Supplementary Materials

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Supplementary Methods

At IRCCS Istituto delle Scienze Neurologiche di Bologna, the whole exome sequencing (WES) sample library was prepared from genomic DNA and enriched using xGen Exome Hyb Panel v2 (IDT), except for family F which was enriched with Twist Human Comprehensive Exome (Twist Bioscience). The sequencing was performed on a NovaSeq 6000 instrument (Illumina) with 100 or 150 bp paired-end reads. Bioinformatic analysis followed the GATK Best Practices workflow for germline variant discovery, aligning to reference genome GRCh38/hg38; called variants were annotated using Ensembl Variant Effect Predictor (VEP).[1] Rare variants in genes included in the Panel for Optic Neuropathy of Genomics England PanelApp (https://panelapp.genomicsengland.co.uk) were prioritized, followed by rare variants in genes encoding mitochondrial proteins.

At IRCCS Istituto Neurologico Carlo Besta, a panel-based target capture using probes for 230 or 300 genes associated with mitochondrial disorders (including 16 inherited optic neuropathies) was used for library preparation. The sequencing was performed on a MiSeq instrument (Illumina) with 150 bp paired-end reads. Single nucleotide variants (SNVs) and small insertions/deletions (INDELs) calling were performed using GATK4.1; Variant Interpreter software (Illumina) was used for variants annotation and filtering.[2]

At IRCCS Ospedale Pediatrico Bambino Gesù, sequencing was performed using a Trusight One Expanded Sequencing panel (Illumina), run on NextSeq 500 platform (Illumina) with a 150 bp pair-end reads. The bioinformatic analysis was performed using BWA aligner together with GATK Best Practice for germline variant discovery, then data were annotated and analyzed using Geneyx software.

At Moorfields Eye Hospital, the whole genome sequencing was performed through the 100,000 Genomes Project, following its guidelines and data quality requirements,[3,4] while the clinical exome/oculome sequencing was performed as described in previous work.[5]

At Charles University and General University Hospital in Prague, sequencing libraries were generated using Agilent SureSelect Human All Exome V6 capture kit. Libraries were sequenced with 150 bp pair-end reads on NovaSeq 6000 instrument (Illumina). The entire bioinformatics pipeline from FASTQ files to final results was performed using the Franklin platform which uses its own bioinformatical pipeline as previously described.[6,7] The *NDUFA10* gene (NG_031855.2; NM_004544.4) pathogenic variant was confirmed by Sanger sequencing of exon 10 in the proband and his brother as well as both parents.

For complete mtDNA sequencing at Charles University and General University Hospital in Prague, a library was prepared using SeqCap EZ Design: Mitochondrial Genome Design (Roche NimbleGen, Pleasanton, CA, United States) enrichment kit and KAPA Hyper Prep Kit (Roche), followed by sequencing analysis on the MiSeq system (Illumina) with 150 bp paired-end reads.

At University Hospital Munich, single WES was performed on genomic DNA isolated from whole blood. Enrichment of target regions was carried out using the Agilent SureSelect 60Mbv6 kit and libraries were sequenced on a NovaSeq 6000 instrument (Illumina) in 100 bp paired-end reads. Following alignment to GRCh37/hg19, data was analyzed and interpreted using an in-house open source NGS pipeline and user interface EVAdb (EVAdb and LIMS version 2022.05 (https://github.com/mri-ihg/EVAdb/releases/tag/1.0.0). The identified *NDUFAF8* variant was confirmed by Sanger sequencing in proband, his parents and three siblings.

For Italian families, identified variants were validated on probands and all available family members by Sanger sequencing on a 3500 Dx Genetic Analyzer (Applied Biosystems).

