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# Gene

## Next generation sequencing identifies novel PMPCA variants in patients with dominant optic atrophy --Manuscript Draft--

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<b>Corresponding Author:</b>	majida charif, Ph.D Universite Mohammed I Oujda: Universite Mohammed Premier Oujda oujda, Oujda MOROCCO
<b>First Author:</b>	majida charif, Ph.D
<b>Order of Authors:</b>	majida charif, Ph.D Arnaud Chevrollier Naïg Gueguen Selma Kane Céline Bris David Goudenège Valerie Desquiret-Dumas Isabelle Meunier Fanny Mochel Luc Jeanjean Fanny Varenne Vincent Procaccio Pascal Reynier Dominique Bonneau Patrizia Amati-Bonneau Guy Lenaers
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	progressive degeneration.
<b>Suggested Reviewers:</b>	Audrey Meunier, MD Department of Ophthalmology , Centre Hospitalier Universitaire Saint-Pierre , Brussels , Belgium audreymeunier@gmail.com request reviewer
	Chiara La Morgia, Ph.D IRCCS Institute of Neurological Sciences of Bologna: IRCCS Istituto Delle Scienze Neurologiche di Bologna chiara.lamorgia@unibo.it request reviewer
	Michael Larsen, MD Copenhagen University Hospital: Rigshospitalet lars.michael.larsen@regionh.dk request reviewer
	SMIRNOV VASILY, MD Faculté de Médecine de Lille: Université de Lille Faculté de Médecine VASILY.SMIRNOV@CHRU-LILLE.FR request reviewer
	DRUMARE Isabelle, MD The Hospital for Sick Children Department of Ophthalmology and Vision Sciences Isabelle.DRUMARE@CHRU-LILLE.FR request reviewer
<b>Opposed Reviewers:</b>	

**Abstract**

Dominant Optic Atrophy (DOA) is one of the most common inherited mitochondrial diseases, responsible for sensorineural blindness. It is caused by the chronic degeneration of the retinal ganglion cells (RGCs), the axons of which form the optic nerve. Until now, DOA has been mainly associated with genes encoding proteins involved in mitochondrial network dynamics. Using next generation and exome sequencing, we identified in five patients with a late onset primary DOA, the first heterozygous variants in *PMPCA*, which encodes an alpha subunit of the mitochondrial peptidase (MPP), responsible for the cleavage and maturation of the mitochondrial precursor proteins imported from the cytoplasm into mitochondria. Recently, *PMCPA* has been identified as the gene responsible for Autosomal Recessive Cerebellar Ataxia type 2 (SCAR2) and another severe recessive mitochondrial disease. In this study, four *PMPCA* variants were identified, two are frame-shift (c.309delA and c.820delG) classified as pathogenic and two missenses (c.1363G>A and c.1547G>A) classified with uncertain pathological significance. Functional assays on patient's fibroblasts show a hyperconnection of the mitochondrial network and revealed that these variants reduced  $\alpha$ -MPP levels, while not significantly affecting the respiratory machinery was noticed. These results suggest that alterations of mitochondrial peptidase function can affect the fusion-fission balance, a key element in maintaining the physiology of retinal ganglion cells, and consequently lead to their progressive degeneration.

**Next generation sequencing identifies novel *PMPCA* variants in patients with dominant optic atrophy**

Majida Charif<sup>1,2</sup>, Arnaud Chevrollier<sup>1</sup>, Naïg Gueguen<sup>1,3</sup>, Selma Kane<sup>1</sup>, Céline Bris<sup>1,3</sup>, David Goudenège<sup>1,3</sup>, Valerie Desquiret-Dumas<sup>1,3</sup>, Isabelle Meunier<sup>4</sup>, Fanny Mochel<sup>5</sup>, Luc Jeanjean<sup>6</sup>, Fanny Varenne<sup>7</sup>, Vincent Procaccio<sup>1,3</sup>, Pascal Reynier<sup>1,3</sup>, Dominique Bonneau<sup>1,3</sup>, Patrizia Amati-Bonneau<sup>1,3</sup> and Guy Lenaers<sup>1,\*</sup>

<sup>1</sup> Université d'Angers, MitoLab team, UMR CNRS 6015 - INSERM U1083, Unité MitoVasc, SFR ICAT, Angers, France.

<sup>2</sup>Genetics and immuno-cell therapy Team, Mohammed First University, Oujda, Morocco.

<sup>3</sup>Departments of Biochemistry and Genetics, University Hospital Angers, Angers, France.

<sup>4</sup>Institut des Neurosciences de Montpellier, INSERM U1051, Université de Montpellier, France

<sup>5</sup> INSERM U 1127, CNRS UMR 7225, Sorbonne Universités, UPMC Univ Paris 06 UMR S 1127, Institut du Cerveau et de la Moelle épinière, ICM, F-75013 Paris, France; AP-HP, Pitié-Salpêtrière University Hospital, Department of Genetics, Paris, France; University Pierre and Marie Curie, Neurometabolic Research Group, Paris, France.

