Impact of mutations within the [Fe-S] cluster or the lipoic acid biosynthesis pathways on mitochondrial protein expression profiles in fibroblasts from patients.

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Abstract:

Lipoic acid (LA) is the cofactor of the E2 subunit of mitochondrial ketoacid dehydrogenases and plays a major role in oxidative decarboxylation. *De novo* LA biosynthesis is dependent on LIAS activity together with LIPT1 and LIPT2. LIAS is an iron-sulfur (Fe-S) cluster-containing mitochondrial protein, like mitochondrial aconitase (mt-aco) and some subunits of respiratory chain (RC) complexes I, II and III. All of them harbor at least one [Fe-S] cluster and their activity is dependent on the mitochondrial [Fe-S] cluster (ISC) assembly machinery. Disorders in the ISC machinery affect numerous Fe-S proteins and lead to a heterogeneous group of diseases with a wide variety of clinical symptoms and combined enzymatic defects. Here, we present the biochemical profiles of several key mitochondrial [Fe-S]-containing proteins in fibroblasts from 13 patients carrying mutations in genes encoding proteins involved in either the lipoic acid (*LIPT1* and *LIPT2*) or mitochondrial ISC biogenesis (*FDX1L*, *ISCA2*, *IBA57*, *NFU1*, *BOLA3*) pathway. Ten of them are new patients described for the first time. We confirm that the fibroblast is a good cellular model to study these deficiencies, except for patients presenting mutations in *FDX1L* and a muscular clinical phenotype. We find that oxidative phosphorylation can be affected by LA defects in *LIPT1* and *LIPT2*

40 patients due to excessive oxidative stress or to another mechanism connecting LA and
41 respiratory chain activity. We confirm that NFU1, BOLA3, ISCA2 and IBA57 operate in the
42 maturation of [4Fe-4S] clusters and not in [2Fe-2S] protein maturation. Our work suggests a
43 functional difference between IBA57 and other proteins involved in maturation of [Fe-S]
44 proteins. IBA57 seems to require BOLA3, NFU1 and ISCA2 for its stability and NFU1
45 requires BOLA3. Finally, our study establishes different biochemical profiles for patients
46 according to their mutated protein.

Introduction

Lipoic acid (LA) is an important antioxidant used in human therapeutics [1, 2]. It is also found as a covalent cofactor of the H subunit of the glycine cleavage system (GCS) and of E2 subunits of mitochondrial ketoacid dehydrogenase complexes, namely pyruvate dehydrogenase (PDHc), α -ketoglutarate dehydrogenase (α -KGDHc), branched-chain ketoacid dehydrogenase (BCKDHc) and ketoadipate dehydrogenase (KADHc). Starting from malonyl-coenzyme A, mammalian mitochondrial fatty acid synthesis type II enables the modification of the acyl-carrier protein by a covalently linked octanoic acid. Then, the octanoic acid is transferred to the H subunit of GCS with the help of the lipoyltransferase LIPT2, and two thiolate groups are added to form LA. The next step is performed by the lipoic acid synthase (LIAS), a [4Fe-4S] cluster-containing protein. Finally, LA is transferred by LIPT1 to target E2 subunits of ketoacid dehydrogenase complexes [3].

LIAS, a key protein of the LA synthesis pathway, coordinates two [4Fe-4S] clusters, which are critical for its activity [4]. The [Fe-S] cluster is a widespread prosthetic group found in proteins localized in most mammalian organelles, including mitochondria, cytosol and nucleus, but also in all the kingdoms of life. Their numerous functions vary from electron transfer to sulfur donor or gene expression regulation [5]. They are composed only of iron and inorganic sulfur and are found with different nuclearities. The two major [Fe-S] clusters are the [2Fe-2S] and [4Fe-4S] clusters. In many cases, the [Fe-S] cluster is necessary for enzymatic activity, as for LIAS [6]. Moreover, several subunits of respiratory chain (RC) complexes as complex I (eight subunits), complex II (SDHB subunit) and complex III (UQCRFS1 or Rieske protein) also assemble [Fe-S] clusters, which are essential for OXPHOS electron transfer. Furthermore, ferrochelatase, a mitochondrial enzyme involved in the heme synthesis pathway, carries a [2Fe-2S] cluster [7], and mitochondrial aconitase (mt-aco), an enzyme of the tricarboxylic acid cycle, carries a [4Fe-4S] cluster [8].

The biogenesis of all mammalian [Fe-S] clusters starts in the mitochondrion, independently of the subcellular localization of the [Fe-S] protein (Figure 1). Using inorganic sulfur provided by the cysteine desulfurase NFS1 complexed to ISD11 and ferrous ions, a [2Fe-2S] cluster is built on the scaffold protein ISCU with the help of ferredoxin (FDX1L) and frataxin (FXN). In a second step, the [2Fe-2S] cluster is transferred from ISCU to the glutaredoxin (GLRX5) with the help of the chaperone proteins HSPA9 and HSC20 [9]. Finally, the cluster is either transferred to target mitochondrial proteins or exported to the cytosol for the maturation of extra-mitochondrial proteins through the iron-sulfur exporter system. However, the exact nature of the exported component (ISC or sulfur) remains unknown [5]. This last step of the maturation of mitochondrial [Fe-S] proteins involves transfer factors, including IBA57, BOLA3, NFU1 and ISCA2 [10]. The specific roles of these proteins are still debated. It is proposed that all of them are required only for the maturation of [4Fe-4S]-containing mitochondrial proteins [11], with NFU1 and BOLA3 being more specifically involved in the specific maturation of LIAS, mt-aco and SDHB [8, 9].

