

A mutation in *MT-TW* causes a tRNA processing defect and reduced mitochondrial function in a family with Leigh Syndrome

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Abbreviations used are: LS, Leigh Syndrome; MT-TW, mitochondrial tRNA tryptophan; OXPHOS, oxidative phosphorylation; rRNA, ribosomal ribonucleic acid; tRNA, transfer ribonucleic acid; NGS, next generation sequencing; IUGR, intrauterine growth retardation.

ABSTRACT

Leigh syndrome (LS) is a progressive mitochondrial neurodegenerative disorder, whose symptoms most commonly include psychomotor delay with regression, lactic acidosis and a failure to thrive. Here we describe three siblings with LS, but with additional manifestations including hypertrophic cardiomyopathy, hepatosplenomegaly, cholestatic hepatitis, and seizures. All three affected siblings were found to be homoplasmic for an m. 5559A>G mutation in the T stem of the mitochondrial DNA-encoded *MT-TW* by next generation sequencing. The m.5559A>G mutation causes a reduction in the steady state levels of tRNA^{Trp} and this decrease likely affects the stability of other mitochondrial RNAs in the patient fibroblasts. We observe accumulation of an unprocessed transcript containing tRNA^{Trp}, decreased *de novo* protein synthesis and consequently lowered steady state levels of mitochondrial DNA-encoded proteins that compromise mitochondrial respiration. Our results show that the m.5559A>G mutation at homoplasmic levels causes LS in association with severe multi-organ disease (LS-plus) as a consequence of dysfunctional mitochondrial RNA metabolism.

Key words: Leigh syndrome, multi-organ mitochondrial disease, tRNA, MT-TW, tRNA tryptophan, mitochondrial disease, OXPHOS

1. Introduction

Mitochondrial diseases are the most common form of inherited metabolic disorders (Skladal et al., 2003; Suzuki et al., 2011), and most often affect multiple tissues and organs with high-energy demands such as the brain, skeletal muscle and the heart. These diseases are a result of mitochondrial dysfunction, which manifests with a wide range of clinical presentations including neurodegeneration, cardiomyopathies, visual impairment, muscle defects and exercise intolerance (Brzezniak et al., 2011; Chinnery and Schon, 2003; Lopez Sanchez et al., 2011; Taylor and Turnbull, 2005; Vafai and Mootha, 2012). Mitochondrial diseases may be caused by mutations in mitochondrial DNA (mtDNA) or nuclear DNA encoding mitochondrial proteins, resulting in defective oxidative phosphorylation (OXPHOS) and energy metabolism. There are numerous downstream pathological effects as well, such as changes in cell signalling, increased oxidative damage and initiation of cell death (Barić et al., 2013; Mkaouar-Rebai et al., 2009; Montoya et al., 1981; Smits et al., 2010; Taylor and Turnbull, 2005; Vafai and Mootha, 2012). The mtDNA is a compact, circular, double-stranded genome encoding only 11 mRNAs that direct the synthesis of 13 electron transport chain protein subunits, 2 rRNAs and 22 tRNAs (Gardner et al., 2007; Mkaouar-Rebai et al., 2009; Montoya et al., 1981). Point mutations in the mtDNA can affect tRNA genes, mitochondrial polypeptide genes or rRNA genes causing diseases such as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, maternally inherited Leigh syndrome or aminoglycoside-induced non-syndromic deafness, respectively. In addition, mtDNA deletions or rearrangements can cause Kearns-Sayre syndrome, diabetes and deafness (Dimauro and Schon, 2003; Tang et al., 2010). MtDNA and consequently diseases caused by mutations in mtDNA are maternally inherited and like mutations in nuclear genes encoding mitochondrial proteins can cause a range of disorders with varying age of onset and severity (Rackham et al., 2009; Vafai and Mootha, 2012).

Leigh syndrome is an early-onset progressive neurodegenerative disorder with a characteristic neuropathology consisting of focal, bilateral lesions in one or more areas of the central nervous system, including the brainstem, thalamus, basal ganglia, cerebellum, and spinal cord (Davies et al., 2009; Rahman et al., 1996). The most common underlying cause is a defect of oxidative phosphorylation (OXPHOS). The symptoms of LS vary but most commonly include severe developmental delay presenting in infancy with regression, central hypotonia and failure to thrive. Lactic acidosis, dysphagia, optic disc abnormalities, dystonia, ataxia and peripheral neuropathy are also common features. The condition is severe and usually results in death in early childhood (Baertling et al., 2014; Frazier and Thorburn, 2011). Mutations in nuclear genes encoding mitochondrial proteins account for approximately 75% of LS and the remaining cases are a result of mutations in the mtDNA (Kuznetsov et al., 2008; Rahman et al., 1996). Mutations in *MT-ATP6* are the most frequent cause of mitochondrial DNA inherited LS (Mkaouar-Rebai et al., 2009; Thorburn and Rahman, 2014). However mutations in other mitochondrial mRNA coding genes (*MT-ND2*, *MT-ND3*, *MT-ND4*, *MT-ND5* and *MT-ND6* genes) and tRNA genes (*MT-TV*, *MT-TV1*, *MT-TW* and *MT-TK* genes) also have been reported (Mkaouar-Rebai et al., 2009; Thorburn and Rahman, 2014).

