Alteration of mitochondrial membrane inner potential in three Italian patients with megaconial congenital muscular dystrophy carrying new mutations in CHKB gene.

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Abstract

Congenital Muscular Dystrophies (CMDs) are a heterogeneous group of autosomal recessive disorders presenting at birth with psychomotor delay, cognitive impairment, muscle weakness and hypotonia. Here we described an alteration of mitochondrial inner membrane potential and mitochondrial network in cells derived from Italian patients carrying three novel mutations in CHKB gene, recently associated with "megaconial CMD". On the bases of our findings, we hypothesize that the mitochondrial membrane potential alteration, presumably as a consequence of the altered biosynthesis of phosphatidylcholine, could be responsible for the peculiar morphological aspect of mitochondria in this disease and might be involved in the disease pathogenesis.

Keywords: CHKB, megaconial CMD, JC-1, mitochondria, membrane phospholipids

Abbreviations used in this paper: PI=patient 1, PII=patient 2, PIII=patient3, CMDs= Congenital Muscular Dystrophies, ΔΨm=differential membrane potential, MRC=Mitochondrial Respiratory Chain.

1 Introduction

CMDs refer to a clinically and genetically wide group of muscular disorders, inherited both as autosomal dominant or recessive trait (1,2), typically manifesting since birth or early infancy with muscle weakness and hypotonia, delay of gross motor development, joint and/or spinal deformities or contractures. Heart involvement could be occasionally present. Three major categories of CMDs are commonly recognized including collagenopathies, merosinopathies and dystroglycanopathies even if a systematic and comprehensive classification is not available. Other rare CMDs do not fit into any of the above reported categories (3). Muscle morphology might be helpful in diagnosis, recognizing quite variable signs of dystrophy or myopathy in different stages of the disorder (4). In the last few years, the so-called "CHKB-related CMDs", rare CMDs characterized by giant mitochondria (OMIM #602541), have been identified in about thirty patients. These disorders were

also called “megaconial type CMDs”, a term derived from “megaconial myopathy”, as reported in 1964 and 1966 by Shy and Gonatas (5, 6) in myopatic children with giant (mega) mitochondria. More recently, Nishino and co-workers (7) described megaconial CMDs in four patients with CMD and altered mitochondria, which were not only gigantic but also peculiarly distributed toward the periphery of muscle fibers, leaving the center devoided of organelles. The authors suggested that mitochondrial enlargement could represent a functional compensation for mitochondrial depletion in the central sarcoplasm, where myofibrillar degeneration occurred. The gene associated to this disorder, identified in a cohort of 15 individuals by Mitsuhashi, was CHKB (8). To shed light on the pathogenesis of this disease, we investigated the presence of mitochondrial alterations in myoblasts of our Italian patients cohort, collected from two unrelated families and carrying three new mutations in CHKB gene.

2 Material and Methods

Informed consent for morphological, biochemical and genetic studies was obtained from patients' caregivers by the Neurological Institute C. Besta of Milan and the Pediatric Neurology and Muscular Disorders Institute G. Gaslini of Genoa. All procedures followed are in accordance with the ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983).

2.1 Case report

Patient 1. PI is an 11 years old girl born at 36 weeks by premature caesarean delivery from unrelated healthy parents. No family history for muscle disease or neurological disorders was documented. The pregnancy was complicated by threat of abortion in the first trimester. The weight at birth was 2350 gr. Since birth she presented psychomotor delay, she could stay seated at 6 months, she could stand up at 16 months, she was able to walk at 30 months with a characteristic anserine gait. Since the first month of life she showed ichtyosis associated with a light increase of IgE, obstinate constipation and pollakiuria without urinary infections. The neurological examination at 14 months showed a global hypotonia associated with hypotrophy in shoulder muscle and weakness. She presented an improvement in motor function without a substantial acquisition of expressive speech since she could only vocalize. At 6 years old, the neurological evaluation showed pectus excavatum, hypotrophic proximal upper limbs muscles, hyposthenic head flexors and lower limbs, diffuse hypotonia, confirmed by computed tomography and by muscle MRI that documented diffused upper legs muscle tropism associated with the increase of adipose tissue. Tendons reflexes of upper limbs were weak. She was not able to stand up from a chair by herself, she could stand up and walk with wide base gait. ECG did not reveal any heart alterations. Brain MRI was normal. EEG presented epileptic anomalies in right occipital lobe and in left frontoparietal lobes in *siesta* however she never presented any crisis. Neuropsychic evaluations by Griffith’s scale revealed an important psychomotor development delay

due to cognitive impairment, in fact she understood only simple orders and could not express intelligible words. Blood tests showed normal level of lactate, pyruvate and amino acids, while CK and LDH levels were mildly increased(CK 268 U/L; normal value of 24-150 U/L; LDH 597 U/L; normal value of 230-480 U/L). At present, her general conditions are stable.

Patient 2. PI had an older sister presenting the same clinical features. She was born at 40 weeks with uncomplicated pregnancy and delivery. Since the first month of life she presented with ichthyosis, psychomotor delay, cognitive impairment, muscle weakness and hypotonia. She started to walk at 18 months with an serine gait and frequent falls. A difficult to speech was noticed. At 5 years of age a clinical examination demonstrated a global hypotonia, axial and proximal arts hypostenia, swinging gait. She was unable to run and she needed help to stand up. The CK level was increased (944 U/L with normal value of 24-150 U/L) but at 9 years of age she developed a severe dilated cardiomyopathy and died one year later.