Since the first report of Cameron et al. in 2011 [12], various multiple mitochondrial dysfunctions syndromes (MMDS) have been reported involving mutations in genes encoding proteins involved in the Fe-S cluster biogenesis pathway, namely MMDS1 (NFU1), MMDS2 (BOLA3), MMDS3 (IBA57) and MMDS4 (ISCA2) [3, 12–17], or in the lipoic acid synthesis pathway (LIAS, LIPT1, and LIPT2) [16, 18–21]. Recently, a new MMDS was described with mutations in *ISCA1* [22]. The clinical phenotypes of all these patients are very similar to those frequently observed in disorders concerning mitochondrial energy metabolism. In general, the presenting symptoms occur during the neonatal period or infancy. Common clinical presentations are neurological symptoms, including hypotonia, leukoencephalopathy, and psychomotor regression, but extraneurological symptoms have also been reported [23]. Biochemical studies show low PDHc enzymatic activity, but defects in RC complex activities are variably observed.

In this report, we describe 13 patients carrying mutations in genes encoding proteins involved either in lipoic acid synthesis (*LIPT1* and *LIPT2*) or in mitochondrial [Fe-S] cluster biogenesis (*NFU1*, *BOLA3*, *IBA57*, *ISCA2*, *FDX1L*). Ten of these patients are described here for the first time. Using their cultured skin fibroblasts, we established biochemical profiles (protein level and enzymatic activity) of several key mitochondrial proteins carrying either a [Fe-S] cluster or bound to lipoic acid. Since the development of next-generation sequencing technologies, molecular diagnosis of rare diseases is accelerating, but biochemical data are needed to improve our knowledge of these proteins and to help the formulation of new therapeutic approaches for present and future patients.

1. Materials and methods

a) Patients and cell culture

Thirteen patients with mutations in genes encoding proteins involved in either [Fe-S] cluster biogenesis or the LA synthesis pathway were included. The genotype of each patient is described in Table 1, patients P1, P2 and P8 having already been reported [19–21, 24]. The integrated software ALAMUT V.2.7 (Interactive Biosoftware; <http://www.interactive-biosoftware.com>) was used for *in silico* analysis of missplicing effects. No novel mutations were reported as SNP variants in Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (<http://evs.gs.washington.edu/EVS/>) [March 2017 accessed]. Cultured skin fibroblasts from these patients and from individuals without mitochondrial disease (controls) were grown in HAM'S F-10 medium (Eurobio, Les Ulis, France) supplemented with antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin), 50 mg/L uridine and 12% fetal bovine serum. Cells were cultured at 37°C in a humidified incubator with 5% CO₂. Clinical and biochemical data and brain magnetic resonance imaging (MRI)

findings were collected retrospectively. Informed consent was obtained from all patients before inclusion in the study.

b) Biochemical studies

All biochemical analyses were performed on cultured skin fibroblasts. PDHc activity was measured as described [25]. RC complexes, α -KGDHc and citrate synthase activities were measured by spectrophotometric assay [26]. Assays of complex I and of mt-aco activity were performed on mitochondria-enriched fractions prepared from permeabilized cells with digitonin, as described previously [27, 28]. RC complex and mt-aco activities were normalized to citrate synthase activity. Glutathione measurement was carried out on total fibroblast lysates [29].

c) Western blot and BN-PAGE analyses

Western blot analysis was performed on mitochondria-enriched fractions or on total fibroblast lysates. About 30 μ g of protein extracts was separated by SDS-PAGE and transferred to PVDF membranes, blocked in PBS-Tween containing 5% non-fat milk and then incubated overnight with primary antibodies. Antibodies against lipoic acid (LA) (Abcam, ab18724), PDH-E2 subunit (SantaCruz Biotechnology, sc-271352), NFU1 (kindly provided by Dr. T. Rouault, NICHD, NIH, Bethesda, USA), IBA57 (kindly provided by Prof. R. Lill, Marburg, Germany), SDHB (Abcam, ab14714), Rieske protein (Abcam, ab14746), LIAS (ThermoFisher Scientific, PA5-22387), ferrochelatase (kindly provided by Dr. H. Puccio, Strasbourg, France), mt-aconitase (kindly provided by Dr. R. B. Franklin, University of Maryland, Baltimore, USA) and prohibitin (Novusbio, NBP2-37563) were used. Secondary peroxidase-conjugated anti-mouse (Sigma-Aldrich, A4416) and anti-rabbit (Sigma-Aldrich, NA934V) antibodies were used. Detection was achieved using the ECL chemiluminescence kit (GE Healthcare) and G-Box (Sysgene) associated with GeneSNAP software. For some experiments, goat anti-rabbit or anti-mouse IRDye® 800-conjugated secondary antibodies

(LI-COR, Lincoln, USA) were used. Membranes were scanned with the Odyssey infrared imaging system and images were processed with the Image Studio Software (LI-COR, Lincoln, USA).

Blue-native PAGE (BN-PAGE) analysis was carried out on mitochondria-enriched fractions from fibroblasts [30]. Immunoblotting was performed using antibodies raised against the complex I subunit GRIM19 (Abcam, ab10240), the 70kDa subunit of complex II (SDHA, Abcam, ab14715) and the complex III subunit core2 (UQCRC2, Abcam, ab14745).