Mitochondrial ribosomes (mitoribosomes) decode the 11 mt-mRNAs using tRNA adaptor molecules. In animals the full complement of mt-tRNAs is encoded by the mtDNA providing the minimum set of tRNAs required for all sense codons, whereas the mitochondrial gene expression machinery is encoded on nuclear genes and imported into mitochondria post-translationally (reviewed in (Bindoff et al., 1993; Rackham et al., 2012)). The genetic code is established by aminoacyl-tRNA synthetases, which load specific amino acids onto the 3' end of tRNAs. Modifications of tRNA residues, particularly at the first anticodon position (wobble position), are crucial for tRNA decoding (Suzuki et al., 2011).

Mutations in mitochondrial tRNA genes cause a variety of mitochondrial diseases with over 260 different identified pathogenic mutations (Dimauro et al., 2013) affecting the stability of tRNAs, modification of tRNA bases, aminoacylation or processing of polycistronic transcripts (Suzuki et al., 2011) where the cleavage of tRNAs enables the release of mRNAs and rRNAs (Brzezniak et al., 2011; Lopez Sanchez et al., 2011).

Mutations in *MT-TW* have been associated with mitochondrial myopathy, LS, neurogastrointestinal syndrome, myoclonic epilepsy, encephalomyopathy and a separate phenotype characterised by dementia, chorea, deafness and ataxia, as a result of combined complex I, III and IV deficiencies caused by a general mitochondrial translation defect and reduced OXPHOS complex assembly (Barić et al., 2013; Mkaouar-Rebai et al., 2009; Smits et al., 2010). There is only a single report of LS caused by an m.5559A>G mutation in *MT-TW* with a 43% load of the mutant mtDNA compared to wild type mtDNA, a state known as heteroplasmy. Although *in silico* prediction has suggested this mutation may cause secondary structure disruption of the tRNA (Mkaouar-Rebai et al., 2009), no further studies beyond the identification of the mutation have been carried out and functional studies on the effects of the m.5559A>G *MT-TW* mutation are lacking.

Here we have identified three siblings who are homoplasmic for the m.5559A>G mutation in *MT-TW* resulting in decreased tRNA^{Trp} levels and generalized reduction of *de novo* mitochondrial protein synthesis. The siblings have LS associated with reduction in respiratory complexes and decreased OXPHOS. Interestingly we identify that the stability of mitochondrial RNAs is reduced and the mutation causes the accumulation of an unprocessed RNA intermediate containing the tRNA^{Trp}.

2. Materials and Methods

2.1 Approval

All sample collection and experimentation was performed with appropriate informed consent according to approvals given by the Human Research Ethics Office at the University of Western Australia.

2.2 Mitochondrial genome sequencing

MtDNA was extracted from patient blood using a Qiagen DNA extraction kit and amplified in two overlapping fragments using the following primer pairs (Tang et al., 2010): Fragment 1: forward 5'-AACCAAACCCCAAAGACACC-3' and reverse 5'-GCCAATAATGACGTGAAGTCC-3'. Fragment 2: forward 5'-TCCCCTCCTAACACATCC-3' and reverse 5'-TTTATGGGGTGATGTGAGCC-3' using an Expand LR kit (Roche) following manufacturers recommended protocols. The two fragments were combined in equimolar amounts for library preparation and sequencing on an IonTorrent sequencer (Life Technologies) at the Lotteries West State Biomedical Facility, Genomics node (LSBFG). Sequences were aligned to the mitochondrial genome (NC_012920) and variants called using the IonReporter software suite. Pyrosequencing was performed by AGRF (Perth Node) according to the manufacturer's protocol and quantification of the heteroplasmy level was carried out using the PyroMark Q24 Method 012 that compared the peak heights at the wild-type and mutant nucleotides at position 5559.

2.3 Cell culture

Fibroblasts were obtained from a skin biopsy of patient (II-3) and a control subject and were cultured at 37°C under humidified 95% air/5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies) containing glucose (4.5 g.l⁻¹), 1 mM pyruvate, 2 mM glutamine, penicillin (100 U.ml⁻¹), streptomycin sulfate (100 µg.ml⁻¹) and 10 % fetal bovine serum (FBS).

2.4 Mitochondrial isolation

Mitochondria were prepared from 10^7 cells grown overnight in 15 cm² dishes and isolated as described previously (Rackham et al., 2009) with some modifications. Mitochondria were lysed for 30 min in buffer containing 250 mM sucrose, 100 mM KCl, 20 mM magnesium acetate, 10 mM Tris-HCl pH 7.5, 0.5 % Triton X-100 and EDTA-free Complete protease inhibitor cocktail (Roche).

2.5 Immunoblotting

Specific proteins were detected using mouse monoclonal antibodies against: β -actin, porin, NDUFA9 (a subunit of Complex I), Complex II subunit 70, Complex III subunit core 2 (UQCRC2), COX1, COXII, COXIV and ATP synthase subunit alpha (ATP5A) (Abcam, diluted 1:1000), in Odyssey Blocking Buffer (Li-Cor). IR Dye 800CW Goat Anti-Rabbit IgG or IRDye 680LT Goat Anti-Mouse IgG (Li-Cor) secondary antibodies were used and the immunoblots were visualized using an Odyssey Infrared Imaging System (Li-Cor).

