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High frequency of mitochondrial DNA rearrangements in the peripheral blood of adults with intellectual disability

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Abstract

Background Mitochondrial DNA (mtDNA) rearrangements are recognised factors in mitochondrial disorders and ageing, but their involvement in neurodevelopmental disorders, particularly intellectual disability (ID) and autism spectrum disorder (ASD), remains poorly understood. Previous studies have reported mitochondrial dysfunction in individuals with both ID and ASD. The aim of this study was to investigate the prevalence of large-scale mtDNA rearrangements in ID and ID with comorbid ASD (ID-ASD).
Method We used mtDNA-targeted next-generation sequencing and the MitoSAlt high-throughput computational pipeline in peripheral blood samples from 76 patients with ID (mean age 52.5 years, 37% female), 59 patients with ID-ASD (mean age 41.3 years, 46% female) and 32 healthy controls (mean age 42.4 years, 47% female) from Catalonia.
Results The study revealed a high frequency of mtDNA rearrangements in patients with ID, with 10/76 (13.2%) affected individuals. However, the prevalence was significantly lower in patients with ID-ASD 1/59 (1.7%) and in HC 1/32 (3.1%). Among the mtDNA rearrangements, six were identified as deletions (median size 6937 bp and median heteroplasmy level 2.3%) and six as duplications (median size 10 455 bp and median heteroplasmy level 1.9%). One of the duplications, *MT-ATP6* m.8765-8793dup (29 bp), was present in four individuals with ID with a median heteroplasmy level of 3.9%.
Conclusions Our results show that mtDNA rearrangements are frequent in patients with ID, but not in those with ID-ASD, when compared to HC. Additionally, MitoSAlt has demonstrated high sensitivity and accuracy in detecting mtDNA rearrangements, even at very low heteroplasmy levels in blood samples. While the high frequency of mtDNA rearrangements in ID is noteworthy, the role of these rearrangements is currently unclear and needs to be confirmed with further data, particularly in post-mitotic tissues and through age-matched control studies.

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Keywords Autism spectrum disorder, Deletion, Duplication, Intellectual disability, Mitochondrial DNA, MitoSAlt

Introduction

Mitochondria, the major cellular organelles responsible to produce adenosine triphosphate (ATP), vary in number from hundreds to thousands per cell depending on the energy needs of the tissue (Verge *et al.* 2011; Nissanka & Moraes 2020). Mitochondria have their own genome called mitochondrial DNA (mtDNA). Human mtDNA is a circular, 16 569 bp double-stranded DNA molecule that contains 37 genes encoding 13 essential polypeptides for the oxidative phosphorylation system (OXPHOS), 22 transfer RNAs (tRNAs) and two ribosomal RNAs (rRNAs) involved in mtDNA transcription and protein synthesis. mtDNA also contains a non-coding region called the displacement loop (D-loop). The D-loop plays a critical role in mtDNA replication and transcription by containing the origin of replication for the heavy strand (OH), the light strand promoter (LSP) and the H-strand promoters (HSP1 and HSP2) (Krishnan *et al.* 2008; Verge *et al.* 2011; Basu *et al.* 2020b). Notably, mtDNA has a mutation rate 10 to 20 times higher than nuclear DNA (nDNA) due to its less efficient DNA repair capacity, lack of protective histone proteins and exposure to elevated levels of reactive oxygen species (ROS) (Verge *et al.* 2011; Nissanka & Moraes 2020; Chen *et al.* 2022). In humans, cells gradually accumulate somatic mutations in mtDNA as a natural part of the ageing process, leading to mitochondrial dysfunction (Chinnery *et al.* 2002), mtDNA alterations include single nucleotide variants (SNVs), short insertions and deletions (indels) and large rearrangements of mtDNA fragments that are involved in primary mitochondrial disorders. While SNVs are commonly inherited (Verge *et al.* 2011; El-Hattab & Scaglia 2016; Chen *et al.* 2022), large mtDNA rearrangements (mostly deletions) tend to occur sporadically and are associated with many mitochondrial disorders. They also accumulate in post-mitotic tissues during ageing (Krishnan *et al.* 2008; Taylor *et al.* 2014). The mechanisms behind mtDNA deletions remain unknown; however, approximately 90% of clinically relevant deletions are flanked by repeat sequences generated by copy-choice recombination during active synthesis of the mtDNA light (L) strand (Persson *et al.* 2019). In addition, the polyploid nature of mtDNA allows for the coexistence of different mtDNA species within a cell, a phenomenon known as heteroplasmy. The level of heteroplasmy can vary between tissues within an individual and is typically characterised by the proportion of mutant mtDNA compared to normal mtDNA. In contrast, homoplasmy is a state in which all mtDNA molecules are identical (Nissanka & Moraes 2020).

Intellectual disability (ID) and autism spectrum disorder (ASD) are etiologically heterogeneous neurodevelopmental disorders with a combined prevalence of approximately 3% worldwide (Lai *et al.* 2014; Srour & Shevell 2014). Individuals with ID have difficulties with cognitive and adaptive skills that can affect their ability to function independently in daily life, while individuals with ASD have difficulties with social communication and interaction, as well as restricted and repetitive patterns of behaviour, interests or activities (Thurm *et al.* 2019). Approximately 4–40% of individuals with ID are thought to have comorbid ASD, while 50–70% of individuals with ASD also have comorbid ID (Goldin *et al.* 2014). While many of the causes of ID are unknown, the aetiology is largely of genetic origin (Jansen *et al.* 2023). Genetic abnormalities include single nucleotide variants (SNVs), copy number variants (CNVs) or chromosomal abnormalities that cause an inborn error of metabolism, neurodevelopmental defects and neurodegeneration. Historically, trisomy 21 was found to create a diagnostic uplift as a cause of ID (Jansen *et al.* 2023), but now *RNU4-2* and *MECP2* have been shown to be the most prevalent monogenic causes of neurodevelopmental disorders (Greene *et al.* 2024). Other genetic disorders such as fragile X syndrome, phenylketonuria, Lesch–Nyhan syndrome, Rett syndrome and 22q11.2 deletion syndrome also present with ID (Chinnery 2021). The recent introduction of clinical exome sequencing has revealed a high degree of genetic heterogeneity and a predominance of autosomal dominant de novo variants with a significant diagnostic yield of 34% (Ballesta-Martínez *et al.* 2023).

