ONLINE SUPPLEMENTAL DATA

Expression pattern of mitochondrial respiratory chain enzymes in skeletal muscle of patients with mitochondrial myopathy associated with the homoplasmic m.14674T>C variant

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MATERIAL AND METHODS

Patient 1-7

Family 1

P1 was born at term after an normal pregnancy and delivery. At 4-5 weeks, there was increasing vomiting, feeding difficulties, and failure to thrive. He developed pneumonia and sepsis at 2 months of age. At this time, muscle weakness and generalized hypotonia were noticed. He managed without ventilatory support, but had swallowing difficulties necessitating a transient period of tube feeding. Laboratory investigations showed increased urinary excretion of lactate, increased serum creatine kinase (CK) and a blood lactate that was increased to more than 4X upper normal levels. Muscle biopsy was performed at 4 months of age. Gross motor development was slightly delayed and he walked unsupported at 18 months of age. Motor function and muscle strength at rest were normal at 2.5 years of age and there were no signs of cognitive impairment. He has experienced exercise intolerance throughout childhood and adolescence. An exercise stress test was performed at 18 years of age, in which his exercise capacity was 40% of normal compared to healthy subjects [1]. Exercise-induced hyperlactatemia was found, with a maximum value of 2X the upper limit of normal 10 minutes after completing the test. A resting CK value was slightly elevated. Clinical examination at this time showed normal motor function, normal muscle strength at rest, and normal deep tendon reflexes. Repeated ophthalmological and cardiological investigations have been normal. A second muscle biopsy was also performed at age 18 years.

The patient’s sister has high-functioning autism. Neurological examination at 20 years of age demonstrated epicanthus, ocular hypotelorism, and some foot dorsiflexion weakness. The clinical neurological examination was otherwise normal.

The patient’s mother (I:2, Fig. 1) is healthy and has never had any signs or symptoms of myopathy nor exercise intolerance.

Family 2

P2 was born after a normal pregnancy and delivery. From 1 month of age, he presented with increasing irritability and tiredness. At 2 months of age, he had generalized muscular hypotonia, feeding difficulties, and failure to thrive. Laboratory investigations showed severe hyperlactatemia (12X upper normal levels) and mildly increased CK levels. Muscle biopsy was performed at 2 months of age. There were also signs of mild liver involvement and a liver biopsy at age 4 months showed moderate steatosis but normal enzyme histochemical COX activity. At 2 years of age, mental and fine motor development were normal, while gross motor development was delayed to a developmental age of 12 months. He walked without support at 3 years of age. At age 5 years, symptoms had improved, but he still showed muscle weakness and a second biopsy was performed. At 14 years of age, he still showed signs of muscle weakness with walking difficulties, and he was unable to run. A third biopsy was performed. Laboratory investigation showed elevation of serum CK to >16X upper normal levels, and to >25X six months later. Blood lactate levels were essentially normal after the rise in the neonatal period. At age 32 years, he had a successive worsening of symptoms with increased exercise intolerance with myalgia and muscle cramps. He also experienced a decline in ambulation with a maximal walking distance of 500 m. The 6-min walk test showed reduced capacity (65% of expected), and the patient rated the exercise as very hard on the Borg’s perceived exertion scale. Serum CK was elevated to 6X upper normal levels.
The patient’s mother (I:2, Fig. 1) did not experience any symptoms of myopathy.

P3 is the maternal nephew of P2. He was admitted to the hospital at 4 weeks of age because of a respiratory syncytial virus infection, feeding difficulties and tiredness. Clinical examination revealed muscular hypotonia, swallowing difficulties, and respiratory failure. He was transiently treated with CPAP and needed gastrostomy feeding until 2 years of age. Muscle biopsy was performed at 1 month of age. Investigation of organic acids in urine revealed a massive excretion of lactate (110X upper normal level). Blood lactate was more than 4X upper normal levels, but serum CK level was normal. Gross motor development was delayed, but he walked without support at 18 months of age. Speech development was delayed because of dysarthria. At last follow-up at 12 years of age, he had residual muscle weakness with a positive Gower’s sign and exercise intolerance. He managed to walk 400 m, but needed repeated pauses. Clinical examination showed mild ptosis, increased lumbar lordosis, and weak tendon reflexes. Laboratory follow-up showed normal serum CK level and clinical follow-up with cardiological and ophthalmological investigations were normal.

