

## Mitochondrial Genome Profile in Demyelinating Diseases

Durastanti V<sup>1\*</sup>, Monaco A<sup>2,3</sup>, Caronti B<sup>1</sup>, Cortese A<sup>1</sup>, Fustaino V<sup>3</sup>, Wang E<sup>2</sup>, Berardelli A<sup>1</sup>, Marincola FM<sup>2</sup> and Millefiorini E<sup>1</sup>

<sup>1</sup>Department of Neurology and Psychiatry, Sapienza University, Rome, Italy

<sup>2</sup>Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD, USA

<sup>3</sup>Department of Cellular Biotechnology and Ematology, Sapienza University, Rome, Italy

### Abstract

Multiple sclerosis and neuromyelitis optica are chronic inflammatory diseases of the central nervous system. These pathologies share clinical similarities with Leber hereditary optic neuropathy, which is primarily due to mutations of mitochondrial DNA. Mitochondrial genetic variations may influence susceptibility to develop multiple sclerosis and neuromyelitis optica. In order to explore the possible correlation between mitochondrial DNA specific patterns and demyelinating diseases involving central nervous system, mitochondrial DNA from 13 patients with relapsing-remitting multiple sclerosis, 4 patients with neuromyelitis optica, 1 patient with myelitis, 2 patient with optic neuritis, and 7 healthy controls were analyzed by sequencing the full length 16 Kbs of the mitochondrial DNA genome. Common variants presence in healthy controls and patients showing no clinical impact on diseases development were not further explored. Analyzing 414 patient specific variants, six nonsense mutations, causing early stop-codon formation, and nine previously described variants, associated with demyelinating/degenerative disease of central nervous system were identified. Some of these variants are linked to disease development through known and previously described mechanisms. We report for the first time other truncating mutations leading to incomplete proteins involved in Oxidative Phosphorilation complexes and we speculate their role in demyelinating diseases development.

**Keywords:** Mitochondrial diseases; Multiple sclerosis; Neuroophthalmology

### Introduction

Multiple sclerosis (MS) is a chronic inflammatory neurological disease of the central nervous system (CNS), characterized by demyelination, neurodegeneration and astroglial proliferation [1], with complex genetic factors exerting a profound influence [2]. Despite major advances in the current understanding of the pathogenesis of MS, the inflammatory cascade involved in MS remains unknown; although there is considerable evidence implicating the involvement of mitochondria in axonal and glial injury mechanisms [3,4].

Similarly to MS, neuromyelitis optica (NMO), or Devic's disease, is a demyelinating disease of the CNS characterized by optic neuritis (ON) and myelitis (My) [5] associated with anti-aquaporin 4 (AQP4) antibodies detectable in the serum [6]. Several siblings with NMO have been reported [7,8], raising the possibility of a genetic predisposition, but no pathogenic mutations have been identified in the AQP4 gene on chromosome 18q11.2-q12.1 [9].

MS and NMO have similarities with Leber hereditary optic neuropathy (LHON, MIM 535 000), which is the commonest cause of isolated blindness in young men. LHON is primarily due to mutations of mitochondrial DNA (mtDNA) that disrupt complex I of the respiratory chain [10,11]. Moreover, some patients, with mtDNA mutations causing LHON, develop a demyelinating disease, which is clinically and radiologically indistinguishable from MS [12].

Mitochondria are unique amongst cellular organelles for having their own distinct genome, separate from nuclear DNA (nDNA). This mtDNA, which is 16,569 base pairs in length, contains a total of 37 genes - 2 ribosomal RNAs (rRNAs), 22 encoding transfer RNAs (tRNAs) and 13 encoding polypeptides. The polypeptides subunit are components of the respiratory chain, including complex I (NADH dehydrogenase-ubiquone oxidoreductase), complex III (ubiquinone-cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthetase) [13].

