using in-house analysis pipelines and the Genoox data analysis platform (Tel Aviv, Israel) utilizing a family-based paradigm for variant filtering. Due to consanguinity and multiple affected children, recessive inheritance pattern was prioritized (homozygous and compound heterozygous variants). In addition, variants were filtered based on their effect on the protein (missense, nonsense, frameshift, splice-site) and a minor allele frequency < 0.001 in general population databases (*e.g.* gnomAD), the Rambam Genetics Institute internal database, and the RGC database. Variant pathogenicity was assessed by multiple algorithms, including SIFT [10], PolyPhen-2 [11], MutationTaster [12], and Align GVGD [13], and genomic evolutionary rate profiling (GERP) scores were obtained [14]. The candidate variant was validated by Sanger sequencing.

2.3. In silico protein analysis

The protein crystal structure of mitochondrial Fe/S cluster synthesis heterohexameric complex, including NFS1, ISD11, ACP and ISCU, was previously determined (PDB: 5WKP) [15]. We explored this structure using UCSF Chimera software in order to study the potential effect of the p.Arg72Gln variant on NFS1 structure and function, and on complex formation.

2.4. Haplotype analysis

We used KING 2.1.5 [16] with the –ibdseg command to identify pairwise identity-by-descent (IBD) segments between carriers of the hg38 chr20:g.35697793C>T *NFS1* variant using Illumina GSA genotyping array data for each sample. We subsetted down and extracted from the KING results file all IBD segments on chromosome 20 overlapping the region containing the variant of interest to analyze and look for overlapping haplotypes.

2.5. Gas chromatography mass spectrometry analysis

Chromatography of urinary organic acids using Agilent HP-5MS (30 m, 0.25mmX 0.25 μm) column was performed by gas chromatography mass spectrometry (Agilent GC 7890A/MS 5975). Urine organic acids were extracted into ethyl acetate/ether and converted to trimethylsilyl derivatives in a sample volume are equivalent to a creatinine volume of 2 mmol/L. Three internal standards 2-phenylbutyric, Tropic and Margaric Acids were used.

2.6. Enzymatic analysis

Enzymatic analysis of the mitochondrial respiratory chain complexes was performed in muscle mitochondria and liver homogenates by spectrophotometry, as we previously described [6,17].

Complex I (CI) was measured as rotenone sensitive NADH-CoQ reductase, complex I + III (CI + III) as rotenone sensitive NADH-cytochrome *c* reductase, complex II (CII) as succinate dehydrogenases, complex II + III (CII + III) as succinate cytochrome *c* reductase, complex III (CIII) as antimycin sensitive ubiquinol cytochrome *c* reductase, complex IV (CIV) cytochrome *c* oxidase, and complex V (CV) as oligomycine sensitive $Mg^{2+}ATPase$.

3. Results

3.1. Case description

A consanguineous couple of Christian Arab descent was referred to genetic counseling due to the death of two children (II-2 and II-5, Fig. 1B, Table 1) following acute metabolic crises, including infantile progressive hypotonia, acute life-threatening lactic acidosis and abnormal urinary organic acids profiles (Fig. 2), suggesting a mitochondrial disorder of unknown etiology. Both children presented in critical condition at ages 40 days (II-2) and 7 months (II-5), following a brief incurrent illness and poor feeding. Pregnancy and delivery were reported to be normal with a normal initial perinatal period for both children. Patient II-5 had mildly elevated lactate at birth with a normal echocardiogram and achieved all developmental milestones for his age.