2. Results

a) Patients

Thirteen children from 12 unrelated families were included in our study. Patients P3 and P4 were first cousins. The clinical, MRI and biochemical data are summarized in Table 2:. Clinical presentations were mostly severe encephalopathy features with hypotonia, psychomotor delay since birth (n=2/13) or neurological regression (n=7/13). Epilepsy was noted in five patients. Pulmonary arterial hypertension was also reported in two of the four (P6 and P8) NFU1 patients. The only patient with normal neurological development was an FDX1L-deficient patient (P13), who presents recurrent acute episodes of rhabdomyolysis and muscular weakness. Onset began between four and five years of age. In most patients, first symptoms began during the first days or months of life. All the patients with *BOLA3* (P3 and P4), *NFU1* (P5 to P8), *IBA57* (P9 and P10) mutations and one *ISCA2* patient (P12) died before 3 years of age. The second *ISCA2* patient (P11) has a mild disease with isolated and non-progressive spastic paraplegia at 12 years of age. White matter abnormalities were usually found in patients with encephalopathy. Magnetic resonance spectroscopy showed a lactate peak in three (*LIPT1*, P1; *LIPT2*, P2; *NFU1*, P7) of the six tested patients (P1, P2, P5, P7, P8 and P13).

Patient	Gene	Reference sequence	Allele 1			Allele 2		
			Nucleotide change	Protein change	Location	Nucleotide change	Protein change	Location
P1 [§]	<i>LIPT1</i>	NM_145199	c.535A>G	p.Thr179Ala	Exon 2	c.875C>G	p.Ser292*	Exon 2
P2 [§]	<i>LIPT2</i>	NM_001144869	c.89T>C	p.Leu30Pro	Exon 1	c.377T>G	p.Leu126Arg	Exon 1
P3	<i>BOLA3</i>	NM_212552	c.136C>T	p.Arg46*	Exon 2	c.136C>T	p.Arg46*	Exon 2
P4	<i>BOLA3</i>		c.136C>T	p.Arg46*	Exon 2	c.136C>T	p.Arg46*	Exon 2
P5	<i>NFU1</i>	NM_001002755	c.146del	p.Pro49Leufs*8	Exon 3	c.303-1988_c.369+1021del	p. ?	Intron 3 to intron 4
P6	<i>NFU1</i>		c.622G>T	p.Gly208Cys	Exon 7	c.629G>T	p.Cys210Phe	Exon 7
P7	<i>NFU1</i>		c.179G>T	p.Phe60Cys	Exon 3	c.179G>T	p.Phe60Cys	Exon 3
P8 [§]	<i>NFU1</i>		c.565G>A	p.Gly189Arg	Exon 7	c.622G>T	p.Gly208Cys	Exon 7
P9	<i>IBA57</i>	NM_001010867	c.335T>G	p.Leu112Trp	Exon 1	c.437G>C	p.Arg146Pro	Exon 2
P10	<i>IBA57</i>		c.316A>G	p.Thr106Ala	Exon 1	c.738C>G	p.Asn246Lys	Exon 3
P11	<i>ISCA2</i>	NM_194279	c.154C>T	p.Leu52Phe	Exon 2	c.154C>T	p.Leu52Phe	Exon 2
P12	<i>ISCA2</i>		c.313A>G	p.Arg105Gly	Exon 4	c.313A>G	p.Arg105Gly	Exon 4
P13	<i>FDX1L</i>	NM_001031734	c.1A>T	p.Met1?	Exon 1	c.1A>T	p.Met1?	Exon 1

Table 1: Mutational spectrum of the thirteen patients.

Mutations in bold are described for the first time in this publication. [§] P1, P2 and P8 were already reported [19–21, 24]. Nomenclature of variants is in accordance with the recommendations of the Human Genome Variation Society [31].

Patient	Gene	Gender	Age at onset/ Age at death	Clinical presentation	Brain MRI	Lactate (mmol/L)		Glycine (μmol/L)		Other
						Plasma (RV <2)	CSF (RV<1.8)	Plasma (RV<300)	CSF (RV<16)	
P1	LIPT1	Male	15 months/ alive at 8 years	PMD, hypotonia, tetraparesis. Steady aortic root dilatation [19].	Cortical atrophy, delayed myelination. MRS: peak of lactate during decompensation.	8 at onset N at 8 years	6.6	<300	<16	increase of 2 keto-glutarate (urine)
P2	LIPT2	Male	Neonatal period/ alive at 7 years	PMD, hypotonia, microcephaly, epilepsy [20, 21].	Cortical atrophy, delayed myelination. MRS: peak of lactate.	4.5	<1.8	419	ND	
P3	BOLA3	Male	5 months/ 8 months	Neurological degradation, tetraparesis.	Diffuse demyelination.	3.2	2.4	600	43	increase of pyruvate (blood) and glycine (urine)
P4	BOLA3	Male	7 months/ 8 months	Hypotonia, pyramidal syndrome.	Diffuse demyelination. Leigh syndrome.	5.4	3.9	922	16	
P5	NFU1	Male	3 months/ 4 months	Hypotonia, seizures, apnea.	White matter abnormalities. MRS: no lactate peak.	3.6	<1.8	374	15 (RV 4-11)	increase of glycine and fumaric acid (urine)
P6	NFU1	Female	3.5 months/ 4 months	PMD, hypotonia, seizures, acute heart failure, PAHT.	No abnormalities.	>2	ND	>300	ND	increase of 2-amino-adipate (plasma) increase of 2 keto-adipate (urine)
P7	NFU1	Male	7 months/ <3 years	Progressive neurological degradation. PMD, spasticity and epileptoid trepidation.	Necrotizing leukodystrophy. MRS: lactate peak.	>2	ND	ND	ND	
P8	NFU1	Female	5 months/ 3 years	PMR, PAHT, hypotonia, tetraparesis, seizures [24].	Kystic leukoencephalopathy. MRS: no lactate peak.	2.7 (acute period)	<1.8	1178	20	
P9	IBA57	Male	8 months/ 11 months	PMR, hypotonia, episodes of general hypertonia, pyramidal syndrome.	Periventricular leukodystrophy.	7.7	1.9	<300	<16	
P10	IBA57	Female	3 weeks/ 2.5 months	Hypotonia, myoclonus, apnea.	Myelination delay.	2	5.5	ND	ND	
P11	ISCA2	Male	ND/Still alive at 12 years	Non-progressive spastic paraplegia.	White matter atrophy at 12 years.	<2	<1.8	ND	ND	
P12	ISCA2	Male	Neonatal/12 days	Hypotonia, apnea.	Leukodystrophy.	>2	5	ND	ND	
P13	FDX1L	Female	5 years/ Still alive at 18 years	Acute episodes of rhabdomyolysis, muscular weakness.	Normal	Up to 23 in acute episodes	3	<300	<16	increase of serum creatine kinase in acute episodes

