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ORIGINAL ARTICLE

Neonatal onset of mitochondrial disorders in 129 patients: clinical and laboratory characteristics and a new approach to diagnosis

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Abstract
Introduction Mitochondrial disorders (MD) may manifest in neonates, but early diagnosis is difficult. In this study, clinical and laboratory data were analyzed in 129 patients with neonatal onset of MD to identify any association between specific mitochondrial diseases and their symptoms with the aim of optimizing diagnosis.
Materials and methods Retrospective clinical and laboratory data were evaluated in 461 patients (331 families) with confirmed MD.
Results The neonatal onset of MD was reported in 28% of the patients. Prematurity, intrauterine growth retardation and hypotonia necessitating ventilatory support were present in one-third, cardiomyopathy in 40%, neonatal seizures in 16%, Leigh syndrome in 15%, and elevated lactate level in 87%. Hyperammonemia was observed in 22 out of 52 neonates. Complex I deficiency was identified in 15, complex III in one, complex IV in 23, complex V in 31, combined deficiency of several complexes in 53, and PDH complex deficiency was identified in six patients. Molecular diagnosis was confirmed in 49 cases, including a newborn with a 9134A>G mutation in the *MTATP6* gene, which has not been described previously.
Conclusion The most significant finding is the high incidence of neonatal cardiomyopathy and hyperammonemia. Based on our experience, we propose a diagnostic flowchart applicable to critically ill neonates suspicious for MD. This tool will allow for the use of direct molecular genetic analyses without the need for muscle biopsies in neonates with Alpers, Barth, MILS and Pearson syndromes, *SCO1*, *SCO2, TMEM70, ATP5E, SUCLG1* gene mutations and PDH complex deficiency.

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Abbreviations

ATP5E	Epsilon subunit of F_1F_O-ATP synthase
BCAA	Branched-chain amino acids
CMP	Cardiomyopathy
IEM	Inborn errors of metabolism
IUGR	Intrauterine growth retardation
LHON	Leber hereditary optic neuropathy
L/P	Lactate/pyruvate ratio
MD	Mitochondrial disorders
MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes

MERRF	Myoclonic epilepsy with ragged-red fibers
MILS	Maternally inherited Leigh syndrome
MMA	Methylmalonic acid
MNGIE	Mitochondrial neuro-gastrointestinal encephalomyopathy
MTATP6	Mitochondrially encoded ATP synthase 6 subunit
mtDNA	Mitochondrial DNA
MTND1	Mitochondrially encoded NADH dehydrogenase (complex I) 1 subunit
nDNA	Nuclear DNA
NARP	Neuropathy, ataxia and retinitis pigmentosa
NDUFA1, NDUFS1, NDUFS4, NDUFS6	Complex I subunits
NDUFAF2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2
OXPHOS	Oxidative phosphorylation system
PDHc	Pyruvate dehydrogenase complex
POLG	Polymerase gamma
SCO1	Synthesis of cytochrome *c* oxidase 1, cytochrome *c* oxidase assembly protein
SCO2	Synthesis of cytochrome *c* oxidase 2, cytochrome *c* oxidase assembly protein
SDH	Succinate dehydrogenase
SUCLA2	Succinate-CoA ligase, ADP-forming, beta subunit
SUCLG1	Succinate-CoA ligase, alpha subunit
SURF1	Surfeit 1, cytochrome *c* oxidase assembly protein
TMEM70	Transmembrane protein 70, F_1F_O-ATP synthase biogenesis
3-MGA	3-methylglutaconic acid

Introduction

Mitochondrial disorders (MD) of energy metabolism represent a heterogeneous group of diseases presenting at any age, including the early neonatal period, and they typically present with a wide variety of clinical symptoms (Munnich and Rustin 2001). More than 90% of cellular ATP is produced by the mitochondrial oxidative phosphorylation system (OXPHOS) composed of four respiratory chain complexes and F_1F_O-ATP synthase. At birth, an adequate capacity of the mitochondrial energetic metabolism is of utmost importance for successful adaptation to extra-uterine life in all neonates. The recruitment of OXPHOS in the neonatal tissues reflects changes in hormonal status and also the shift from glucose alone to glucose and fat as the major energy sources after birth (Schagger et al. 1995). The cellular competence for ATP provision relies on the adequate biosynthesis of OXPHOS complexes. Consequently, it is not surprising that neonatal manifestations of primary MD are quite common (Skladal et al. 2003, von Kleist-Retzow et al. 2003, Garcia-Cazorla et al. 2005, Debray et al. 2007, Gibson et al. 2008, Honzik et al. 2010). However, it can be difficult, especially in critically ill neonates, to properly distinguish the primary disorders of energy provision from secondary mitochondrial disturbances due to delayed mitochondrial biogenesis in very premature neonates (Sperl et al. 1992, Honzik et al. 2008, Pejznochova et al. 2010) or the increased degradation of mitochondrial proteins due to sepsis or hypoxia (Vary and Hazen 1999, Azevedo 2010).