Mitochondrial disorders are a clinically heterogeneous group of disorders that result from

dysfunction of the mitochondrial respiratory chain caused by pathogenic variants in genes encoding the mitochondrial respiratory chain and related proteins, encoded in either nDNA or mtDNA. Common clinical features of mitochondrial disorders include ptosis, external ophthalmoplegia, proximal myopathy and exercise intolerance, cardiomyopathy, sensorineural deafness, optic atrophy, pigmentary retinopathy and diabetes mellitus. Central nervous system findings often include fluctuating encephalopathy, seizures, dementia, migraine, stroke-like episodes, ataxia and spasticity, as well as chorea and dementia (Chinnery 2021). Some of these conditions associated with mitochondrial disorder, are commonly observed in ID and ASD, suggesting that systemic mitochondrial defects may play a potential role in the development of these disorders (Valiente-Pallejà *et al.* 2018; Frye 2020). In addition, mitochondrial dysfunction has also been reported in individuals with ID (Valenti *et al.* 2014; Wilson *et al.* 2020) and in patients with ID-ASD (Valiente-Pallejà *et al.* 2018; Varga *et al.* 2018; Scuderi *et al.* 2023). Many nuclear variants have been associated with ID and ASD; however, while defects in mtDNA may also lead to or be involved in these disorders, there are currently very few studies in this area (Pei & Wallace 2018; Citrigno *et al.* 2020). Whole-genome sequencing (WGS) and whole-exome sequencing (WES) are crucial techniques in the current identification of the underlying genetic defects in ID and ASD; however, mtDNA is not usually specifically assessed, with a few exceptions (de Boer *et al.* 2021; Trost *et al.* 2022). Notably, specific analytical pipelines such as 'mity' have been developed to analyse mtDNA from WGS, which can detect very low levels of heteroplasmy in blood (Davis *et al.* 2022).

In a recent systematic review, we found that most of the mtDNA rearrangements reported in the brain are also present in the blood, albeit at lower cumulative read rates (Valiente-Pallejà *et al.* 2022). We therefore hypothesised that mtDNA rearrangements may be present in blood samples from patients with ID and may be involved in the complex genetic mechanisms underlying this heterogeneous neurodevelopmental disorder. We therefore used mtDNA-targeted next-generation sequencing technology combined with the MitoSAlt (Mitochondrial Structural Alterations) pipeline (Basu *et al.* 2020a) to investigate the presence of mtDNA rearrangements (duplications and deletions) in peripheral blood samples from adult patients with ID and in healthy controls (HC).

Methods

Participants

The study was carried out in patients with ID and patients with ID-ASD institutionalised in the Villablanca Care Services and in HC from the same geographical area. All subjects were adult Caucasians. The Institut Pere Mata manages the Villablanca Care Services (http://www.serveisvillablanca.cat/cat.html) as a reference centre in Catalonia for the care of people with severe and profound ID. They have cared for more than 600 people with ID, with the aim of improving their functional and adaptive autonomy, as well as their quality of life in a substitute home environment. The study was approved by the Ethics Committee of the Hospital Universitari Sant Joan de Reus, and data were coded for anonymity in accordance with current Spanish legislation on biomedical research. Informed written consent was obtained from the patient's relatives or other legal guardians and the HC. In three cases, face-to-face meetings were arranged with relatives to address concerns or doubts about the study. Although informed consent was obtained from relatives or legal guardians, samples were not collected if the participant refused to participate, which happened in two cases. The study was explained in simple language to ensure that participants and their relatives or legal guardians fully understood the procedures and objectives. Efforts were made to use simple terms and visual aids when necessary to facilitate understanding and to respect participants' autonomy and preferences. The main characteristics of the participants in the three study groups (ID, ID-ASD and HC) are shown in Table 1.

Patients' clinical records, diagnoses and physical examination results were obtained by a trained psychiatrist, as previously described (Valiente-Pallejà *et al.* 2018). Briefly, ID and ASD were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR). Weight, height and tobacco use were collected from clinical records, and BMI was calculated using the formula weight (kg)/height (m^2).

Table 1 Main characteristics of the participants in the three study groups: intellectual disability (ID), intellectual disability with comorbid autism spectrum disorder (ID-ASD) and healthy controls (HC)

	ID ***N* = 76**	**ID-ASD** ***N* = 59**	**HC** ***N* = 32**	**Compared groups**	**Statistics**	***P* value**
Sex, *N* (%)						
Male	48 (63)	32 (54)	17 (53)	ID *vs.* ID-ASD	$\chi^2 = 0.95$	0.295
Female	28 (37)	27 (46)	15 (47)	ID *vs.* HC	$\chi^2 = 0.95$	0.331
				ID-ASD *vs.* HC	$\chi^2 = 0.01$	0.919
Age, years	52.5 ± 10.2	41.3 ± 8.7	42.4 ± 12.4	ID *vs.* ID-ASD	$t = 6.75$	**<0.001**
				ID *vs.* HC	U = 650	**<0.001**
				ID-ASD *vs.* HC	$t = -0.487$	0.627
BMI, kg/m^2	26.8 (17.6–45.8)	23.4 (17.1–40.8)	23.6 (17.5–31-1)	ID *vs.* ID-ASD	U = 1604	**0.007**
				ID *vs.* HC	U = 581	**<0.001**
				ID-ASD *vs.* HC	U = 753	0.141
Tobacco use, *N* (%)						
No	69(92)	58 (98)	23 (72)	ID *vs.* ID-ASD	FET	0.134
Yes	6 (8)	1 (2)	9 (28)	ID *vs.* HC	$\chi^2 = 7.54$	**0.006**
				ID-ASD *vs.* HC	FET	**<0.001**
ID severity, *N* (%)						
Moderate	1 (1.3)	0	—	ID *vs.* ID-ASD	$\chi^2 = 21.4$	**<0.001**
Severe	45 (59.2)	12 (20.7)	—			
Profound	30 (39.5)	46 (79.3)	—			
CARS score	21 (14.5–39.5)	41 (28.0–55.0)	—	ID *vs.* ID-ASD	U = 78.5	<0.001
Genetic alterations, *N* (%)						
nDNA	7 (9)	15 (25)	—	ID *vs.* ID-ASD	$\chi^2 = 6.4$	**0.011**
mtDNA	10 (13.2)	1 (1.7)	1 (3.1)	ID *vs.* ID-ASD	FET	**0.023**
				ID *vs.* HC		0.169
				ID-ASD *vs.* HC		1

ASD, autism spectrum disorder; BMI, body mass index; CARS, Childhood Autism Rating Scale; FET, Fisher's exact test; ID, intellectual disability; mtDNA, mitochondrial DNA; N, number of cases; nDNA, nuclear DNA.
Data are shown as the *N* (%), mean ± standard deviation or median (range). Significant differences are indicated in bold.

