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ORIGINAL ARTICLE



Infectious stress triggers a POLG-related mitochondrial disease

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Abstract

A 3-year-old girl presented with severe epilepsy in the context of *Borrelia* infection. After ceftriaxone/lidocaine administration, she showed secondarily generalized focal crises that led to neurological and motor sequelae. Genetic studies identified in the patient two heterozygous *POLG* mutations (c.2591A>G; p.Asn864Ser and c.3649G>C; p.Ala1217Pro). Through analysis of POLG activity in cultured fibroblasts, we confirmed that the mutations altered the mtDNA turnover. Moreover, patient fibroblasts were more sensitive than controls in the presence of a mitochondrial replication-affecting drug, the antiretroviral azidothymidine. To test if ceftriaxone treatment could worsen the deleterious effect of the patient mutations, toxicity assays were performed. Cell toxicity, without direct effect on mitochondrial respiratory function, was detected at different antibiotic concentrations. The clinical outcome, together with the different in vitro sensitivity to ceftriaxone among patient and control cells, suggested that the mitochondrial disease symptoms were hastened by the infection and were possibly worsened by the pharmacological treatment. This study underscores the benefit of early genetic diagnosis of the patients with mitochondrial diseases, since they may be a target group of patients especially vulnerable to environmental factors.

Keywords Mitochondrial disease . Mitochondrial DNA . Mitochondrial DNA polymerase . Azidothymidine . Ceftriaxone

# Introduction

Human mitochondria contain their own genome, the mito- chondrial DNA (mtDNA), encoding for two ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and a subset of proteins required for the oxidative phosphorylation [[1](#_bookmark2)]. The replicase DNA polymerase γ (pol γ) is the responsible for mtDNA replication and repair in mitochondria. Accordingly, pol γ is important for mtDNA maintenance and cellular ener- gy supply, and reduced pol γ activities lead to mtDNA

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depletion and impairment of cellular metabolism. The pol γ holoenzyme consists of a catalytic subunit (encoded by *POLG*), which possesses both polymerase and proofreading exonuclease activities in separate domains, and a dimeric form of its accessory subunit (encoded by *POLG2*), which in- creases enzyme processivity [[2](#_bookmark3)].

In 2001, the first disease mutations were identified in *POLG* [[3](#_bookmark4)]. Since then, over 200 mutations in *POLG* have been associated with mitochondrial diseases ([http://tools.niehs](http://tools.niehs/). [nih.](http://nih.gov/polg) [gov/polg](http://nih.gov/polg)/) [[4](#_bookmark5)–[6](#_bookmark7)]. The identification of disease-causing *POLG* mutations is challenging because *POLG* gene locus is highly polymorphic and the appearance of disease symptoms can be dependent on epigenetic and environmental factors [[5](#_bookmark6), [7](#_bookmark8)]. Pathogenic mutations have been found to affect the activity of the polymerase, the fidelity of replication, and the forma- tion of the holoenzyme [[2](#_bookmark3)]. The clinical identification of POLG-related diseases is difficult because patients present an overlapping range of symptoms and signs with multiple organ system involvement and with variable severity [[6](#_bookmark7)]. Clinical phenotype expresses habitually in the central and pe- ripheral nervous, musculoskeletal, and gastrointestinal sys- tems [[5](#_bookmark6), [6](#_bookmark7), [8](#_bookmark9)]. The disease onset is very variable, ranging from infancy to late adulthood, being the most frequent age of onset during childhood and adolescence. Both autosomal dominant

and recessive transmission have been documented for POLG deficient patients [[4](#_bookmark5)].

Environmental factors, like infection or pharmacological treatment, can precipitate the onset of POLG disease. For ex- ample, infection with human herpesvirus 6 unmasked the POLG mutation phenotypes in two young children, contribut- ing to a more rapid clinical deterioration [[9](#_bookmark10)]. The use of anti- viral therapies based on nucleoside reverse-transcriptase in- hibitors (NRTIs) provides an example of pharmacological in- duction of a POLG disease phenotype, since inhibition of mitochondrial pol γ is a common side effect of NRTIs, leading to azidothymidine (AZT)-induced mtDNA depletion [[10](#_bookmark11)–[12](#_bookmark12)]. Thus, certain POLG mutations facilitate the NRTI-induced toxicity [[13](#_bookmark13), [14](#_bookmark14)].

Here we report the identification of two *POLG* mutations in a 3-year-old girl that presented with severe epilepsy in the context of an infectious process and developed sudden neuro- logical signs during the treatment of the infection. We analyze the pathogenicity of the *POLG* mutations encountered in the patient and analyze the possible contribution of the pharma- cological treatment to the disease progression.

