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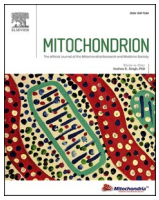

Original Research Article

An inherited mtDNA rearrangement, mimicking a single large-scale deletion, associated with MIDD and a primary cardiological phenotype

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ABSTRACT

Aim: To identify the genetic cause in a previously unsolved pedigree, with mother and two daughters suffering of dilated cardiomyopathy with prevailing arrhythmic burden associated with diabetes mellitus and sensorineural hearing loss, without clear evidence of progressive external ophthalmoplegia.
Methods: Several genetic tests were performed over the years including single-gene sequencing, mitochondrial DNA (mtDNA) sequencing, NGS panel for mitochondrial diseases and cardiomyopathies, clinical exome sequencing and whole exome sequencing. Specific amplifications and long-read NGS were used to evaluate mtDNA structural alterations.
Results: By means of whole exome sequencing we found a novel heteroplasmic 12 kb-long single deletion in the mtDNA in all affected family members, confirmed by long-range PCR. However, a deeper investigation by long-read NGS revealed indeed the presence of rearranged mtDNA species, formed by a wild-type plus a deleted molecule. This mtDNA duplication turned out to be inherited in our pedigree and present in all tested specimens.
Conclusion: While mtDNA single large-scale deletions are generally considered sporadic, few old reports described maternally inherited mtDNA duplication We suggest that mtDNA large rearrangements should be considered as possible disease causes in familial cases with unusual mitochondrial phenotypes. Long-read sequencing is useful for the detection of these variants, particularly mtDNA duplications.

1. Introduction

Primary mitochondrial disorders (PMDs) are a heterogeneous group of genetic disorders that affect the structure or function of the mitochondrial oxidative phosphorylation system and are caused by either mitochondrial DNA (mtDNA) or nuclear DNA pathogenic variants. Chronic progressive external ophthalmoplegia (cPEO), Kearns-Sayre syndrome (KSS) and Pearson marrow-pancreas syndrome (PMPS) are the three classical overlapping phenotypes caused by mtDNA single large-scale deletions (SLDs), which were the first mtDNA aberration to be associated with human PMDs (Holt et al., 1988). mtDNA deletion syndromes usually occur as simplex cases and are considered sporadic,

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as SLDs mostly arise de novo. For offspring of affected women harboring a SLD, the recurrence risk is low but not zero. In the only comprehensive study on this topic, it has been estimated at 4% (3/73), with only 1 out of 3 mothers with an affected child who showed a duplication of mtDNA (Chinnery et al., 2004).

Large-scale mtDNA rearrangement can also occur as tandem direct duplication: old anecdotal reports described maternally inherited mtDNA duplication, associated with myopathy (Poulton et al., 1991), complex phenotypes (Rötig et al., 1992), KSS or normal aging (Damas et al., 2014). SLDs and duplications can also coexist in the same patient. Unlike mtDNA SLDs, duplications can be maternally transmitted (Rötig et al., 1992). mtDNA duplications are difficult to detect with either standard approaches based on restriction enzymes (e.g. Southern blot), possibly cutting twice the duplicated molecule, or by short-read sequencing, which relies on DNA fragmentation hampering the maintenance of the whole mtDNA structure.

Ptosis and/or PEO are hallmark symptoms of cPEO and represent the common basis of the syndromic continuum related to mtDNA SLDs. Within this continuum, cardiomyopathy is a phenotypic feature which defines the "KSS spectrum" but has rarely been reported to occur alone or as a cardinal aspect (Grady et al., 2014; Mancuso et al., 2015). Here we present the cases of an Italian 71-year-old woman (patient I-1) and her two daughters (patients II-1 and II-2) suffering from severe dilated cardiomyopathy associated with early-adulthood onset diabetes mellitus and bilateral sensorineural hearing loss (MIDD). Despite an initial diagnosis of inherited heteroplasmic 12 kb mtDNA SLD, by long-read sequencing we were able to identify a mtDNA rearrangement in all three family members.