Table 2: Clinical and biochemical features of the thirteen patients.

CSF, Cerebrospinal fluid; MRS, magnetic resonance spectroscopy; PAHT, pulmonary arterial hypertension; PMD, psychomotor delay; PMR, psychomotor regression; RV: reference values; ND, not determined.

Patient	Gene	Enzymatic activities (nmol/min/mg protein)			Relative mt-aco and respiratory chain complex activities					Glutathione (pmol/mg protein) (RV:7-21)
		PDHc (RV:1.20-3.10)	α -KGDHc (RV:3.7-7.1)	Citrate synthase (CS) (RV:61-93)	mt-aco/CS (RV:0.40-0.59)	Complex I/CS (RV:0.15-0.41)	Complex II/CS (RV:0.20-0.32)	Complex III/CS (RV:0.70-1.40)	Complex IV/CS (RV:1.00-1.50)	
P1	LIPT1	0.08	0.5	59	0.20	0.04	0.24	1.18	1.61	18
P2	LIPT2	0.28	0.5	58	0.33	0.07	0.30	2.28	1.05	11
P3	BOLA3	0.05	1.6	70	0.18	<0.01	0.08	0.82	0.93	13
P4		0.07	0.7	84	0.34	0.03	0.09	1.18	0.78	25
P5	NFU1	0.15	0.1	64	0.35	0.01	0.12	2.66	0.90	20
P6		0.04	0.6	64	0.13	0.03	0.02	1.69	0.85	11
P7		0.56	11.5	120	0.24	0.02	0.34	1.47	1.45	14
P8		0.35	<0.1	81	0.30	<0.01	0.06	0.86	1.38	9
P9	IBA57	0.39	2.3	81	0.44	0.04	0.15	1.48	1.0	17
P10		0.54	1.1	105	0.40	0.04	0.07	0.9	1.3	19
P11	ISCA2	0.95	2.8	66	0.33	0.05	0.21	2.67	2.03	20
P12		0.09	0.3	101	0.20	0.04	0.15	4.18	1.38	12
P13	FDX1L	0.95	8.4	89	0.46	0.09	0.33	1.48	1.43	19

Table 3: PDHc, α -KGDHc, mt-aco, respiratory chain complex activities and cellular glutathione concentration.

All the analyses were performed on cultured fibroblasts from the patients. Citrate synthase activity served as a reference for mitochondrial content in tested samples. mt-aco and RC complex activities were expressed relative to citrate synthase activity. Reduced glutathione measurement was determined in total lysates of fibroblasts. Gray shading indicates results in the normal value range. Other results were abnormal compared with reference values. CS, citrate synthase; RV, reference values.

168

169 *b) Biochemical results*

170 *Mitochondrial lipoylated proteins*

171 PDHc activity was decreased in fibroblasts of all patients (Table 3). It extended from 0.04 to
172 0.95 nmol/min/mg of protein, corresponding to 3% to 79% of the lowest reference value
173 (reference values: 1.20-3.10 nmol/min/mg of protein). α -KGDHc activity was decreased for all
174 patients except for one NFU1 patient (P7) and the FDX1L patient (P13). PDHc and α -KGDHc
175 activities were only moderately decreased in one ISCA2 patient (P11). We measured the level
176 of lipoylation of the E2 subunit of PDHc and α -KGDHc with an antibody against LA (Figure
177 2A). We found a severely altered lipoylation in all patients except for one NFU1 patient (P7),
178 one ISCA2 patient (P11) and the FDX1L patient (P13), with the E2 subunit of PDHc detectable
179 for all patients. This alteration of protein lipoylation is in accordance with decreased enzymatic
180 activity (Table 3).

181

182 *Mitochondrial Fe-S proteins*

183 We focused on two [4Fe-4S] proteins, LIAS and mt-aco, and two [2Fe-2S] proteins,
184 ferrochelatase and Rieske protein. LIAS activity is directly dependent on the assembly of its
185 [4Fe-4S] cluster, which is essential for incorporation of sulfur residues into octanoic acid. LIAS
186 protein was detectable for all patients and controls (Figure 2B). LIAS activity, which was
187 indirectly measured by the level of lipoylation of target proteins, was decreased in most
188 patients with defects in [Fe-S] biogenesis (Figure 2).

189 Then, we looked more closely at mt-aco, the stress-sensitive [4Fe-4S] cluster of which is also
190 crucial for its enzymatic activity and stability [28, 32]. Its activity was normalized to citrate
191 synthase activity to avoid bias due to between-sample variability in mitochondrial content
192 (Table 3). While mt-aco protein level was little affected (Figure 3), its activity was decreased
193 for all patients except for both IBA57 patients (P9 and P10) and the FDX1L patient (P13)
194 (Table 3). Thus, low mt-aco activity is a consequence of the alteration of its [4Fe-4S] cluster.