The diagnosis of ASD was confirmed using the Autism Diagnostic Interview-Revised (ADI-R). In addition, the Childhood Autism Rating Scale (CARS) was used to measure the severity of autism in the ASD group and to examine autism features in the ID group. Due to the challenges of diagnosing ASD in individuals with severe and profound ID, only patients who scored positive on both assessments (DSM-IV-TR and ADI-R) were included in the ID-ASD group. Severity of ID was determined by IQ range (mild, IQ 52–69; moderate, IQ 36–51; severe, IQ 20–35; and profound, IQ 19 or below). In addition, patients underwent a comprehensive laboratory workup, including standard karyotyping, subtelomeric and targeted multiplex ligation probe amplification (MLPA) screening for chromosomal abnormalities, fragile X molecular testing and array comparative genomic hybridisation (aCGH), as previously described (Cuscó *et al.* 2009). The main results of the genetic tests are shown in Table S1.

HC were assessed by an experienced psychiatrist using the Spanish adaptation of the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) (Vázquez-Barquero *et al.* 1994) to exclude a past or current history of psychiatric disorders. Information on clinical conditions commonly associated with mitochondrial disorders was also obtained from the controls.

mtDNA-targeted next-generation sequencing and prediction of mtDNA rearrangements

Total DNA was extracted from participants' peripheral blood mononuclear cells, with all samples

processed in our centre's biobank (https://www.iispv.cat/plataforma-de-suport/biobanc-iispv/) using standardised protocols and the Gentra® PureGene reagent (Qiagen, Barcelona, Spain). DNA isolation and storage conditions were consistent across all participants. Total mtDNA was amplified in overlapping fragments and sequenced using an Ion Torrent Personal Genome Machine (PGM, Fisher Scientific, Madrid, Spain), as previously described (Valiente-Pallejà *et al.* 2018, 2020).

Read quality was assessed using the FastQC (Andrews 2010) and MultiQC (Ewels *et al.* 2016) tools. Cutadapt (v.3.4) (Martin 2011) was used to remove 10 bases from the start of reads and bases with Phred quality scores less than 20 (sequencing error greater than 1%) from the 3′ and 5′ ends, and to crop reads at 200 bps (Harvey *et al.* 2019; Cortes-Figueiredo *et al.* 2021). Read depth at each position and mean coverage of sequences were calculated using SAMtools (v.1.13) (Li *et al.* 2009). Sequences with coverage greater than 99% and mean read depth greater than 100x were included in the analysis, as recommended (Yao *et al.* 2018; Harvey *et al.* 2019). The statistics of BAM files are shown in Table S2.

The MitoSAlt (v.1.1.1) pipeline (Basu *et al.* 2020a) was then used to detect and quantify mtDNA rearrangements. This tool allows the detection of mtDNA rearrangements even at extremely low levels of heteroplasmy, as is common in blood samples (Taylor *et al.* 2014; Hjelm *et al.* 2019). MitoSAlt is available on SourceForge at https://sourceforge.net/projects/mitosalt to identify, quantify and visualise both deletions and duplications of mtDNA, as it predicts rearrangements based on split alignments to the linear mitochondrial genome (Basu *et al.* 2020a). The MitoSAlt parameters used in this study are listed in Table S3. The MitoSAlt plots of patients carrying more than one mtDNA rearrangement are also shown in Fig. S1. All identified mtDNA breakpoints were adjusted for the presence of direct repeats by using the MitoBreak Classifier tool (Damas *et al.* 2014a), and we checked whether they had been previously reported in the MitoBreak database (Damas *et al.* 2014a) and the Splice-Break dataset (Hjelm *et al.* 2019).

Statistical analyses

Statistical tests were chosen according to the nature of the variables. Normality of continuous variables was tested using the Shapiro–Wilk test. In cases where the data were normally distributed, *t*-tests were used to compare groups, and in cases of non-normal distribution, the Mann–Whitney *U* test was used. Discrete data were evaluated by using the Pearson chi-squared test when less than 20% of cells had fewer than five observations. Otherwise, Fisher's exact test was used (Kim 2017). *Post hoc* power evaluations were performed using the *post hoc* power calculator, https://clincalc.com/stats/Power.aspx. The significance level was set at $P < 0.05$. All data were processed using jamovi 2.3.24 (https://www.jamovi.org/) (The jamovi 2022).

Results

mtDNA rearrangements

We obtained mtDNA sequences from 195 patients with ID, 98 of whom had comorbid ASD (ID-ASD), and 39 HC subjects. After quality control, we excluded four ID and one ID-ASD sample because we did not obtain a complete mtDNA sequence (less than 99% coverage of the mitochondrial genome). In addition, 17 ID, 38 ID-ASD, and 7 HC samples were excluded due to low mean read depth (<100×). Therefore, the final dataset consisted of 76 mtDNA sequences from patients with ID (mean age 52.5 years old; standard deviation [SD] 10.2), 59 from patients with ID-ASD (mean age 41.3 years; SD 8.7) and 32 from HC (mean age 42.4 years; SD 12.4), with a mean read depth of 241 (range 103–1677) (Fig. 1).