P4 is the maternal half-sister of patient P3. Pregnancy and birth history were normal. Development was normal until 2 months of age when she presented with feeding difficulties, dysphagia, and failure to thrive, and tube feeding was required for nutrition. Clinical examination revealed muscular hypotonia. Laboratory investigations showed normal serum CK level and increased blood lactate level (2X upper normal levels). Muscle biopsy was performed at 4 months of age. Gross motor and speech development were slightly delayed. At last follow-up at 3.5 years of age, she had exercise intolerance, dysarthria and difficulties swallowing solid food. Clinical examination showed normal motor function, normal muscle strength at rest and normal deep tendon reflexes. Blood lactate at rest was normal, while serum CK was slightly increased (1.2X upper normal levels).

The mother of P3 and P4 (II:2, Fig. 1) did not have any symptoms of myopathy.

Family 3

P5 presented with failure to thrive with poor feeding, vomiting and lactic acidosis at 3 weeks of age. Muscle biopsy was performed at 1 month of age. At age 3 months, she had profound muscular hypotonia and swallowing difficulties which required gastrostomy. The early gross motor development was slightly delayed and she walked unsupported at 19 months of age. At 8 years of age, mental and fine and gross motor development were normal. She required persistent feeding by gastrostomy and had exercise intolerance into adolescence, but has been reported asymptomatic since the age of 15 years, until the age of 25 when she had an episode of rhabdomyolysis (serum myoglobin 2X upper reference concentration and serum CK >4X upper normal level). At age 27 years of age, clinical examination showed normal motor function, normal muscle strength at rest, and normal deep tendon reflexes, although serum CK level was increased more than 3X upper levels of normal. At the last follow-up at age 29 years of age, no clinical examination was performed, but she reported being asymptomatic.

Her mother (R1) has had no clinical signs or symptoms of myopathy, and the biopsy did not show any signs of mitochondrial myopathy or any other muscle disease.
Family 4

The two patients (P6 and P7) in this family are the two youngest of four children of consanguineous parents of Iraqi origin. The mother had three miscarriages before the birth of the oldest sibling. Both parents and the two oldest siblings are healthy and have no signs or symptoms of myopathy.

P6 was born after an normal pregnancy and delivery. Development was normal until about 4 years of age, when she presented with stumbling gait. At six years of age, she had poor balance and complained of leg pain. Muscle biopsy was performed at 9 years of age. At 10 years of age, she experienced muscle fatigue which deteriorated after periods of activity, feeding difficulties, and a thin stature. Her cognitive skills are assessed as being in the borderline range.

P7, the younger sister of P6, was born at term after a normal pregnancy. At birth, she was notably tired, with generalized hypotonia and absence of deep tendon reflexes. She had flexion contractures of fingers, adducted thumbs, bilateral clubfeet, and bilateral hip dysplasia. She required nasogastric tube feeding because of difficulties in sucking and swallowing. Blood lactate was normal. Muscle biopsy was performed at 1 month of age. During her first year of life, she had hypotonia, decreased movement, and a weak voice. At 3 months of age, she had an upper respiratory tract infection that required continuous assisted ventilation, which later could be phased out to be used only during sleep. Her fine and gross motor development were delayed. She received a gastrostomy at age 11 months because of swallowing difficulties. On the most recent examination at 2 years and 3 months of age, mental and fine motor development were normal. She still had generalized hypotonia, more proximally than distally. She could sit but not stand. Muscles were very soft upon palpation. She needed breathing support during sleep. Weight and length were – 3 SD below the mean compared with a standardized growth chart.
**Supplementary Table 1.** Antibodies used in immunohistochemistry and western blot.