Previous studies reported that various mitochondrial mechanisms

are involved in the pathogenesis of MS and other demyelinating disease of CNS [14-17]. In particular, several studies, linking mitochondrial genome abnormalities to oxidative damage and inflammation, strongly suggest that an acquired mitochondrial dysfunction may contribute to neurodegeneration in MS [15,16,18]. This finding is supported by a decreased expression of several mitochondrial proteins and a reduced activity of complexes I and III detected in the MS motor cortex [4]. Furthermore, mitochondria is one of the prime cellular sources of reactive oxygen species (ROS) which play a role together with reactive nitric species (RNS) in the development of axonal degeneration [19,20]. Since the extent of ROS formation is a function of the oxygen consumption, higher levels of ROS are produced by neurons mitochondria with higher metabolic activity or by neuronal segments enriched in mitochondria, such as synapses. Neurons are particularly vulnerable to the oxidative stress induced by ROS. Furthermore, the overproduction of nitric oxide (NO) and its oxidative metabolites is one of the distinct characteristics of inflammatory CNS diseases including MS and EAE [21-24]. Thus, in conclusion, it is likely that oxidative damage in CNS inflammatory diseases may originates mainly from mitochondria [25].

Given the clinical similarities between NMO and LHON, previous investigators have also looked for specific mtDNA mutations in patients with NMO [26-30], while others have studied polymorphic variation of mtDNA in NMO cases [27,28,31,32]; a subtle increased susceptibility conferred by rare mtDNA variants could not be excluded

**\*Corresponding author:** Valentina Durastanti, Department of Neurological Sciences, Viale Università 30, 00185 Rome, Italy, Tel: +39-06-4457711; Fax: +39-064457711; E-mail: [valentina.durastanti@uniroma1.it](mailto:valentina.durastanti@uniroma1.it)

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[30], although the recruitment of samples sizes large enough to show a statistical significant association between specific mtDNA variants and complex diseases may be extremely challenging [33].

Despite results previously described, the exact role of mtDNA and especially mtDNA's polymorphism in MS and NMO has not been fully characterized.

In our study we aimed at determining a possible correlation between mtDNA specific patterns and demyelinating diseases involving medulla and optic nerve. Particularly, we have sequenced and analyzed the whole mtDNA of a cohort of patients with relapsing-remitting (RR) MS, NMO, My and ON in order to determinate a putative panel of deleterious mutations with a possible correlation with neurodegenerative diseases and establish their penetrance and their actual weight in the pathogenesis.

## Methods

### Population

The present explorative study included a total of 27 Caucasian individuals, 20 of which were patients (13 were diagnoses with RRMS, 4 with NMO, 2 with ON and 1 with My,) and 7 were healthy controls (HC). NMO and MS patients satisfied Wingerchuk [5] and Poser [34] diagnostic criteria, respectively. All subjects gave the informed consent.

### Specimen collection and genetic analysis

Blood samples were collected into CPT sterile tubes (BD) and were centrifuged at 3,000 rpm for 10 minutes at 4°C for peripheral blood mononuclear cell (PBMC) isolation according the manual. Genomic DNA was extracted from PBMC using the DNeasy blood and tissue DNA extraction kit (Qiagen) according to manufacturer's instructions. Extracted Genomic DNA was then diluted to 30 ng/μl, aliquoted and stored at -20°C.

The full coverage of mtDNA were amplified using mitoSEQr™ Resequencing Primers Set (RSS000056015\_01 mitoALL™, Life Technologies), a system specifically designed and optimized for the amplification of 100% of human mitochondrial genome. This primer set consists 46 primers pairs designed in order to standardize the Polymerase Chain Reaction (PCR) conditions and the following sequence reaction. Each pre-designed primer pair generates a resequencing amplicon (RSA) marked with a M13 nucleotide sequence at both 5' ends, useful for simplifying the development of a sequence reaction. Through M13 primers the sequencing reaction can be set up using a single mastermix aliquoted into each well.

The PCR reaction included AmpliTaq Gold® 2x Master Mix, primers set (forward+reverse) and 30 ng of the DNA sample in 20 μl of total volume. Each amplicon was amplified in a single well of a 96-well plate for each sample; each plate also included negative controls to ensure no possible contamination. Thermalcycler conditions were set up according to the manufacturer specifications. 2% agarose gel electrophoresis was performed to test PCR products, and then the PCR was cleaned up from unused primers and dNTPs with the enzymatic method ExoSAP-IT™ (Affymetrix) according to the manufacturer's instructions.