We identified a total of 12 unique mtDNA rearrangements in 10 individuals with ID (13.2%), one individual with ID-ASD (1.7%) and one HC (3.1%). Although we found a higher frequency of mtDNA rearrangements in patients (ID and ID-ASD) than in HC, this difference was not statistically significant (U = 3.4; $P = 0.465$). We observed significant differences in the number of mtDNA rearrangements between the three study groups, as it can be seen in Table 2 ($\chi^2 = 7.523$, $P = 0.023$); however, in the *post hoc* analysis, we found no differences between ID and HC, probably due to the low power (*post hoc* power = 31%, considering $\alpha = 0.05$ and $P = 0.05$). The median size of the detected mtDNA rearrangements was 8332 bp (range 29–13 521 bp), with a median heteroplasmy level of 2.8% (range 1.1–6.7%). Six mtDNA rearrangements (50%) were identified as deletions with a median size

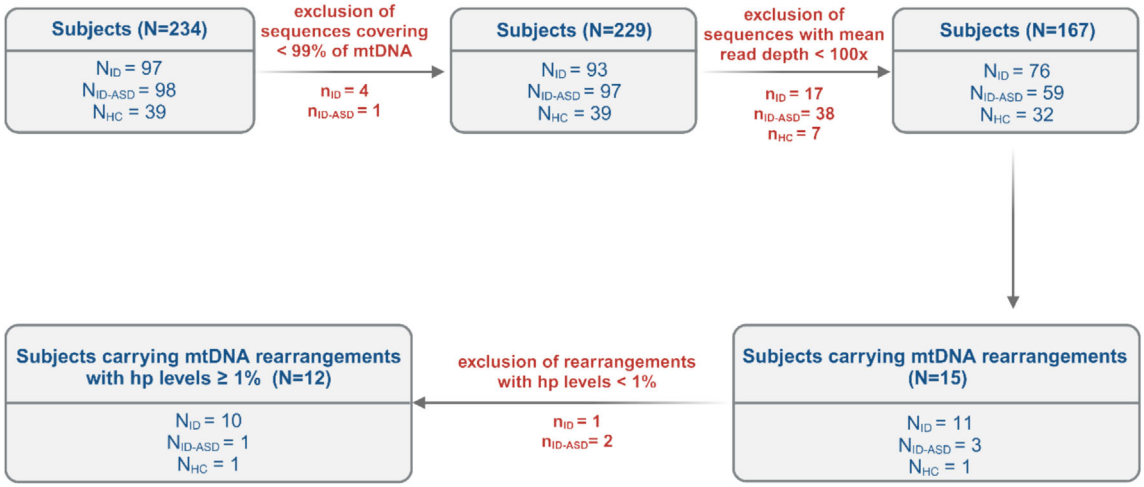

Figure 1. Study workflow including the initial number of participants and those included (in blue)/excluded (in red) after each quality control step. Created with Biorender.com.

Table 2 mtDNA rearrangements identified in the three study groups: intellectual disability (ID), intellectual disability with comorbid autism spectrum disorder (ID-ASD) and healthy controls (HC)

	ID (*N* = 76)	ID-ASD (*N* = 59)	HC (*N* = 32)	Statistic	*P* value
Individuals presenting rearrangements, *n* (%)	10 (13.2)	1 (1.7%)	1 (3.1)	χ^2 = 7.523	**P = 0.023**
Number of subjects presenting deletions	3	1	1	χ^2 = 0.583	*P* = 0.747
Heteroplasmy level (%), median	3.1	1.4	2.8	—	—
Size (bp), median	8332	6128	6517	—	—
N of subjects presenting duplications	8	0	0	—	—
Heteroplasmy level (%), median	2	—	—		
Size (bp), median	8,698	—	—		

bp, base pairs.

Post hoc comparisons (ID *vs.* ID-ASD, χ^2 = 5.832, *P* = 0.016; ID *vs.* HC, χ^2 = 2.478, *P* = 0.116; ID-ASD *vs.* HC, χ^2 = 0.197, *P* = 0.576). Significant p-values are highlighted in bold.

of 6444 bp and a median heteroplasmy level of 2.6%, and another six rearrangements (50%) were identified as duplications with a median size of 10 455 bp and a median heteroplasmy level of 3.0% (Table 3). A 29 bp duplication, m.8765-8793dup, was present in four individuals with ID. In addition, two individuals with ID (ID-101 and ID-104) and one with comorbid ASD (ID-ASD-152) carried two mtDNA rearrangements. The highest levels of heteroplasmy were observed in the 29 bp duplication carried by patients ID-101 (6.7%) and ID-112 (5.3%). Also of note are m.9422-15606del, a 6185 bp deletion with a heteroplasmy level of 5.2% present in patient ID-036, and m.61-13581dup, a 13 521 BP duplication with a heteroplasmy level of 4.6% present in patient ID-008.

Regions involved in mtDNA rearrangements

In seven of the 11 patients (63.6%), mtDNA rearrangements involved at least one of the mtDNA replication origins: 4/7 patients (57.2%) had a duplication in the OH, while 3/7 patients (42.8%) had a deletion in the OH. Two of them had duplications in both the OH and the OL. In addition, ID-104

Table 3 Characteristics of the mtDNA rearrangements identified in the participants of the study

Group	Rearrangement N	Subject ID	Mean read depth	5′ bkp	3′ bkp	Hp % (range)	Rearrangement size	Final event
ID	1	ID-008	190	61[§]	13581[§,†,‡]	4.6	13 521	dup
	2	ID-011, ID-019, **ID-101**, ID-112	190	8765[§]	8793[§,†]	4.1 (2.0–6.7)	29	dup
	3	ID-036	255	9422	15 606[†,‡]	5.2	6185	del
	4	ID-074	284	9003[§,‡]	621	3.1	8186	del
	5	ID-075	241	7112[†]	638	1.7	10 096	dup
	6	ID-083	378	348[§]	11 391[§]	1.3	11 044	dup
	7	**ID-101**	180	8895[§]	805[§,‡]	1.4	8478	del
	8	**ID-104**	176	8764	892[‡]	1.1	8698	dup
	9			8580[§,†]	2823[§,‡]	2.0	10 813	dup
ID-ASD	10	**ID-ASD-152**	284	6075[†,‡]	11 962[‡]	1.1	5886	del
	11			11 317[‡]	1117	1.7	6370	del
HC	12	HC-019	161	9090	15 606	2.8	6517	del

ASD, autism spectrum disorder; bp, base pair; bkp, breakpoint; del, deletion; dup, duplication; hp, heteroplasmy level; ID, intellectual disability; n.d., not detected.

Patients carrying more than one mtDNA rearrangement are highlighted in bold.

[§]Corrected breakpoint according to MitoBreak Classifier.

[†]Breakpoint reported in the MitoBreak database.

[‡]Breakpoint reported in the Splice-Break dataset.

carried two different duplications in the OH (Fig. 2). The deletion present in the HC did not involve any of the replication origins or promoter regions.

The genes *MT-ATP6*, *MT-CO3*, *MT-TT*, *MT-ND3*, *MT-TW*, *MT-ND4L* and *MT-ND4* were affected in eight patients with ID. Indeed, these genes were deleted in four subjects with an average heteroplasmy level of 2.5% and duplicated in another four subjects with an average heteroplasmy level of 2.2%. In addition, the 29 bp duplication, m.8765-8793dup, present in four patients with an average heteroplasmy level of 4.1%, involved the *MT-ATP6* gene.