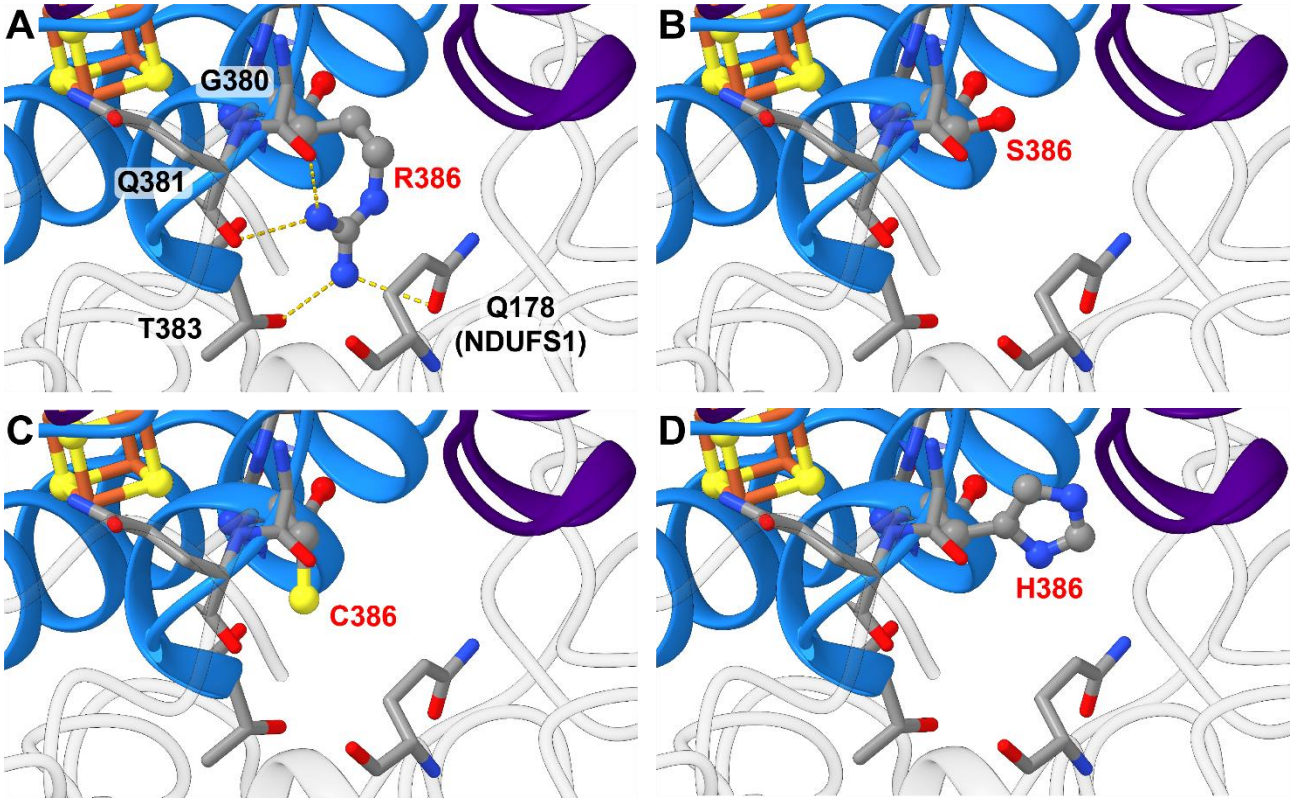
For complete mtDNA sequencing at IRCCS Istituto delle Scienze Neurologiche di Bologna, a PCR amplification was performed using two sets of primers (MT1F: CCCATAACACTTGGGGGTAG/MT1R: TAGAAGTGTGAAAACGTAGGCTTG and MT2F: GCCCTAGCCCACTTCTTACC/MT2R: TGTAAGTTGGGTGCTTTGTGTT) and the PrimeSTAR Max DNA Polymerase (Takara Bio). A library was constructed from purified amplicons using xGen

DNA Library Prep Kit (IDT) and then sequenced on a MiSeq instrument (Illumina) with 150 bp paired-end reads. The sequences were analysed through the mtDNA-Server 2 pipeline.[8]

A similar approach using PCR amplification was followed at IRCCS Istituto Neurologico Carlo Besta, as previously described.[9]