Patient 3. PIII was a 13 years old girl, born from non-consanguineous, healthy parents, from uneventful full-term pregnancy, presented at birth with hypotonia and congenital neurosensorial deafness. She was able to sit at 1 year of age and to walk at 2 years. She also had profound cognitive impairment with behavioral disturbance and severe speech delay, pronouncing only few words at current age. At age 7 years she manifested first epileptic seizure characterized by vocal spasm, face twitching and subsequent loss of consciousness for few minutes. After ten days a second episode with similar features occurred but was followed by generalized hypertonia, upward gaze and vomiting. EEG recordings confirmed epileptic discharges on left temporal lobe, boosted during sleep. Neurological examination at 9 years of age acknowledged proximal weakness and wasting of limb muscles, waddling gait and hyperlordosis. Tendon reflexes were ubiquitously reduced. She was able to rise from floor with Growers maneuver. Skin alterations were not detected. CK was mildly elevated (180-250 U/L with normal value of 24-150U/L). Lactic acid was within normal range. Cardiac assessment including ultrasound investigation revealed no alterations of heart morphology and function. Brainstem evoked potential confirmed neurosensorial deafness. EMG revealed signs of myopathic involvement. Nerve conduction studies were normal. Muscle MRI performed at age 9 detected generalized hypotrophy and fatty infiltration of lower limbs, particularly evident in quadriceps and in gastrocnemii muscles.

2.2 Laboratory analysis

Fibroblasts from skin biopsies and myoblasts from quadriceps muscular biopsies of patients and healthy controls were grown following standard laboratory procedures (9, 10). DNA was extracted from peripheral blood of PI and PII and from fibroblasts pellet from PIII, using standard methods (11). The coding sequence and flanking intronic regions of 11 exons of CHKB gene were analyzed by PCR amplification. Amplicons were stained with ethidium bromide and visualized on 2%

agarose gels, cycle-sequenced using BigDye chemistry 3.1, and run on an ABI 3130XL automatic sequencer (Applied Biosystems). PCR primers and conditions are available upon request. Morphological analysis in skeletal muscle tissues collected from all patients and in cardiac autoptic tissue of PII was carried out using standard histological techniques (12). The reactions for COX and SDH were performed as previously described (13). For immunohistochemical evaluations muscle and heart biopsy specimens were frozen and stored in isopentane cooled in liquid nitrogen. Immunohistochemical staining was performed on 8-μm thick cryosections by using primary antibodies direct against α-dystroglycan clone VIA4-1 (Merk), LAMP1 (Sigma-Aldrich), LAMP2 clone H4B4 (DSHB), LC3B (Cell Signalling) and p62/SQSTM1 (Sigma-Aldrich). For the specific detection and quantification of apoptotic cells, TUNEL assay (Roche) was carried out according to the manufacturer's instructions on skeletal muscular sections of PI and PII and on cardiac sections of PII. Ultrastructural analyses were achieved on glutaraldehyde-fixed muscles biopsies of PI and PII and on the autoptic heart specimen of PII, post-embedded in epoxide resin, as previously described (13). Measurement of the MRC enzymes activity was accomplished by standard spectrophotometric techniques in muscle homogenate of PI and PII (14). Mitochondrial network staining was conducted on fibroblasts of PI and PIII using MitoTracker Red Mitochondrion-Selective probes (Invitrogen) according to manufacturer's instruction. Morphometric analysis (Feret's diameter and circularity) were carried out using ImageJ, an open source Java image processing program inspired by NIH Image. Detection of altered mitochondrial inner ΔΨm in myoblasts of PI and PIII was performed using JC-1 staining kit (Mitochondria staining kit for mitochondrial potential changes detection; Sigma) according to manufacturer's instruction. MitoTracker Red and JC-1 data were expressed by means ± S.D. Statistical significance of differences was determined by the Student's t test. All determinations were performed in at least 3 replicates for each sample.

3 Results

We identified a homozygous mutation c.565_568delTTTG/p.Leu188Glyfr*7 in exon 4 in PI and PII inherited from heterozygous parents (Fig.1A) and two different mutations: c.140_146del/p.Arg47Pro fs*21 in exon 1 inherited from the father and c.1066_1067delTG/p.Trp356Val fs*72 in exon 10 inherited from the mother in PIII (Fig.1B). All three mutations had never been described before according to ExAC, Exom variant server, gnomAD databases. Histological examination of patients' muscles biopsies showed high variability of fibers size and increase of connective tissue, several necrotic/regenerating fibers, multiple centralized nuclei and splitting. At histochemical analysis, the COX activity was reduced at the centre of the muscle fibers. Enlarged mitochondria were present at the periphery of the fibers (Fig. 2A, 2C). The same giant mitochondria were present in autoptic heart tissue (Fig. 2B, 2D). Immunohistochemical studies on muscle samples did not show any abnormality. Antibodies directed against principal autophagic epitopes (LAMP1, LAMP2, LC3B