Only a few studies investigating larger groups of neonates with MD are available: Gibson et al. described the neonatal manifestations in 32 out of 107 patients with MD (Gibson et al. 2008); Debray et al. observed neonatal-onset lactic acidosis in 7 out of 73 patients (Debray et al. 2007), and Skladal et al. reported 75 MD patients, of whom 31 demonstrated a neonatal-onset form, and of these, 11 had „lethal infantile mitochondrial diseases“ (Skladal et al. 2003). A study by Garcia-Cazorla et al. characterized 57 neonates with tachypnea, refusal to eat, hypotonia, cardiac insufficiency (30%) and lactic acidosis (77%) (Garcia-Cazorla et al. 2005). In addition, prenatal manifestations of mitochondrial respiratory chain deficiencies were noted including IUGR, multiple congenital malformations and prematurity (in 68 of 300 patients) (von Kleist-Retzow et al. 2003). The prognosis for symptomatic newborns with MD is often unfavorable. We recently reported on the poor outcomes of 25 neonates with mitochondrial encephalocardiomyopathy due to a mutation in the *TMEM70* gene (Honzik et al. 2010).

The aims of the present study were to analyze the clinical and laboratory characteristics of neonatal onset MD in the largest cohort of patients published to date to identify a possible association between clinical findings and specific mitochondrial diseases and to optimize the diagnostic approaches applicable to neonates with a suspicion of MD, allowing for the use of direct mutation analysis without need for biochemical studies involving muscle biopsy.

Material and methods

We reviewed the clinical and laboratory records of 461 patients from 331 families with respiratory chain, F_1F_O-ATP synthase and pyruvate dehydrogenase complex (PDHc) disorders diagnosed on the enzymatic and/or molecular-genetic level in two centers in Prague (401 patients) and Salzburg (60 patients) between 1992 and 2010 (Table 1). All the patients met published diagnostic criteria for MD (Bernier et al. 2002, Wolf and Smeitink 2002, Morava et al. 2006), but only 129 patients with apparent neonatal onset (<28 days of life) of MD (28% out of 461 patients) were included in this study (Table 1). There were 72 males and 57 females (male/female ratio 1.26:1).

In 123 patients, we found a deficiency of one or more respiratory chain complexes or of F_1F_O-ATP synthase; in the remaining six patients, we diagnosed PDHc deficiency. Respiratory chain complex I deficiency was identified in 15 patients, complex III deficiency was identified in one, complex IV deficiency was identified in 23, complex V (F_1F_O-ATP synthase) deficiency was identified in 31, and combined deficiency of more respiratory chain complexes was identified in 53 of the patients. Thus far, the diagnosis on a molecular-genetic level was established in 49 neonates; 36 had nuclear DNA (nDNA) encoded diseases, and 13 had mutations or large rearrangements in mitochondrial DNA (mtDNA) (Table 1). Mutations in mtDNA and/or 1-15 nDNA-encoded genes were excluded in the remaining 80 patients.

Apart from standard laboratory investigations, we performed mutation testing by PCR-RFLP and direct sequencing of selected genes in the nuclear and mitochondrial DNA.

In skeletal muscle biopsies and/or cultivated fibroblasts, activities of respiratory chain complexes (Rustin et al. 1994, Mayr et al. 2004) and PDHc (Constantin-Teodosiu et al. 1991, Strassburg et al. 2006) were measured and steady-state levels of OXPHOS complexes and PDHc were analyzed by blue-native and SDS-electrophoresis (Schagger and von Jagow 1991, Klement et al. 1995) resp. followed by western blot.

Results

In the group of 129 patients with neonatal onset of MD (Table 1), 66 patients (51%) manifested MD early after birth or during the first two days of life. The other 63 patients manifested between the 3rd and 27th day of life (median of 21 days).

Early neonatal manifestation (from birth until two days of life) was recognized in all patients with *TMEM70* mutations, in both patients with *DLD* mutations and in one patient with a mutation in the *SCO2* gene and Pearson syndrome. From the group of OXPHOS-deficient patients without a known genetic basis, we observed early neonatal presentation in 30 patients with combined complex deficiency, in eight neonates with complex IV and in two neonates with complex I deficiency.

Clinical symptoms in the neonatal period

Clinical symptoms of the 129 neonates according to diagnosis are summarized in Table 2. To provide a complete overview of phenotypes, we included and updated data from several of our patients reported on earlier (Cerna et al. 2001, Capkova et al. 2002, Tesarova et al. 2002, Vesela et al. 2004, Bohm et al. 2006, Stiburek et al. 2009, Honzik et al. 2010, Mayr et al. 2010). The mean birth weight was 2651±746 g (range 1000–4630 g), and the mean gestational age was 37±3 weeks (range 28–42 weeks). Premature delivery (<37 weeks of gestation) was present in 30%, and IUGR was present in 35% of children. Full resuscitation or ventilatory support was necessary in 30% of the patients after delivery. Overall, hypotonia and poor feeding were observed in 90% and apneic spells in 69% of the neonates.

Congenital malformations were noted in 25 neonates: nine hypospadias (seven boys with *TMEM70* mutations and two boys with isolated complex IV deficiency), five with agenesis or hypogenesis of the corpus callosum, four pontocerebellar hypoplasias, four congenital stridors, two hydronephrosis, two arthrogryphosis and single cases of syndactyly, pes equinovarus, heart defect and Dandy-Walker syndrome.