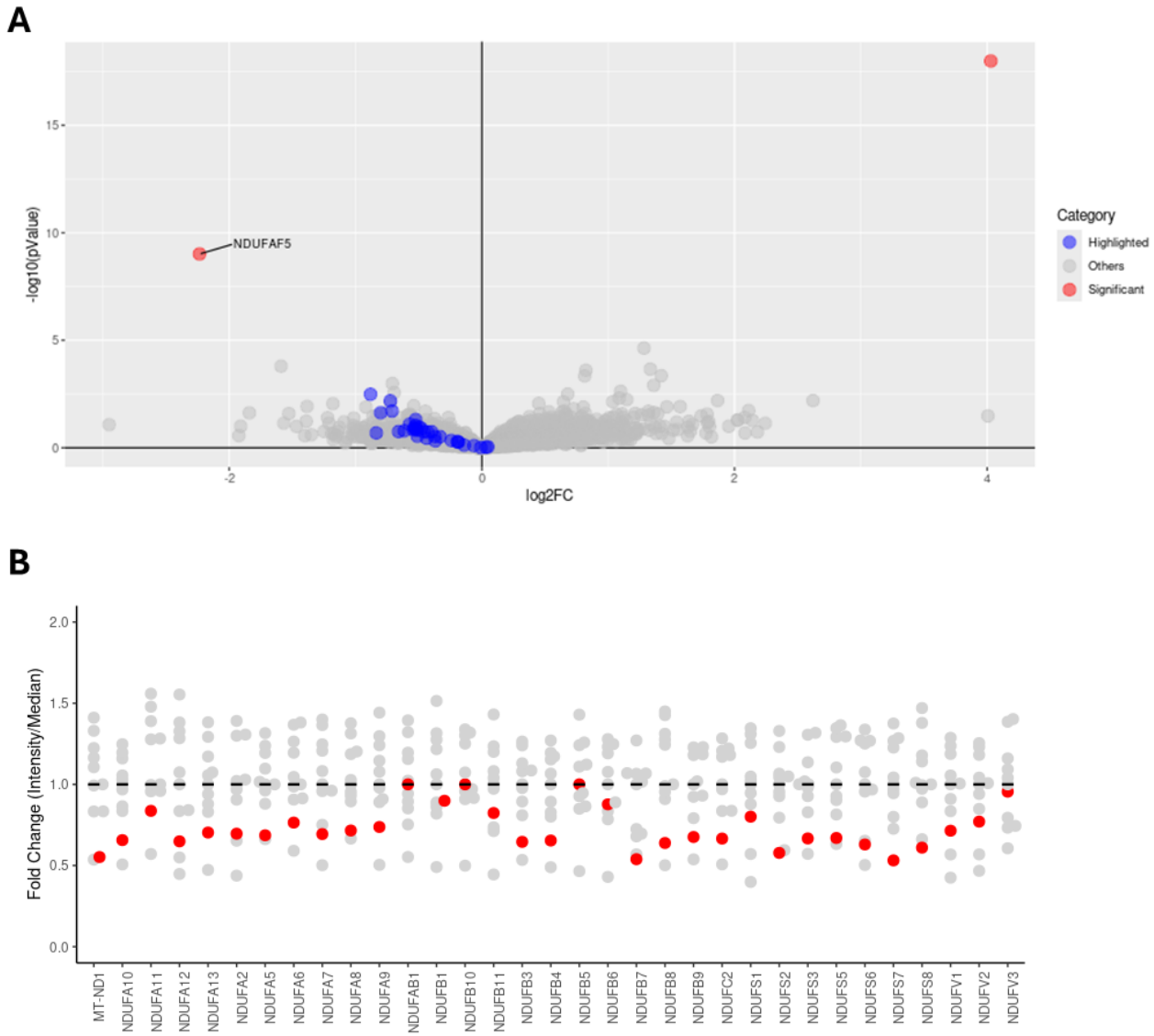
Complex I-related genes

The following list of genes was considered to assess the cumulative prevalence of CI-related genes among the diagnosed cases screened for inherited optic neuropathies: *ACAD9, COA1, DNAJC30, ECSIT, FOXRED1, NDUFA1, NDUFA2, NDUFA3, NDUFA4, NDUFA5, NDUFA6, NDUFA7, NDUFA8, NDUFA9, NDUFA10, NDUFA11, NDUFA12, NDUFA13, NDUFAB1, NDUFAF1, NDUFAF2, NDUFAF3, NDUFAF4, NDUFAF5, NDUFAF6, NDUFAF7, NDUFAF8, NDUFB1, NDUFB2, NDUFB3, NDUFB4, NDUFB5, NDUFB6, NDUFB7, NDUFB8, NDUFB9, NDUFB10, NDUFB11, NDUFC1, NDUFC2, NDUFS1, NDUFS2, NDUFS3, NDUFS4, NDUFS5, NDUFS6, NDUFS7, NDUFS8, NDUFV1, NDUFV2, NDUFV3, NUBPL, SFXN4, TIMMDC1, TMEM126A, TMEM126B, TMEM186*.



Supplementary Figure 1. Detail of the human CI structure.

(A) Detail of the human CI structure (PDB id 5XTD)[10] in the region of NDUFV1 Arg386 residue. NDUFV1 and the nearby NDUFV2 subunits are in ribbons colored in light blue and indigo, respectively. Arg386 is in ball-and-stick representation colored according to atom type. Residues H-bonded to Arg386 guanidino moiety (NDUFV1 Gly380, Gln381, and Thr383 as well as NDUFS1 Gln178) are in stick representation colored according to the atom type. Panels B, C, and D report the same region of the human CI in which Arg386 was mutated in silico to serine, cysteine and histidine, respectively. In each panel, the mutated residue is in ball-and-stick representation.



Supplementary Figure 2 Proteomics analysis for patient II-4 Family U.

(A) Volcano plot of the patient fibroblasts' quantitative proteomics, showing NDUFAF5 subunit as expression outlier. (B) Beeswarm plot including detected subunits of CI in the TMT 11-plex batch that are mostly reduced.

Supplementary references

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