2. Material and methods

After informed consent, total DNA was extracted from blood and urinary epithelial cells of the three affected subjects (I-1, II-2, II-2) and from muscle biopsy of the mother (I-1). Mother's husband (I-2, daughters' father) DNA was not available. We performed whole exome sequencing (WES) on genomic DNA extracted from blood after enrichment with the TWIST Exome 2.0 and TWIST Mitochondrial Panel Kits. Data analysis was conducted in the Institute of Human Genetics (TUM) in-house bioinformatics pipeline and the CDSS (clinical decision support system) EVAdb (Exome Variant Annotation Database), combining publicly available and self-developed analysis tools. Variant calling was done using the Genome Analysis Toolkit (GATK). To confirm the single mtDNA deletion, long-range PCR reactions (LR-PCR) were used to amplify mtDNA. The PCR products were purified using a commercial kit (QIAquick PCR & Gel Cleanup Kit, Qiagen) and analysed by sequencing (ABI-Prism 3500 Analyzer, Applied Biosystem). These results were also confirmed on all available DNA samples from the family using a mitochondrial gene panel containing the xGen™ Human Mitochondrial DNA (mtDNA) Hybridization Panel (IDT). STAR was used for alignment. The bioinformatics analysis was performed as previously described (Legati et al., 2021). MitoSAlt tool was used to predict the presence of structural alteration in mtDNA (Basu et al., 2020).

For long-read sequencing of native mtDNA, we exploited the Oxford Nanopore Technologies (ONT). The ONT Rapid Sequencing Kit (SQK-RAD004) was used for library preparation and samples loaded on MinION Flow Cell (R9.4.1) or Flongle Flow Cell. The bioinformatics analysis was performed at the Institute of Neurology Besta, as previously described (Frascarelli et al., 2023). Amplifications by PCR with mtDNA-specific oligos, followed by processing according to the ONT ligation sequencing kit (SQK-LSK109) and sequencing on MinION with Flongle flow cell were performed to define the rearranged mtDNA molecules. The mtDNA revised Cambridge Reference Sequence (rCRS, NC_012920) was used as wild-type (wt) sequence and for numbering of nucleotide positions throughout the text.

3. Results

3.1. Patients

A 51-year-old Italian woman (patient I-1) was referred to our center because she started developing myopathic involvement manifesting as slight bilateral eyelid ptosis, moderate exercise intolerance, and mild hyperCKemia. She suffered from early-adulthood onset diabetes mellitus and severe bilateral sensorineural hearing impairment. After about 5 years, she started developing a dilated cardiomyopathy phenotype with ventricular extrasystoles and ventricular tachycardia episodes, which required an automatic defibrillator and a pacemaker implantation, subsequently associated with first-degree atrio-ventricular block. Furthermore, at 66 years of age, she developed parkinsonism with moderate global bradykinesia, mild left-lateralization of motor symptoms with left hand and leg tremor, and postural instability. She started levodopa treatment with modest benefit, without levodopa-induced dyskinesias and motor fluctuations. Brain CT revealed basal ganglia calcification and cerebral atrophy, and SPECT DaTScan demonstrated bilateral nigrostriatal degeneration. No other peculiar mitochondrial red flags and/or multisystem involvement were detected. Her family history was unremarkable, with no sudden cardiac death reported.

Her elder daughter (patient II-1) suffered from severe cardiomyopathy (late gadolinium enhancement pattern at heart MRI) that required an automatic defibrillator implantation, ventricular extrasystoles with ventricular tachycardia episodes (which required focus ablation), and later, first-degree atrio-ventricular block. She underwent heart transplantation at 37 years of age because of the severity of her heart failure, which later complicated with cardiac allograft vasculopathy. Interestingly, she complained about vertical diplopia without signs of any ocular movement abnormalities. Brain MRI (plus orbits) was unremarkable. I-1′s younger daughter (patient II-2) developed an analogous cardiological phenotype soon after (at 31 years of age), with less severe heart failure, comparable heart MRI findings, and similar arrhythmic burden (without atrio-ventricular block). Both daughters suffered from early-adulthood onset diabetes mellitus and severe bilateral sensorineural hearing impairment, similar to their mother. Imaging performed over the years and phenotypes are summarized in Fig. 1.

3.2. Genetic studies

Considering the pedigree and phenotypes, autosomal dominant or mitochondrial inheritances were suspected. Several genetic tests performed over the years (including single-gene sequencing, mtDNA sequencing, NGS panel for mitochondrial diseases and cardiomyopathies, clinical exome sequencing) were negative (Fig. 1).