Given that glutathione (GSH) production is typically increased by severe oxidative stress [33], we measured total reduced GSH in cell lysates of fibroblasts, but found no significant variation between control and patient cells (Table 3).

Ferrochelatase assembles a [2Fe-2S] cluster, which is crucial for its stability and presents some sensitivity to oxidative stress [32, 34]. We found that ferrochelatase level, and consequently the presence of the cluster, was moderately reduced for patients 1, 9 and 11 only (Figure 4A).

OXPHOS analysis

Several subunits of RC complexes I, II and III carry [Fe-S] clusters that are essential to their activities. We looked first at the activity of RC complexes relative to citrate synthase activity (Table 3) and then at two [Fe-S] proteins of the RC complex, SDHB (complex II) (Figure 4C) and Rieske protein (complex III) (Figure 4B). Finally, we checked the integrity of RC complexes containing [Fe-S] clusters when activities were affected by BN-PAGE using antibodies against subunits that do not coordinate an [Fe-S] cluster (Figure 5).

Complex I activity was decreased in all patients (Table 1) and its assembly was also clearly affected in all of them except three patients, P1 (LIPT1 patient), P11 (ISCA2 patient) and P13 (FDX1L patient) (Figure 5). Complex II activity and integrity were normal in LIPT1 (P1) and LIPT2 (P2) patients, in one NFU1 patient (P7) and in the FDX1L patient (P13) (Table 1 and Figure 5). For these four patients, the amount of SDHB subunit was moderately decreased or normal (Figure 4C). Concerning the other patients, complex II activity and assembly were compromised for eight patients (the two BOLA3 patients, P3 and P4; three of the four NFU1 patients, P5, P6, P8; the two IBA57 patients, P9 and P10; one of the two ISCA2 patients, P12) while one ISCA2 patient (P11) showed low normal activity of complex II and its assembly seemed to be only moderately affected. For these nine patients, SDHB was undetectable (Figure 4C). So, complex II activity and/or integrity seems to be affected by defects in NFU1, BOLA3, IBA57 and ISCA2, proteins involved in the late step of mitochondrial [Fe-S] protein

maturation. Conversely, complex III activity was normal in all patients, as was the amount of Rieske protein (Figure 4B). Complex IV activity was moderately affected by defects in BOLA3 or NFU1 (P3 to P6).

The biochemical profile of patient P7 with a mutation in *NFU1* differed from that of other NFU1 patients. First, lipoylation of PDH-E2 and α -KGDH-E2 and α -KGDHc activity were normal, while PDHc and mt-aco activities were clearly affected (Figure 3 and Table 3). RC complex analysis showed only an effect on the activity and integrity of complex I (Table 3 and Figure 5). These results contrasted with the findings concerning other NFU1 patients (P5, P6 and P8) and underscored the various biochemical profiles in the same deficiency despite similar severe clinical features and a fatal outcome for all the NFU1 patients.

NFU1 and IBA57 protein level

Finally, we looked at the level of NFU1 and IBA57 proteins in the fibroblasts of patients presenting a mutation in one protein involved in the late steps of mitochondrial [Fe-S] cluster maturation (P3 to P12)(Figure 6). As expected, NFU1 protein was undetectable for the P5 NFU1 patient, who carries one intragenic deletion and one point deletion. NFU1 protein level was decreased for the two other NFU1 patients (P6 and P8) presenting a point mutation in their *NFU1* gene and, more surprisingly, for both BOLA3 patients (P3 and P4). NFU1 level was not changed for the last NFU1 patient (P7) or for IBA57 (P9 and P10) and ISCA2 (P11 and P12) patients. IBA57 protein was seriously decreased in all patients presenting a mutation in a gene encoding a protein involved in the late steps of mitochondrial [Fe-S] cluster maturation (P3 to P12).

3. DISCUSSION

We studied 13 patients with a mutation in a gene encoding a mitochondrial protein involved either in LA biosynthesis (*LIPT1* and *LIPT2*) or in [Fe-S] cluster biogenesis (*BOLA3*, *NFU1*,

IBA57, *ISCA2* and *FDX1L*). Among these patients, ten are described for the first time in this manuscript with mutations in *BOLA3* (P3 and P4), *NFU1* (P5, P6, and P7), *IBA57* (P9 and P10), *ISCA2* (P11 and P12), and the second case with a mutation in *FDX1L* (P13). Deficiencies in those proteins are known to cause diseases with severe encephalopathy due to defects in mitochondrial OXPHOS and keto-dehydrogenases (PDHc and α -KGDHc) [23]. Conversely, *FDX1L* deficiency leads to a muscular phenotype with normal neurological development [35]. In fact, PDHc and α -KGDHc are both active only when bound to LA, whose synthesis involves LIAS, a [4Fe-4S] cluster-containing protein. As a consequence, LA synthesis and keto-dehydrogenases activity are dependent on the mitochondrial [Fe-S] cluster biogenesis pathway. We performed biochemical analysis to assess the precise impacts of each mutation on both lipoylated proteins, but also on key mitochondrial [Fe-S] proteins (several subunits of respiratory chain complexes, ferrochelatase and mt-aco). As the analyses were carried out in the same conditions and tissue, i.e. skin fibroblast cultures, for all the patients, biochemical profiles can be compared to each other.