<sup>6</sup>Department of Ophthalmology, Nîmes University Hospital, Nîmes Cedex 9.

<sup>7</sup> Department of Ophthalmology, Hôpital Pierre Paul Riquet CHU Purpan, Toulouse, France.

(\*). Corresponding author: Guy LENAERS.

MitoLab team, Mitochondrial Medicine Research Centre, Institut MitoVasc, Université d'Angers, UMR CNRS 6015, INSERM U1083, CHU Bât IRIS/IBS

Rue des Capucins, 49933 Angers cedex 9, FRANCE.

e-mail: [guy.lenaers@inserm.fr](mailto:guy.lenaers@inserm.fr)

Tel : 33/0 244 68 84 87

Fax: 33/0 244 68 65 94

## Highlights

- We identified four novel *PMPCA* variant in DOA patient.
- Hyperconnection of mitochondrial network and reduced PMPCA levels were disclosed.
- Alterations of mitochondrial peptidase function can affect mitochondrial dynamics.

## Abstract

Dominant Optic Atrophy (DOA) is one of the most common inherited mitochondrial diseases, responsible for sensorineural blindness. It is caused by the chronic degeneration of the retinal ganglion cells (RGCs), the axons of which form the optic nerve. Until now, DOA has been mainly associated with genes encoding proteins involved in mitochondrial network dynamics. Using next generation and exome sequencing, we identified in five patients with a late onset primary DOA, the first heterozygous variants in *PMPCA*, which encodes an alpha subunit of the mitochondrial peptidase (MPP), responsible for the cleavage and maturation of the mitochondrial precursor proteins imported from the cytoplasm into mitochondria. Recently, *PMCPA* has been identified as the gene responsible for Autosomal Recessive Cerebellar Ataxia type 2 (SCAR2) and another severe recessive mitochondrial disease. In this study, four *PMPCA* variants were identified, two are frame-shift (c.309delA and c.820delG) classified as pathogenic and two missenses (c.1363G>A and c.1547G>A) classified with uncertain pathological significance. Functional assays on patient's fibroblasts show a hyperconnection of the mitochondrial network and revealed that these variants reduced  $\alpha$ -MPP levels, while not significantly affecting the respiratory machinery was noticed. These results suggest that alterations of mitochondrial peptidase function can affect the fusion-fission balance, a key

element in maintaining the physiology of retinal ganglion cells, and consequently lead to their progressive degeneration.

**Abbreviations:**

CS: citrate synthase; DOA: dominant optic atrophy; ION: inherited optic neuropathy; mtDNA: mitochondrial DNA; LHON: Leber's hereditary optic neuropathy; RGC: retinal ganglion cell; SCAR2: Autosomal Recessive Cerebellar Ataxia type 2. SCA28: Spinocerebellar ataxia 28.

**Keywords**

Dominant optic atrophy, mitochondrial peptidase, mitochondrial dynamic, heterozygous variants, retinal ganglion cell degeneration.

## 1. Introduction:

Dominant optic atrophy (DOA, MIM \*605290) is an inherited optic neuropathy with a prevalence estimated to 1 in 25,000 (Carelli et al., 2004; Newman and Biouesse, 2004; Yu-Wai-Man et al., 2011; Lenaers et al., 2012). DOA affects the visual acuity by altering the central visual field and the color vision, due to a progressive loss of retinal ganglion cells (RGCs) and their axons that form the optic nerve, ensuring the transmission of visual information from the retina to the brain. Most patients have an age of onset in the first decade of life, some may experience functional blindness and others can be relatively asymptomatic (Eleni Bagli, Anastasia K Zikou, Niki Agnantis, 2017). DOA can be also a syndromic disorder called DOA *plus* in 20% of cases, with secondary symptoms affecting auditory, neuronal and muscular functions.

Sixty to seventy percent of DOA cases harbor pathogenic variants in nuclear *OPA1* gene (Le Roux et al., 2019), which was the first gene identified to cause DOA (Alexander et al., 2000; Delettre et al., 2002). But recently, an increasing number of novel DOA genes were identified through the introduction of next generation sequencing technologies (Jurkute et al., 2019), including *OPA3*, *MFN2*, *SPG7*, *AFG3L2*, *DNM1L*, and *SSBPI* (Charif et al., 2015; Charif et al., 2020; Gerber et al., 2017; Klebe et al., 2012; Piro-Mégy et al., 2020; Reynier et al., 2004; Rouzier et al., 2012; Lenaers et al., 2021). These genes are all involved directly in mitochondrial function, mostly mitochondrial dynamics, with the exception of *SSBPI*, which is involved in mtDNA replication. In addition, the *WFS1* gene, responsible for the Wolfram syndrome, is also responsible for DOA associated with neuro-sensorial deafness (Rendtorff et al., 2011), and for isolated recessive isolated optic atrophy (Grenier et al., 2016).