Hypertrophic cardiomyopathy was diagnosed in 40% of the neonates, and in 80% of them, it was recognized within the first days of life. In two newborns with complex IV deficiency, dilated cardiomyopathy was found. The frequency of cardiomyopathy was not significantly different between groups of patients with respiratory chain complex I, IV, V or combined deficiencies. Cardiomyopathy was not an isolated symptom but was always accompanied with hypotonia and/or with encephalopathy. Hypotonia was present in 90% of patients (Table 2), apneic spells representing central nervous system involvement was found in 69% and liver impairment in 35% of the neonates.

Leigh syndrome was documented in 15% of the neonates examined either by an early MRI or by autopsy. Neonatal seizures developed in 16% of our patients.

Metabolic analyses

The laboratory data are summarized in Table 3. Elevated blood lactate levels (>2.3 mmol/l) were documented in 87% of neonates and, in 63% of them, were accompanied by hyperalaninemia (>500 μmol/l) and increased urinary excretion of Krebs cycle intermediates (mainly 2-oxoglutaric aciduria, which was documented in half of the patients). Hepatopathy and elevated muscle creatine kinase activity (>6.6 μkat/l) were found in 35% and 20% of the neonates,

Table 1 Mitochondrial disorders with neonatal onset among 461 patients with mitochondrial disorders diagnosed in the Czech Republic and Austria between 1992 and 2010

Mitochondrial disorder	Patients n=461	Families n=331	Neonatal manifestation n=129 (%)
OXPHOS deficiency caused by mutations of mtDNA			
LHON syndrome	101	26	0
MELAS syndrome	57	31	1
Kearns-Sayre syndrome	22	22	0
MERRF syndrome	15	11	0
NARP/MILS syndrome	13	8	7 (54)
other mtDNA mutations	13	11	3 (23)
Pearson syndrome	2	2	2
OXPHOS deficiency caused by mutations of nuclear DNA			
TMEM70 gene	25	19	22 (88)
SURF1 gene	15	15	0
SCO2 gene	11	9	3 (27)
SCO1 gene	1	1	1
POLG gene	4	4	1
Barth syndrome (*TAZ* gene)	2	2	1
MNGIE syndrome (*TYMP* gene)	2	2	0
NDUFA1 gene	1	1	0
NDUFA12L gene	1	1	0
NDUFS1 gene	1	1	0
NDUFS4 gene	1	1	1
ATP5E gene	1	1	1
SUCLG1 gene	1	1	1
OXPHOS deficiency without known genetic basis			
Complex I deficiency	36	36	12 (33)
Complex III deficiency	1	1	1
Complex IV deficiency	28	25	19 (68)
Complex V deficiency	1	1	0
Combined deficiencies	90	85	47 (52)
Disorders of pyruvate metabolism			
PDHA1 gene	11	10	3 (27)
DLD gene	2	1	2
PDHB gene	1	1	0
PDHc deficiency	2	2	1

OXPHOS: oxidative phosphorylation system

mtDNA: mitochondrial DNA

LHON: Leber hereditary optic neuropathy

MELAS: Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes

KSS: Kearns-Sayre syndrome

MERRF: Myoclonic epilepsy with ragged-red fibers

NARP: Neuropathy, ataxia and retinitis pigmentosa; MILS: maternally inherited Leigh syndrome

TMEM70: transmembrane protein 70, F_1F_O-ATP synthase biogenesis

SURF1: surfeit 1, cytochrome *c* oxidase assembly protein

SCO1: synthesis of cytochrome *c* oxidase 1, cytochrome *c* oxidase assembly protein

SCO2: synthesis of cytochrome *c* oxidase 2, cytochrome *c* oxidase assembly protein

POLG: polymerase gamma (phenotype of Alpers syndrome)

MNGIE: Mitochondrial neurogastrointestinal encephalomyopathy

NDUFA1; *NDUFS1; NDUFS4*: complex I subunits

NDUFA12L: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2

ATP5E: epsilon subunit of F_1F_O-ATP synthase

SUCLG1: succinate-CoA ligase, alpha subunit

PDHA1: pyruvate dehydrogenase E1-alpha subunit

DLD: dihydrolipoamid dehydrogenase

PDHB: pyruvate dehydrogenase complex, E1-beta subunit

PDHc: pyruvate dehydrogenase complex

Table 2 Clinical symptoms in 129 newborns with neonatal onset of mitochondrial disorders