2.6 Quantitative RT-PCR

The abundance of mitochondrial RNAs and unprocessed transcripts was measured on RNA isolated from cells using the miRNeasy RNA extraction kit (Qiagen). Levels of mitochondrial transcripts were measured from RNA isolated from cells or purified mitochondria. cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen) and used as a template in the subsequent PCR that was performed using a Corbett Rotorgene 6000 using SensiMix SYBR mix (Bioline) and normalised to 18S rRNA.

2.7 Northern blotting

RNA (5 µg) was resolved on 1.2% agarose formaldehyde gels, then transferred to 0.45 µm Hybond-N⁺ nitrocellulose membrane (GE Lifesciences) and hybridized with biotinylated oligonucleotide probes specific to mitochondrial mRNAs, rRNAs and tRNAs. The hybridizations were carried out overnight at 50°C in 5x SSC, 20 mM Na₂HPO₄, 7% SDS and 100 µg.ml⁻¹ heparin, followed by washing. The signal was detected using either streptavidin-linked horseradish peroxidase or streptavidin-linked infrared antibody (diluted 1: 2000 in 3x SSC, 5% SDS, 25 mM Na₂HPO₄, pH 7.5) by enhanced chemiluminescence (GE Lifesciences) or using an Odyssey Infrared Imaging System.

2.8 Mitochondrial protein synthesis

Cells were grown in six-well plates until 80% confluent and *de novo* protein synthesis was analyzed as described previously (Davies et al., 2009).

2.9 OXPHOS enzyme assays and respiration

Spectrophotometric enzyme assays assessing mitochondrial OXPHOS activity in skeletal muscle and liver biopsies were performed as described previously (Frazier and Thorburn, 2011). Mitochondrial respiration was measured in permeabilized cells according to Kuznetsov *et al* (Kuznetsov et al., 2008).

3. Results

The family consists of two males and one female with different fathers. The mother, who is heteroplasmic for the identified mutation, is clinically unaffected.

Patient II-1

The proband in this family was a newborn male born prematurely at 34 weeks gestation weighing 1150 grams. Pregnancy was complicated by illicit drug and alcohol use and

symmetrical intrauterine growth retardation (IUGR) throughout gestation. The infant demonstrated a failure to thrive, with height and weight consistently under the 3rd percentile. He had cholestatic jaundice and progressive hepatosplenomegaly from birth. Blood lactate levels were elevated to a maximum of 4.1 mmol/L. Magnetic resonance imaging (MRI) of the brain at 10 weeks showed oedema within the parietal and occipital white matter and increased lactate peak in the basal ganglia (Fig 1A-C). A further MRI of the brain aged 3 years showed an increased T2 signal within the white matter bilaterally and increased bilateral thalamic signal, corresponding with lactate peaks on MR spectroscopy (MRS). Several seizures have been documented and although no epileptic discharges were recorded on EEG, there was an abnormal background rhythm. A liver biopsy at 4 months showed active hepatitis with non-specific cholestasis. Respiratory enzyme analysis of liver tissue indicated low activity of complexes I, III and IV (Supplementary Table 1). Liver function tests showed some improvement by 5 years of age. There was global developmental delay, especially in the area of speech. Neuropsychometric testing placed his intellectual functioning at the extremely low range. At 8 years of age there is an absence of speech, however he is able to use Makaton signing to communicate a few words.

Patient II-2

The male sibling of patient II:1 was born at 35 weeks gestation weighing 1300 grams. Again there was illicit substance abuse and IUGR throughout pregnancy. Blood lactate levels were recorded at levels up to 4.3 mmol/L. Hypospadias and bilateral undescended testes were present at birth. Assessment at 13 months showed severe global developmental delay again with speech being the most distinctive area of delay. An MRI of the brain showed reduced white matter in the frontal regions, posterior corpus callosum, caudate nucleus and thalami. An MRS demonstrated a significant lactate peak at the level of the thalami. Cholestatic hepatitis with abnormal liver function resulting in cirrhosis was present. The right kidney was

malformed with a Pelvi-ureteric junction (PUJ) obstruction, resulting in hypertension. A right nephrectomy was performed at 5 years of age. Hypertrophic cardiomyopathy was diagnosed at 3 years of age. Respiratory enzyme analysis of liver and muscle tissue indicated low activity of complexes I, III and IV (Supplementary Table 1).

Patient II-3

The female sibling of these two males was born at 30 weeks gestation with a birth weight of 996 grams. Again there was severe IUGR and drug and alcohol use throughout pregnancy. Blood lactate levels were measured several times with a maximum result of 27.5 mmol/L (ref range < 2.1mmol/L). Psychomotor assessment at 13 months demonstrated a severe developmental delay, with hypotonia. There is a severe persisting failure to thrive, with all measurements well below the 3rd percentile. At the age of 10 months liver function was within normal.

