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► **To cite this version:**

Saskia Hoefs, Francjan van Spronsen, Ellen Lenssen, Leo Nijtmans, Richard Rodenburg, et al.. NDUFA10 mutations cause complex I deficiency in a patient with Leigh disease. *European Journal of Human Genetics*, 2010, 10.1038/ejhg.2010.204 . hal-00598937

HAL Id: hal-00598937

<https://hal.science/hal-00598937>

Submitted on 8 Jun 2011

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***NDUFA10* mutations cause complex I deficiency in a patient with Leigh disease**

Saskia J.G. Hoefs¹, Francjan J. van Spronsen², Ellen W.H. Lenssen¹, Leo G. Nijtmans¹,
Richard J. Rodenburg¹, Jan A.M. Smeitink¹, Lambert P. van den Heuvel^{1,3,*}

¹Department of Paediatrics, Nijmegen Centre for Mitochondrial Disorders, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

²Beatrix Children's Hospital, University Medical Centre of Groningen, University of Groningen, Groningen, The Netherlands

³Department of Paediatrics, Catholic University Leuven, Leuven, Belgium

*Corresponding author

Dr. L.P. van den Heuvel

Nijmegen Centre for Mitochondrial Disorders

Department of Paediatrics

Radboud University Nijmegen Medical Centre

P.O. Box 9101

6500 HB Nijmegen

The Netherlands

E-mail: B.vandenHeuvel@cukz.umcn.nl

Phone: +31 24 3617983 or 31 24 3614428

Fax: +31 24 3618900

Running title: Leigh syndrome caused by *NDUFA10* mutations

Abstract

Mitochondrial complex I deficiency is the most common defect of the oxidative phosphorylation system. We report a patient with Leigh syndrome who showed a complex I deficiency expressed in cultured fibroblasts and muscle tissue. To find the genetic cause of the complex I deficiency, we screened the mitochondrial DNA and the nuclear encoded subunits of complex I. We identified compound heterozygous mutations in the *NDUFA10* gene, encoding an accessory subunit of complex I. The first mutation disrupted the start codon, and the second mutation resulted in an amino acid substitution. The fibroblasts of the patient displayed decreased amount and activity, and a disturbed assembly of complex I. These results indicate that *NDUFA10* is a novel candidate gene to screen for disease-causing mutations in patients with complex I deficiency.

Keywords: Complex I deficiency; *NDUFA10* gene; Leigh syndrome

Introduction

NADH:ubiquinone oxidoreductase (E.C.1.6.5.3.), or complex I, is the first and largest of the five complexes of the oxidative phosphorylation (OXPHOS) system. Its function is binding and oxidizing NADH to free electrons, which are transferred to the electron acceptor ubiquinone. The energy released during this electron transfer is used to translocate protons across the inner mitochondrial membrane, generating a proton gradient, which can be used for the synthesis of ATP. Complex I consists of 45 subunits of which 7 are encoded by the mitochondrial DNA (mtDNA). It is an L-shaped complex consisting of a hydrophobic membrane arm embedded in the mitochondrial inner membrane and a hydrophilic peripheral arm protruding into the matrix. The complex can be divided into 3 functional modules. The dehydrogenase module is important for the oxidation of NADH, the hydrogenase module plays a role in the transport of electrons to ubiquinone, and the proton translocation module is involved in proton pumping.^{1,2}

Isolated complex I deficiency is the most common defect of the OXPHOS system, accounting for approximately 23% of all patients with childhood respiratory chain deficiency.³ It has a wide clinical variety affecting one or more tissues or organs.⁴ The organs with the highest energy demand like heart, brain, skeletal muscle tissue and liver are the most affected organs. Due to the bigenomic control of the OXPHOS system, mutations causing complex I deficiency can be found in either the mtDNA or in genes encoded by the nuclear DNA. Previous studies identified disease-causing mutations in nuclear structural genes encoding for the seven core subunits (NDUFS1, NDUFS2, NDUFS3, NDUFS7, NDUFS8, NDUFV1, and NDUFV2) and five accessory subunits of complex I (NDUFS4, NDUFS6, NDUFA1, NDUFA2, and NDUFA11).⁵⁻⁸