Mitochondrial disorder	Prematurity	IUGR	Apneic spells	Hypotonia	HCMP	Leigh syndrome	Seizures	Died <3 months	Reference
OXPHOS deficiency caused by mutations of mt DNA									
MILS syndrome (8993T>G; n=7)♣	1/7	4/7	4/7	7/7	2/7	1/1	0/7	0/7	Tesarova et al. 2002
MTND1 gene (3697G>A; n=2)	0/2	0/2	0/2	2/2	0/2	0/2	0/2	0/2	
Pearson syndrome (n=2)♦	0/2	0/2	1/2	2/2	0/2	n.d.	0/2	0/2	
MELAS (3243A>G; n=1)	0/1	1/1	1/1	1/1	1/1	0/1	0/1	0/1	
MTATP6 (9134A>G; n=1)	0/1	1/1	0/1	1/1	1/1	n.d.	0/1•	0/1	
OXPHOS deficiency caused by mutations of nuclear DNA§									
TMEM70 gene (n=22)#	16/22	15/22	19/21	22/22	18/21	0/12	0/21	10/22	Honzik et al. 2010
ATP5E gene (n=1)	0/1	1/1	1/1	1/1	0/1&	0/1	0/1	0/1	Mayr et al. 2010
SCO1 gene (n=1)*	0/1	1/1	0/1	1/1	1/1	0/1	0/1	0/1	Stiburek et al. 2009
SCO2 gene (n=3)	1/3	0/2	3/3	3/3	3/3	0/3	1/3	3/3	Vesela et al. 2004
NDUFS4 gene (n=1)	0/1	0/1	0/1	1/1	1/1	n.d.	0/1	0/1	
SUCLG1 gene (n=1)	0/1	1/1	0/1	1/1	0/1	n.d.	0/1	0/1	
Alpers syndrome (*POLG* gene, n=1)	1/1	0/1	1/1	1/1	0/1	0/1	0/1	0/1	
Barth syndrome (*TAZ* gene, n=1)	0/1	0/1	0/1	1/1	1/1	0/1	0/1	0/1	
OXPHOS deficiency without known genetic basis									
Complex I deficiency (n=12)	3/11	5/11	6/12	11/12	2/8	2/6	3/10	2/12	
Complex III deficiency (n=1)	0/1	0/1	0/1	0/1	n.d.	1/1	0/1	0/1	
Complex IV deficiency (n=19)	7/17	7/17	12/18	16/18	4/19♠	3/8	5/19	7/19	Bohm et al. 2006
Combined deficiencies (n=47)	9/47	8/47	36/47	39/47	14/46	2/19	10/47	11/47	
Disorders of pyruvate metabolism§									
PDHA1 gene (n=3)	0/3	0/2	1/3	1/2	0/2	0/2	0/2	0/3	
DLD gene (n=2)	0/2	0/2	2/2	2/2	0/2	0/2	0/2	0/2	Cerna et al. 2001
PDHc deficiency (n=1)	0/1	0/1	1/1	1/1	n.d.	n.d.	1/1	0/1	
Total	38/126	44/124	88/127	114/127	48/120	9/61	20/125	33/129	

n.d.- not determined, HCMP: hypertrophic cardiomyopathy; IUGR: intrauterine growth retardation; mtDNA: mitochondrial DNA; OXPHOS: oxidative phosphorylation system; MILS syndrome (maternally inherited Leigh syndrome) ♣ all patients with neonatal onset died early, on average at 5.8+2.6 months of age (range 3-10 months); ♦ for severe sideroblastic anemia, a red blood cell transfusion was administered in the neonatal period in both patients; • neonatal seizures were not observed, but hypsarrhythmia developed during infancy; # hypospadias and cryptorchidism were present in 7 out of 11 boys; & hypertrophic cardiomyopathy was documented at 4 years of age; * exitus was at the age of 5 months; ♠ in two other newborns, dilated CMP was documented; § genotypes of patients: *TMEM70*: c.[317-2A>G]+[317-2A>G], *ATP5E*: c.[35A>G]+[35A>G], *SCO1*: c.[394G>A]+[394G>A], *SCO2*: c.[1487_1488insC]+[1541G>A]; c.[1518delA]+[1541G>A]; c.[1280C>T]+[1541G>A], *NDUSF4*: c.[462delT]+[462delT], *SUCLG1*: novel mutation (publication under preparation), *POLG1*: c.[926G>A]+[1881G>A], *TAZ*: c.[280C>T], PDHA1: novel mutation (publication under preparation), *DLD*: c.[1081A>G]+[1123G>A]

respectively. Hypoglycemia (<2.5 mmol/l) was observed in 14 neonates. All 22 neonates with *TMEM70* mutations exhibited mild to moderate 3-methylglutaconic aciduria (18-460 mg/g of creatinine, controls <15), and 80% of them had hyperammonemia (100-520 μmol/l, controls <80) early after birth. Moderate methylmalonic aciduria (461 mg/g creatinine, controls <15) was found in the neonate with the *SUCLG1* mutation.

Histological and histochemical analyses (mostly from muscle biopsies) were performed in 30% of the neonates. Abnormal findings were documented in 60% of analyzed patients. Accumulation of SDH reaction product indicating mitochondrial proliferation was observed in majority of them.

Prognosis of neonates with mitochondrial disorders

Twenty-six percent of our patients died within the first three months of life, with a median age of 42 days; 60% of them died within the neonatal period. Ten out of 22 patients with *TMEM70* mutations died during the first three months of life (Table 2). All three patients with cytochrome *c* oxidase deficiency due to compound heterozygous mutations in the *SCO2* gene (which is involved in the assembly of complex IV) died within the first three months of life (Vesela et al. 2004). Similarly, the prognosis of the patients with MILS syndrome was also poor; all of them succumbed during infancy (range 3-10 months). In addition, none of our patients with Pearson and Alpers syndromes or with *SCO1*

Table 3 Laboratory data in 129 newborns with neonatal onset of mitochondrial disorders