and p62) on skeletal and cardiac biopsies did not show any significant increase of tissue expression in patients compared to controls. TUNEL reaction did not identify any apoptotic nuclei in skeletal and heart muscle biopsies. Electron microscopy confirmed the presence of enlarged, vesicular, peripheral mitochondria with few altered cristae in the majority of fibers, disorganized and rarefied myofibrillary apparatus, vesicled sarcoplasmic reticulum and loss of alignment of sarcomeres (Figs.2E, 2F). Spectrophotometric biochemical assays of the respiratory chain complexes activities in the skeletal muscle homogenate (Fig.2G) revealed an important defect in CI. This complex showed a residual activity of about 30% in PI and of about 20% in PII as compared to the average of controls. Citrate synthase activity was normal. Utilizing MitoTracker Red probes, fibroblasts from controls displayed a typical normal filamentous network (Fig 3A) while almost all the fibroblasts of PI and PIII exhibited an altered mitochondrial network, characterized by an organization in extremely numerous small dots (Fig. 3B, 3C). MitoTracker Red fluorescence quantification was performed on confocal images by ImageJ and both circularity ($p<0.001$ both PII and PIII versus controls) and Feret's diameter ($p<0.005$ PII versus controls, $p<0.001$ PIII versus controls) parameters were evaluated (Fig. 3D; 3E). Through the JC-1 dye the vast majority of the myoblasts from controls showed a prevalent red fluorescence (Fig.3F), while there was a diffuse green fluorescence in a great percentage of patients myoblasts (Figs. 3H, 3I). To validate the assay, we treated myoblasts from controls with uncoupling agent valinomycin to induce $\Delta\Psi m$ loss; as expected, treated myoblasts displayed exclusively green fluorescent signals (Fig.3G). Ratio red/green, both in PI and PIII, resulted statistically lower ($p<0.001$) as compared to controls (Fig. 3L).

4 Discussion

We investigated the mitochondrial activities and morphological changes in cell lines and tissues (skeletal muscle and heart) from patients affected by "megaconial CMD" harbouring a homozygous and two compound heterozygous frameshift mutations in CHKB gene. One of the patients died for an acute and severe cardiomyopathy while cardiac assessments revealed no alterations of heart morphology and function in the others two patients. Cardiac involvement is relatively frequent (around 50%) in patients affected by megaconial CMD (15). One patient manifested epileptic seizures and the other two presented with ichthyosis-like skin changes similarly the two siblings studied by Yis in which was underlined the importance of this clinical aspect in the differential diagnosis of CMD (16). We observed a peculiar rearrangement of mitochondrial network and alterations of the mitochondrial inner $\Delta\Psi m$ in patients' derived cells. These observations led us to hypothesize that these findings could be related to an anomalous biosynthesis of phosphatidilcholine. In fact, it has been recently described a new pathogenic pathway for CMDs involving phospholipids metabolism caused by mutation in CHKB. In humans, choline kinase consists of three isoforms, CHKa1, CHKa2 and CHKb, encoded by two separate genes (CHKA and CHKB). CHKB gene is located on chromosome 22 and encodes for a protein, which has a key role in phospholipids

biosynthesis. It catalyzes the first step in phosphatidylethanolamine biosynthesis, phosphorylating ethanolamine, and can also act on choline *in vitro*. Nevertheless the pathogenesis of these diseases is still unknown. Some authors supposed a pivotal role of mitophagy because of the decreased number of mitochondria in skeletal muscle that could be due to increased mitochondrial clearance (17, 18). To explore this hypothesis, we tested the available tissues of the patients by immunohistochemistry for P62, LAMP1, LC3 antibodies, validated markers for autophagy (19), but we could not identify any relevant tissue expression. It is known that apoptosis starts with a redistribution of phospholipids in the plasma membrane and that inhibition of phosphatidylcholine synthesis leads to cell cycle arrest at G2 and subsequent apoptosis (20). Moreover, it seems that one cell reaction to metabolic stresses is the shift of the balance of mitochondrial fission and fusion toward the development of mega-mitochondria to acquire resilience to apoptosis (21). Using TUNEL reaction we searched for apoptosis in skeletal and cardiac muscles, but we didn't found any apoptotic nuclei, as previously demonstrated in the mouse model *rmd/rmd* hind limb fresh-frozen muscle sections employing cleaved caspase-3 antibodies (20). The peculiar morphological features characterized by enlarged mitochondria located at the periphery of the fibers strongly suggest an active role of mitochondria in the pathogenesis of this type of dystrophy. We examined in depth this observation evaluating the MRC complexes activity in two of our patients identifying a consistent reduction of CI in their skeletal muscles. Single alterations in CI activity had just been described by Quinlivan et al (22) while other authors reported combined deficiencies involving CI, CIII and CIV (23) or CII and CIV (24). Until now a mild decrease of all OXPHOS complexes was found in the skeletal and cardiac muscles of only one individual (25). The profile of combined deficiencies had been attributed to mtDNA depletion, however the activities of the MRC enzymes are also markedly influenced by the composition of the phospholipids environment of the inner mitochondrial membrane. It had been proved that an increase of mitochondrial membrane phospholipids content lowers the enzymatic activity of electron transport complexes (26). We demonstrated an alteration of the $\Delta\Psi m$ and a consequent alteration of the mitochondrial network on cells of the patients. Our experiments on myoblasts reported a modification in the distribution of the JC-1: the ratio green/red fluorescence signal in myoblasts of patients, indicating an intact $\Delta\Psi m$, was statistically lower in respect to the controls one. MitoTracker Red staining allowed to distinguish between live labelled fibroblasts presenting a preserved tubular mitochondrial network, from that presenting an altered fragmented network. As expected, controls' fibroblasts were more tubular in respect to the patients' fibroblasts that showed several small dots. MitoTracker Red dye is a cationic fluorophore that accumulates electrophoretically into mitochondria in response to the highly negative $\Delta\Psi m$. Because of dissipation of $\Delta\Psi m$ usually leads to mitochondrial fragmentation in healthy cells, we can deduce that network fragmentation observed in the patients' fibroblasts could be consequence of a reduction of the $\Delta\Psi m$. Modifications of the mitochondrial membrane potential were