Furthermore, mutations have been described in eight assembly factors (NDUFAF1, **5b** NDUFAF2, NDUFAF3, NDUFAF4, C8orf38, C20orf7, ACAD9, and NDUBPL) of this complex and in an uncharacterized protein (FOXRED1) causing complex I deficiency.⁹⁻

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Although pathogenic mutations have been described in accessory subunits, the function of these subunits is not exactly known yet. It has been suggested that some are important for the biogenesis of complex I. One of these subunits is NDUFA10. The predicted 355-amino acid human protein is for 80% identical to the 42-kDa bovine homolog. This subunit is located in the hydrophobic protein fraction of complex I and might therefore be involved in the transfer of protons. Furthermore, NDUFA10 is one of the subunits that undergoes post-translational modification: it can be phosphorylated at **9** a single amino acid, serine-59.^{17,18}

6a In the present communication, we describe two compound heterozygous mutations in a patient with a complex I deficiency expressed in cultured fibroblasts and muscle tissue. Cell biological studies were performed to show the functional consequences of the genetic variations. A new gene responsible for human complex I deficiency has been identified.

Materials and Methods

Case report

- 1 The patient, a boy, was born after a normal pregnancy of 32 weeks with a section caesaria due to fetal distress. His birth weight was 2715 gram. He had a normal start and neonatal period. From early on, he showed hypotonia. His milestones were uneventful with regard to laughing, contact, grabbing things, and rolling over to his back, but he did not reach sitting position, and head control remained poor. At 10 months of age he was referred for evaluation of the cause of his retarded development and hypotonia. Tendon reflexes were somewhat increased. Therefore, it was concluded that there was a central cause of hypotonia together with retarded development. His blood and cerebrospinal fluid lactate were 8.6 and 4.9 mmol/l, respectively (reference value 0.5-2.2 mmol/l), with increased lactate to pyruvate ratios (being around 20 on more than one occasion and the one measurement in cerebrospinal fluid). His cerebral MRI showed symmetrical lesions in especially the basal ganglia and substantia nigra. Based on the high lactate concentrations and the increased lactate: pyruvate ratio, a defect of pyruvate dehydrogenase complex or within the oxidative phosphorylation was considered likely. Biochemical investigations were performed in muscle and fibroblasts (Table 1). We started with thiamine, and a ketogenic diet administered by gastrointestinal tube feeding. Due to analyses of blood gasses showing a pH of 7.12 with 4 mmol/l of bicarbonate, sodium bicarbonate was started, resulting in normalisation of pH with some respiratory compensation ($p\text{CO}_2$ usually between 3.5 and 4.0 kPa). With these strategies, growth showed adequate response so that his early fragility disappeared. His development improved with uttering noises rather than clear words. During the next months he displayed periods of abnormal breathing with hypo- and

hyperventilation. At one of the occasions he seemed to have a convulsion. Routine cardiac evaluation by ultrasound suggested some hypertrophic cardiomyopathy without clear clinical relevance at that moment. During one such episode of convulsions he experienced a severe cardiac respiratory arrest. His X ray of the thorax showed massive pleural fluid most probably as a result cardiac decompensation by hypertrophic cardiomyopathy. Notwithstanding treatment with mechanical ventilation with high pressures, and the use of cardiovascular agents to stimulate contractility followed by resuscitation procedures were unsuccessful, and he died at 23 months of age. In the last days of life he also showed hypoglycaemia (glucose 1.6 mmol/l), a finding not being observed before, possibly due to multi organ failure rather than isolated liver failure. At autopsy besides the already known cardiomyopathy degenerative abnormalities were reported in the liver, spleen, kidneys, and pancreas. Apart from these findings the most outspoken abnormalities were seen in the brain: in the thalamus, the pons, and also in the hippocampus degradation of myeline, decreased myelinisation, proliferation of vessels, some gliosis and some foam cell response was reported. Especially from the abnormalities found in the thalamus and the pons the diagnosis classical Leigh syndrome was concluded.