3.1 A homoplasmic m.5559A>G mutation in the MT-TW gene causes Leigh Syndrome

To identify the disease causing mutation in this family with LS, we carried out next generation sequencing of the mitochondrial genome of one affected sibling in two overlapping fragments of 7 kb and 9 kb. Dataset analysis identified a previously reported (Mkaouar-Rebai et al., 2009) mutation m.5559A>G in the *MT-TW* gene that affects the A48 residue in the T-stem of tRNA^{Trp}. We confirmed that the identified mutation was homoplasmic in all three affected siblings, by Sanger and pyrosequencing, and heteroplasmic in the asymptomatic mother (Figure 1C). The mutation was homoplasmic in the blood of all three patients, in the liver of II-1 and also in fibroblasts from II-3 (Figure 1D), indicating that different tissues maintained the homoplasmic mutation.

3.2 Mutation in MT-TW affects mitochondrial RNA metabolism processing and abundance

We investigated the consequences of the m.5559A>G MT-TW mutation on mitochondrial function in fibroblasts from patient II-3 relative to control fibroblasts. Investigation of the abundance of mature and unprocessed mitochondrial transcripts by northern blotting showed that the m.5559A>G mutation causes a significant loss of the mature tRNA^{Trp} and enrichment in the unprocessed polycistronic transcript containing this tRNA (Fig 2A). In addition, we observe a varying decrease in other mitochondrial DNA encoded RNAs including other tRNAs, some mRNAs such as *MT-CO1*, but no change in the two rRNAs (Fig 2A), likely as a consequence of the dramatic reduction in tRNA^{Trp} (Fig 2A) that may have downstream effects on overall mitochondrial RNA stability. We investigated if the mutation affects the processing of mitochondrial polycistronic transcripts by qRT-PCR across the entire mitochondrial transcriptome and we show accumulation of an intermediate transcript, ND2-tRNA^{Ala}, that contains the unprocessed tRNA^{Trp} in the patient compared to control cells (Fig 2B). We do not observe significant accumulation of other pre-processed intermediates suggesting that the m.5559A>G mutation causes a specific processing defect for this tRNA. Furthermore we observe that there is a slight decrease in some mt-mRNAs indicating decreased stability of these mRNAs (Fig 2C), likely as a consequence of decreased protein synthesis given the mutation is in a tRNA.

3.3 The m.5559A>G mutation leads to reduced mitochondrial protein synthesis and oxygen consumption

We investigated the effect of the mutation on translation of mt-mRNAs by ³⁵S-labeled *de novo* incorporation of methionine and cysteine into mitochondrial DNA encoded proteins. We observed an overall decrease of mitochondrial protein synthesis in the patient compared to controls, albeit to varying levels (Fig 3A). Immunoblotting against mitochondrial DNA encoded polypeptides that form the OXPHOS complexes showed a decrease in their

abundance, but not in nuclear encoded components of these complexes (Fig 3B). We observed a slight increase in the nuclear encoded complex II subunit in the patient, likely in response to lowered mitochondrial DNA encoded proteins. Interestingly we observed an increase in mtDNA copy number in the patient cells compared to control (Fig 3C), likely as a compensatory response to decreased mitochondrial DNA gene expression and biogenesis.

The effect of the m.5559A>G mutation on mitochondrial oxygen consumption was measured in digitonin-permeabilized patient and control cells. Mitochondrial oxygen consumption from substrates entering at complexes I, III and IV was significantly lowered when compared to control cells (Fig 4), likely as a direct consequence of decreased mitochondrial protein synthesis and consequent reduction in the protein components of the OXPHOS system.

4. Discussion

Here we used next generation sequencing of mtDNA to identify an m.5559A>G mutation in a family with LS-plus and investigated the functional consequences of the mutation on mitochondrial function. The mutation is at residue A48 that is part of the mitochondrial tRNA^{T^{TP}} T-stem. This mutation has been reported previously at 43% heteroplasmy in a single affected child with psychomotor delay, vomiting and lactate acidosis and symmetric hyperintense signal on MRI of the head of the caudate nucleus and putamen (Mkaouar-Rebai et al., 2009). In our study, the 3 affected individuals were homoplasmic and their unaffected mother was 46% heteroplasmic for the mutation. The mutated residue is highly conserved in mammals and the predicted effect on the tRNA is a loss of the T-stem and disruption of the secondary structure and function.

There is only one pathogenic mutation m.3302A>G in tRNA^{Leu(UUR)} shown to date that causes accumulation of a specific pre-processed transcript, RNA19, in patients with

mitochondrial myopathy and decreased OXPHOS (Bindoff et al., 1993). Our findings indicate that the m.5559A>G mutation causes a significant decrease of tRNA^{Trp} but also impaired processing of the polycistronic transcript that contains tRNA^{Trp} suggesting that changes in the secondary structure of this tRNA may obscure the processing site required for excision of this tRNA. *In silico* structural predictions for tRNA^{Trp} containing the m.5559A>G mutation suggest the formation of a larger loop in the T-stem (Mkaouar-Rebai et al., 2009), which may affect the 3' processing site required for cleavage by the ELAC2 enzyme (Brzezniak et al., 2011; Lopez Sanchez et al., 2011). Therefore our work describes an additional pathogenic mutation that causes a processing defect in another mitochondrial RNA processing intermediate.