In mitochondria, the mitochondrial processing peptidase (MPP) play the most important role in preprotein processing compared to other mitochondrial peptidases (Gakh et al., 2002). *PMPCA* (MIM \* 613036), a nuclear gene localized on human chromosome 9q34.3, encodes the  $\alpha$ -subunit of mitochondrial processing peptidase ( $\alpha$ -MPP), a protein that participates in the cleavage of the mitochondrial targeting peptide of nuclear-encoded mitochondrial precursor proteins upon their import into mitochondria (Teixeira and Glaser, 2013). Without its function, abnormal nuclear-encoded mitochondrial precursor proteins accumulate inside mitochondria, disrupts mitochondrial functions and halts cell growth. In 2015, *PMCPA* has been identified as the gene responsible for Autosomal Recessive Cerebellar Ataxia type 2 (SCAR2), a severe mitochondrial diseases and later to a Leigh-like syndrome with spastic ataxia (Jobling et al., 2015; Choquet et al., 2016; Joshi et al., 2016; Takahashi et al., 2020)

In the present study, we identified the first heterozygous variants of *PMPCA* in 5 families with primary DOA. Fibroblasts characterization show a tendency to mitochondrial network hyper-connection in *PMPCA* patient fibroblasts, and revealed decreased levels of PMPCA protein.

## **2. Materials and Methods:**

### *2.1. Consent for Genetic investigations:*

Written informed consent to perform genetic analyses was obtained from each subject involved in this study or from the parents of individuals under 18 years of age, according to protocols approved by the Ethical Committees of the different Institutes involved in this study, and in agreement with the Declaration of Helsinki (Institutional Review Board Committee of the University Hospital of Angers, Authorization number: AC-2012-1507).

### *2.2. Genetic analysis*

Genomic DNA was extracted from peripheral blood cells from cohorts of DOA and sporadic cases of optic atrophy, initially screened for *OPA1*, *OPA3* and *WFS1* exonic sequences and all mutations in the mitochondrial DNA responsible for LHON. Negative cases were analysed using a resequencing gene panel dedicated to the clinical molecular diagnosis of inherited optic neuropathies (ION). Further negative samples, among which the first patient with a *PMPCA* variant (Family 3) were analyzed by whole exome sequencing. Then *PMPCA* molecular screening was included in the ION panel and led to the discovery of family 1, 2, 4 and 5. Library preparation, sequencing, bioinformatics and variants analysis were done as previously described (Charif et al., 2020; Gerber et al., 2017; Charif et al., 2021)

### *2.3. Fibroblasts study*

Fibroblasts from *PMPCA* individuals P1: I:1 and P2: II:2 from family 1 and 3 respectively, were generated from skin biopsies and cultured in 2/3 Dulbecco's Minimum Essential Medium (DMEM, Gibco) supplemented with 1/3 AmnioMAX (Gibco), 10% fetal calf serum (Lonza)

and 1% Penicillin-Streptomycin-Amphotericin B (Lonza). Respiratory chain enzymatic activities and Western blot were assessed as described (Jobling et al., 2015; Gerber et al., 2017).

### 2.3.1. *Time lapse and Deconvolution microscopy.*

To assess the mitochondrial network dynamic (Codron et al., 2018), cells were incubated during 20 minutes with MitoTracker green FM (Invitrogen™) to visualize mitochondria (green). Coverslips were mounted in housing and placed on the stage of an inverted wide-field microscope ECLIPSE Ti-E (Nikon, Tokyo, Japan) equipped with a 100X oil immersion objective (Nikon Plan Apo100x, Tokyo, Japan, N.A. 1.45) and an Andor NEO sCOMS camera controlled by Metamorph7.7 software (Molecular Devices, Sunnyvale, CA). A precision, piezoelectric driver mounted underneath the objective lens allowed faster Z-step movements, keeping the sample immobile while shifting the objective lens. 35-one image planes were acquired along the Z-axis at 0.1mm increments. For mitochondrial network characterization, acquired images were iteratively deconvolved using Huygens Essentialsoftware (Scientific Volume Imaging, Hilversum, The Netherlands), with a maximum iteration scored 50 and a quality threshold at 0.01. Imaris 8.0 ® software (Bitplane) was used for 3D processing and morphometric analysis. Time lapse images of 90ms duration (5sec interval) were acquired at fixed temperature 25°C.

### 2.3.2. *Immunofluorescence*

Human skin fibroblasts were seeded at a density of ~90,000 cells per well in a six-well plate containing 20-mm coverslips and incubated overnight. Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min. After fixation, cells were quickly washed 3 times in PBS and then cells were incubated in the blocking buffer (BF; PBS with 5% BSA) for 15

min. Coverslips were then washed in PBS three times and were incubated overnight with primary antibody diluted in the BF at 4°C on a rocking platform providing a gentle “wave” effect. Coverslips were then washed in BF three times for 5 min and subsequently incubated for 90 min at room temperature with goat anti-mouse Alexa 647 IgG (H+L) secondary antibody diluted in BF in dark chamber. Finally, coverslips were washed in PBS two times for 5 min, and keep in PBS at 4 degrees up to the assembly for the STORM acquisition.