To further investigate the pedigree, a trio-WES from blood-derived DNA was performed. It did not reveal pathogenic variants in nuclear genes but demonstrated a drop of about 40% in the coverage depth in the 3350-15750 mtDNA region (Fig. 2). LR-PCR revealed a large heteroplasmic mtDNA SLD in both urine and blood in the three patients (Fig. S1) and in patient I-1′s muscle. Sanger sequencing showed a 12412 bp SLD (m.3336-15749del), removing part of mtDNA minor and major arcs and origin of light strand replication (OriL) (Fig. 2). These results were also confirmed by short-read sequencing with a different pool of mtDNA probes on all DNA samples from the family. At this point a diagnosis of inherited mtDNA SLD was made.

However, since the diagnosis of mtDNA SLD did not really fit with the clinical presentation and inheritance pattern of the family, we decided to perform additional investigations. To better detect and identify structural alterations affecting the mitochondrial genome, we exploited long-read sequencing by ONT, analyzing the I-1′s DNA from blood. Alignment and visualization on the rCRS showed again a 12 kb SLD (Fig. 2); however, some reads were longer than 16.5 kb, the length of a wt mtDNA molecule. By looking in detail at these reads, they turned out to be duplicated mtDNA species, formed by a wt plus a deleted

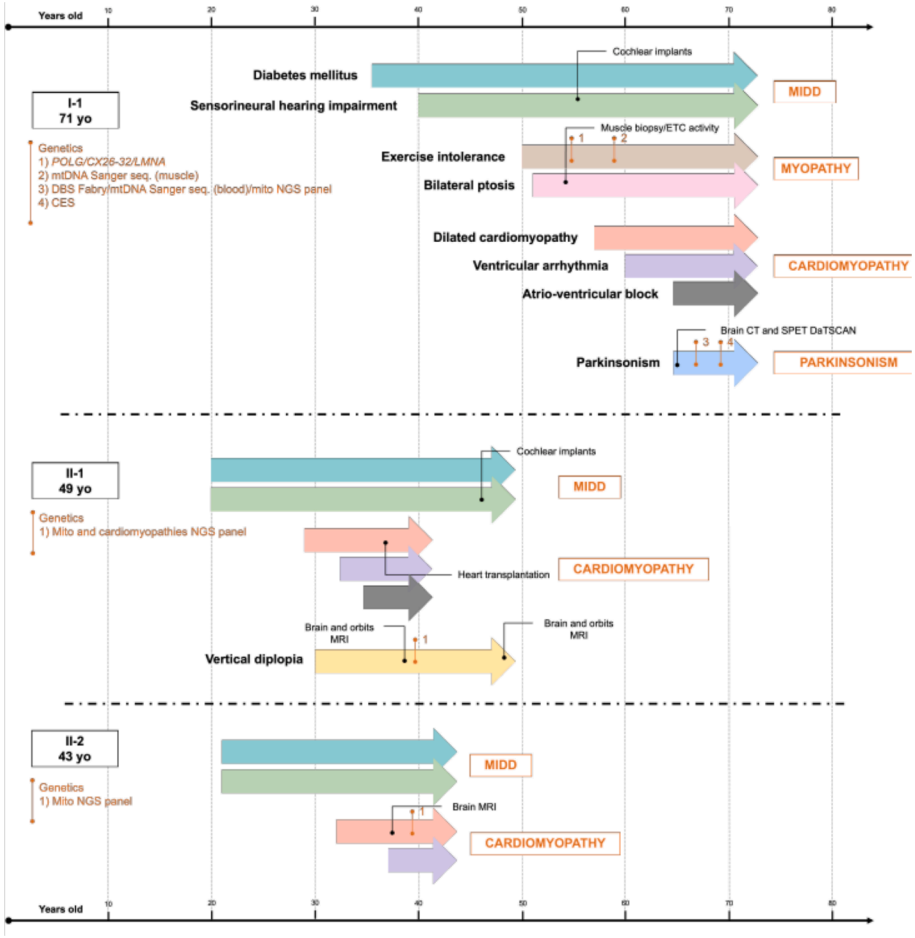

Fig. 1. Timeline of pedigree phenotypes, with genetic tests and imaging performed. I-1: mother; II-1 and II-2: daughters. CES: clinical exome sequencing; DBS: dried blood spot; MIDD: mitochondrial inherited diabetes and deafness; mtDNA: mitochondrial DNA; NGS: next-generation sequencing.

molecule. A new alignment of the long-reads to a reference sequence corresponding to the duplicated mtDNA showed a uniform coverage, with few reads containing only the wt sequence (Fig. 2).