already described in some types of mitochondrial encephalomyopathies such as those due to mutation in TMEM 70, a supposed assembling factors of complex V (27). Mitochondria are dynamic organelles, which continuously fuse and divide and it had just been reported that membrane fusion defect could underlie some dystrophies types (20). We hypothesize that also in fibroblasts and myoblasts from our patients with megaconial CMD the fission/fusion mitochondrial mechanism could be malfunctioning. One of possible cause could be the alteration of the ΔΨm conducting to the formation of giant mitochondria, as observed both by histochemistry and electron microscopy. We believe that our data could show the fundamental role of the altered mitochondrial inner ΔΨm, as a consequence of an anomalous biosynthesis of phosphatidilcholine in the CHKB related-CMDs providing useful additional information in the comprehension of the pathogenesis of the disease. The giant mitochondria present in muscle resemble the same scenario observed in the mouse model and in the patients of another human disorder named PKAN (pantothenate kinase-associated neurodegeneration), caused by mutations in the PANK2 gene, coding for the mitochondrial enzyme pantothenate kinase type 2, responsible for the phosphorylation of pantothenate or vitamin B5 in the biosynthesis of co-enzyme A (28). It is an autosomal recessive disorder characterized by dystonia, dysarthria, rigidity, pigmentary retinal degeneration and brain iron accumulation. The hallmark of this disease is the eye of-the-tiger signal in the globus pallidus on T2-weighted MRI (28, 29). It may be possible that the mitochondrial alterations visible both in patients' muscles affected by mutations in CHKB and in PANK2 genes, even if belonging to different metabolic pathways, could be due to a dysfunction in the converging phospholipids biosynthesis steps (30, 31).

5 Conclusions

Our data suggest that the mitochondrial alterations observed in three patients affected by megaconial CMDs with mutations in CHKB gene could play a relevant role in the pathogenic mechanism of the disease. The mitochondrial membrane potential alteration, most likely consequence of the altered biosynthesis pathway of phosphatidylcholine, one of the major membrane phospholipid, could be one of the reasons for the peculiar morphology and distribution of mitochondria.

Authors contribution

SM performed autophagic and apoptotic analysis, mitochondrial network staining, analyzed the data and drafted the paper. FI performed molecular analysis. FB performed histochemical, immunocitochemical and ultrastructural analysis. VB performed biochemical analysis. SD and PV performed JC-1 staining. CF performed clinical evaluations and provided laboratory documentation of one patient. GB and EL performed clinical evaluations on two patients. MM analyzed morphological data. VT and FP revised the manuscript. CL received funding, designed the research study, performed clinical evaluations, drafted the paper and carefully revised the manuscript.

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Conflict of interest

Authors have no conflict of interests.