Furthermore we observed a varied decrease in the stability of other mitochondrial transcripts in the patient cells, which may be a downstream effect of the mutation, possibly a consequence of reduced mitochondrial protein synthesis. Decreased protein synthesis resulted in reduced steady state levels of mitochondrial DNA encoded polypeptides and significant reduction of oxygen consumption at the three electron pumping complexes, confirming that mutation of tRNA^{Trp} causes general OXPHOS dysfunction. This defect is a key feature of mitochondrial diseases, including the Leigh syndrome identified in this family. Similar OXPHOS defects have been identified in patients carrying mutations at different positions in the *MT-TW* gene, indicating that functional tRNA^{Trp} is required for OXPHOS complex biogenesis and function (Barić et al., 2013; Smits et al., 2010). Based on the findings in this study and the additional independent report of this mutation (Mkaouar-Rebai et al., 2009) we conclude that the this mutation is definitely pathogenic with a score of 15 according to the pathogenicity scoring system (Blakely et al., 2013; Yarham et al., 2011).

The functional analyses in our study provide mechanistic support that the m.5559A>G mutation identified previously in a single affected individual (Mkaouar-Rebai et

al., 2009) and in our three patients, is a disease-causing mutation. Furthermore, the clinical features described in our family are more widespread. All three patients have typical features of LS, but in addition, organs such as liver, kidney, and heart are affected. This more widespread involvement is most likely due at least in part to the homoplasmy detected in the affected tissues of the children in this family, compared to the 43% heteroplasmy detected in the affected individual described previously (Mkaouar-Rebai et al., 2009). The liver dysfunction observed in the three individuals with the m.5559A>G mutation is noteworthy as hepatopathies have been identified in many mitochondrial disorders, commonly involving defects in the mitochondrial translational system, although usually in nuclear genes rather than mtDNA (Sasarman et al., 2011; Schara et al., 2011; Zeharia et al., 2005). Many mutations in nuclear genes that encode protein components of the mitochondrial translational machinery result in compromised biogenesis of specific or all mitochondrial OXPHOS complexes and lead to decreased OXPHOS. Some of these have been shown to cause accumulation of lipids in hepatocytes (Boczonadi and Horvath, 2014; Lee and Sokol, 2007), including a mouse model of mitochondrial dysfunction that involves hepatic steatosis (Richman et al., 2015).

5. Conclusions

Identification and functional validation of mutations in mitochondrial disease remains an important priority to enable the correct diagnosis and management of the disease. Next generation sequencing has facilitated faster identification of DNA variants, however the functional analyses of these variants remain an important priority for validation of disease pathogenicity.

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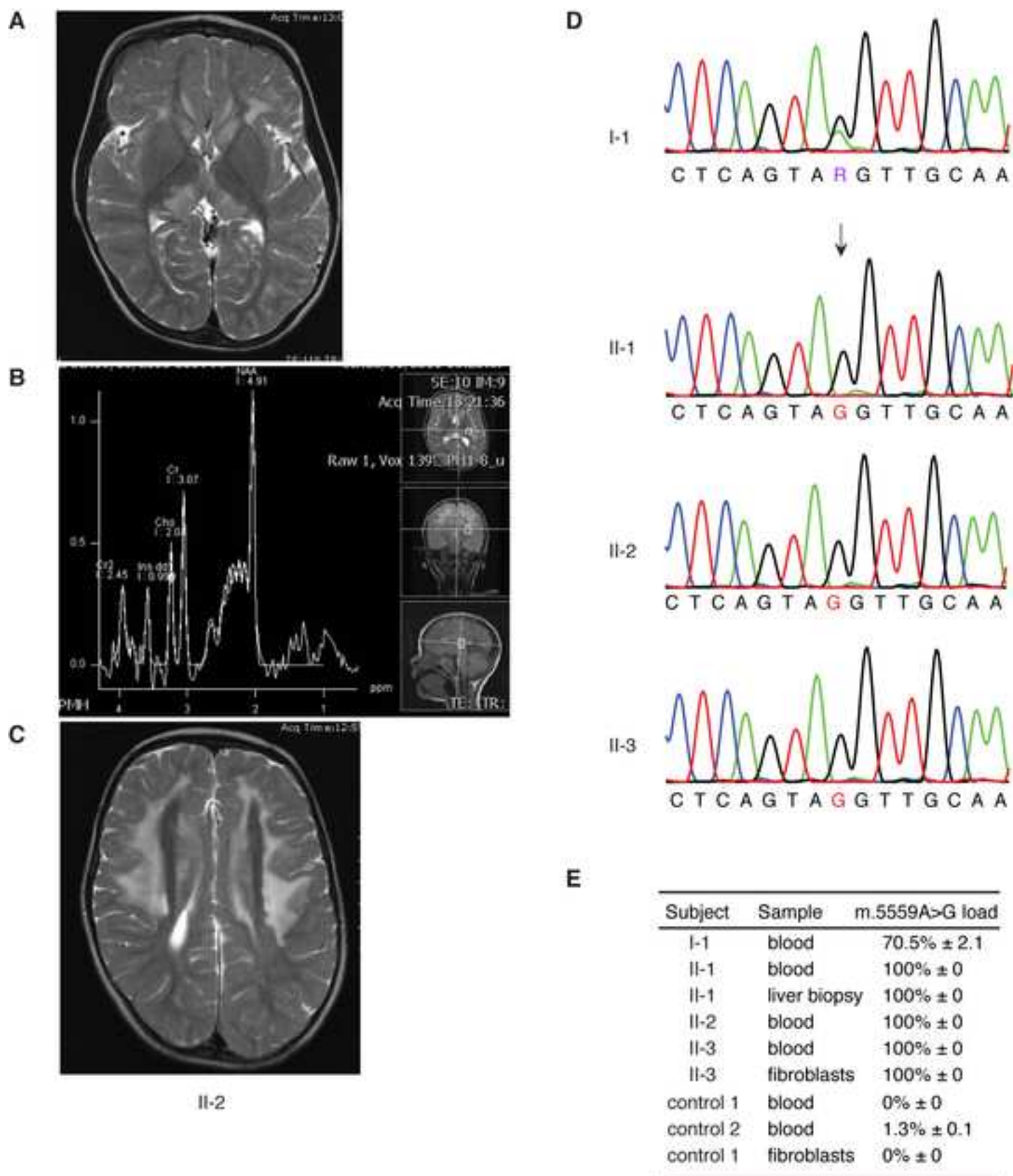
FIGURE LEGENDS