One HC subject had a deletion between the *MT-ATP6* and *MT-CYB* genes with a heteroplasmy level of 2.8%, affecting eight protein genes and six tRNA genes; however, the replication and transcription capacity was not altered as it did not involve the replication and transcription origins of the mtDNA.

Demographic and clinical characteristics between mtDNA rearrangement carriers and non-carriers

The mean age of the ID group was significantly higher than that of the ID-ASD group ($P < 0.001$) and the HC group ($P < 0.001$). Similarly, subjects with ID had a significantly higher BMI than the ID-ASD and HC groups ($P = 0.007$ and $P < 0.001$, respectively). However, we did not observe significant differences between carriers and non-carriers of mtDNA rearrangements in age (U = 754, $P = 0.276$), sex ($\chi^2 = 3.31e\text{-}4$, $P = 0.985$), BMI (U = 915, $P = 0.958$) or tobacco use ($\chi^2 = 0.0253$, $P = 0.874$).

We observed that mtDNA carriers have a significantly lower CARS score (U = 392, $P = 0.028$) and lower ID severity ($P = 0.036$) compared to non-carriers. In addition, we observed no association between CARS scores or ID severity and the presence of mtDNA rearrangements in replication or transcription origins. Considering only the ID group, no significant differences were observed in the classification of ID severity (moderate, severe and profound) between carriers and non-carriers of mtDNA rearrangements ($P = 0.165$) and in heteroplasmy levels ($P = 0.286$).

Clinical characteristics of the participants with mtDNA rearrangements

Some notable clinical features were present in patients with mtDNA rearrangements (Fig. 3). None

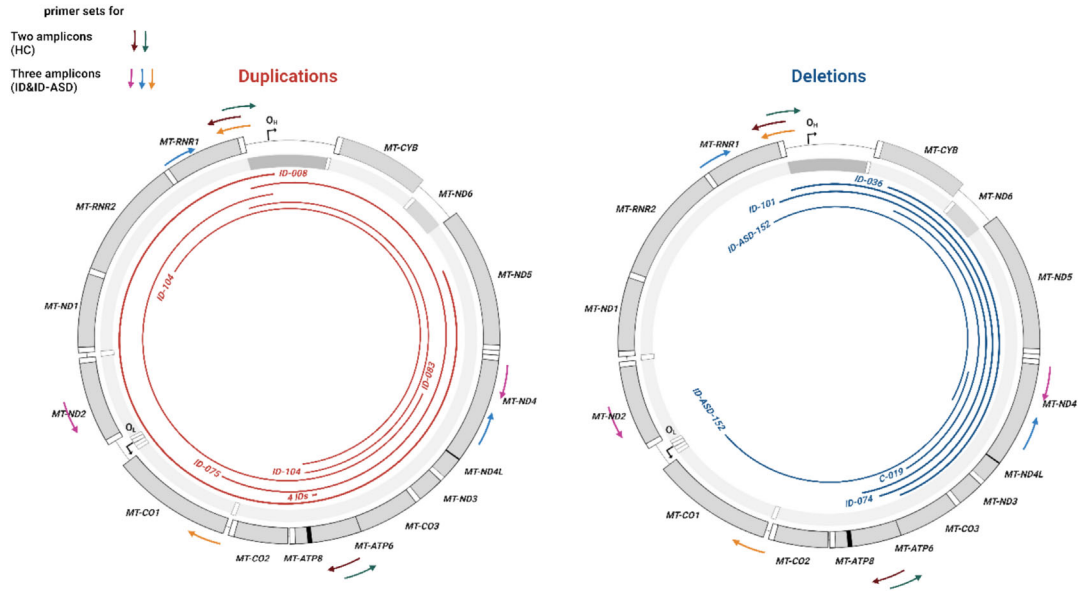

Figure 2. Illustrative plots showing the location of the mtDNA duplications (left) and deletions (right) detected by MitoSAlt. The four IDs represent ID-011, ID-019, ID-101 and ID-112, which carried the 29 bp duplication. Primer binding sites used for mtDNA amplification are also indicated. Created with BioRender.com.

of these patients, apart from ID-083, had nuclear DNA rearrangements. ID-008 was a 50-year-old woman with severe ID who carried a 13 521 bp duplication and had a diagnosis of a psychotic disorder not otherwise specified, Hashimoto's thyroiditis, high cholesterol, obesity (BMI 31.6) and constipation. ID-036, a 39-year-old woman with profound ID, carried a 6185 bp deletion and had comorbid obesity (BMI 33.1), constipation, seizures, hypertension and nocturnal enuresis. ID-074, a 48-year-old overweight man (BMI 27.8) with profound ID, carried an 8186 bp deletion and had comorbid conditions including psychotic disorder with onset in infancy, cerebral palsy, congenital mitral stenosis and arthritis. ID-075 was a 53-year-old man with severe ID who carried a 10 096 bp duplication and had a history of acute hepatitis B, anaemia and immune thrombocytopenia. ID-083 was a 48-year-old man with profound ID and a diagnosis of cri-du-chat syndrome (5p14 deletion), who carried an 11 044 bp duplication and was also diagnosed with situs inversus, congenital cataract and optic atrophy. This individual also had congenital cardiopathy due to the persistence of the patent ductus arteriosus and a heart murmur. He also showed symptoms of multiple depressive episodes and had a history of suicide attempts. ID-101 was a 50-year-old woman with severe ID carrying both an 8480 bp deletion and a 29 bp duplication. She was reportedly obese (BMI 45.8) and had comorbid diagnoses of epilepsy with generalised seizures, hirsutism, constipation and lymphatic filariasis. ID-104 was a 53-year-old woman with severe ID carrying two duplications of 8689 and 10 813 bp. She had a grade II/IV systolic heart murmur, spinal osteoarthritis, constipation and obesity (BMI 30.6). ID-ASD-152, the only individual diagnosed with severe ID and ASD, was a 21-year-old male carrier of two duplications (5886 bp and 6370 bp), who had comorbid constipation, thrombocytopenia and Becker nevus syndrome. Thus, among the 11 patients with mtDNA rearrangements, constipation was present in six patients, while seizures and uncorrected visual impairment were present in three patients each.