Mitochondrial disorder	↑B-lactate (>2.3 mmol/l)	↑NH_3 (>80 μmol/l)	↑ALT, AST (>0.73; >1.21 μkat/l)	↑CK (>6.6 μkat/l)	↑U-Krebs cycle intermediates	↑S-alanine (>500 μmol/l)
OXPHOS deficiency caused by mutations of mtDNA						
MILS syndrome (8993T>G; n=7)	7/7	1/2	2/7	n.d.	2/2	2/2
MTND1 gene (3697G>A; n=2)	2/2	n.d.	0/2	0/2	2/2	2/2
Pearson syndrome (n=2)	2/2	0/1	1/2	0/2	1/1	2/2
MELAS (3243A>G; n=1)	1/1	0/1	1/1	0/1	0/1	0/1
MTATP6 (9134A>G; n=1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
OXPHOS deficiency caused by mutations of nuclear DNA§						
TMEM70 gene (n=22)	19/19	9/11	4/14	10/12	14/18	17/19
ATP5E gene (n=1)	1/1	n.d.	n.d.	n.d.	1/1	1/1
SCO1 gene (n=1)	1/1	n.d.	1/1	0/1	1/1	1/1
SCO2 gene (n=3)	3/3	n.d.	0/3	1/3	2/3	3/3
NDUFS4 gene (n=1)	1/1	n.d.	n.d.	n.d.	n.d.	n.d.
SUCLG1 gene (n=1)	1/1	0/1	1/1	0/1	0/1•	1/1
Alpers syndrom (*POLG* gene, n=1)	1/1	n.d	1/1	0/1	1/1	1/1
Barth syndrome (*TAZ* gene, n=1)	1/1	n.d.	0/1	0/1	1/1	1/1
OXPHOS deficency without known genetic basis						
Complex I deficiency (n=12)	8/9	1/7	3/9	0/7	5/8	3/7
Complex III deficiency (n=1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Complex IV deficiency (n=19)	14/18	5/10	4/15	2/7	8/15*	9/15
Combined deficiencies (n=47)	30/40	6/16	16/43	3/42	17/34	13/31
Disorders of pyruvate metabolism§						
PDHA1 gene (n=3)	2/2	0/1	0/1	n.d.	n.d.	1/1
DLD gene (n=2)	2/2	0/2	2/2	0/2	2/2	2/2
PDHc deficiency (n=1)	1/1	n.d.	n.d.	n.d.	1/1	n.d.
Total	97/112	22/52	36/103	16/82	58/92	57/90

n.d.- not determined; ALT: alanine aminotransferase; AST: aspartyl aminotransferase; B: blood; CK: creatine kinase; S: serum; U: urine

mtDNA: mitochondrial DNA; • increase methylmalonic acid excretion; *: in another patient, isolated 3-methylglutaconic aciduria was documented; § genotypes of patients:

TMEM70: c.[317-2A>G]+[317-2A>G], *ATP5E*: c.[35A>G]+[35A>G], *SCO1*: c.[394G>A]+[394>A], *SCO2*: g.[1487_1488insC]+[1541G>A]; g.[1518delA]+[1541G>A]; g.[1280C>T]+[1541G>A], *NDUSF4*: c.[462delT]+[462delT], *SUCLG1*: novel mutation (publication under preparation), *POLG1*: c.[926G>A]+[1881G>A], *TAZ*: c.[280C>T], PDHA1: novel mutation (publication under preparation), *DLD*: c.[1081A>G]+[1123G>A]

mutations (involved in the assembly of complex IV) survived beyond 1.5 years of life.

Optimized approach to diagnosis

Based on the clinical findings and laboratory data of the affected neonates, we proposed a diagnostic flowchart applicable for critically ill neonates with a suspicion of MD (Fig. 1). This approach involves the application of 14 genetic tests in addition to screening for the most common mtDNA mutations before performing a muscle biopsy. This approach is designed to speed up the diagnostic process.

Since 2008, we have applied this model in 12 neonates with a high suspicion of MD. Four of them were diagnosed prospectively with MD on a molecular-genetic level using DNA isolated from blood or buccal swab cells within 2 - 6 weeks after sample collection. Subsequently, it significantly shortened the wait period before genetic counseling could be provided to affected families. The *TMEM70* mutation was recognized in two patients, and the *SCO2* mutation and NARP/MILS syndrome were recognized in one neonate each. Two neonates (one girl) with *TMEM70* mutations were of Roma origin. The mutation analysis was indicated when lactic acidosis and 3-methylglutaconic aciduria was recognized in neonates with hypotonia and hypertrophic cardiomyopathy. In addition, the boy had penile hypospadia common in boys homozygous for c.317-2A>G *TMEM70* mutation. In the girl with neonatal onset of severe muscle hypotonia and lactic acidosis, the diagnosis of NARP/MILS was molecularly confirmed at the age of 3 months after early recognition of Leigh