Figure 1. Mutation in m.5559A>G causes LS in three siblings. (A) MRI of II-2 aged 3.5 years, showing symmetrical increased signal within the thalami bilaterally, corresponding to lactate peaks on spectroscopy (B); and a pattern of leukoencephalopathy (C). (D) Sanger sequencing chromatograms showing the heteroplasmic load of the m.5559A>G mutation in the mother and homoplasmic distribution of the same mutation in the three siblings. (E) Mutation load of the m.5559A>G mutation in different tissues determined by pyrosequencing.

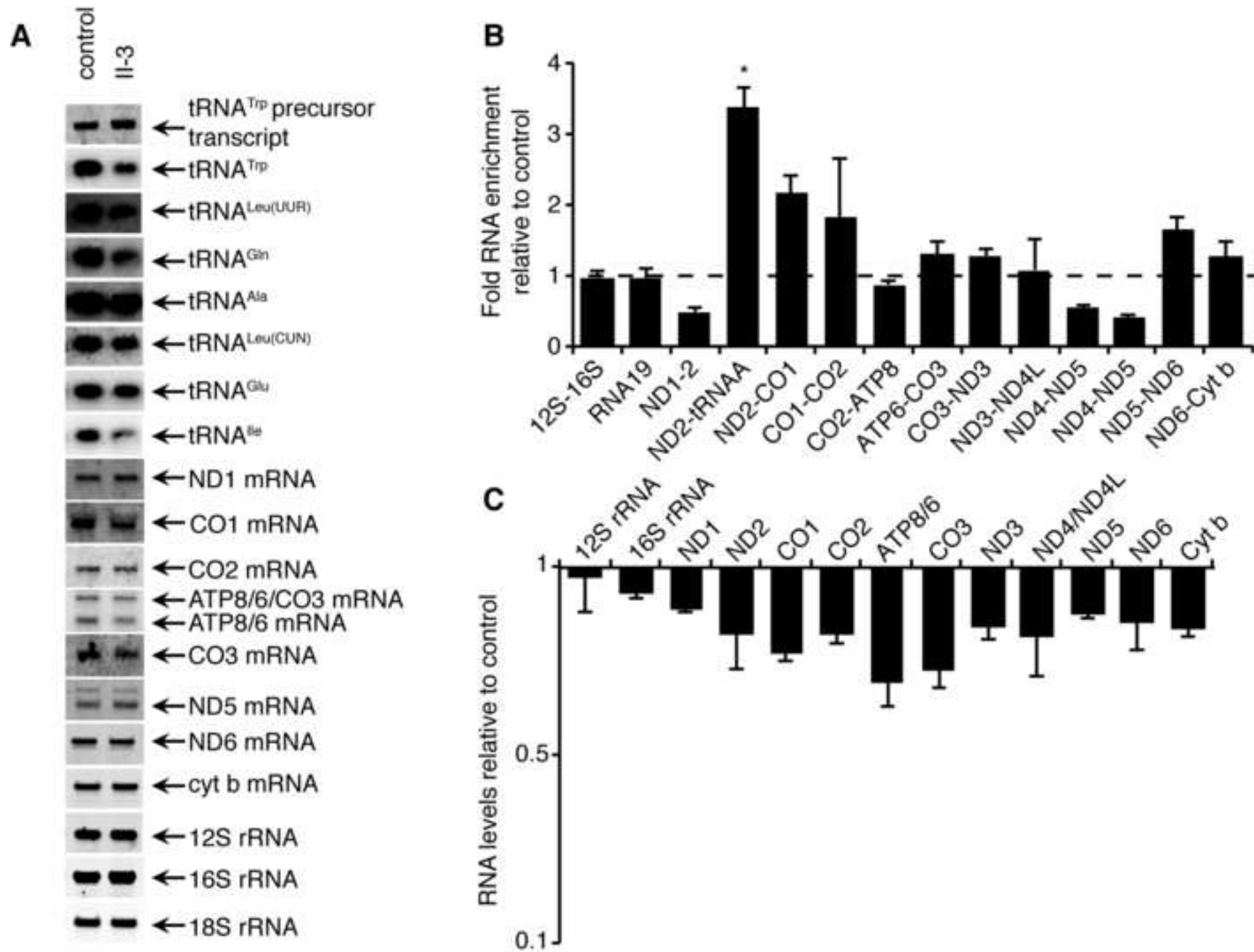
Figure 2. The m.5559A>G mutation causes misprocessing of tRNA^{Trp}. (A) The abundance of mitochondrial RNAs was analyzed by northern blotting and 18S rRNA was used as a loading control. (B) The processing intermediates and mitochondrial mRNA levels (C) were measured by qRT-PCR and expressed as fold change relative to control fibroblasts. Data were normalized to 18S rRNA levels and are means \pm SD of three separate experiments.