Fibroblasts from LIPT1 and LIPT2 patients

Both patients present similar brain imaging and clinical features with early-onset encephalopathy [19–21]. Up to now, four LIPT1 patients have been described: P1 of our study [19] and three others [18, 36], and three LIPT2 patients have been reported one of whom was included in our study (P2, corresponding to Patient 1 described by Habarou et al.) [20, 21]. P1 and P2 presented a decrease in PDHc and α -KGDHc activities due to a defect of lipoylation of the E2 subunits (Table 3, Figure 2). Mt-aco is well known to be highly sensitive to cellular oxidative stress, which degrades its [4Fe-4S] cluster to a [3Fe-4S] cluster, and, consequently, decreases its enzymatic activity [37]. We observed an unexpected decrease in mt-aco activity for both subjects (Table 3), despite a normal amount of the protein (Figure 3), indicating a probable increase in oxidative stress in the fibroblasts of these patients. On the other hand, the [2Fe-2S] cluster of ferrochelatase is not necessary for catalytic activity, but is required for

protein stability [34]. No strong variation in the level of ferrochelatase between patients and controls was observed (Figure 4A). The hypothesis of increased oxidative stress in patients with lipoylation defects has previously been suggested to explain their clinical pulmonary hypertension [18]. There was no significant variation in the level of total reduced glutathione (GSH) between patient and control fibroblasts (Table 3). While the amounts of the oxidized form of glutathione (GSSG) were under the limit of detection of our assay, variations in the ratio between GSH and GSSG cannot be excluded. Surprisingly, complex I was also affected in both patients (Table 3, Figure 5). These results confirm those obtained by polarographic study in both patients which showed low consumption of oxygen with pyruvate and malate [19, 21]. To our knowledge, none of the subunits of this complex are lipoylated. However, a decrease of complex I following down-regulation of acyl-carrier protein, which is involved in octanoic acid synthesis, has been observed [38]. Moreover, LA supplementation is known to improve mitochondrial function [39], probably by increasing the efficiency of RNase P processing and, consequently, tRNA^{Pro} processing, which is essential for mitochondrial protein expression [40, 41]. Lastly, the decreased activity and stability of complex I could also be a consequence of oxidative stress in these patients. Taken together, our results suggest that moderate oxidative stress may be present in the fibroblasts of these patients and leads to dysfunction of [Fe-S] proteins, which are highly sensitive to oxidative stress, like mt-aconitase and complex I, but has no effect on other [Fe-S] proteins that are less sensitive to oxidative stress, such as ferrochelatase. Clearly, LA deficiency induces OXPHOS dysfunction and more studies are needed to elucidate the molecular mechanism.

Fibroblasts from the FDX1L patient

Here we report the second case of ferredoxin deficiency. Both patients (P13 and [35]) carry the same homozygous mutation c.1A>T and present similar clinical features, with late onset of metabolic myopathy, recurrent episodes of rhabdomyolysis, hyperlactacidemia and muscular weakness. Neurological development is normal. FDX1L is clearly involved in the first steps of

mitochondrial [Fe-S] cluster biogenesis, steps that are necessary for the maturation of all the [Fe-S] clusters independently of their subcellular localization. The FDX1L patient (P13) presented with mild biochemical abnormalities (Table 3, Figure 2) and a moderate decrease in SDHB protein level (Figure 4C). Spiegel et al. observed an SDHA defect in fibroblasts and muscle of their patient with stronger OXPHOS deficiencies in muscle, with lower activity of complex I and II in muscle than in fibroblasts [35]. Similar observations were reported previously with patients with *FXN* and *ISD11* defects [44, 45], patients with motor deficiency as the major clinical feature, as observed in FDX1L patients. Muscle biopsy was not available for our FDX1L patient and we could not check whether deficiencies, particularly lipoylation of proteins, were also stronger in muscle than in fibroblasts. In conclusion, for FDX1L patients it would be appropriate to look at the deficiencies not only in fibroblasts but also in muscle to obtain a more significant biochemical signature.

Patients with defects in the late steps of the mitochondrial ISC machinery fibroblasts

We looked at 10 patients carrying mutations in four different proteins involved in the delivery of the [Fe-S] cluster to mitochondrial target proteins, namely NFU1, BOLA3, IBA57 and ISCA2. As with other patients previously described, they presented encephalopathy with early-onset and a fatal outcome for nine of them. One ISCA2 patient (P11) was more moderately affected and still alive at 12 years (Table 2). In the current model for eukaryotic cells [10, 41, 46], NFU1 and BOLA3 serve for the maturation of some [4Fe-4S]-containing proteins such as LIAS, mt-aco and SDHB. Interestingly, all the patients we have studied are affected in complex I activity (Table 3) and integrity (except one ISCA2 patient: P11) (Figure 5). As for the LIPT1 and LIPT2 patients, the amount of ferrochelatase (Figure 4A) and the GSH concentration (Table 3) are not significantly affected. Thus, for ISCA2, IBA57, and BOLA3, these results are in accordance with the model proposed by Lill's group, which positions these proteins downstream of IND1 in the maturation of complex I [10]. But, according to this model, NFU1 is only dedicated to the

maturation of SDHB and LIAS. The effects we observed in NFU1 patients indicate that NFU1 is involved in complex I maturation, as proposed by Cameron et al. [12].

Interestingly, all BOLA3, NFU1 and ISCA2 patients have a decreased amount of IBA57 (Figure 6). It was shown previously that, in yeast, Iba57p physically interacts with Isa1p and Isa2p [47]. Due to the high homology of sequences between yeast and mammals, it is probable that IBA57 interacts with ISCA2 in mammals too, an interaction that would explain the effects of mutation in *ISCA2* on IBA57 protein level. The effects of mutations in *BOLA3* on IBA57 protein level indicate a link (cellular or physical interaction) between these two proteins. Concerning [2Fe-2S] protein maturation, our results suggest that mutations in these four genes would not affect the maturation of the [2Fe-2S]-containing proteins (Rieske protein, ferrochelatase). This is in agreement with the model for eukaryotic cells, which does not involve Nfu1 in the delivery of [2Fe-2S] clusters [9, 48–50]. It seems that BOLA3, ISCA2 and IBA57 are not involved in this function either.