Cell culture and biochemical analysis

Skin fibroblasts were cultured in M199 medium (Life Technologies) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Measurement of the mitochondrial respiratory chain enzyme activities in muscle tissue and skin fibroblasts were performed according to established

procedures.^{19,20} The values were expressed relative to the activity of cytochrome *c* oxidase and/or citrate synthase.

2a *Genetic analysis of the mtDNA and nuclear complex I genes*

Patient DNA was extracted from cultured skin fibroblasts by standard procedures.²¹

- 3** Oligonucleotide primers were designed from the mtDNA sequence and from the gene sequences of all nuclear encoded complex I subunits. The Genbank accession number of the *NDUFA10* gene sequence is NM_004544.3). Other Genbank accession numbers and primer sequences are available upon request. The size of the amplification products were checked with a 1.5% agarose gel and the amplicons were sequenced directly with the BigDye terminator cycle sequencing chemistry on a Applied Biosystems ABI PRISM 3130xl Genetic Analyzer. The nucleotide numbering follows cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

BN-PAGE analysis and in-gel activity assay

Blue native-gradient gels (5-15%) were cast and run with 40 µg of digitonin-isolated mitochondria as described previously.^{22,23} After electrophoresis, the gels were further processed for in-gel activity assay or second-dimension 10% SDS-PAGE followed by immunoblot analysis as described by Calvaruso et al.²²

SDS-PAGE analysis

Mitochondrial lysates were separated on a 10% SDS-PAGE gel. Next, the proteins were transferred to PROTAN nitrocellulose membrane (Schleicher & Schuell) for immunodetection.

Antibodies and ECL detection

Western blotting was performed with primary antibodies raised against the complex I subunits NDUFA9 (Invitrogen), NDUFS3 (Invitrogen), NDUFA10 (Sigma-Aldrich), ND1 (Dr. A. Lombes, Paris), and the complex II 70 kDa subunit (SDHA; Invitrogen).

- 11** Secondary antibodies used were peroxidase-conjugated anti-mouse (Invitrogen), anti-rabbit (Invitrogen), or anti-chicken IgGs (SouthernBiotech) and detection of the signal performed using ECL Plus (Amersham Biosciences) according to the manufacturer's instructions.

Results

Biochemical studies

Investigation of the activities of the respiratory enzyme complexes in cultured fibroblasts and fresh muscle tissue of the index patient revealed a severely reduced complex I activity (Table 1). The enzymatic activities were 29% and 7% from the lowest value of the control range in the fibroblasts and muscle tissue, respectively. Furthermore, the activity of complex III in muscle tissue was slightly decreased (68% of the lowest control value).

*Molecular genetic studies in the *NDUFA10* gene*

7 We searched for the molecular defect causing the complex I deficiency by screening the entire mtDNA, followed by screening the genes for all 38 nuclear encoded subunits. We excluded the presence of disease-causing mutations in the mtDNA. Sequence analysis of the nuclear *NDUFA10* gene demonstrated the presence of 2 heterozygous substitutions (Figure 1A). The first mutation is a substitution from A-to-G (c.1A>G) in exon 1, which disrupts the start codon methionine (p.Met1?). The next possible start codons can be found 116 and 118 nucleotides upstream. However these alternative start codons are either not in frame or abolish the mitochondrial target sequence, which makes both unlikely to be functional. The father of the index patient was carrier of this mutation. The second mutation was found in exon 3 (c.425A>G), causing a substitution of amino acids (p.Gln142Arg). This mutation affects a highly conserved amino acid (Figure 1B), and the mutation software SIFT predicted this mutation to be pathogenic, 2b because it affects the protein function with a score of 0.00. Substitutions with score less than 0.05 are predicted to affect protein function. The mother of the patient was found

to be heterozygous for this mutation. Both mutations were not present in 108 healthy controls or 21 other patients with isolated complex I deficiency expressed in cultured fibroblasts. From these observations we conclude that it is highly likely that both mutations are pathogenic.