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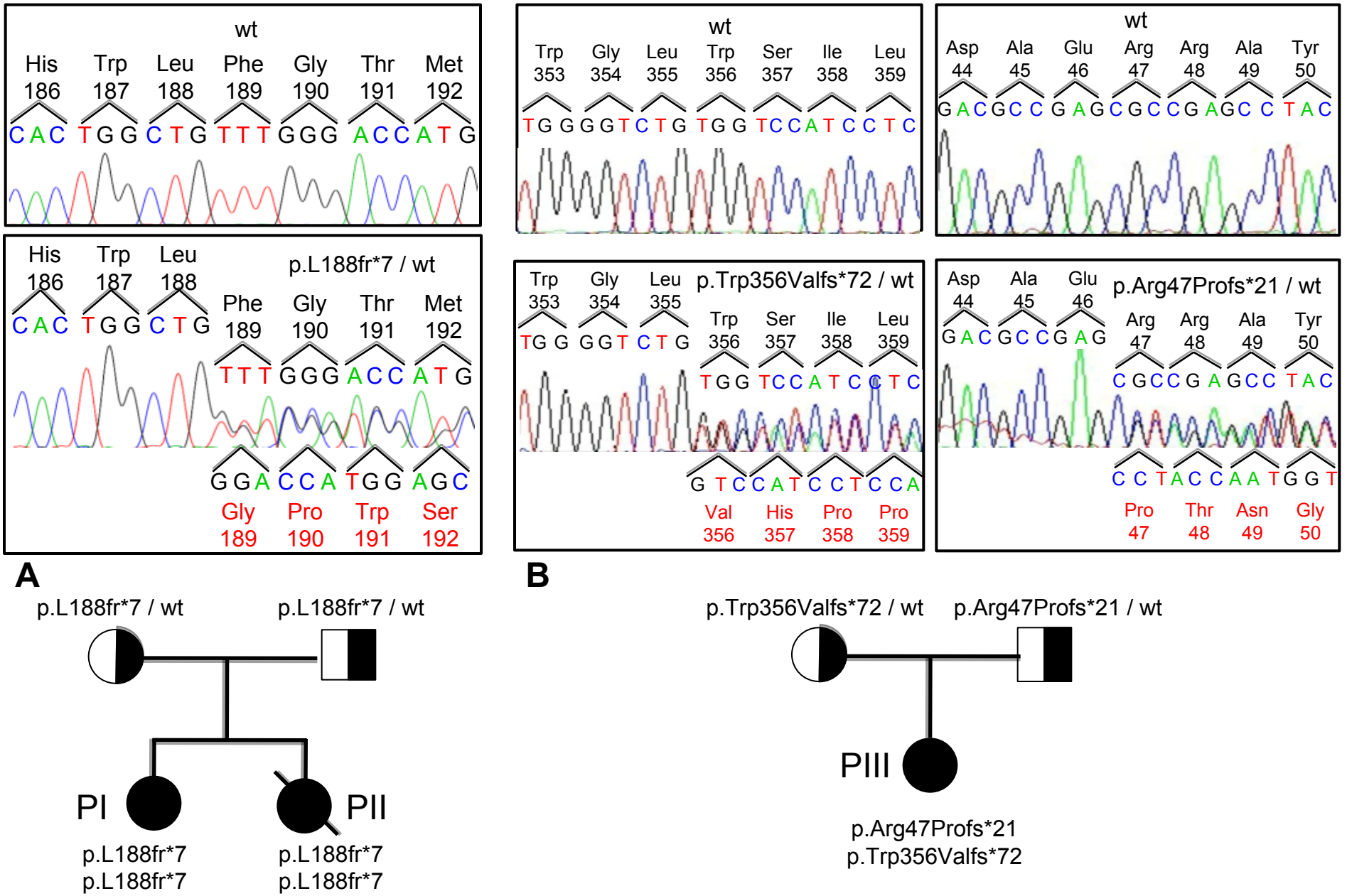
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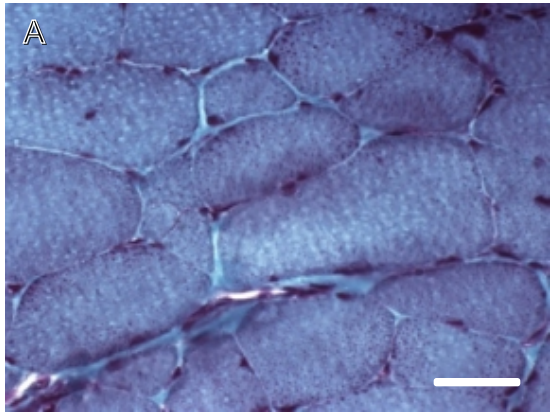
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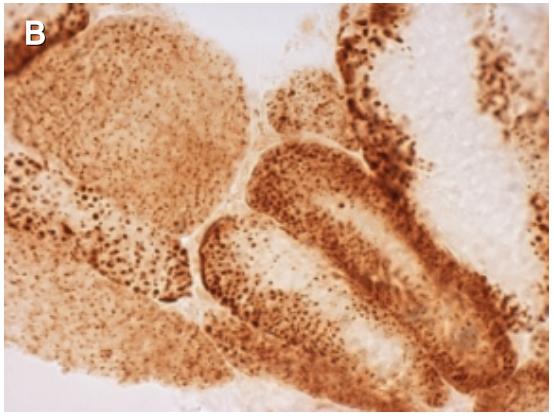
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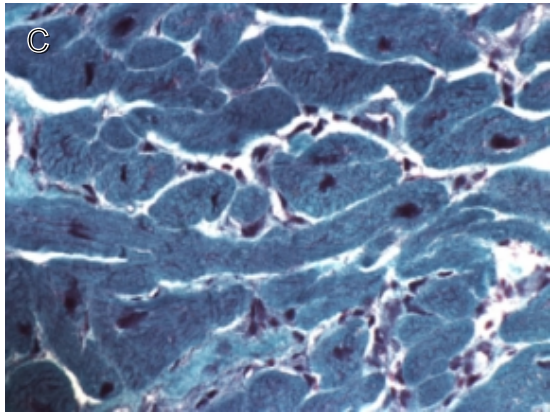