Immunodetection of nucleoids was achieved on fixed fibroblasts using antibodies against DNA (clone AC-30-10) Progen GmbH and co-stained with Citrate synthase antibodies. Image treatment was performed using Imaris spot detection software (Bitplane) to visualize and quantify mtDNA/nucleoid number per cell and mtDNA/nucleoid spacing within mitochondria. For mitophagy exploration, co-staining with autophagosomes marker anti-microtubule-associated protein 1 light chain 3 $\alpha$  (LC3, ab48394, 1:500; Abcam) and mitochondrial marker citrate synthase were performed. Images acquisitions were performed using deconvolution microscopy.

### *2.3.3. STORM Acquisition*

For super resolution imaging, the cavity of a clean single depression slide (Paul Marienfeld, Lauda-Königshofen, Germany) was filled with 50  $\mu$ L of switching buffer (Abbelight, Paris, France), and covered by the coverslip, the sample side facing downward. The device was placed on the stage of an inverted motorized microscope NIKON ECLIPSE Ti-E (Nikon Instruments Europe, Amsterdam, The Netherlands) equipped with a CFI SR APO TIRF 100X ON1.49 objective, a Perfect Focus System, and a total internal reflection fluorescence (TIRF) ILas2 module (Roper Scientific, Martinsried, Germany).

Acquisition of images were proceeded using Metamorph 7.7 software (Molecular Devices, CA, USA). Image sequences were acquired with a single-photon sensitive camera Evolve 128TM EMCCD 512 x 512 imaging array, 16 x 16  $\mu\text{m}$  pixels (Photometrics, Tucson, AZ, USA). Acquisitions were performed at fixed temperature 25°C in a dark heating chamber (Okolab NA, Pozzuoli, Italy). Phase contrast was first used for orientation and focus adjustment. Prior to STORM imaging, a multichannel TIRF fluorescence microscopy image was acquired for subsequent comparison with STORM image. Images were acquired with an integration time of 60 ms per frame. The total acquisition time points for each sequence were adapted to the observed structure and to the labelling density (5,000 to 20,000 frames). Images were analyzed and reconstructed using the WaveTracer module integrated into Metamorph software (Kechkar et al., 2013).

### 3. Results

#### 3.1. Identification of *PMPCA* variants in individuals with primary dominant optic atrophy

Using whole exome sequencing, we screened for mutations in unrelated patients presenting an inherited optic neuropathies (ION) without a negative molecular diagnosis after analyzing *OPA1*, *OPA3*, *WFS1*, *SPG7*, *AFG3L2*, *DNM1L*, *MFN2* exonic sequences, and mutation in the mitochondrial DNA responsible for LHON. After eliminating frequent ( $>1/10.000$ ) and non-pathogenic variants, according to the Sift, Polyphen and Mutation-Taster prediction tools, a first heterozygous mutation was identified in *PMPCA*, which led to include this gene in the ION panel. Three additional heterozygous *PMPCA* variants were identified in four families, two unreported and two being referenced with an allele frequency of  $4,01.e^{-6}$  and  $8,01.e^{-6}$  (Table1). Two variants (c.309delA and c.820delG) are pair base deletions causing a frame-shift (p.Lys103AsnfsTer74 and p.Val274SerfsTer27); and two are missense mutations (c.1363G>A and c.1547G>A) causing the p.Ala455Thr and p.Arg516His amino-acid changes, respectively (Table1). These variants were confirmed by Sanger sequencing and analyzed for segregation whenever DNA samples were available (Figure 1).

#### 3.2. Clinical manifestations of *PMPCA* patients

All individuals included were referred to ophthalmology departments for poor visual acuity. All patients are male aged between 30 and 69 years who were primarily diagnosed with an isolated dominant optic atrophy, three had a normal brain MRI imaging. Patients II.1 from family 1 and II.1 from family 4 disclosed a severe optic atrophy with visual acuities ranging from 0.5/10 to counting fingers (Table 1). Additional symptoms were observed, as a multiple sclerosis for patient II.1 from family 4 and a peripheral neuropathy for patient II.1 from family 2 (Table 1).

### *3.3. Functional effects of PMPCA variants*

To evaluate the consequences of *PMPCA* variants on mitochondrial physiology, two fibroblast cell lines were derived from individuals P1: I:1 and P2: II:2 from family 1 and 3. Mitochondrial network overlaid on phase contrast revealed hyper-connected structures in cells from *PMPCA* patients, compared to control (Figure 2 A). Western blots experiments, using *PMPCA*, *PMPCB*, *OPA1* and citrate synthase (*CS*) antibodies revealed a 25% reduction of *PMPCA* level, lower than expected for frame-shift mutations, and equal levels of *PMPCB* and *OPA1* proteins (Figure 2 B). Enzymatic activities of the respiratory chain complexes did not reveal significant difference between control and mutated fibroblasts (Figure 2 C). In addition, no alteration was noticed in the analysis of *PMPCA* distribution in the mitochondrial network (Figure 2 D).