On presentation, both children were encephalopathic with generalized hypotonia and signs of dehydration. Laboratory results were significant for metabolic acidosis with elevated blood lactate and alanine, low bicarbonate, hypoglycemia with low cortisol (II-2), hepatocellular dysfunction with elevated transaminases and abnormal INR, as well as hyperCPKemia. Further, metabolic work-up showed increased plasma and CSF lactate. Serum and CSF amino acids revealed elevated alanine and glycine, while urinary organic acids revealed increased secretion of lactate, 3-hydroxyisovaleric, keotnes, 3-methyllgutaconic and 3-methyllgutaric acids (Fig. 2). Electrolytes, renal function, uric acid and ammonia were normal. Both patients were treated initially with fluid resuscitation, 10% dextrose drip and empirical antibiotics following appropriate cultures. Nevertheless, both continued to deteriorate. They were subsequently treated empirically with carnitine, vitamin B12, thiamine and 10% dextrose with bicarbonate. Patient II-2 required mechanical ventilation and circulatory support; echocardiogram showed poor ventricular function but seemingly without evidence of cardiomyopathy. He received high-dose hydrocortisone due to laboratory evidence of adrenal insufficiency. He died after 3 days of hospitalization; the parents refused postmortem examination but agreed to liver and muscle biopsies which revealed multiple mitochondrial respiratory chain complexes deficiencies (26%–70%) of complexes I, II and III, with complexes IV and V within or above control range. The decreases were more prominent when normalized to CIV (17–60%) (Fig. 3, Table 1). Such a combination, which includes complex II, raised the possibility of a mitochondrial defect involving Fe—S containing respiratory chain complexes [1,2]. Patient II-5 received only supportive care as the parents opted not to escalate treatment. Echocardiogram showed hypertrophic cardiomyopathy with reduced systolic function; he did not have adrenal insufficiency. He died of cardiac arrest within 24 h of hospitalization.

The couple's fourth child (II-4) had a single episode of lactic acidosis, elevated creatine kinase and liver dysfunction during a urinary tract infection at age 1 month. Echocardiogram was normal, urine showed increased lactic acid and Krebs cycle metabolites. Her serum glycine level was within the high normal level. She was treated with carnitine, vitamins B1 and B2, and fully recovered. On last follow-up at 6 years of age, she was healthy and well developed. She had no reported recurrent episodes suggestive of acute exacerbation. All studies performed during the asymptomatic period, including ECG, echocardiography, liver and muscle function tests and cortisol levels following ACTH test were normal, as were plasma lactate, amino acids and urinary organic acids profiles.

3.2. Exome sequencing and variant analysis

Family-based WES analysis revealed three homozygous missense variants in the genes *NFS1*, *RBM12* and *GDAP1L1*, which were shared by the affected siblings but observed in heterozygous or wild-type state in healthy family members; no compound heterozygous variants were observed. Of these, the main candidate variant was Chr20(GRCh37):

Table 1
Clinical and biochemical features of patients with homozygous NFS1 p.Arg72Gln.

	Current study			Farhan et al. (2014)		
	Patient II-2	Patient II-4	Patient II-5	Patient IV-I	Patient IV-II	Patient IV-III
Age at presentation	40 days	7 months	1 month	7 months	6 weeks	6 months
Age at last assessment	43 days (deceased)	7 months (deceased)	6 years	7 months (deceased)	7 months (deceased)	12 years
Gender	M	M	F	F	M	M
Clinical features						
Lethargy/hypotonia	+	+	+	+	+	+
Respiratory failure	+	+	−	+	+	−
Cerebral infraction	N/A	N/A	−	−	+	−
Seizures	−	−	−	−	+	−
Cardiac failure	+	+	−	+	+	−
HCM	+	−	−	N/A	N/A	N/A
Adrenal insufficiency	+	−	−	−	−	−
Hemorrhagic pancreatitis	−	−	−	+	−	−
Renal failure	−	−	−	+	+	−
DIC	+	+	−	+	+	−
Biochemical features						
Hypoglycemia	+	+	−	+	+	−
Elevated serum lactate	+	+	+	+	+	+
Elevated serum AST	+	+	+	+	+	+
Elevated serum amylase	−	−	−	+	−	N/A
Elevated serum CK	+	+	+	+	+	+
Elevated plasma AAs	Alanine, Glycine	Alanine, Glycine	Glycine	Most amino acids	+	Alanine
Urine organic acids	Lactate, 3-hydroxyisovaleric, keotnes, 3-methyllgutaconic and 3-methyllgutaric acids	Ethylmalonic, krebs cycle metabolites	Lactate, ethylmalonic, ketones, 3-methyllgutaconic	+	−	+
Amino aciduria	+	+	N/A	N/A	+	+
Respiratory chain enzymes						
Muscle mitochondria						
Complex I + III (nmoles/min/mg)	81 (range 141–692, mean 312)	N/A	N/A	23 (range 37–99, mean 71)	39 (range 37–99, mean 71)	N/A
Complex II + III (nmoles/min/mg)	9 (range 27–93, mean 88)	N/A	N/A	5 (range 85–214, mean 152)	27 (range 85–214, mean 152)	N/A
Complex IV (nmoles/min/mg)	777 (range 436–2896, mean 1126)	N/A	N/A	156 (range 193–354, mean 264)	111 (range 193–354, mean 264)	N/A
Citrate synthase (nmoles/min/mg)	N/A	N/A	N/A	229 (range 170–481, mean 339)	540 (range 170–481, mean 339)	N/A
Liver mitochondria						
Complex I + III (nmoles/min/mg)	N/A	N/A	N/A	7 (range 2–14, mean 7)	19 (range 2–14, mean 7)	N/A
Complex II + III (nmoles/min/mg)	15 (range 8–26, mean 88)	N/A	N/A	133 (range 18–70, mean 45)	13 (range 18–70, mean 45)	N/A
Complex IV (nmoles/min/mg)	134 (range 48–119, mean 77)	N/A	N/A	39 (range 15–100, mean 41)	35 (range 15–100, mean 41)	N/A
Citrate synthase (nmoles/min/mg)	N/A	N/A	N/A	37 (range 15–53, mean 33)	14 (range 15–53, mean 33)	N/A