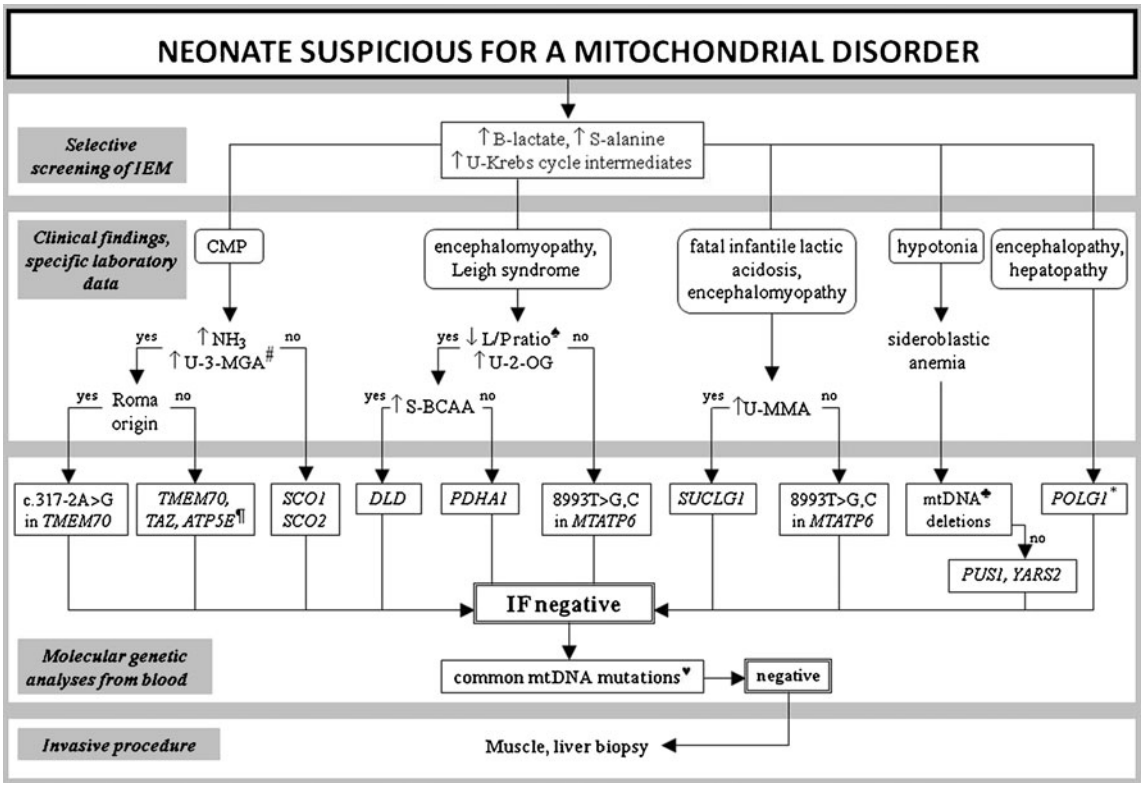

Fig. 1 Diagnostic flowchart for a critically ill neonate suspicious for mitochondrial disorders. # - 3-methylglutaconic acid can be present intermittently; therefore, repeated organic acid analysis is required. ♠ - in PDHc deficient patients, normal or borderline L/P ratio can be present. ¶ - ammonium concentration was not determined in the first published patient. ♣ - analyses should also be performed using buccal swab and urinary epithelial cells. * - rarely, blood and cerebral spinal fluid lactate can be normal in neonates and infants with *POLG1* gene mutations. ♥ - 3243A>G; 13513G>A; 8344A>G; 8363G>A. **Abbreviations:** ***ATP5E*** epsilon subunit of ATP synthase; ***B*** blood; ***BCAA*** branched-chain amino acids; ***CMP*** cardiomyopathy; ***DLD*** dihydrolipoamid dehydrogenase; ***IEM*** inborn errors of metabolism; ***L/P*** lactate/pyruvate ratio; ***3-MGA*** 3-methylglutaconic acid; ***MMA*** methylmalonic acid; ***mtDNA*** mitochondrial DNA; ***MTATP6*** mitochondrial ATP synthase subunit 6; ***2-OG*** 2-oxoglutaric acid; ***PDHA1*** pyruvate dehydrogenase complex, E1-alpha subunit 1; ***POLG1*** polymerase gamma; ***PUS1*** pseudouridine synthase 1; ***S*** serum; ***SCO1, SCO2*** cytochrome *c* oxidase assembly protein sco1, 2; ***SUCLG1*** succinate-CoA ligase, alpha subunit; ***TAZ*** tafazzin; ***TMEM70*** transmembrane protein 70; ***U*** urine; ***YARS2*** tyrosyl-tRNA synthetase 2

syndrome (using MRI). Similarly, molecular analysis in the girl with mutations in *SCO2* gene was initiated early for the neonatal onset of progressive hypotonia, low tendon reflexes, laryngeal stridor, hypertrophic cardiomyopathy and lactic acidosis without 3-methylglutaconic aciduria. The girl died at the age of 3 months.

Discussion

Detailed clinical and laboratory characteristics in large cohort of patients with neonatal onset of MD enables us to summarize the prominent clinical symptoms and to propose an optimized diagnostic flowchart for newborns with a suspicion of MD.

The most important significant finding was the high incidence of neonatal cardiomyopathy (40%) in our group of patients. Observed incidence of neonatal cardiomyopathy was higher than the rates reported in previous studies (16%, 5%, and 0%) (Skladal et al. 2003, Garcia-Cazorla et al. 2005, Gibson et al. 2008). Yaplito-Lee et al. recognized cardiac involvement in 29 out of 89 (33%) patients with MD; however, cardiomyopathy was the presenting finding during the neonatal period in only nine of their patients (Yaplito-Lee et al. 2007). Only Scaglia et al. found similar frequency of cardiomyopathy (40%) in patients with MD from their group of 113 patients exhibited cardiac disease between 2 weeks and 18 years, but the age of manifestation was not specified (Scaglia et al. 2004). Neonatal cardiomyopathies due to OXPHOS defects have been recently summarized (Schiff et al. 2011). The high incidence of neonatal cardiomyopathy in our group of 129 neonates with MD might be explained by the high number of patients with *TMEM70* mutations, in whom the cardiac involvement was the main symptom (86%). However, even after excluding these patients, the incidence of cardiomyopathy remained high (29%). The explanation may be in the constantly increasing

awareness of neonatologists in the field of inherited metabolic disorders and early transport and centralization of the care for critically ill newborns in specialized NICU centers (Lui et al. 2006) with higher rate of early survival necessary for successful diagnostic procedures.