#### 4. Discussion

Dominant optic atrophy is a heterogeneous group of diseases caused by selective loss of retinal ganglion cells (RGCs) and ascending degeneration of the optic nerve. In this study, we describe, the first four *PMPCA* heterozygous variants in five unrelated families with primary DOA. These variants are either novel or referenced with a very low frequency in gnomAD database. Recessive mutations in *PMPCA* have been described in the context of non-progressive cerebellar ataxia and a severe Leigh like syndrome associated to spastic ataxia (Jobling et al., 2015; Joshi et al., 2016; Choquet et al., 2016; Takahashi et al., 2020). Thus, except for *OPAI*, all other DOA genes were identified initially in severe mitochondrial diseases essentially recessive, then causing DOA by dominant variants. Again, this is the case for *PMPCA*, for which we disclosed the involvement of heterozygous variants in a rather late onset optic atrophy. Because among the four mutations identified, two were frame shifts, it should be advised to examine the parents of *SCAR2* patients who are carriers of other frame-shift mutations.

Interestingly, among the five patients identified with *PMPCA* variants, two had additional neurological symptoms, as peripheral neuropathy for the index case of family 2 and multiple sclerosis for the index case from family 4. This parallels the observations that multisystemic manifestations, including neurological symptoms, are reported in up to 20% of *OPAI* mutation carriers and in LHON patients (Amati-Bonneau et al., 2008; Amati-bonneau and Pasquier, 2003; Hudson et al., 2008; Yu-Wai-Man et al., 2016; Priglinger et al., 2019). Thus, functional alterations of *PMPCA* might predispose to the emergence of neurological symptoms, in addition to jeopardizing RGC function and survival.

*PMPCA* encodes the alpha subunit of the mitochondrial processing peptidase (MPP), which

cleaves the targeting peptide of nuclear-encoded mitochondrial precursor proteins upon their import into mitochondria (Teixeira and Glaser, 2013). Our observation of *PMPCA* fibroblasts revealed decreased level of PMPCA protein, although not reaching a 50% decrease, as it would be expected for cells with frame-shift mutations, and deeply contrasting with the observation of a severe PMPCA depletion in SCAR2 patient lymphoblasts, associated to impaired frataxin production and processing (Jobling et al., 2015; Joshi et al., 2016). In addition, a hyper-connected mitochondrial network, was observed in PMPCA conditions, as already reported in fibroblasts from DNM1L and OPA3 patients affected by DOA (Reynier et al., 2004; Gerber et al., 2017). Conversely, we did not disclose alteration of the respiratory chain, nor of PMPCA distribution in the mitochondrial network, but well some rare events of mitochondria-autophagosome contact sites suggestive of increased mitophagy (data not show).

From these results, we conclude that the identification of *PMPCA* heterozygous variants points to a novel gene responsible for DOA, and a novel pathophysiological mechanism responsible for retinal ganglion cell degeneration, most likely milder from the one responsible for SCAR2 presentation caused by bi-allelic *PMPCA* mutations.

## **5. Conclusion**

In conclusion, we report the first DOA patients with heterozygous *PMPCA* variants, confirming the heterogeneity of these pathologies, and the important role of mitochondrial import in the maintenance of retinal ganglion cells integrity.

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## **Contribution of each author to the study**

- 1) Conception and design of the study: P. Amati-Bonneau and Guy Lenaers.
- 2) Clinical investigation and phenotyping : Isabelle Meunier, Fanny Mochel, Fanny Varenne, Luc Jeanjean, Dominique Bonneau.
- 3) Acquisition and analysis of data : Majida Charif, Arnaud Chevrollier, Naïg Gueguen, Selma Kan, Céline Bris, David Goudenège, Valerie Desquirit-Dumas, Vincent Procaccio, Patrizia Amati-Bonneau, Pascal Reynier,
- 4) Drafting a significant portion of the manuscript and figures: Majida Charif, Arnaud Chevrollier and Guy Lenaers.

## **Conflicts of Interest:**

All authors declare that they have no conflict of interest with the publication of the data presented here.

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## **Legends to the figures:**

### **Figure 1: *PMPCA* pedigrees and amino acid changes localization**

**A:** Description of the pedigrees with *PMPCA* variants and their segregation among the families.

**B:** The structure of the *PMPCA* protein with the amino acid changes associated to DOA on the top, and associated to Spinocerebellar ataxia, autosomal recessive 2 (*SCAR2*) and a progressive mitochondrial encephalopathy, on the bottom.