12 *Effect of the NDUFA10 mutations on the activity and steady state level of complex I*

The effect of the mutations on the activity of complex I was examined by performing BN-PAGE analysis followed by Western blotting with an anti-NDUFA9 antibody to detect complex I amounts, and in-gel complex I activity measurements with mitochondria enriched fractions of the cultured patient fibroblasts. Figure 2A shows a marked decrease of complex I activity in patient fibroblasts compared to the control cells. Furthermore, the expression of NDUFA9, indicative of the amount of fully assembled complex I, was severely decreased. These results are in agreement with the spectrophotometric complex I observations in the patient fibroblasts.

Next, we performed SDS-analysis to study the effect of the mutations on the expression of complex I. Figure 2B shows a clear decrease in the protein level of NDUFA10 in the patient compared to the control. The expression of the NDUFA9 subunit was also decreased.

Effect of the NDUFA10 mutations on the assembly of complex I

To test whether the assembly of complex I is disturbed, we performed two-dimensional BN/SDS-PAGE analysis followed by immunoblotting with antibodies directed against NDUFS3 and ND1 (Figure 3). This figure shows a decreased amount of fully assembled complex I and an increased amount of subcomplexes in cultured fibroblasts of the

patient compared to the control, suggesting a disturbed assembly or stability of complex I. Remarkably subcomplexes 4 and 5, as previously described²⁴ are accumulated, indicating that subunit NDUFA10 is incorporated in complex I after the formation of subcomplex 5. This suggests that NDUFA10 will be incorporated in a late stage of complex I assembly.

Discussion

6b This is the first report describing compound heterozygosity for two mutations in the *NDUFA10* gene in a patient with complex I deficiency expressed in both cultured fibroblasts as well as in muscle tissue. One of the mutations is located in the sequence encoding start codon for translation and the other mutation introduced an amino acid substitution from glutamine to arginine. Several lines of evidence suggest that these compound heterozygous mutations are the underlying cause of the complex I deficiency. First, the start codon disappeared and the second mutation affected a highly conserved amino acid. Second, the father was carrier of the first mutation and the mother of the second mutation. Third, both mutations were not present in 108 controls and 21 patients with isolated complex I deficiency. Fourth, the patient fibroblasts showed decreased amount and activity of complex I, and last, we demonstrated a disturbed assembly of complex I in the patient fibroblasts.

Besides the severely reduced complex I deficiency, the activity of complex III in muscle tissue was also somewhat reduced. This observation has been described before in several complex I deficiencies, and could be due to the fact that in the normal situation, supercomplexes can be found in which complex I and III occur together (CI/CIII₂).²⁵⁻²⁸

13 Therefore, screening of *NDUFA10* should not be limited to only patients with isolated complex I deficiencies, but also patients with a reduced complex I deficiency combined with a reduction in complex III activity should be screened for the presence of disease-causing mutations.

The 7 mtDNA encoded subunits together with 7 nuclear encoded subunits form the minimal constitution of complex I, since these are involved in catalysis of electron transfer from NADH to ubiquinone and for the generation of the proton gradient. Most

mutations causing complex I deficiencies have been described in these genes. However, genes encoding accessory subunits, like *NDUFS4*, *NDUFS6*, *NDUFA1*, *NDUFA2* and *NDUFA11* may also contain pathogenic mutations.^{8-10,29,30} *NDUFA10* also belongs to these accessory subunits.