| Antigen         | Host  | Dilution IHC | Dilution WB | Company (Catalogue Number)       |
|-----------------|-------|--------------|-------------|----------------------------------|
| NDUFB8 (C1)     | Mouse | 1:100        | 1:1000      | Abcam (ab110242)                 |
| SDHB (CII)      | Mouse | 1:500        | 1:1000      | Abcam (ab14714)                  |
| UQCRC2 (CIII)   | Mouse | 1:4000       | 1:2000      | Abcam (ab14745)                  |
| MTCO1 (CIV)     | Mouse | 1:2000       | 1:1000      | Abcam (ab14705)                  |
| ATPB (CV)       | Mouse | 1:500        | 1:2000      | Abcam (ab14730)                  |
| VDAC1           | Mouse | 1:2000       |             | Abcam (ab14734)                  |
| VDAC (D73D12)   | Rabbit| 1:2000       | 1:1000      | Cell Signaling Technology (4661) |
| Nesprin-1       | Mouse | 1:3          |             | Wolfson Centre for Inherited Neuromuscular Disease (CIND) [2] |
| (MANNES1A)      |       |              |             |                                  |
| Nesprin-1       | Mouse | 1:50         |             | Wolfson Centre for Inherited Neuromuscular Disease (CIND) [3] |
| (MANNES1E)      |       |              |             |                                  |

**References**

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2. Randles KN, Lam le T, Sewry CA, Puckelwartz M, Furling D, Wehnert M, McNally EM, Morris GE. Nesprins, but not sun proteins, switch isoforms at the nuclear envelope during muscle development. Dev Dyn 2010; 239: 998-1009
3. Holt I, Duong NT, Zhang Q, Lam le T, Sewry CA, Mamchaoui K, Shanahan CM, Morris GE. Specific localization of nesprin-1-alpha2, the short isoform of nesprin-1 with a KASH domain, in developing, fetal and regenerating muscle, using a new monoclonal antibody. BMC Cell Biol 2016; 17: 26
RESULTS

Supplementary figures and legends for light microscopy (Figure S1-11)

**Figure S1.** Patient 1, quadriceps muscle at 4 months of age. There is increased variability of fibre size (A, H&E), multiple ragged red fibres and lipid vacuoles (B, Gomori trichrome) and profound COX deficiency (C, COX; D, COX-SDH) Bar = 50 µm

**Figure S2.** Patient 1, quadriceps muscle at 18 years of age. There is slight variability of fibre size (A, H&E), occasional ragged red fibres (B, Gomori trichrome) and numerous fibres with COX deficiency (C, COX; D, COX-SDH) Bar = 100 µm
Figure S3. Patient 2, quadriceps muscle at 2 months of age. There is increased variability of fibre size (A, H&E), multiple ragged red fibres and lipid vacuoles (B, Gomori trichrome) and profound COX deficiency (C, COX; D, COX-SDH) Bar = 50 µm

Figure S4. Patient 2, quadriceps muscle at 5 years of age. There is slight variability of fibre size and occasional central nuclei (A, H&E), no apparent ragged red fibres (B, Gomori trichrome) but occasional fibres with COX deficiency (C, COX) and occasional fibres with mitochondrial proliferation (D, SDH) Bar = 100 µm
**Figure S5.** Patient 2, quadriceps muscle at 14 years of age. There is marked variability of fibre size and numerous fibres with internal nuclei and a slight increase in connective tissue (A, H&E), no apparent ragged red fibres (B, Gomori trichrome) but numerous fibres with complete or partial COX deficiency (C, COX; D, COX-SDH) Bar = 100 µm

**Figure S6.** Patient 3, quadriceps muscle at 1 month of age. There is increased variability of fibre size (A, H&E), multiple ragged red fibres and lipid vacuoles (B, Gomori trichrome) and profound COX deficiency (C, COX; D, COX-SDH) Bar = 50 µm
Figure S7. Patient 4, quadriceps muscle at 4 months of age. There is increased variability of fibre size (A, H&E), multiple ragged red fibres and lipid vacuoles (B, Gomori trichrome) and profound COX deficiency (C, COX; D, COX-SDH) Bar = 50 µm