Abbreviations: AAs, amino acids; AST, aspartate aminotransferase; CK, creatine kinase; DIC, disseminated intravascular coagulation; HMC, hypertrophic cardiomyopathy.

g.34285715C>T in the *NFS1* gene (NM_ 021100.5): c.215G>A; p. Arg72Gln (Fig. 1B–D) due to its relation to assembly of mitochondrial iron-sulfur clusters and a previous publication implicating this variant in a similar phenotype in an Old Order Mennonite family [8]. The variant's aggregated MAF in publically available databases was <0.0001 (gnomAD: 0.00006, ESP 6500: 0.0002); it was not observed in the Rambam internal database, and did not appear in the homozygous state in any of these databases. The variant was found in the RGC database at a MAF = 0.000081, interestingly, including one homozygous individual. The variant was predicted to be deleterious by all bioinformatic algorithms and has a CADD score of 31. The affected amino acid (arginine at position 72) is highly conserved (GERP 5.75), as well as larger in size and

positively charged compared to the smaller and neutral glutamine. This physiochemical difference between the amino acids may lead to loss of internal interactions, disturbing the NFS1 protein structure. Moreover, Arg72 was previously shown to form a hydrogen bond with Tyr31 of ISD11 [15]; therefore, the p.Arg72Gln substitution may hinder NFS1-ISD11 complex formation or its stability and proper function (Fig. 1E).