It is also worth mentioning quite high frequency of Leigh syndrome in our study representing nearly 15% of analyzed neonates. Leigh syndrome was found in only one patient out of 57 by Garcia-Cazorla (Garcia-Cazorla et al. 2005).

Concerning laboratory findings, all of our newborns with *TMEM70* mutations and a patient with an *ATP5E* mutation exhibited mild to moderate 3-methylglutaconic aciduria. In addition, high frequency of critical hyperammonemia was observed in our group of patients with *TMEM70* mutations (Honzik et al. 2010). Nevertheless, hyperammonemia is not frequently used as a laboratory marker in patients with MD. Life-threatening hyperammonemia was described in only a small number of patients with Barth syndrome (Donati et al. 2006, Yen et al. 2008). Excluding the neonates with *TMEM70* mutations, we also observed hyperammonemia in 13 out of the remaining 41 analyzed neonates in our present study. In addition, the combination of encephalomyopathy and hyperlactacidemia with moderately increased methylmalonic acid excretion was the pivotal indication for *SUCLG1* gene sequencing leading to positive results (Ostergaard et al. 2007). These findings led us to include the assessment of ammonia, methylmalonic acid, and 3-MGA excretion level in the diagnostic concept. Most of the other clinical symptoms, the laboratory findings and the severity of prognosis were similar to those found in previously reported studies (Skladal et al. 2003, Garcia-Cazorla et al. 2005, Gibson et al. 2008). However, it is worth noting that the worst prognosis, observed in patients with *TMEM70* mutations (45%), might be improved significantly by adequate management in intensive care units (Honzik et al. 2010).

In our group of neonates with MD, combined deficiencies of several respiratory complexes were the most common findings (47% of patients), while isolated complex I deficiency was noted in only 9% (vs. 47% in (Gibson et al. 2008). Isolated complex IV was found in 18% (vs. none of 57 and 32 neonates in (Garcia-Cazorla et al. 2005) and (Gibson et al. 2008), respectively) of the neonates. Mutations in the *COX10* and *COX15* genes associated with severe neonatal presentation (Antonicka et al. 2003a, 2003b) were not found in our patients with isolated complex IV deficiency.

The yield of molecular-genetic testing with positive results in neonates with MD is generally low. In the previously published studies summarizing larger groups of neonates with MD (Skladal et al. 2003, Garcia-Cazorla et al. 2005, Gibson et al. 2008), a molecular-genetic diagnosis was found in only 20 out of 120 cases (17%), including neonates with mtDNA depletion (6x), Alpers (6x), NARP (4x), Barth (2x), and Pearson (1x) syndromes, and a single case of a *NDUFS6* mutation. In our study, a molecular-genetic diagnosis was confirmed in 38% of patients, including mutations in both the nuclear genes and in the mtDNA. Even when the patients with *TMEM70* mutations due to a single founder mutation were omitted, the molecular confirmation rate stayed at 25%, similar to the rates generally found in the diagnosis of mitochondrial disease. We found, in agreement with our previous studies and the works of other authors, early neonatal onset of MD in newborns with Alpers (Spinazzola et al. 2009), Barth (Barth et al. 2004), NARP (Garcia-Cazorla et al. 2005) and Pearson (Morel et al. 2009) syndromes, PDHc deficiency (Zand et al. 2003, Soares-Fernandes et al. 2008, Barnerias et al. 2010) and *TMEM70* (Honzik et al. 2010), *ATP5E,* and *SUCLG1* mutations (Mayr et al. 2010, Rouzier et al. 2010); two neonates were found to have complex I deficiency (Moslemi et al. 2008, Saada et al. 2009) due to mutations in the mtDNA gene encoding the ND1 subunit. Furthermore, isolated cytochrome *c* oxidase deficiency caused by mutations in *SCO1* (Valnot et al. 2000, Stiburek et al. 2009) or compound heterozygous mutations in *SCO2* genes (Taylor 2004, Vesela et al. 2004, Verdijk et al. 2008) resulted in an early neonatal onset of MD as well.

Moreover, we present for the first time a *MTATP6* 9134A>G mutation in a neonate with muscle hypotonia, lactic acidosis, and defect in ATP synthesis in muscle observed by measurement of energy-generating capacity in intact mitochondria (Janssen et al. 2006). Hypertrophic cardiomyopathy developed during early infancy. The mutation changes highly conserved Glu^{203} into Gly in Atp6 subunit and results in 50% decrease of oligomycin-sensitive ATP hydrolytic activity of F_1F_O-ATP synthase. Furthermore, we observed the neonatal onset of MELAS syndrome due to a common mtDNA mutation of 3243A>G in one of the 57 diagnosed patients in our centers. Manifestations of MELAS syndrome in the neonatal period are extremely rare (Wortmann et al. 2007), and the first clinical findings and typical attacks of MELAS syndrome usually occur in school-age children. We are aware that our group of neonates has a large number of patients of Roma origin, a population with a high prevalence of *TMEM70* pathology. Although this particular group is probably not represented to the same degree at every diagnostic center, there is increasing evidence that *TMEM70* mutations also occur in non-Roma populations (Shchelochkov et al. 2010, Cameron et al. 2011, Spiegel et al. 2011).