### **Figure 2: Mitochondrial dynamic and *PMPCA* distribution studies of fibroblasts from control and *PMPCA* mutated patient.**

**A:** Representative fluorescent images of mitochondrial network structure overlaid on phase contrast (on the left) showed a mitochondrial network hyper-connection in *PMPCA* fibroblasts. Mitochondrial volume (in purple on black background in the middle) was assessed using the mitotracker Green fluorescent signal by Imaris software and color-coded on the right. The inset illustrates the classification code. To present the changes in mitochondrial morphology in patient's cells, types of mitochondria were classified in 5 groups according to mitochondrial length: blobs<1um, fragmented<5um, tubular<10um, filamentous<20um, mitochondrial network>20um. Bar graphs show the distribution of the mitochondrial population of Control, P1 and P2. Mean  $\pm$  SEM. Scale bar: 10  $\mu$ m.

**B:** Western blots (left) against *PMPCA*, *PMPCB*, *OPA1* and citrate synthase (*CS*) on control (C1) and two patients (P1 and P2) fibroblasts reveal decreased levels of *PMPCA* and equal levels of *PMPCB* and *OPA1* in the pathological conditions, as shown on the histogram (right).

**C:** Enzymatic activities of the respiratory complexes (CI to CV) from the control and the *PMPCA* mutated fibroblast strains related to the citrate synthase (*CS*) enzymatic activity, did

not reveal significant difference between control and mutated fibroblasts. Results are Mean  $\pm$  S.E.M. from four independent experiments.

**D:** Single-molecule localization microscopy dSTORM was used to analyze PMPCA distribution, correlated to total internal reflection fluorescence TIRF microscopy for mitochondrial staining. Using Imaris software, the dSTORM PMPCA immunofluorescence signal was used to quantify their mitochondrial surface protein distribution.

**Table 1: Clinical and molecular data of the *PMPCA* patients.**

Family	Patient	Sex	Age	VA	other symptoms	brain MRI	ORF mutation	protein change	rs #	gnomAD Freq.	ACMG classification
1	(II.1)	M	30	counting fingers	-	normal	c.309delA	p.Lys103AsnfsTer74	unknown	-	PVS1, PM2 and PP3 Class 5
2	(II.1)	M	53		peripheral neuropathy	normal					
3	(II.2)	M	45	4/10	-	normal	c.820delG	p.Val274SerfsTer27	rs777445198	4,01.e-6	PVS1, PM2 and PP3 Class 5
4	(II.1)	M	35	0.5/10	multiple sclerosis	ND	c.1363G>A	p.Ala455Thr	unknown	-	PM2, PP2 and BP4 Class 3
5	(II.1)	M	69	6/10	-	ND	c.1547G>A	p.Arg516His	rs768196711	8,01.e-6	PM2, PP2 and BP4 Class 3

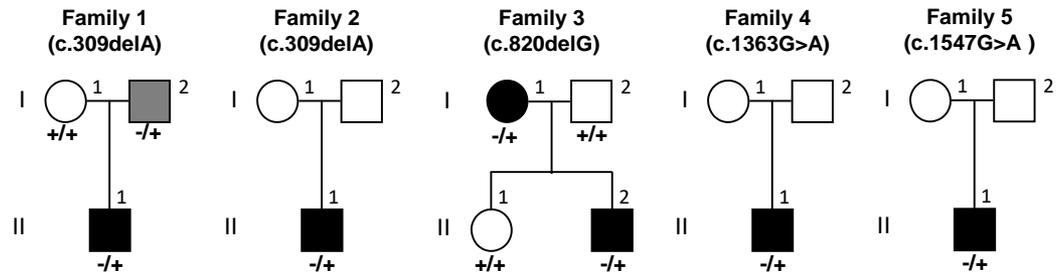
Abbreviations: VA: visual acuity; M: male; rs #: reference sequence number; gnomAD Freq.: Frequency in the Genome Aggregation Database,

ACMG: American College of Medical Genetics and Genomic.

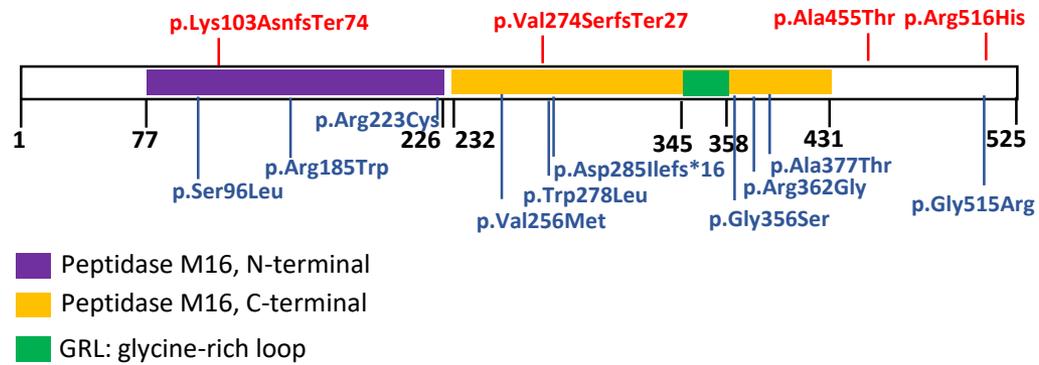
**Abbreviations:**

CS: citrate synthase; DOA: dominant optic atrophy; ION: inherited optic neuropathy; mtDNA: mitochondrial DNA; LHON: Leber's hereditary optic neuropathy; RGC: retinal ganglion cell; SCAR2: Autosomal Recessive Cerebellar Ataxia type 2. SCA28: Spinocerebellar ataxia 28.