NFU1 and BOLA3 patients

Six BOLA3 patients [12, 16, 17] and more than 25 patients with *NFU1* mutations have been reported to date [12, 13, 24, 51, 52]. The clinical and biochemical phenotypes of these two deficiencies are quite similar, with early onset of severe encephalopathy, variable cardiomyopathy, PDHc and mitochondrial RC defects (Tables 2 and 3). Our study includes two new BOLA3 patients (P3 and P4) carrying the known homozygous mutation p.Arg46* [12]. The four NFU1 patients presented early-onset encephalopathy and died before the age of three years. Two of them had pulmonary hypertension (P6 and P8), as is usually observed in patients carrying the same mutation p.Gly208Cys [51]. Patient P5 carries two heterozygous deletions in the *NFU1* gene, leading to undetectable amounts of NFU1 protein in his fibroblasts and this can be considered as an almost complete NFU1 deficiency (Figure 6A). This patient presents severe RC and mt-aco dysfunctions, and keto-dehydrogenases deficiency, secondary to lipoylation defects, resulting in severe clinical encephalopathy. This

confirms that NFU1 is clearly involved in at least the maturation of [4Fe-4S] proteins such as LIAS, mt-aco and SDHB. In the case of patient P7, we observed a discrepancy between biochemical profiles (with unexpected normal α -KGDHc activity and protein lipoylation) and clinical features as previously reported for some patients [52]. But, our patient presents a point mutation (p.Phe60Cys) that does not affect NFU1 protein level. This mutation in the β 1 strand of the protein is predicted to be deleterious by *in silico* predicting softwares (SIFT, MutationTaster and PolyPhen-2). The recent NMR structure of NFU1 highlights the formation of a trimer of dimers of holo-NFU1 and the authors proposed that the β 1 strand might be involved in interaction with chaperones, which would help cluster transfer [49]. Thus, this mutation, which does not affect the stability of NFU1, might decrease the efficiency of cluster delivery to target proteins, which would explain the mild effects on biochemical profiles of the patient.

Both BOLA3 patients (P3 and P4) present low levels of NFU1 protein (Figure 6A). These results are in accordance with studies in yeast showing that Bol3 forms a complex with Nfu1, stabilizes it and is involved in [4Fe-4S] cluster delivery [41]. Thus, in these patients, the mutation in *BOLA3* affects both BOLA3 and NFU1 proteins. This explains the very severe clinical phenotype of all reported BOLA3 patients, with a cumulative effect of lack of both BOLA3 and NFU1 [17].

IBA57 and ISCA2 patients

In our study, both IBA57 patients carry two heterozygous missense mutations, have no detectable IBA57 protein (Figure 6B), and present a severe clinical phenotype with early onset and a fatal outcome. Of the 18 IBA57 patients already described, some suffer from severe diseases (n=4), while others, all members of the same family, present chronic spastic paraplegia [14, 53, 54]. Clinical presentations of our two ISCA2 patients (P11 and P12), who both present novel missense mutations, are quite different, with neonatal onset and early death for patient P12, whereas patient P11, at 12 years of age, suffers from non-progressive

spastic paraplegia (Table 2). Only 7 cases, from six unrelated families, of patients with *ISCA2* mutations have been described before [15, 55]. They presented spasticity and psychomotor delay, a clinical presentation quite similar to that observed for P11 (Table 1). One *ISCA2* patient (P12) showed marked decreases in the enzymatic activities of PDHc, α -KGDHc and RC complexes and in SDHB protein level, while the other one (P11) showed more limited effects in accordance with their respective clinical phenotypes. The molecular relation between *IBA57* and *ISCA* proteins is still debated. Initially, it was considered that *IBA57* acts with *ISCA1* and *ISCA2* in the maturation of mitochondrial [4Fe-4S] proteins [11]. Then, it was proposed that *ISCA* proteins might form heterocomplexes without the help of *IBA57* [56]. Finally, proteomic studies proposed that *ISCA2* interacts with *ISCA1*, but also with *GLRX5* and *IBA57* [57]. As for patients with *NFU1* and *BOLA3* disorders, *IBA57* patients have impaired lipoylation (Figure 2) with decreased activities of PDHc and α -KGDHc (Table 3). RC complex defects were reported in most of the previous published cases of *IBA57* deficiencies [14, 53, 58] and dysfunction of complexes I and II was observed in our two *IBA57* patients. As for *BOLA3* and *NFU1* disorders, they are likely due to abnormal assembly of the [Fe-S] clusters held by subunits of these complexes. But, as previously observed in *IBA57*-depleted HeLa cells [11], mt-*aco* is not affected in these patients (Table 3 and Figure 3). Thus, *IBA57* protein may not be essential for [4Fe-4S] transfer to mt-*aco* protein, even if one *IBA57* patient, carrying mutations compared with our patients, was recently reported with decreased mt-*aco* protein in fibroblasts [58].