Selected oligonucleotides across the mtDNA were used to specifically amplify the wt and/or the duplicated molecules. Gel electrophoresis and long-read sequencing of the targeted PCR products confirmed the expected rearrangements in all family members (Fig. S1).

With 40% of reads having the deletion after alignment on the rCRS, we can calculate that ≈80% of mtDNA molecules are duplicated (for each deletion one complete mtDNA), so we can estimate a heteroplasmy of 80% for the rearranged mtDNA molecule. In the ONT data from I-1′s DNA, we found reads with a size of ≈4.1 kb and containing the deletion (7% of total reads >4 kb), possibly corresponding to a mtDNA SLD. Finally, we tested the prediction tool MitoSAlt for identification and visualization of mtDNA deletions and duplications from short-read NGS data. In all samples from the affected family members, a single duplication was called (Fig. S2).

4. Discussion

mtDNA SLDs account for approximately 16% of all adult mtDNA pathogenic variants related to PMDs (Gorman et al., 2015). Despite a specific ~5 kb SLD is commonly reported, SLDs have variable size (from 1 kb to 10 kb) (Grady et al., 2014; Schon et al., 1989). They mostly arise *de-novo*, presenting as sporadic cases. However clinical recurrence has been noted in a small number of human pedigrees, with or without concomitant inherited mtDNA duplication (Chinnery et al., 2004; Shanske et al., 2002). All these reports were based on experiments using restriction enzymes plus mtDNA probes or specific PCR amplification. Although SLDs were the first mtDNA aberration to be associated with human PMDs, there are still many open questions regarding their origin, propagation and the pathological mechanisms underlying them (Gellerich et al., 2002; Rocha et al., 2018), as well as their link with other mtDNA rearrangements (Damas et al., 2014).

To our knowledge, there is no previous case with mtDNA duplication or rearrangement identified by long-read sequencing. In our family, this approach allowed us to refine an "inexact" initial diagnosis of SLD identified by WES into the final molecular diagnosis of a complex mtDNA rearrangement. mtDNA duplication have been thought to be the explanation for inheritance of mtDNA SLDs in the offspring of affected individuals (Rötig et al., 1992; Poulton et al., 1989), although this hypothesis was ruled out in other familial cases (Chinnery et al., 2004; Shanske et al., 2002). Nevertheless, technical difficulties leading to a wrong diagnosis (mtDNA SLD instead of mtDNA duplication and vice versa) should be considered for all published cases. We recently described how sequencing technologies based on long-reads are a valuable approach to find structural alterations in mtDNA (Frascarelli et al., 2023). These rearrangements usually had required long and cumbersome analyses for their identification; notably, most of the reports describing mtDNA duplications date back to the nineties or early 2000s. It is possible to speculate that most of the mtDNA duplications have been missed in recent years, or misinterpreted as SLDs, due to the massive introduction of short-read NGS.

Often flanking repeats facilitate formation of mtDNA SLDs or rearrangements, with one of those repeats which is lost in the process (Samuels et al., 2004). Mitobreak is an on-line resource with curated