Figure 3. The m.5559A>G mutation affects mitochondrial protein synthesis. (A) Mitochondrial translation was measured by *de novo* pulse incorporation of ³⁵S-labelled methionine and cysteine. Equal amounts of cell lysate protein (50 μ g) were separated by SDS-PAGE and visualized by autoradiography using Coomassie staining as a loading control. (B) Immunoblotting of mitochondrial proteins in patient and control cells. (C) MtDNA copy number was measured by qPCR and expressed as a ratio to the nuclear encoded β -globin gene in patient and control cells.

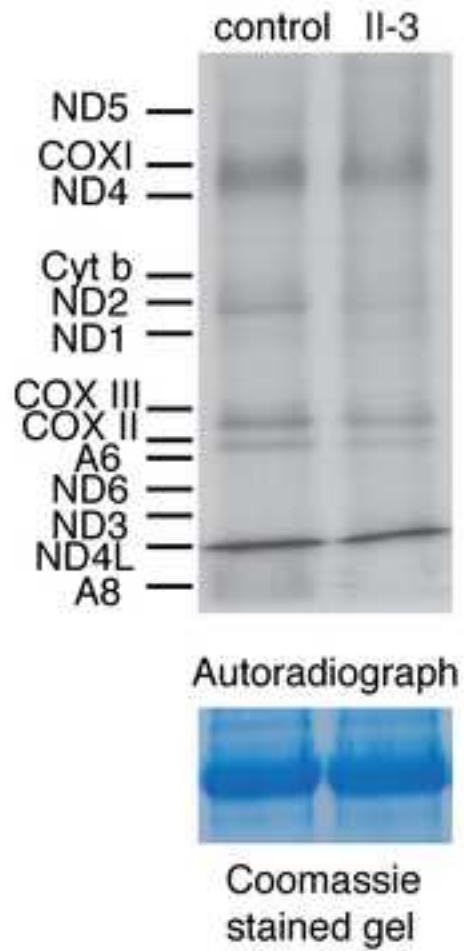
Figure 4. Decreased mitochondrial oxygen consumption in patient II-3 fibroblasts compared to control was measured using a high resolution OROBOROS respirometer.