Of note, both deceased brothers (II-2 and II-5) also shared a homozygous variant in *NDUFAF5*, a gene related to mitochondrial complex I deficiency nuclear type 16 [18]. This variant (NM_024120.5: c.1024A>G; p.Lys342Glu) is located in the gene's last exon and predicted to be benign by all pathogenicity assessment software with a CADD score of 11.67, as it affects a weakly conserved amino acid (GERP
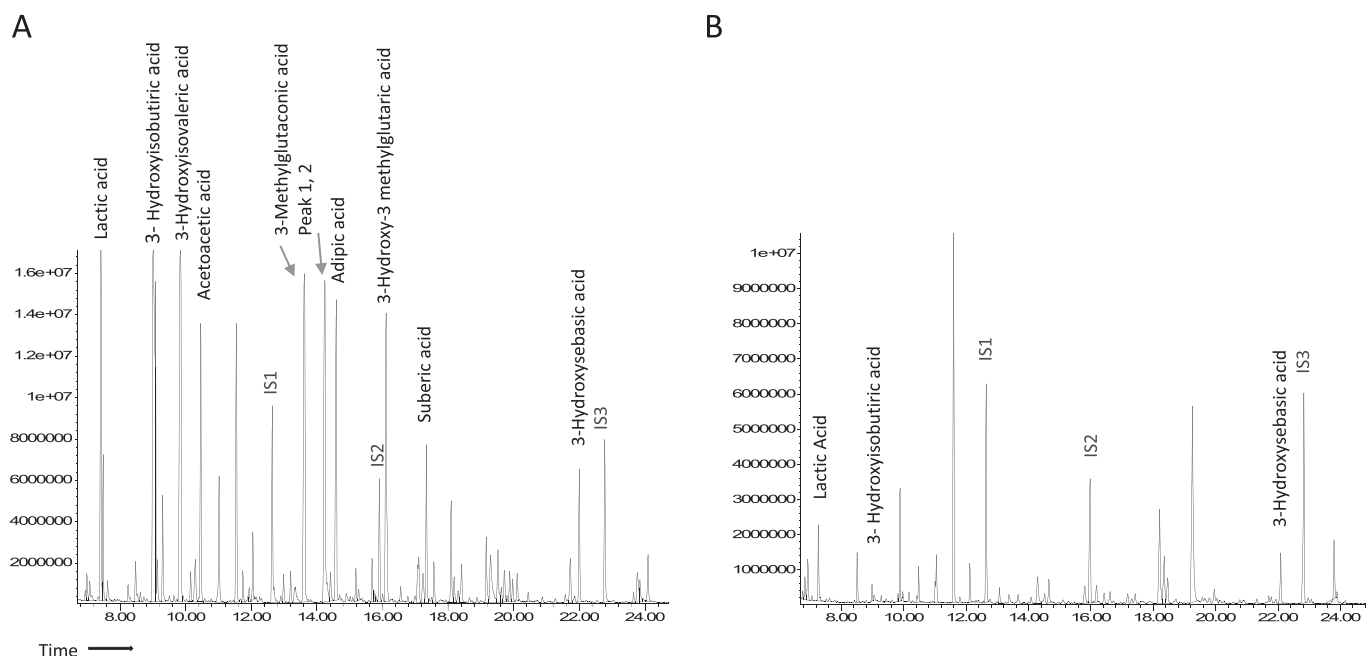
Fig. 2. Urine organic acids analysis. Typical urine organic acids profile for Patient II-2 using gas chromatography–mass spectrometry of affected individual, (A) during acute crises showing increased excretion of lactic acid, 3-hydroxybutyric acid, 3-hydroxyisovaleric acid, aetoacetic acid, 3-methylglutaconic acid, adipic acid, 3-Hydroxy-3 methylglutaric acid, suberic acid and 3-hydroxysebasic acid, and (B) during an intercrisis interval urinary organic acids could be almost normal. IS1, internal standard 1 (2-phenylbutyric acid); IS2, internal standard 2 (tropic acid); IS3, internal standard 3 (margaric acid).
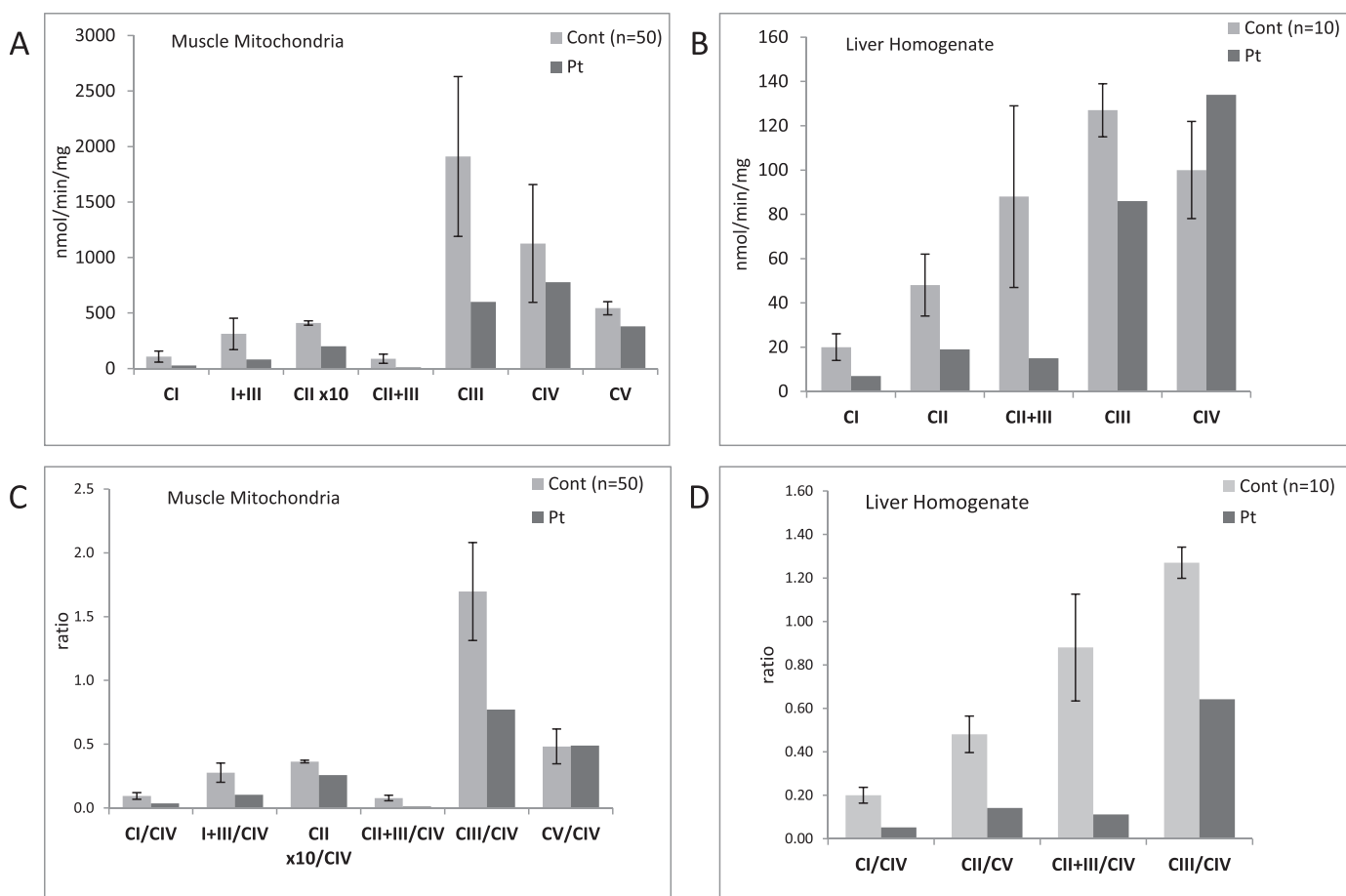