All amplicons were sequenced using universal conditions. BigDye® Terminator v3.1 Cycle Sequencing kit was used for the sequence reaction: in a total volume of 10 μl, sequencing was performed with BigDye® Terminator Mix, purified PCR amplicons and M13 primer forward (or reverse when forward primer did not produced a readable

sequence). Unincorporated dye terminators were removed with DyeEx® 96 (QIAGEN) plate according to manufacturer's instructions.

Sequence reactions were analyzed on the 48-Capillary 3130xl Genetic Analyzer (Life technologies). AB1 files with sequencing info for each sample were extracted at the end of each run.

### Data analysis

Using the software Sequencer 4.10.1 (<http://genecodes.com>) to analyse wild type sequences from mitochondrial genome obtained from healthy samples, we defined a reference wild type sequence to be compared to the samples in order to identify variants potentially associated with the diseases.

Einscluster and Treeview (<http://rana.lbl.gov/EisenSoftware.htm>) softwares were used to generate heatmaps representing clusters of differential genetic profiles. Characterization of known variants (the one for which an *rs* number was available) was performed using an online software Pupasuite (<http://pupasuite.bioinfo.cipf.es/>), an interactive web-based SNP analysis tool that allows for the selection of relevant SNPs within a gene, based on different characteristics of the SNP itself; the algorithm helped in identifying putative variants for which a previous association with specific disease had been described.

The variants identified by comparing the reference sequence with each sample was searched against gene variants information databases available online: GeneCards (<http://www.genecards.org>), Ensembl (<http://www.ensembl.org>), COSMIC (<http://www.sanger.ac.uk/genetics/CGP/cosmic>), LOVD (<http://www.lovd.nl/2.0>) UniProt (<http://www.uniprot.org>) and HGMD (<http://www.hgmd.org>).

## Results

### Variants analysis

Mitochondrial genome Sequencing of 27 samples (13 RRMS, 4 NMO, 2 ON, 1 My and 7 HC) highlighted the presence of total 414 variants present only in patients. Among them 74 were already classified in literature (and therefore registered with *rs* number) and 340 were not previously described. Out of 414 variants, 121 were missense (generating an amino-acid exchange), 108 synonymous (variants occurring in coding region but not generating amino-acid exchange) and 185 intronic (occurred in non-coding regions of DNA sequence) variants. None of the variants described so far ever occurred in a HC patient (data not shown). The variants distribution in our cohort of patients is represented in Figure 1.

### Variants not previously described

Out of 340 not previously described variants, we found 6 missense mutations generating stop codon and consequently a truncated protein with compromised function. These mutations were found in patients with RRMS, ON and My. All truncating mutations occurred in heteroplasmic condition except one. The features of these mutations are described in Table 1.

### Variants previously described

Out of 74 variants already classified in literature we found 9 variants reported to have a clinical impact; none of these variants were present in the HC group (data not shown) analysed in the present study. The features of these variants are summarized in Table 2.

### Mitochondrial haplogroup typing

Haplogroup for each individual of our cohort was established





in several studies with an increased risk of developing MS, LHON, Parkinson and Alzheimer disease [46,47]. Several other mutations in the protein coding gene ND5 have been found in LHON and MELAS (*mitochondrial encephalomyopathy, lactic acidosis, strokelike episodes*) reinforcing the belief that mutations in these genes are related to optical and CNS demyelinating pathologies [13,40].

### Haplotypes analysis

As mentioned in previous publications, specific haplotypes were associated with higher risk of MS development. Interestingly, haplotype J only occurred in samples diagnosed with a degenerative disease. Unfortunately, the small sample size did not allow us to provide statistically significant information concerning haplogroups distribution. However, our preliminary analysis in line with previously published data [2,46].

In conclusion, despite the limited number of samples of our cohort did not allow us to give a statistical significance to our findings, the study highlighted new insights on the clinical impact of mitochondrial genome's variants in neurodegenerative diseases; we looked not only for mutation with evident deleterious features, but also for variants that, occurring in non-coding regions, may affect the protein function through indirect mechanisms (such as interaction with transcription factor or other gene expression regulators such as miRNA). The use of most informative online databases allowed us to compare our results to previous findings and to confirm the presence of variants with a biological significance and likely showing a clinical impact. For some variants, which had not previously been identified, a possible biological deleterious function was provided, consistent with their occurrence in patients with neurodegenerative diseases.

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