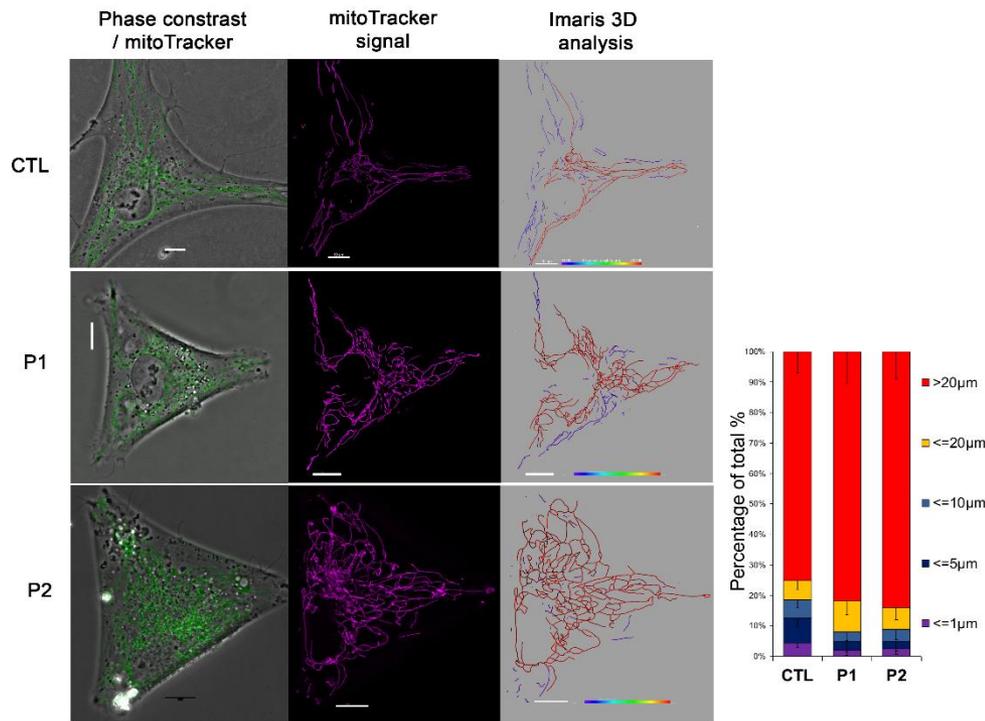
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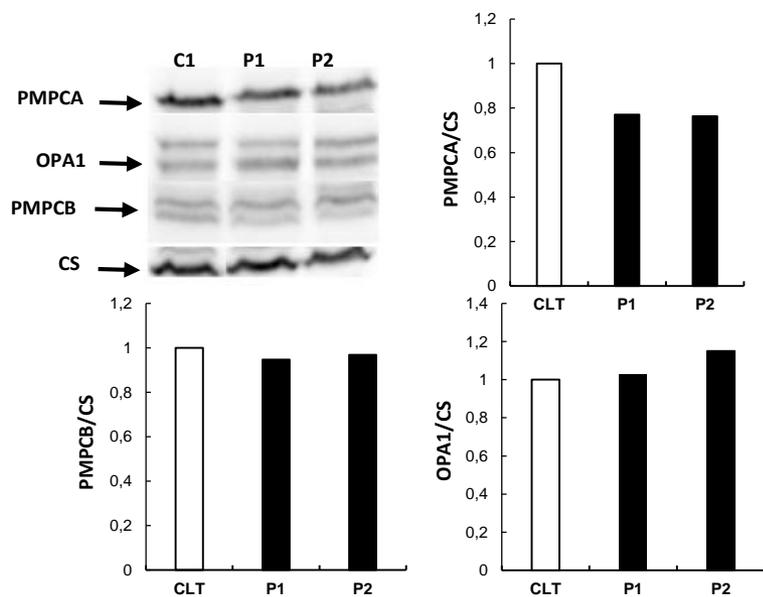
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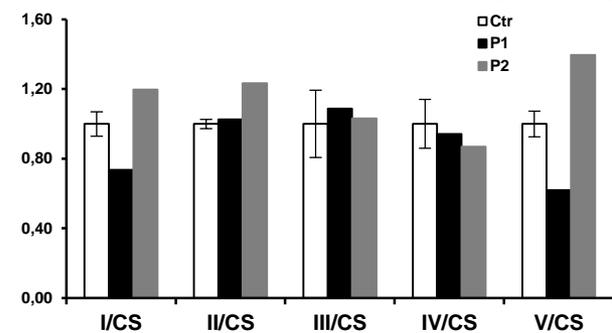
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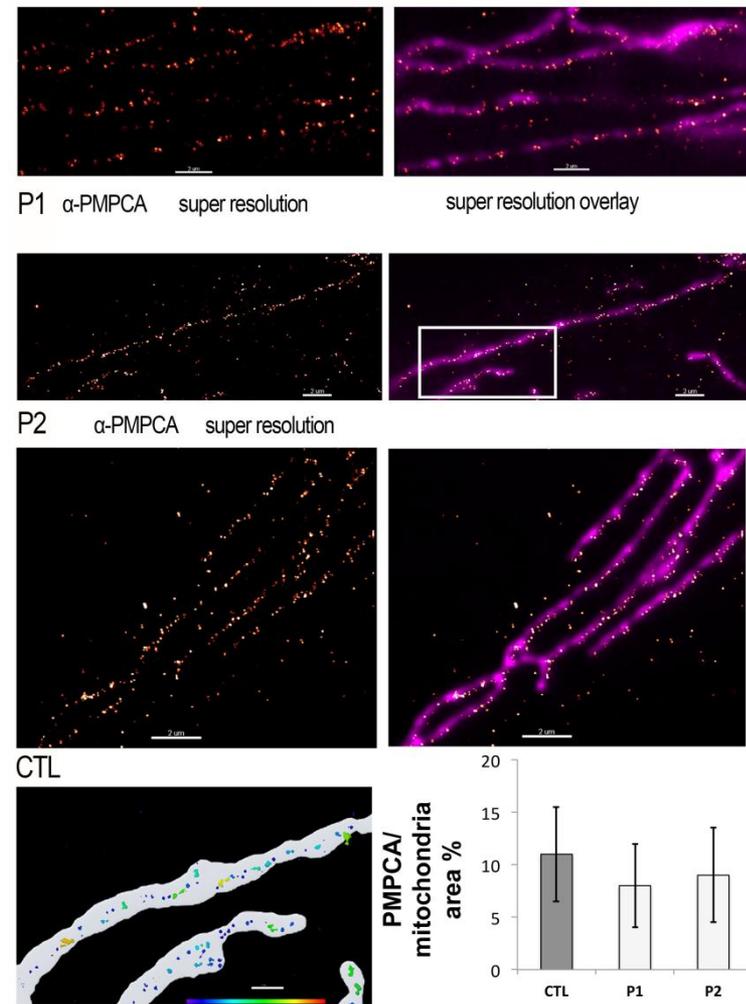
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C