Fig. 3. Mitochondrial respiratory chain activities in muscle and liver. Enzymatic activities were measured in Patient II-2 (A,C) quadriceps muscle mitochondria (CI-CV), and in Patient II-2 (B,D) liver homogenate (CI-CIV). Absolute activities in patient (Pt) and controls (Cont) ± SD are depicted in A and D and ratios to CIV in C and D.

2.1) and there is a small physicochemical difference between lysine and glutamate (Grantham score 56). The mildly affected sister (II-4) is heterozygous for this variant; therefore, this variant cannot be excluded as a modifier of the intra-familial phenotype severity.

3.3. Haplotype analysis

Given the presence of this variant in a number of individuals in the RGC database, including many from the Old Order Mennonite population [19], where this particular variant was first identified, we asked whether the variant resided in a common ancestral European haplotype or perhaps it had occurred independently in the Mennonite and Christian Arab populations. Analysis of the haplotypes in heterozygous carriers and homozygous individuals failed to identify a common shared haplotype between individuals from the two distinct populations. Therefore, we concluded that the variant most likely arose *de novo* and independently in the two populations. Of note, the variant occurs as a C to T transition in a CpG dinucleotide, known to be more prone to deamination and spontaneous mutation. In aggregate, our data supports the conclusion that the c.215G>A; p.Arg72Gln variant in *NFS1* originated as a recurrent *de novo* variant in different population haplotypes.

4. Discussion

The *NFS1* gene encodes a cysteine desulfurase that initiates the first step of FeSC formation by catalyzing the conversion of L-cysteine to L-alanine while generating a sulfide intermediate [20]. This inorganic sulfur binds to cysteine ligands supplied by to the scaffold protein ISCU, and is combined with an iron element, thereby forming the FeSC (Fig. 1A) [1]. Once formed, the FeSC is then incorporated into a variety of proteins most notably complexes I, II and III of the mitochondrial respiratory chain[1,2]. While data linking *NFS1* depletion and mitochondrial dysfunction in tissues derived from animal models has been previously reported, reports of NFS1-related disease in humans are lacking [21,22]. Here we report the second case of mitochondrial disease due to homozygous *NFS1* pathogenic variant (c.215G>A; p.Arg72Gln), observed in an Israeli Christian Arab family. The affected siblings in this family had similar clinical manifestations, and the exact same *NFS1* variant had been reported only once previously in an Old Order Mennonite family, thus corroborating the pathogenicity of this variant [8]. Haplotype analysis of our patients and variant carriers in a Mennonite population dataset did not show a common haplotype; this, as well as the geographic and ethnic differences, indicate that a founder effect is unlikely, implicating this variant as a "hot-spot" variant.