Not much is known about the function of the accessory subunits, but the mutations observed in the *NDUFA10* gene indicate that at least this subunit is important for proper functioning of complex I. *NDUFA10* is one of the subunits that can be phosphorylated, which could be important for the function of this subunit, since phosphorylation of complex I subunits has been involved in regulation of enzymatic activity and possibly affects electron transfer.^{17,18} Therefore, *NDUFA10* might play a role in the control of the activity of complex I through binding of NADH.^{31,32} Furthermore, it has been described that unlike other protein components of the membrane arm, *NDUFA10* is a relatively hydrophilic protein and is thought to be generally weakly associated with complex I.^{33,34} The phosphorylation might influence the binding affinity of *NDUFA10*

14 and regulate the amount of fully active complex. In addition, the loose association of the *NDUFA10* subunit would make it more accessible to external interactions with other proteins like kinases and phosphatases.

Complex I deficiency is one of the most frequently diagnosed defects of the OXPHOS system. At present, the diagnosis is often only based on biochemical measurements of the single enzyme activities of the OXPHOS system, since the genetic cause is still unknown in many patients. Therefore, improved methods like genomic microarray screening or exome sequencing of the nuclear genes encoding subunits and assembly factors of complex I may be useful tools in the near future. Our finding of mutations in accessory subunit *NDUFA10* emphasises that disease causing mutations are not

restricted to a certain set of subunits and genetic screening should involve all subunits. Identifying new mutations causing complex I deficiency is important to improve genetic counselling and to get more insight in the function of complex I.

Acknowledgments

This work was supported by the European Community's sixth Framework Programme for Research, Priority 1 "Life sciences, genomics and biotechnology for health", contract number LSHM-CT-2004-005260 (MITOCIRCLE).

Conflict of interest

The authors declare no conflict of interest.

Web Resources

The URLs for data presented herein are as follows:

Genbank, <http://www.ncbi.nlm.gov/Genbank>

SIFT, <http://sift.jcvi.org>

Tables

Table 1. Respiratory chain enzyme activities in skin fibroblasts and fresh muscle tissue of the index patient. Deficiencies are indicated in bold.

Complex	Patient fibroblasts	Control range	Patient fresh muscle	Control range
15 I	30	104-206 ^a	5	70-250 ^c
II	n.m.	-	119	67-177 ^c
III	1428	1270-2620 ^a	1490	2200-6610 ^c
IV	1026	684-1190 ^b	1067	810-3120 ^c

8a ^a mU/U Cytochrome *c* oxidase

8b ^b mU/mU Citrate synthase

^c mU/U Citrate synthase

n.m. = not measured

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Figure Legends

Figure 1. Molecular analysis of the *NDUFA10* gene. (A) Electropherograms showing the wild-type sequence of *NDUFA10* (top) and the nucleotide changes in fibroblasts of the patient (bottom). Arrows represent the nucleotide substitutions, c.1A>G (left) and, c.425A>G (right). (B) Conservation of the altered amino acid (p.Gln142Arg) is shown in a Clustal W alignment.

Abbreviations are as follows: Wt: wild-type; P: index patient

Figure 2. The mutations in *NDUFA10* result in decreased activity and amount of complex I. (A) BN-PAGE analysis followed by an in-gel activity assay demonstrates a decreased complex I activity in fibroblasts of the patient compared to control cells. The amount of subunit NDUFA9 is also severely reduced, indicating a decreased amount of whole complex I. The amount of complex II is not affected. (B) SDS-PAGE analysis and immunoblotting with an antibody directed against NDUFA10 shows that the amount of NDUFA10 is decreased in patient fibroblasts. The amount of NDUFA9 is also reduced, suggesting a reduction in amount of complex I. The amount of complex II is not affected.