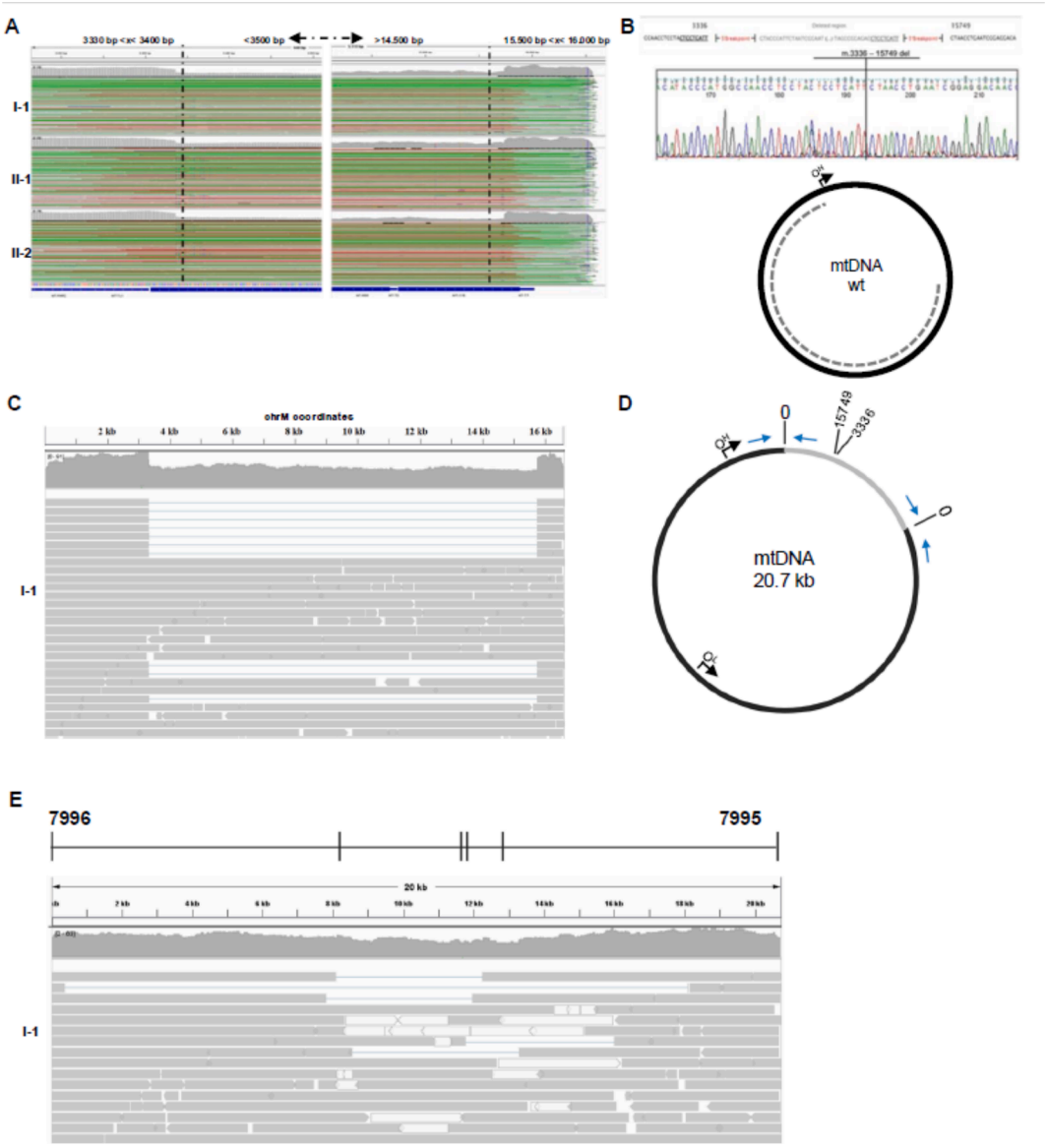

Fig. 2. Genetic studies. A. The WES analysis from blood-derived DNA from I to 1, II-2 and II-2 revealed a lowering in the coverage depth in the mtDNA region from about 3300/3400 bp to 15.500/16.000 bp. B. Breakpoints Sanger sequencing showed a 12412 bp SLD (m.3336-15749 del), removing part of mtDNA minor and major arcs and OL, as cartooned in dashed gray bar the circular mtDNA plot. Note the presence of repeated sequences (CTCCTCATT) flanking the breakpoints. C. Depth of coverage and alignment visualization, using IGV tool, of mtDNA from I to 1 sequenced by long-read NGS (Oxford Nanopore Technologies). The large deletion of 12 kb was confirmed and heteroplasmy levels were comparable with those obtained by WES. D. Graphical representation of the mtDNA complex rearrangements identified analyzing reads longer than 16.5 kb: a duplicated mtDNA species formed by a wt plus an inserted deleted molecule. E. Depth of coverage and alignment visualization, using IGV tool, of mtDNA from I to 1 sequenced by long-read NGS; reads were aligned to the predicted duplicated mtDNA molecule. IGV: Integrative Genomics Viewer; LR-PCR: long-range polymerase chain reaction; mtDNA: mitochondrial DNA; NGS: next-generation sequencing; OL: origin of light strand replication; SLD: single large-scale deletion; WES: whole exome sequencing.