In conclusion, we report two patients with mutations in either *LIPT1* or *LIPT2*, one new patient with a mutation in *FDX1L*, a protein of the early steps of mitochondrial [Fe-S] cluster biogenesis, and ten patients, including nine new patients with deficiency in proteins (*IBA57*, *BOLA3*, *NFU1*, *ISCA2*) involved in the late steps of this same pathway. Clinical presentation was extremely severe with early fatal outcome for eight of them. Ferredoxin deficiency leads to

severe myopathy with moderate mitochondrial protein dysfunction in fibroblasts. In all other deficiencies, PDHc and α -KGDHc activities and lipoylation of these enzymes were affected. OXPHOS was also affected. Interestingly, in all the patients, the integrity of complex I was affected and its activity was greatly reduced even in LIPT1 and LIPT2 patients. Similar observations were made for mt-aco activity. These decreases in activity of several mitochondrial [Fe-S] proteins in patients with mutations in either LIPT1 or LIPT2 may reflect an increase in oxidative stress, which degrades stress-sensitive [Fe-S] clusters. We confirm that ISCA2, NFU1, BOLA3 and IBA57 are involved only in the maturation of mitochondrial [4Fe-4S]-containing proteins and not [2Fe-2S]-containing proteins. Moreover, IBA57 does not seem to be involved in mt-aco maturation, as previously proposed using HeLa cells as model. BOLA3, NFU1 and ISCA2 are required for IBA57 stability and BOLA3 is required for NFU1 stability. These findings, summarized in a proposed scheme (Figure 7), indicate the formation of multiple heterodimers between these four proteins, as already proposed for IBA57 and ISCA2 [11, 56], and BOLA3 and NFU1 [41]. We cannot exclude that they form a larger complex including all four proteins.

Acknowledgements

The authors would like to thank Dr. Annabelle Chaussenot (Inherited Errors of Metabolism Reference Center, Hôpital de l'Archet, Nice, France) for providing clinical data on her patient. We thank Prof. R. Lill (Institut für Zytobiologie und Zytopathologie, Philipps-Universität Marburg, Marburg, Germany), Prof. T. Rouault (Molecular Medicine Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, USA), Dr H. Puccio (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France) and Dr. R. B. Franklin (University of Maryland, Baltimore, USA) for sharing IBA57, NFU1, ferrochelatase and mt-aconitase antibodies, respectively.

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Figure 1: Simplified scheme of human mitochondrial [Fe-S] protein biogenesis [5, 9, 23].

Starting from iron atoms and inorganic sulfur provided by cysteine desulfuration, a [2Fe-2S] cluster is built on the scaffold protein ISCU, with the help of NFS1 and ISD11. Ferredoxin serves as electron donor. In the second step, chaperone proteins transfer a [2Fe-2S] cluster from ISCU to GLRX5. The cluster can then be exported to the cytosol to integrate cytosolic Fe-S assembly (CIA) machinery or transferred to mitochondrial apoprotein, which needs one or several [2Fe-2S] or [4Fe-4S] clusters. For this, ISC targeting factors (ISCA1, ISCA2, IBA57, NFU1, BOLA3) are essential, but the exact role of each one is not yet clearly defined. Among [4Fe-4S] target proteins, lipoic acid synthase (LIAS) is a key enzyme in lipoic acid synthesis, and is covalently linked to mitochondrial ketoacid dehydrogenases such as PDHc and α KGDHc. Mutated proteins (green) found in the patients included in this study. Mitochondrial [4Fe-4S]- and [2Fe-2S]-containing proteins (salmon and blue, respectively) and lipoylated proteins (yellow) studied in this report.

Figure 2: Protein level of lipoylated proteins and lipoic acid synthase in fibroblasts.

Fibroblasts of the 13 patients and 2 controls (C1 and C2) were analyzed by immunoblot after mitochondrial enrichment. Antibodies against lipoic acid (detection of lipoic acid bound to E2 subunits of PDHc and α -KGDHc), E2 subunit of PDHc (A) and LIAS (B) were used.

Figure 3: Protein level of mt-aconitase in fibroblasts. Fibroblasts of the 13 patients and 2 controls (C1 and C2) were analyzed after mitochondrial enrichment by immunoblot with antibody against mt-aconitase. Prohibitin is shown as the loading control of mitochondrial content.

Figure 4: Protein level of mitochondrial [Fe-S] proteins in fibroblasts. Fibroblasts of the 13 patients and 2 controls (C1 and C2) were analyzed after mitochondrial enrichment using antibodies against the [2Fe-2S]-containing ferredoxin (panel A) and Rieske protein (panel B). Prohibitin served as the loading control. Panel C shows the level of the [4Fe-4S] protein SDHB, subunit of RC complex II. Antibody against SDHA, another subunit of respiratory chain complex II, without [Fe-S] clusters, is shown as the loading control of mitochondrial content.

Figure 5: Blue-native PAGE analysis of respiratory chain complexes I and II in fibroblasts. BN-PAGE analysis of mitochondria-enriched fibroblasts of the 13 patients and three controls (C1, C2 and C3) was performed using antibodies against respiratory chain complex subunits that do not coordinate an [Fe-S] cluster (CI: GRIM19, CII: SDHA). Complex III (subunit UQCRC2) served as the loading control, while the activity of complex III was not affected in all patients.

Figure 6: NFU1 and IBA57 protein levels in fibroblasts. Fibroblasts of the 10 patients (patients with mutations in *LIPT1*, *LIPT2* and *FDX1L* were not included) and two controls (C1 and C2) were analyzed after mitochondrial enrichment using antibodies against NFU1 (A) and IBA57 (B). Prohibitin was used as the loading control of mitochondrial content. Unfortunately, the tested antibodies against BOLA3 and ISCA2 were not suitable for immunoblotting.

Figure 7: Proposed scheme of human mitochondrial [Fe-S] protein biogenesis. The scheme summarizes the results obtained in our study. Arrows between BOLA3, NFU1, ISCA2, IBA57 symbolize the stabilizing effects we observed between these four proteins.

Figure 1

A

B

Figure 2

Figure 3

Figure 4

Figure 5

A

B

Figure 6

Figure 7