Three males and a one female with severe ID carried the 29 bp duplication. Within this group, ID-011 also had inactive hepatitis B and hypertension; ID-019 had amblyopia, pleural thickening and pes cavus; and ID-112 had chronic hepatitis B, hypertension, diabetes mellitus and a cataract.

Finally, the HC carrying a 6517 bp deletion was a 50-year-old woman diagnosed with migraine.

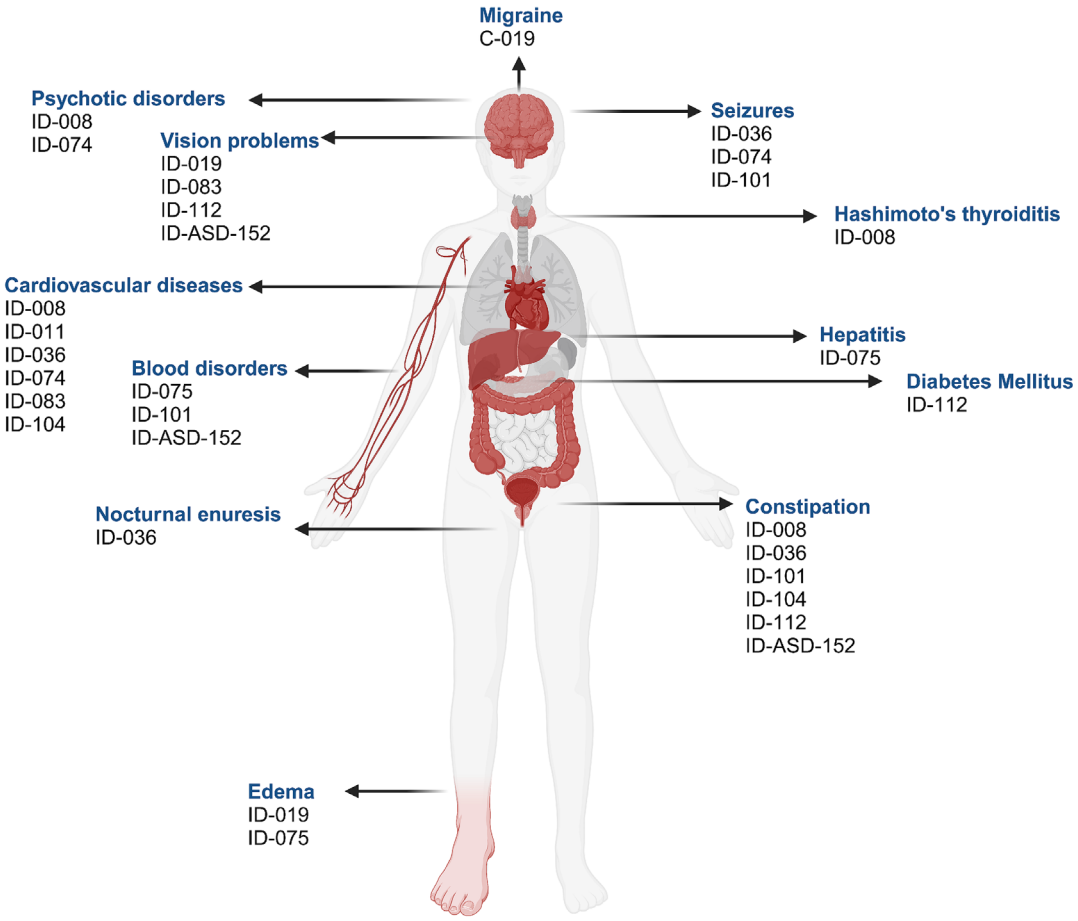

Figure 3. Clinical conditions associated with mitochondrial disorders present in individuals carrying mtDNA rearrangements. ASD, autism spectrum disorder; ID, intellectual disability. Created with BioRender.com.

Because of the large number of clinical conditions associated with mitochondrial disorders observed in patients with ID (Fig. 3), we investigated whether these conditions were more frequent in carriers of mtDNA rearrangements than in non-carriers; however, no significant differences were observed. For example, constipation was reported in 54.5% of the mtDNA rearrangement carriers and 67.7% of non-carriers ($P = 0.505$), and seizures were reported in 51.6% of the mtDNA rearrangement carriers and 27.3% of non-carriers ($P = 0.207$).

Discussion

Despite the predominant focus on nuclear DNA in studies of neurodevelopmental disorders, the observed mitochondrial dysfunction in ID and ID comorbid with ASD (ID-ASD) highlights the importance of assessing mtDNA in individuals with these clinical conditions (Valiente-Pallejà *et al.* 2018; Varga *et al.* 2018; Noda 2022). Two recent informatics tools, eKLIPse and MitoSAlt, analyse major mtDNA rearrangements. Among them, MitoSAlt stands out as the most accurate and sensitive tool to detect, quantify and visualise both mtDNA deletions and duplications (Goudenège *et al.* 2019; Basu *et al.* 2020a). To date, the mechanism underlying mtDNA deletions and their potential impact on mitochondrial dysfunction has been more extensively studied than the mechanism underlying duplication formation (Krishnan *et al.* 2008; Fontana & Gahlon 2020). For example, in MitoBreak, only 44 mtDNA duplications between 150 bp and 13.5 kb in length were reported out of a

total of 1356 mtDNA rearrangements (Damas *et al.* 2014a).