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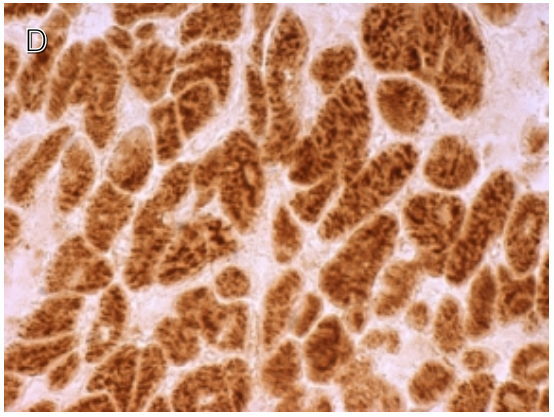
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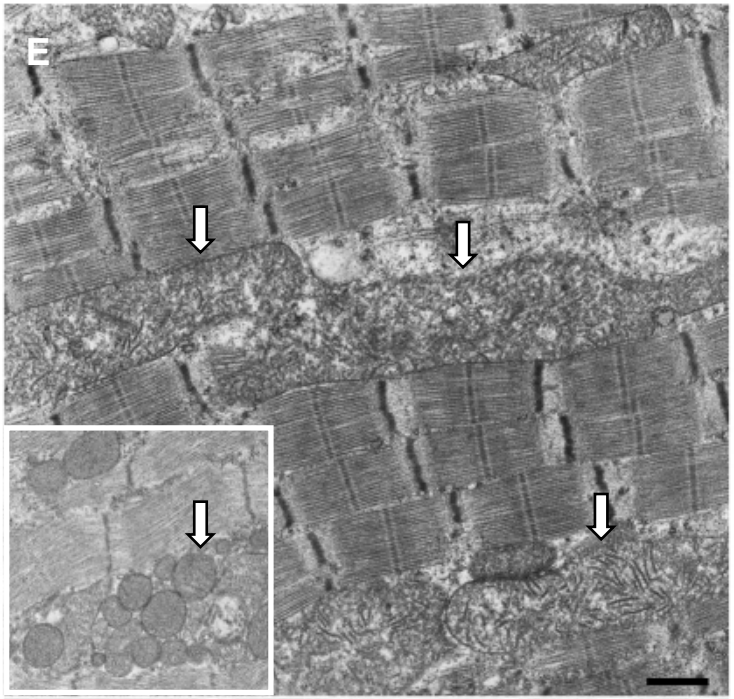
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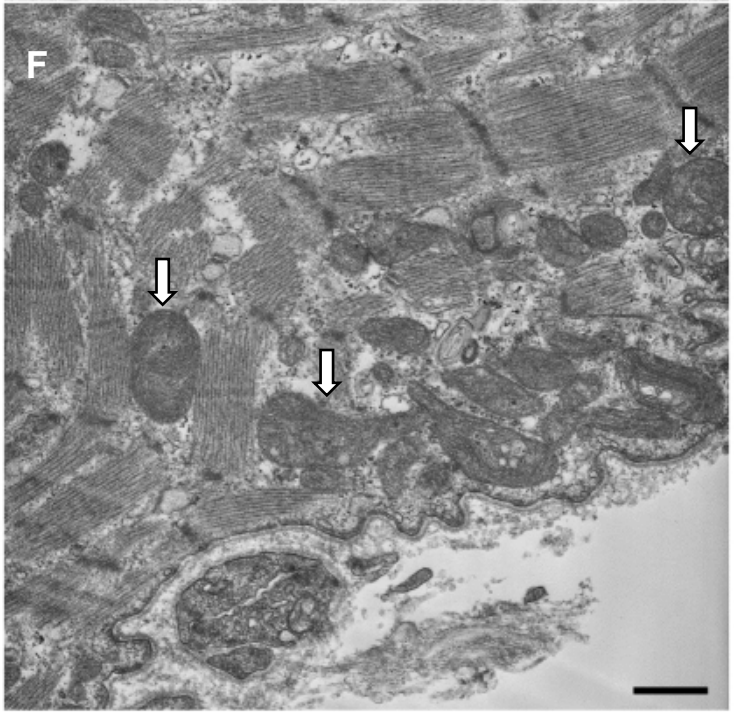
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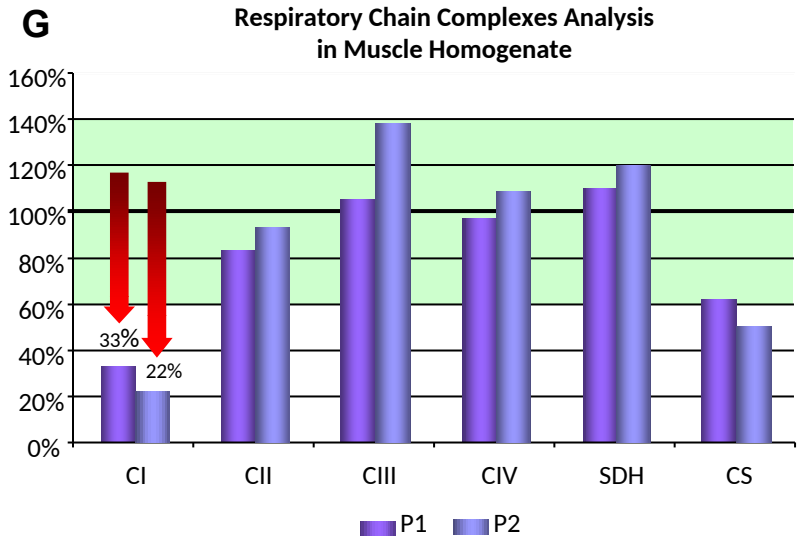
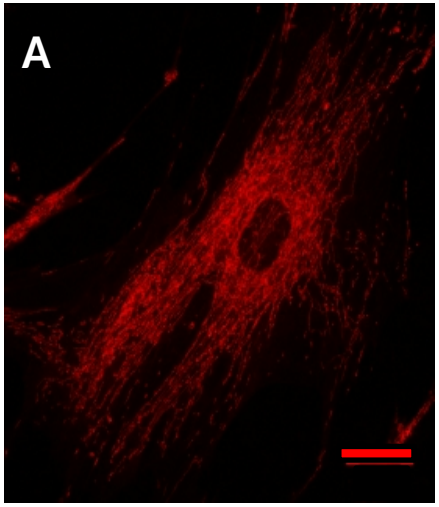
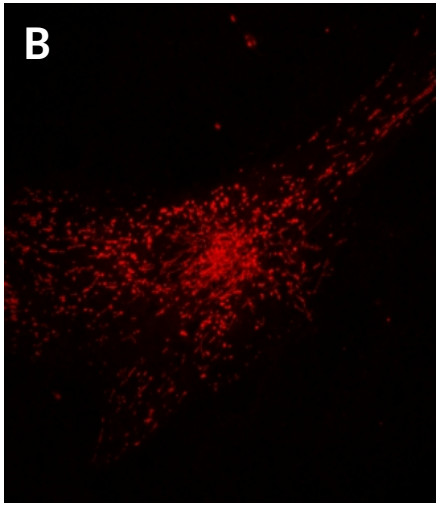