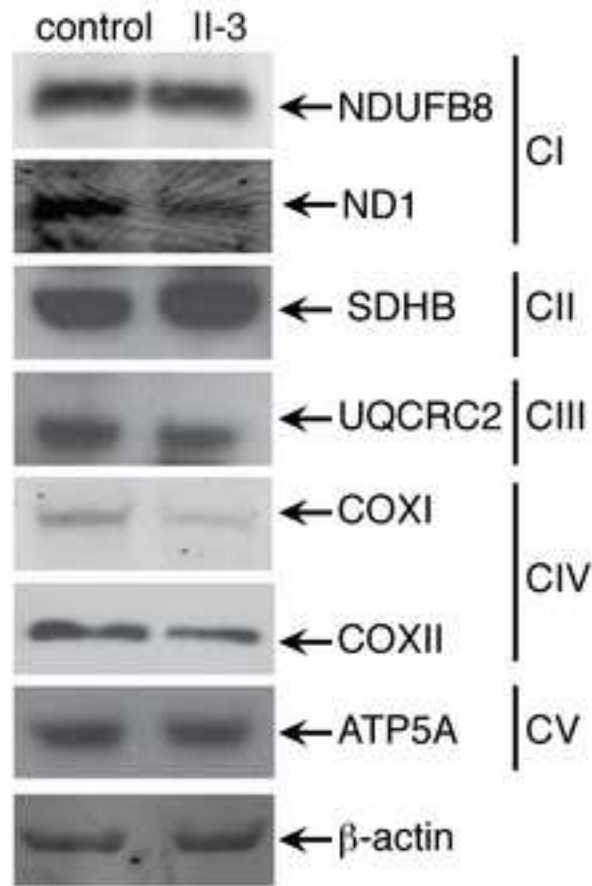
Figure(s)
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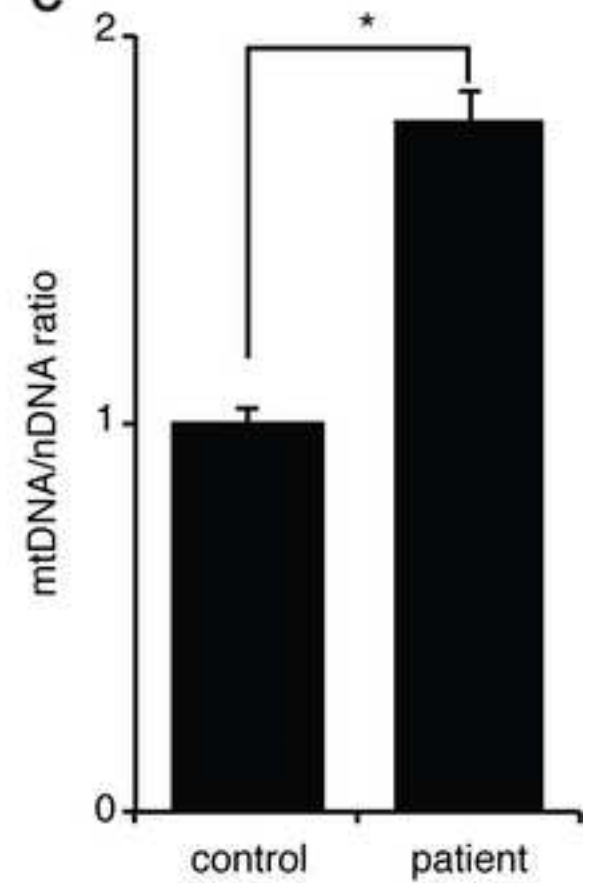
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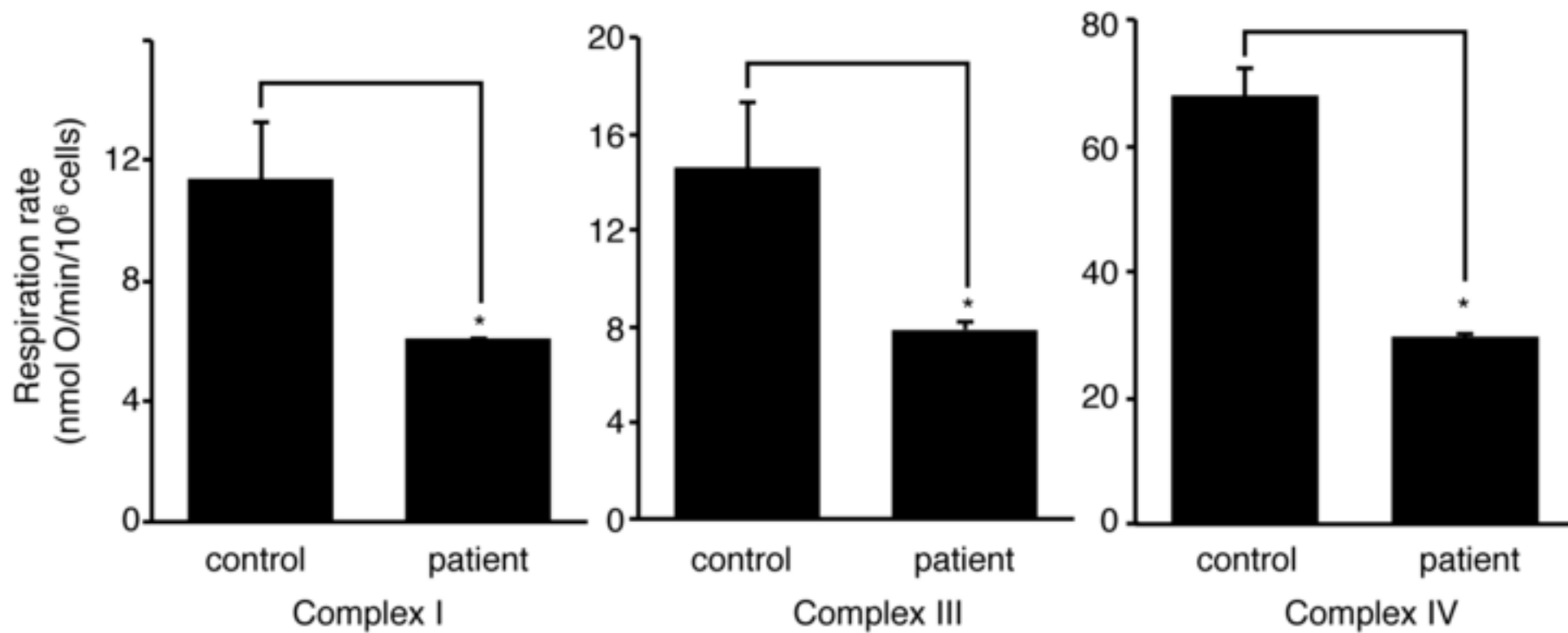


B



C





Supplementary material

[Click here to download Supplementary material: Supplementary Table 1.docx](#)