Here, we used MitoSAlt to identify mtDNA rearrangements in patients with ID, ID-ASD and HC. Our results showed that mtDNA rearrangements were present in 13.2% of individuals with ID, 1.7% of individuals with ID-ASD and 3.1% of HC. This result highlights that patients with ID are more likely to carry mtDNA rearrangements in their blood than HC; however, statistical significance was not achieved, probably due to the small sample size, as our study had low power to detect differences between ID and HC groups. We report 12 new mtDNA rearrangements, six deletions and six duplications. While the rearrangements have not been previously reported in MITOMAP or MitoBreak, most of the identified breakpoints were reported in MitoBreak. Deletions were identified in the range of 5.9–8.4 kb, which is similar to previous results showing mtDNA deletions ranging from 2.4 to 7.9 kb in children with ASD (Varga *et al.* 2018) and in postmortem brain tissue and blood samples from individuals with psychiatric disorders (Varga *et al.* 2018; Valiente-Pallejà *et al.* 2022). Blood is a self-renewing tissue and tends to lose mtDNA species with partial deletions (Taylor *et al.* 2014; Hjelm *et al.* 2015; Filograna *et al.* 2021), which is consistent with the low levels of heteroplasmy observed in our study. However, the fact that we detected mtDNA rearrangements in the blood suggests that they are also present in other tissues, such as the brain, where higher levels of heteroplasmy can be observed (Damas *et al.* 2014b; Valiente-Pallejà *et al.* 2022). It is important to note that mtDNA heteroplasmy is quite common in humans, typically occurring at lower levels ranging from 0.5% to 1.5%. In contrast, the levels reported in this study are notably higher, reaching up to 6.7%. However, it is important to note that these findings relate to mtDNA SNVs and that mtDNA rearrangements have not yet been investigated (Payne *et al.* 2013; Parakatselaki & Ladoukakis 2021). In terms of duplications, we reported a large duplication of 13 521 bp in length, encompassing both replication origins, the entire *MT-ND4* gene and part of the *MT-ND5* gene. Similarly, MitoBreak reported a duplication of 13 491 bp that also included both replication origins and, in this case, the entire *MT-ND5* gene and part of the *MT-ND4* gene. Importantly, three duplication carriers in our study showed optic atrophy, amblyopia and cataracts, and most single duplications reported in MitoBreak were identified in patients with progressive external ophthalmoplegia (Damas *et al.* 2014a).

In terms of genes involved in mtDNA rearrangements, *MT-ND4*, which plays a critical role in complex I assembly (Giachin *et al.* 2016), was deleted (or partially deleted) in most of the deleted mtDNA molecules we identified. In a previous study, *MT-ND4* gene deletion was also found in the frontal cortex of 44% of ASD patients examined by quantitative real-time PCR (Gu *et al.* 2013). Similarly, we found that the *MT-ATP6* gene was deleted in four patients, which could lead to defects in complex V (ATP synthase) activity. The human *MT-ATP6* transcript, m.8527-9207, consists of 681 bp, and the resulting polypeptide subunit has 226 residues. The four duplication carriers we identified had a transcript length of 710 bp, and although the results of the duplication do not change the first 22 amino acids, two new amino acids (K and S) and a stop codon are introduced afterwards, probably resulting in a non-functional ATP6 subunit of 24 residues. *MT-ND3*, *MT-ND4*, *MT-ND4L*, *MT-ND5* and *MT-ND6* (complex I, NADH dehydrogenase) were deleted in five patients who may therefore have defects in ATP generation. This finding also supports the results of a meta-analysis of 300 studies reporting 730 mtDNA deletions and 37 mtDNA duplications, which showed that the mtDNA fragment between the *MT-ATP8*/*MT-ATP6* and *MT-ND5* genes was absent in more than 70% of reported clinical features associated with mitochondrial defects (Damas *et al.* 2014b). Finally, the OH deficiency found in three patients in our study is not commonly observed in wild-type mtDNA molecules, as it results in limited replication capacity (Lujan *et al.* 2020). As expected, no individual in this study was missing both replication origins.

We examined the socio-demographic and clinical characteristics of the patients with mtDNA rearrangements. No differences in age or sex were observed between carriers and non-carriers of mtDNA rearrangements. Patients carrying mtDNA rearrangements had significantly lower CARS scores and lower ID severity than non-carriers; however, it is likely that these significant differences are due to differences between the two clinical groups, ID and ID-ASD, and not to the presence or absence of mtDNA rearrangements, since CARS scores were

lower and ID severity was higher in the ID-ASD group than in the ID group, and only one patient out of 59 (1.7%) in the ID-ASD group had an mtDNA rearrangement. The lower frequency of mtDNA rearrangements observed in individuals with ID comorbid with ASD, compared with those without ASD, reflects differences in the underlying mechanisms of these neurodevelopmental disorders. It is possible that mitochondrial dysfunction plays a different role in the pathophysiology of ID and ASD, with greater mitochondrial involvement in ID. This may explain the higher frequency of mtDNA rearrangements found in individuals with ID alone, suggesting that mtDNA abnormalities may contribute more to the aetiology of ID than ASD.

People with ID have higher prevalence rates of poor social determinants of health, behavioural risk factors, depression, diabetes and suboptimal health status compared with adults without ID (Krahn & Fox 2014). People with ID also have a higher risk of weight gain than the general population (Koritsas & Iacono 2016; Skelly *et al.* 2021), and obesity is a major factor in the development of metabolic disorders, such as hypertension, high cholesterol and hyperglycaemia, which are associated with mitochondrial dysfunction (Todosenko *et al.* 2023). Consistently, six of the 11 patients carrying an mtDNA rearrangement in this study had a BMI in the obese or overweight range. Six patients had constipation, which is a prominent gastrointestinal manifestation in mitochondrial disorders (Finsterer & Frank 2017). However, we could not discard whether constipation is a consequence of patient inactivity or the mtDNA rearrangement itself. Psychotic disorders and schizophrenia, which were present in two patients with mtDNA rearrangements in this study, are also common psychiatric manifestations in mitochondrial diseases (Anglin *et al.* 2012). Three patients had seizures, which have been reported to occur in 35–60% of people with mitochondrial disease (Rahman 2012). We have previously reported mitochondrial dysfunction and low mtDNA copy number in a family with psychosis and chronic fatigue syndrome (Torrell *et al.* 2017). In addition, two mtDNA deletions between the *MT-RNR1* and *MT-CYTB* gene regions have been reported in brain samples from patients with schizophrenia (Hjelm *et al.* 2015), which is consistent with the mtDNA rearrangement present in the individual with schizophrenia in this study. Optic neuropathy is also a hallmark of mitochondrial disorders and is associated with the male sex (Carelli *et al.* 2023). In this study, we found optic atrophy and a congenital cataract in a male individual carrying a 5.5 kb deletion, which is similar to the 6.7 kb mtDNA deletion found in a male subject with a neuromuscular disease who presented with congenital cataract as the first symptom (Bene *et al.* 2003). In addition, one of the patients carrying the 29 bp duplication in the *MT-ATP6* gene also showed amblyopia, which is common in Leber hereditary optic neuropathy (Yu-Wai-Man *et al.* 2011). Three patients with mtDNA rearrangements in the present study had congenital heart disease or a heart murmur. Cardiac involvement in mitochondrial diseases is common through the effect of metabolism in the heart (Bates *et al.* 2012). Similarly, some studies have reported that patients with ID and mitochondrial disorders also have epilepsy (Guevara-Campos *et al.* 2015; Ortiz-González 2021). Finally, epileptic seizures have been reported in 5% of patients with large mtDNA deletion-associated syndromes with deletions of 2–9 kb (Björkman *et al.* 2023). In our study, we found one subject with an 8 kb deletion who presented with epilepsy with generalised seizures. Therefore, common features associated with mitochondrial disorders were present in the study subjects; however, we did not observe that these features were more frequent in mtDNA rearrangement carriers than in non-carriers. In addition, when considering only the ID group, we observed no differences in the severity of ID between carriers and non-carriers, nor in the levels of heteroplasmy. This could be due to the small number of samples in our study or because the levels of heteroplasmy in the blood do not correlate with the levels in other tissues, such as the brain.