G
Respiratory Chain Complexes Analysis
in Muscle Homogenate
160%
140%
120%
100%
80%
60%
40%
20%
0%
33%
22%
CI
CII
CIII
CIV
SDH
CS
P1
P2


A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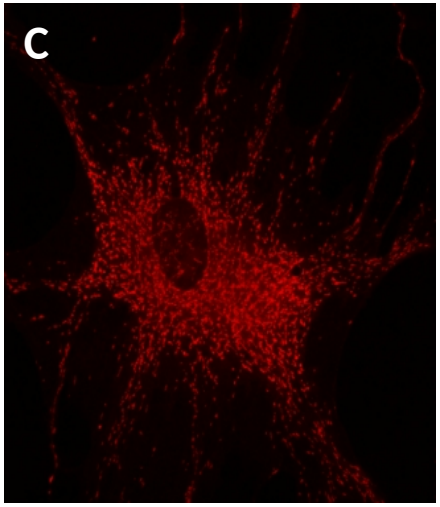
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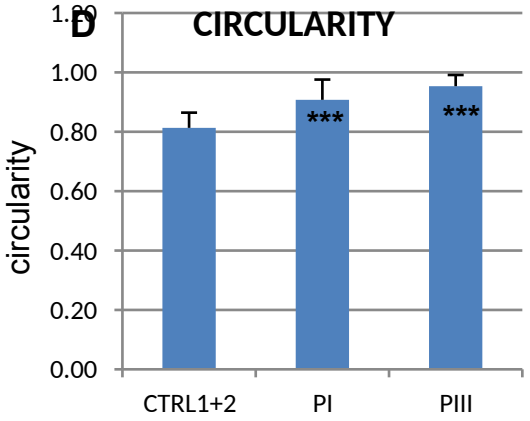
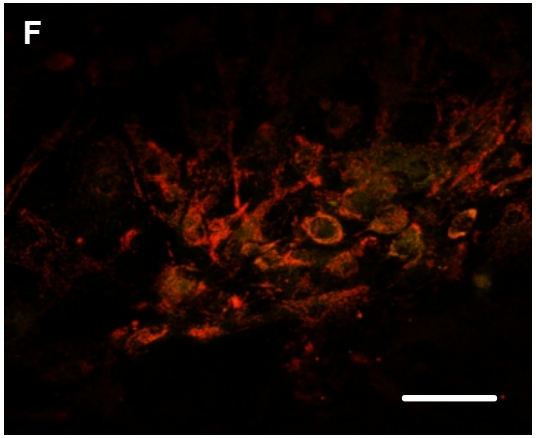