Figure S8. Patient 5, quadriceps muscle at 1 months of age. There is increased variability of fibre size (A, H&E), multiple ragged red fibres and lipid vacuoles (B, Gomori trichrome) and profound COX deficiency (C, COX; D, COX-SDH) Bar = 50 µm
Figure S9. Patient 5, quadriceps muscle at 8 years of age. There is marked variability of fibre size, numerous necrotic and regenerating fibres and a slight increase in connective tissue (A and B, H&E), and numerous fibres with complete or partial COX deficiency (C, COX; D, COX-SDH) Bar = 100 µm

Figure S10. Patient 6, quadriceps muscle at 9 years of age. There is slight variability of fibre size (A, H&E), and numerous fibres with mitochondrial proliferation and occasional ragged red fibres (B, Gomori trichrome). Occasional fibres with mitochondrial proliferation show partial COX deficiency (C, COX; D, COX-SDH) Bar = 100 µm
Figure S11. Patient 7, quadriceps muscle at 1 month of age. There is marked variability of fibre size (range: 5-40 μm) and marked increase in connective tissue (A, H&E; B, Gomori trichrome). Numerous fibres show partial or complete COX deficiency (C, COX; D, COX-SDH) Bar = 50 μm
Figure S12. Repeated immunoblotting shows the same results with decreased levels of the complex I subunits NDUFB8 and NDUFS3 in P7. The decreased level of VDAC in P7 is also confirmed. Coomassie staining of the myosin heavy-chain band (MHC) serves as loading control. C, control, P, patient.
**Supplementary Table 2.** Information about the 13 mtDNA encoded genes with the occurrence of the amino acid Glu

| Complex   | Gene     | Position mtDNA# | UniProtKB | AA | Glu | Glu/AA (%) | Total AA and Glu in the complex | Glu/AA |
|-----------|----------|-----------------|-----------|----|-----|------------|---------------------------------|--------|
| Complex I | MT-ND1   | 3307–4262       | P03886    | 318| 11  | 3.46       | 2114 AA                         |        |
|           | MT-ND2   | 4470–5511       | P03891    | 347| 6   | 1.73       | 53 Glu                          |        |
|           | MT-ND3   | 10,059–10,404   | P03897    | 115| 6   | 5.22       | 53/2114=2.51%                   |        |
|           | MT-ND4L  | 10,470–10,766   | P03901    | 98 | 2   | 2.04       |                                |        |
|           | MT-ND4   | 10,760–12,137   | P03905    | 459| 9   | 1.96       |                                |        |
|           | MT-ND5   | 12,337–14,148   | P03915    | 603| 9   | 1.49       |                                |        |
|           | MT-ND6   | 14,149–14,673   | P03923    | 174| 10  | 5.75       |                                |        |
| Complex III| MT-CYB  | 14,747–15,887   | P00156    | 380| 4   | 1.05       | 380 AA                         | 4 Glu  |
|           |          |                 |           |    |     |          | 4/380=1.05%                     |        |
| Complex IV| MT-CO1   | 5904–7445       | P00395    | 513| 10  | 1.95       | 1001 AA                         | 28 Glu |
|           | MT-CO2   | 7586–8269       | P00403    | 227| 11  | 4.85       | 28/1001=2.80%                   |        |
|           | MT-CO3   | 9207–9990       | P00414    | 261| 7   | 2.68       |                                |        |
| Complex V | MT-ATP8  | 8366–8572       | P03928    | 68 | 1   | 1.47       | 294 AA                         | 4 Glu  |
|           | MT-ATP6  | 8527–9207       | P00846    | 226| 3   | 1.33       | 4/294=1.36%                     |        |

#; mtDNA rCRS is GenBank number (NC_012920.1), AA; amino acid