Previous studies in eukaryotes have shown that NFS1 activity occurs as part of a functional complex with both ISD11, and ACP proteins on the scaffold protein ISCU (Fig. 1A,E) [23,24]. This complex formation is essential for stability and proper function of NFS1 [23,24]. Thus, variants affecting protein binding are likely to be disease-causing *via* decreased complex formation with a concurrent decrease in NFS1 activity. Indeed, *in silico* modeling of the NFS1-ISD11 complex indicates that the identified p.Arg72Gln variant affects an important NFS1-ISD11 binding site, meaning that this amino acid substitution will likely hamper complex formation (Fig. 1E). In fact, reduced NFS1 production was observed in fibroblasts from the previously reported patients [8]. Similarly, a previously reported *LYRM4* pathogenic variants, which encodes the ISD11 protein, led to a similar phenotype to our patients, including acute mitochondrial dysfunction in the first year of life. Concordantly, decrease in ISD11 levels and in FeSC-containing proteins were observed in the tissues from these patients, and *in vitro* assays showed reduced desulfarase activity [5].

Examining databases of genomic variation, including gnomAD and the RGC internal database, revealed that there are no *NFS1* homozygous loss-of-function variants and only few homozygous missense variants in the general population. This suggests that the encoded protein is required for proper development and survival. As such, patients with *NFS1*-related disease are expected to harbor homozygous missense variants that confer residual enzymatic activity, allowing survival at least into infancy.

Interestingly, in both families with homozygous *NFS1* p.Arg72Gln, as well as in the pedigree with ISD11 dysfunction, a metabolic crisis occurred within the first year of life, likely triggered by an acute intercurrent illness. In all three pedigrees, the initial crisis was fatal in all but one affected member who went on to be relatively asymptomatic (Table 1) [5,8]. The one homozygous Mennonite individual identified in the RGC database is also asymptomatic but with a history of an episode of viral infection and lethargy in infancy that eventually resolved favorably with no later concerns. We also report adrenal insufficiency in one of our patients, which has not been described in previous pedigrees. It may be prudent to actively evaluate adrenal function in FeSC-related disorders.

Our report supports previous data implicating the first year of life as the critical period in disorders involving the first steps in the biogenesis of FeSCs and the NFS1-ISD11-APC complex. This is likely due to the fact that cystathionase activity, which is essential in cysteine metabolism, is underactive in the first year of life, making cysteine an essential amino acid in early infant life [25]. As such, it is possible that the combination of low cysteine desulferase activity and the relatively sparse substrate, lead to a "crash" in the first year of life in patients with FeSC assembly dysfunction, especially when exacerbated by acute states with high energy demands. This report adds to the body of evidence supporting a unique natural history, including the possibility of full or nearly complete recovery after the initial acute phase in FeSC disease as opposed to many mitochondrial disorders caused by other mechanisms. This could direct clinical decision making and prompt aggressive treatment with curative intentions.

It has thus been postulated that cysteine supplementation during the first year of life or during acute exacerbation may ameliorate symptoms in this critical period and even save patients [5,26]. A recent report of successful early L-Cysteine in supplementation in a patient with *TRMU*-deficiency [27], a mitochondrial enzyme which catalyzes the addition of a sulfur-containing thiol group to the wobble position of several mitochondrial tRNAs [26]; the treated patient showed recovery of liver function and liver transplantation could be avoided [27]. It is thus tempting to speculate that early cysteine supplementation in NFS1-deficiency patients could bypass the enzymatic defect and prevent the early fatal progression of this disorder. Moreover, "empirical" cysteine supplementation may be warranted as part of the arsenal in the treatment of acutely ill patients with mitochondrial dysfunction particularly when FeSC containing complexes are involved. Rapid analysis for this possible hotspot variant may also be efficient for informing this approach. In this way, genetic diagnosis and understanding of the errant metabolic pathway may facilitate lifesaving treatment.

As demonstrated by this report, knowledge of the specific genotype and biochemical pathway involved in a particular clinical scenario can be vital in predicting the likely course of a disease and thereby directing treatment strategies in rare life-threatening mitochondrial disorders. Testing laboratories should consider *NFS*1 when studying mitochondrial disorders with defects in respiratory complexes I, II and III, as well as hypoglycemia and adrenal insufficiency. As this is only the second report of a *NFS1* variant, future reports of additional families with *NFS1* and FeSC assembly disorders can further contribute to corroborating novel treatment approaches.

Declaration of competing interest

JS and CG-J are full-time employees of the Regeneron Genetics Center and receive stock options as part of compensation. All other authors have no conflicts to declare.

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