datasets of mtDNA rearrangements (Damas et al., 2014), containing 1.369 deletions and 44 duplications in human samples: no duplicated molecule overlapping with the one identified in our family was present. It's important to point out that all the approaches we used (long-read sequencing and targeted PCR amplification) were not able to reveal with confidence a single deleted molecule (deletion monomer) and distinguish it from a piece originating from mtDNA duplication. However, the experimental data overall suggest that the duplicated species is the most represented form in all affected subjects and in all biological samples of this family. This assumption is also supported by the fact that the deletion removed the OriL replication site according to the strand-displacement model of mtDNA replication (Clayton, 1982). Absence of one or both origins makes deleted mtDNA molecule incapable of replicating, hence diluted or lost during cell division. This feature is also used by the tool MitoSAlt to predict the presence of a mtDNA duplication starting from short-read NGS data (Basu et al., 2020); indeed, the deletion in this family encompasses the OriL region (m.5730-5763) and resulted in a duplication called in all affected subjects. However, our data showed the presence of few reads corresponding to possible mtDNA SLD molecules; a de novo formation of deletions during replication has been hypothesized to explain why often duplications and deletions may co-exist.

Phenotypically, excluding the classical associated syndromes (i.e. PMPS, KSS, cPEO), less common phenotypes have been associated with mtDNA SLDs, including cardiomyopathy (Campos et al., 2000; Moslemi et al., 2000). Our case demonstrates a primary cardiological mitochondrial phenotype with prevailing arrhythmic burden, associated with MIDD. No selective or predominant heart involvement was

previously described even for other mtDNA rearrangements. Interestingly, to date none of the daughters have developed PEO or other skeletal muscle involvement as well as any other mitochondrial manifestations. This clinical aspect, together with the inheritance, has complicated the path to reach a molecular diagnosis of a mtDNA structural variant.

5. Conclusion

In conclusion, we suggest that mtDNA large rearrangements should be considered as possible disease causes also in unusual mitochondrial phenotypes and familial cases. Long-read sequencing is nowadays the method of choice for the detection of these variants, particularly mtDNA duplications. Conversely, short-read sequencing presents limitations for the identification of structural mtDNA alterations, which may lead to result misinterpretation. Increasing data on this issue would better define the mechanisms of heritability of these mtDNA rearrangements.

Ethics declaration

This research adheres to the principles set out in the Declaration of Helsinki. The author received and archived written patient consents which are de-identified.

Data availability statement

Data are available under requests. All the literature used for this review is listed in the bibliography.

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CRediT authorship contribution statement

Piervito Lopriore: Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation, Conceptualization. **Andrea Legati:** Writing – review & editing, Software, Methodology, Formal analysis, Conceptualization. **Christiane Michaela Neuhofer:** Writing – review & editing, Methodology, Formal analysis. **Annalisa Lo Gerfo:** Writing – review & editing, Formal analysis. **Robert Kopajtich:** Writing – review & editing, Formal analysis. **Marco Barresi:** Writing – review & editing, Visualization, Software. **Giulia Cecchi:** Writing – review & editing, Formal analysis, Data curation. **Martin Pavlov:** Writing – review & editing, Software, Formal analysis. **Rossella Izzo:** Writing – review & editing, Formal analysis. **Vincenzo Montano:** Writing – review & editing, Data curation. **Maria Adelaide Caligo:** Writing – review & editing, Formal analysis, Data curation. **Riccardo Berutti:** Writing – review & editing, Supervision, Software. **Michelangelo Mancuso:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization. **Holger Prokisch:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Daniele Ghezzi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

The following are the Supplementary data to this article: Supplementary Fig. S1. mtDNA analysis; Supplementary Fig. S2. MitoSAlt analysis. Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2025.102037.

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