D



**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

There is no financial interests/personal relationships which may be considered as potential competing interests

**Next generation sequencing identifies novel *PMPCA* variants in patients with dominant optic atrophy**

Majida Charif<sup>1,2</sup>, Arnaud Chevrollier<sup>1</sup>, NaïgGueguen<sup>1,3</sup>, Selma Kane<sup>1</sup>, Céline Bris<sup>1,3</sup>, David Goudenège<sup>1,3</sup>, Valerie Desquiret-Dumas<sup>1,3</sup>, Isabelle Meunier<sup>4</sup>, Fanny Mochel<sup>5</sup>, Luc Jeanjean<sup>6</sup>, Fanny Varenne<sup>7</sup>, Vincent Procaccio<sup>1,3</sup>, Pascal Reynier<sup>1,3</sup>, Dominique Bonneau<sup>1,3</sup>, Patrizia Amati-Bonneau<sup>1,3</sup> and Guy Lenaers<sup>1,\*</sup>

<sup>1</sup> Université d'Angers, MitoLab team, UMR CNRS 6015 - INSERM U1083, Unité MitoVasc, SFR ICAT, Angers, France.

<sup>2</sup>Genetics and immuno-cell therapy Team, Mohammed First University, Oujda, Morocco.

<sup>3</sup>Departments of Biochemistry and Genetics, University Hospital Angers, Angers, France.

<sup>4</sup>Institut des Neurosciences de Montpellier, INSERM U1051, Université de Montpellier, France

<sup>5</sup> INSERM U 1127, CNRS UMR 7225, Sorbonne Universités, UPMC Univ Paris 06 UMR S 1127, Institut du Cerveau et de la Moelle épinière, ICM, F-75013 Paris, France; AP-HP, Pitié-Salpêtrière University Hospital, Department of Genetics, Paris, France; University Pierre and Marie Curie, Neurometabolic Research Group, Paris, France.

<sup>6</sup>Department of Ophthalmology, Nîmes University Hospital, Nîmes Cedex 9.

<sup>7</sup> Department of Ophthalmology, Hôpital Pierre Paul Riquet CHU Purpan, Toulouse, France.

(\*). Corresponding author: Guy LENAERS.

MitoLab team, Mitochondrial Medicine Research Centre, Institut MitoVasc, Université d'Angers, UMR CNRS 6015, INSERM U1083, CHU Bât IRIS/IBS

Rue des Capucins, 49933 Angers cedex 9, FRANCE.

e-mail: [guy.lenaers@inserm.fr](mailto:guy.lenaers@inserm.fr)

Tel : 33/0 244 68 84 87

Fax: 33/0 244 68 65 94