Abbreviations are as follows: C: control; P-A10: patient with *NDUFA10* mutations; CI, II: complexes I, II; IGA: in-gel activity; SDHA: succinate dehydrogenase complex subunit A

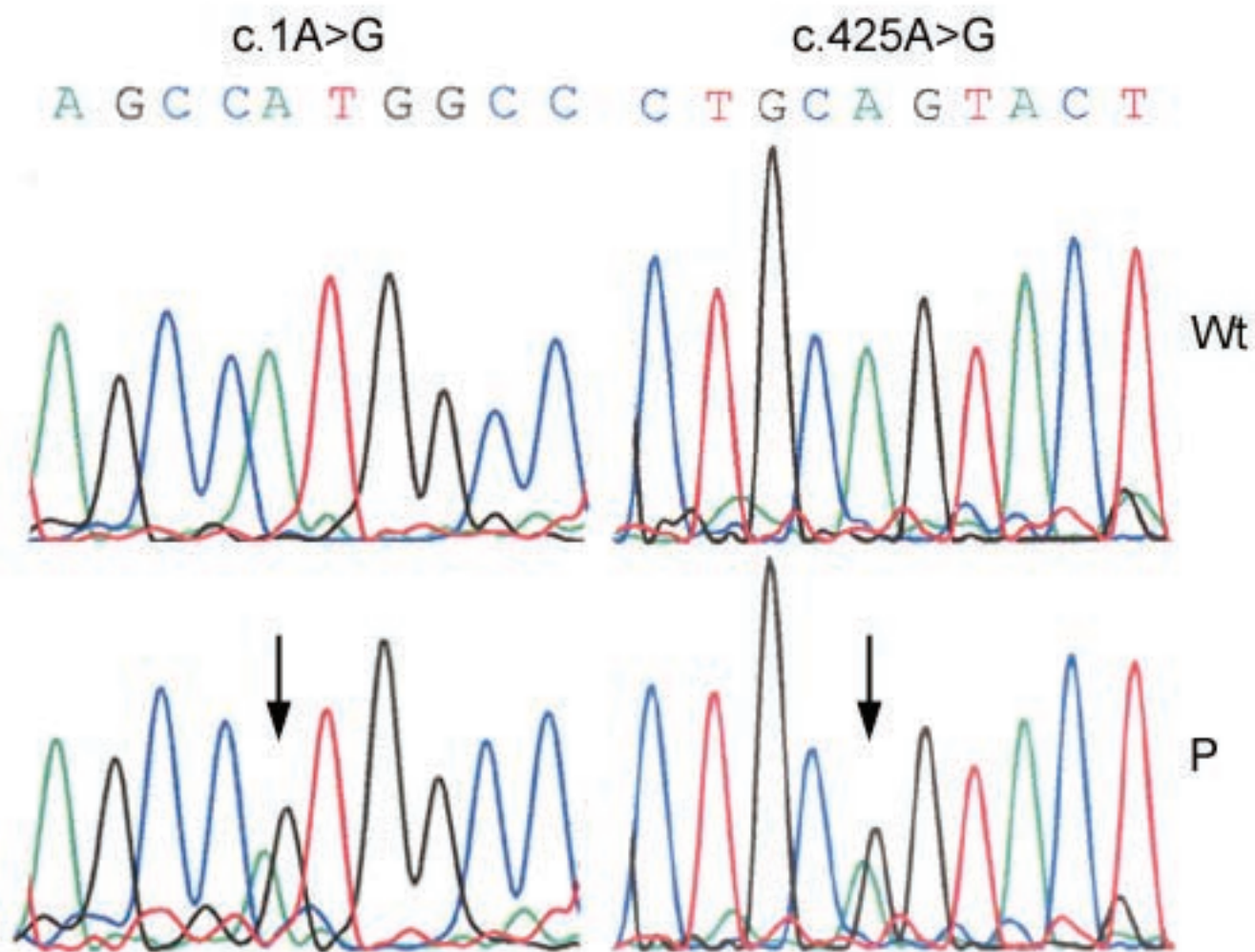
Figure 3. The mutations in *NDUFA10* result in accumulation of subcomplexes of complex I. Two-dimensional BN/SDS-PAGE analysis followed by immunoblotting with antibodies directed against NDUFS3, to investigate the early steps of the complex I

assembly, and ND1, to examine the incorporation of the mtDNA encoded subunits, shows the accumulation of mainly the subcomplexes 4 and 5 in the index patient. The subcomplexes are numbered according the assembly model described by Vogel and colleagues.²⁴