An optimized diagnostic approach

The gold standard for the diagnosis of MD is muscle biopsy. However, this procedure is invasive and has several limitations specific to neonates, in particular, difficult interpretation of enzymatic studies due to lack of age-related controls

and the low muscle mass and unstable clinical status of the neonates. Hence, new possibilities for more rapid and non-invasive diagnostic processes are needed. Even though there are an increasing number of mutated genes leading to MD, extensive molecular-genetic studies should be done before biopsy, as proposed previously (Wong et al. 2010). Unfortunately, the required time and expense of these tests hinder their use in neonatal intensive care units. In addition, all currently used diagnostic mitochondrial disease criteria (Bernier et al. 2002, Wolf and Smeitink 2002, Morava et al. 2006) are not applicable for diagnostic decision-making in neonates with MD.

Using data from the retrospective part of our study we proposed a new diagnostic procedure for critically ill neonates with a suspicion of MD to speed up the diagnostic process and to determine a genetic cause of the phenotype while the patient is still in the intensive care unit. We specifically focused on the most frequent MD with distinct clinical and laboratory characteristics and on disorders with potential treatment strategies (Fig. 1), such as the ketogenic diet in PDHc deficiency (Wexler et al. 1997), the treatment of hyperammonemia and infusion of fat emulsion in *TMEM70* deficiency (Honzik et al. 2010) or the avoidance of valproic acid in Alpers syndrome (Tzoulis et al. 2006). Elevated levels of blood lactate, together with hyperalaninemia, were repeatedly reported as reliable markers of MD (Magner et al. 2011, Suomalainen 2011); therefore, they were selected as an initial criterion in the proposed scheme. Actually, elevated level of lactate was found in 77% of patients with neonatal onset of MD (Garcia-Cazorla et al. 2005). The lactate in our group of 129 patients with neonatal onset of MD was elevated even in 87% of neonates. Subsequent steps add to the utility of several molecular-genetic tests in advance of muscle/liver biopsy. For instance, in the case of a critically ill neonate with hypertrophic cardiomyopathy, muscle hypotonia, hyperammonemia, and 3-methylglutaconic aciduria, the mutation analyses of the *TAZ* gene (Barth syndrome) and the *TMEM70* gene would be directly indicated (Fig. 1). *ATP5E* gene sequencing would be the next step in diagnosis. We suggest a *TMEM70* gene analysis in the case of male patients with hypospadias. In neonates of Roma origin, we would specifically recommend the analysis for the most prevalent mutation (c.317-2A>G) in the *TMEM70* gene. In a neonate with a similar clinical appearance but without cardiomyopathy, MILS/NARP syndrome (8993 T>G,C; *MTATP6* gene) should be investigated. We would also conduct this analysis in neonates with early onset of Leigh syndrome. In neonates with hypertrophic cardiomyopathy but without hyperammonemia and 3-MGA-uria, we would first conduct *SCO2* and *SCO1* gene sequencing, at least in the Slavonic population (Vesela et al. 2004, Bohm et al. 2006). A critically ill neonate with hypotonia and apneic spells, agenesis of the corpus callosum and a decreased or borderline lactate/pyruvate ratio leads to suspicion of PDHc deficiency. On the other hand, sideroblastic anemia may correspond to Pearson syndrome and hepatocerebral manifestation to Alpers syndrome. Moderate methylmalonic aciduria accompanied by encephalomyopathy prompted the investigation for the *SUCLG1* gene. Although neonatal manifestations occur in several mtDNA point mutations (Leshinsky-Silver et al. 2005; Fragaki et al. 2009; Smits et al. 2010), including the 9134A>G substitution found in one of our patients, because of their rarity and possible clinical heterogeneity, mtDNA sequencing was not included in our diagnostic concept. Nevertheless, testing for common mtDNA variants should precede muscle/liver biopsy.

We are aware of the fact, that many of mitochondrial disorders are rare or very rare, therefore cooperation between centers for diagnostics of mitochondrial disorders and prospective multicenter studies are required to further improve and optimize the diagnostic scheme. However, according to our experience, direct mutation analyses suggested in the proposed scheme could be indicated properly, but repeated metabolic testing may be required in smaller group of critically ill newborns with initially negative metabolic tests. A new biomarker FGF-21 for the non-invasive diagnostics for muscle-manifesting MD was described recently (Suomalainen et al. 2011). Moreover, the FGF-21 sensitivity in neonates with MD remains to be elucidated.

Conclusion

Mitochondrial disorders often manifest in the neonatal period; therefore, a diagnostic approach optimized for critically ill neonates with a suspicion of a defect in mitochondrial energy metabolism was suggested. Our concept allows for direct molecular genetic analyses without the need for muscle biopsies in neonates with Alpers, Barth, MILS and Pearson syndromes, *SCO1*, *SCO2, TMEM70, ATP5E, SUCLG1* gene mutations or PDH complex deficiency.

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