To our knowledge, this is the first study to investigate low levels of heteroplasmy in the blood (range of 1.1–6.7%) using these new tools, next-generation sequencing and the MitoSAlt pipeline. We observed a high frequency of mtDNA rearrangements in blood samples from patients with ID. Due to the limitations of the previous sequencing technologies, studies have generally examined mtDNA deletions with a high heteroplasmy threshold, usually above 10% (Guo *et al.* 2013). Therefore, mtDNA rearrangements with low levels of heteroplasmy have not been extensively studied.

Next-generation sequencing allows for high deep reads, and MitoSAlt is able to identify heteroplasmy levels above 0.5%, allowing for an accurate measurement of low heteroplasmy levels. Therefore, in the coming years, with the decreasing cost of high-throughput sequencing technologies and the development of bioinformatics tools such as MitoSAlt, these new methods will provide a powerful tool to study the involvement of low levels of heteroplasmy in diseases where mitochondrial dysfunction is present.

We are aware that our study has some limitations. First, we analysed blood samples, a self-renewing tissue that tends to lose mtDNA deletion variants. However, obtaining blood is a non-invasive procedure, and the presence of mtDNA rearrangements in blood could be indicative of their presence in post-mitotic tissues such as the brain, where they may be present at higher levels of heteroplasmy (Valiente-Pallejà *et al.* 2022). We also recognise that collecting additional samples from other tissues would be beneficial for cross-validation of heteroplasmic rearrangements (Krjutškov *et al.* 2014). Second, although Ion Torrent technology is widely used, it has some shortcomings that require additional manual curation of the data (Seneca *et al.* 2015; Harvey *et al.* 2019), which we addressed by applying several quality control criteria. Third, the difference in the number of amplified mtDNA fragments between controls (two fragments) and individuals with ID and ID-ASD (three fragments). This difference in amplification strategy raises the possibility of introducing technical artefacts during library preparation and sequencing. Such artefacts could affect interpretations when comparing between groups; however, the BAM file statistics did not show differences between data obtained using two and three fragments. Fourth, this study was performed on individuals with a mean age of approximately 47 years old. Although we did not find age differences between carriers and non-carriers of mtDNA rearrangements, the lack of younger participants in our dataset may be a confounding factor in understanding the age-related accumulation of mitochondrial DNA variants. Fifth, although some genetic testing was performed to identify the presence of mutations in the nuclear genome, no exome (or genome) sequencing was performed, and it cannot be excluded that the clinical features of the patients have a nuclear genetic cause. However, the contribution of both genomes to the observed features cannot be excluded either. Sixth, the study mainly evaluated patients with severe and profound ID, and further studies are needed to know whether they can be generalised to mild or moderate ID. Finally, our study had a relatively small sample size, especially in the HC group, which reduced the statistical power. Therefore, we were not able to detect statistically significant differences between the groups or to assess whether the presence of an mtDNA rearrangement was associated with a specific clinical feature.

In this study, we identified mtDNA rearrangements with low levels of heteroplasmy in blood samples of individuals with ID. However, it is challenging to associate it with clinical features. To address it, future mtDNA analysis in neurodevelopmental diseases like ID should consider using databases like MitoPhen (Ratnaike *et al.* 2021), MitoBreak and MITOMAP, which offer valuable insights into the relationship between clinical features and heteroplasmy levels of mtDNA rearrangements, helping to close gaps in our understanding of these diseases.

Conclusions

mtDNA rearrangements with low levels of heteroplasmy were identified in blood samples from individuals with severe or profound ID, suggesting that these rearrangements may be present with higher levels of heteroplasmy in other post-mitotic tissues, such as the brain. This is the first study to investigate the presence of mtDNA rearrangements in blood samples from patients with ID. Future studies are needed to confirm the present findings and to determine whether these rearrangements are a contributing factor to ID or a downstream effect of the neurodevelopmental process. Identifying the presence of mtDNA rearrangements in individuals with neurodevelopmental disorders is likely to have therapeutic relevance, as disorders with different aetiological causes may respond differently to specific treatments. In the present study, we found that mtDNA-targeted next-generation sequencing and the high-throughput MitoSAlt computational pipeline are very sensitive tools for detecting mtDNA rearrangements in blood and low levels of heteroplasmy.

Author contributions

Design: G.M. and L.M. *Methodology*: B.K.B., J.T. and L.T.-E. *Investigation*: A.V.-P., R.M.-L. and E.V. *Data curation*: G.M. *Writing—original draft preparation*: B.K.B. and L.T.-E. *Writing—review and editing*: G.M. and L.M. *Supervision, project management and funding acquisition*: G.M. and L.M. All authors read and approved the published version of the manuscript.

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Conflict of interest

The authors report no competing interests. The funders had no role in the design of the study; in the collection, analysis or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

Ethics statement

The study was conducted in agreement with the guidelines of the Helsinki Declaration as revised in 1975 and approved by the Ethics Committee of the Hospital Universitari Sant Joan de Reus (protocol code 12-06-28/6proj2, date of approval 28/06/2012). Written informed consent was obtained from the relatives or other legal guardians of the patients and the HCs.

Data availability statement

Datasets of mtDNA sequences and clinical data of the participants are available at the European Genome Phenome Archive (EGA, https://ega-archive.org) under the study reference numbers EGAS00001002750 and EGAS00001003269. Other data collected for this study can be provided upon reasonable request.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Representative graphs obtained with MitoSAlt of the mtDNA alterations present in patients ID-104 (carrier of two duplications), ID-ASD-152 (carrier of two deletions) and ID-101 (carrier of one duplication and one deletion).
Table S1. Information for each participant.
Table S2. Summary statistics of BAM files.
Table S3 MitoSAlt parameters used in the analysis.