Abbreviations are as follows: C: control; P-A10: patient with *NDUFA10* mutations; CI: complex I

Figure 1

A



B

Gln142Arg

Patient	RLQSWLYSSRLLRYSDALEHLLTTG
H. sapiens	RLQSWLYSSRLLQYSDALEHLLTTG
P. troglodytes	RLQSWLYSSRLLQYSDALEHLLTTG
B. taurus	RLQAWLYASRLLQYADALEHLLSTG
M. musculus	RLQSWLYASRLLQYADALEHLLSTG
D. rerio	RLQAWMYLMRLLQYSDAVEHLLTTG
D. melanogaster	QFQIRMVYMLRYSQYIDALQHVLTSTG
C. elegans	AMQDRIFNCRFDQYLNALAHILNTG

Figure 2

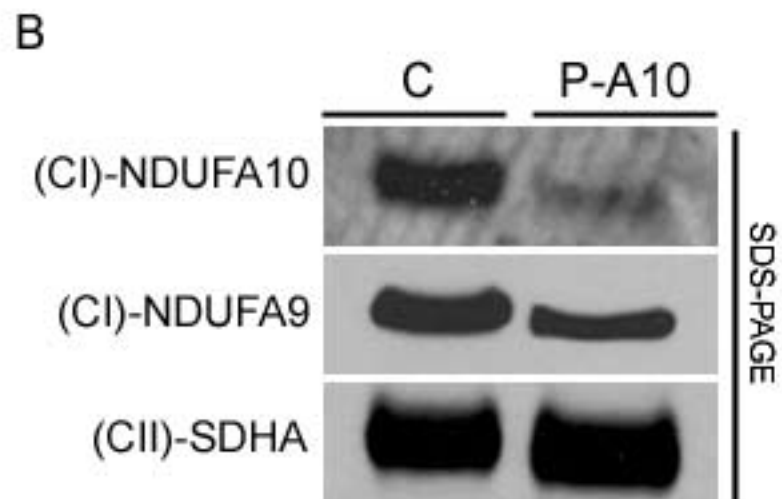
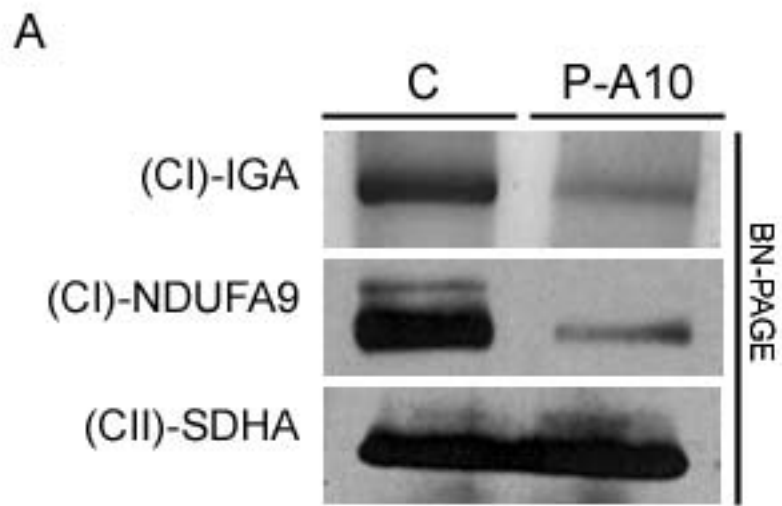


Figure 3