D
CIRCULARITY
circularity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
***
***
CTRL1+2
PI
PIII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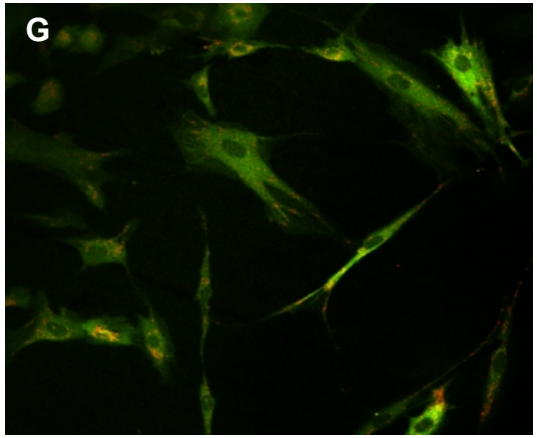
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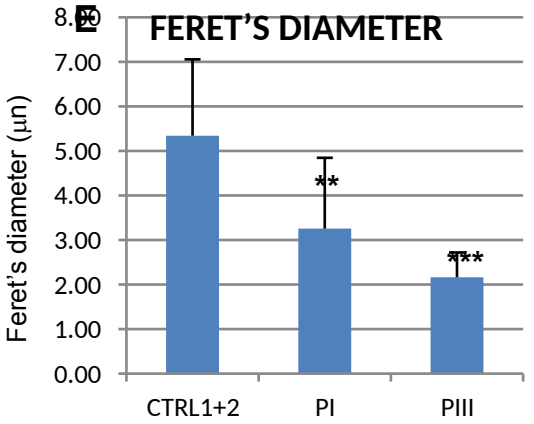
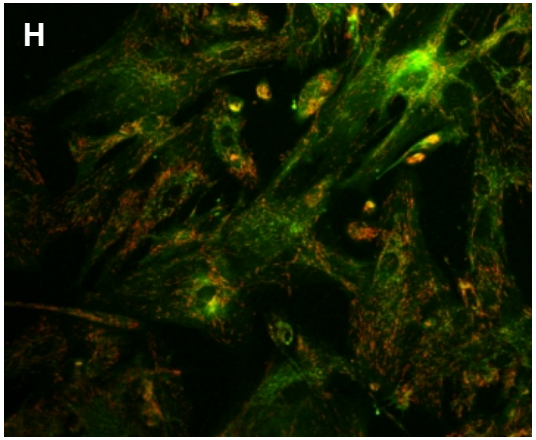
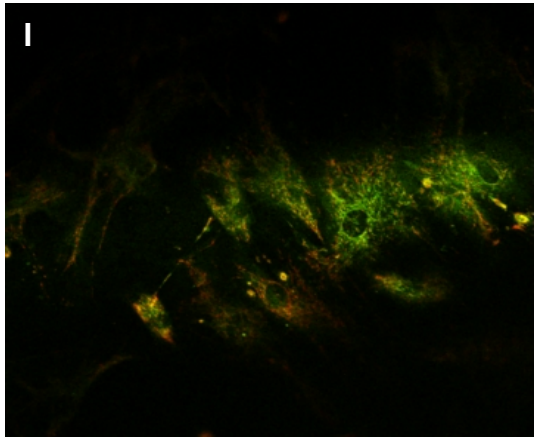
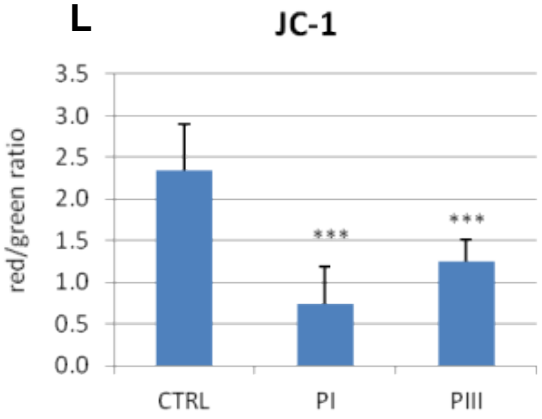

E
FERET'S DIAMETER
Feret's diameter (µn)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
**
***
CTRL1+2
PI
PIII


H


I


L
JC-1
red/green ratio
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
CTRL
PI
PIII

LEGENDS FOR FIGURES

Fig. 1.
Electropherograms of the DNA genomic region encompassing the CHKB gene mutations and pedigrees of PI, PII (Fig.A) and PIII (Fig.B). The mutation c.565_568delTTTG/p.Leu188Glyfr*7 in exon 4, homozygous in PI and PII, was heterozygous in both parents. PIII harbored two different mutations c.140_146del/p.Arg47Pro fs*21 in exon 1 inherited from the father and c.1066_1067delTG/p.Trp356Val fs*72 in exon 10 inherited from the mother .

Fig. 2.
Slides of muscle biopsy from PI and cardiac biopsy from PII stained for Gomori trichrome (Fig. A, Fig. C) and COX (Fig. B, Fig. D) showed large mitochondria. In the muscle, mitochondria were often at the periphery of the fibers with central areas devoid of activity. Scale bar 40 μm. Electron microscopy on skeletal muscle of PII (Fig. E) and PI (Fig. F) showed enlarged mitochondria particularly at the periphery of fibers. In the insert (Fig,3) an example of normal mitochondria. Scale bar 1 μm. Spectrophotometric biochemical assays of the MRC complexes activities in the skeletal muscle homogenate from both PI and PII (Fig.G) revealed an important defect in CI: CI in PI showed a residual activity of about 30%, in PII of about 20% in respect to the average of controls (more marked line in the graph, the green shaded area represents the normal range of activities calculated as mean±s.d.); the activities of the other complexes and of the enzyme citrate synthase (CS) were normal.

Figure 3
Mitochondrial network. MitoTracker Red staining underlined a normal threadlike mitochondrial network in fibroblasts from controls (Fig.3A), and an altered, fragmented network in fibroblasts from PI (Fig.3B) and PIII (Fig.3C). Scale bar 30 μn. Histograms displaying morphometric quantifications of circularity (Fig.3D) and Feret's diameter (Fig.3E) in fibroblasts stained with MitoTracker Red supported this observation. Student's T test was performed on 3 experiments' mean. ** p value<0,005, *** p value<0,001.
Mitochondrial membrane potential (ΔΨm). Representative confocal immunofluorescence images of JC-1 staining on myoblasts from controls (F), PI (H), PIII (I) and on myoblasts from controls treated with valinomycin (G). Red fluorescence, sign of preserved ΔΨm, was observed in almost all non-treated controls, whereas several myoblasts, as well as valinomycin treated cells, displayed green fluorescent signals, index of mitochondrial membrane depolarization. Scale bar 100 μm. (L) Histogram showing that myoblasts from patients presented a higher number of cells with green fluorescence than controls. Student's T test performed on 3 experiments' mean (CTRL vs PI; CTRL vs PIII). *** p value<0,001