# Materials and methods

## Cell culture and cell growth assays

Control (C and C3) and patient (P) primary skin-derived fi- broblasts were obtained from a 1-month-old child, a 3-year- old child, and the patient, respectively. Cells were cultured at 37 °C under a 5% CO2 atmosphere in high-glucose DMEM (Gibco-ThermoFisher Scientific) with 10% fetal bovine serum (FBS, Gibco-ThermoFisher Scientific), or DMEM no glucose (Gibco-ThermoFisher Scientific) supplemented with 5 mM galactose, 1 mM sodium pyruvate, and 10% FBS.

To evaluate cell growth, 2.5 × 104 cells were plated in 6- well 6-cm and 10-cm dishes (for increasing time points), and cells were counted every 2 days using a cell counter Countess II FL Automated Cell Counter (Invitrogen, Thermo Fisher Scientific) for a period of 8–10 days. Cell growth was deter- mined in the presence and absence of AZT (zidovudine) (Ghentham Life Sciences) or ceftriaxone disodium salt hemi(heptahydrate) (Sigma-Aldrich GmbH).

## Biochemical analysis

Cerebrospinal fluid (CSF) 5-methyltetrahydrofolate (5- MTHF) concentrations were analyzed using high- performance liquid chromatography with fluorescence detec- tion procedures as previously reported [[15](#_bookmark15), [16](#_bookmark16)]. CSF lactate and total protein values were analyzed by automated spectro- photometric analysis.

## Multiple sequence alignment

A total of 210 chordate POLG reference sequences were ob- tained from GenBank ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/genbank/) [genbank/](http://www.ncbi.nlm.nih.gov/genbank/)) (accessed July 23, 2019), aligned with Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and the conservation indexes (CIs) of Asn 864 and Ala 1217 were estimated. To estimate the CI of POLG pathological varia- tions, the variations were obtained from Rahman et al. [[17](#_bookmark17)] and from Human DNA Polymerase Gamma Mutation Database (<https://tools.niehs.nih.gov/polg>) (accessed in July 23, 2019). Mutations associated with Alpers- Hutte n loch er syndrome, progressive ex te rn al ophthalmoplegia, ataxia-neuropathy syndrome, sensory ataxia neuropathy with ophthalmoplegia, spinocerebelar ataxia with epilepsy, Friedreich’s ataxia, Charcot-Marie tooth disease, and Leigh syndrome were considered. Variations associated with male infertility, testicular cancer, idiopathic Parkinson disease, NRTI toxicity, and others were considered altogether with single nucleotide polymorphisms (SNPs).

## Genetic analysis

mtDNA pathogenic mutations and mtDNA deletions were analyzed as in [[18](#_bookmark18), [19](#_bookmark19)] respectively. Nuclear DNA was assessed by next-generation sequencing (NGS) using a cus- tomized panel of 132 mitochondrial genes as previously re- ported [[20](#_bookmark20)] in a NextSeq500 sequencer (Illumina). Progenitor studies to evaluate the inheritance model and to confirm the molecular diagnosis were performed by SANGER sequencing.

mtDNA copy number was quantitated by qPCR as previ- ously described [[21](#_bookmark21)], using a StepOne™ Real-Time PCR System (Applied Biosystems™). The mitochondrial probe, labeled with a FAM fluorophore, was targeted to the *MT- RNR1* gene (TGC CAG CCA CCG CG) and the nuclear probe, labeled with a VIC, was targeted to the *RNAsa P* gene. Four independently isolated samples were measured in triplicate.

## Cloning of *POLG* and lentiviral transduction

The *POLG* cDNA (corresponding to RefSeq NM\_002693.2; NP\_002684) was amplified from retrotranscribed total RNA extracted from human fibroblasts as in [[22](#_bookmark22)], using the specific primers F w: GTT TAAACG CCACC AT G AGC C GCCTGCTCT and Rv: GGATCCCTATGGTCCAGGCT

GG. A sequence checked clone was transferred to the lentiviral expression vector pWPXLd-ires-NeoR, which is a modified version of pWPXLd (Tronolab, Addgene #12258). *POLG* cDNA carrying (c.2864A>G, p.Y955C) variant was obtained using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) using the primers Fw: CTACGGCC

GCATCTGTGGTGCTGGGCAGC and Rv: GCTGCCCA

GCACCACAGATGCGGCCGTAG. Lentiviral particles were generated as in [[23](#_bookmark23)] and fibroblastas were transduced with lentiviral particles in 100-mm dishes by adding 5–50 μl of media with viral particles. Twenty-four hours after transduc- tion, transduced cells were isolated by 10-day selection in the presence of 400 μg/ml geneticin (Invitrogen-ThermoFisher Scientific).

## SDS-PAGE and WB analysis

SDS-PAGE and western bloting analysis of proteins was per- formed using standard methods. Primary antibodies used for immunodetection were as follows: anti-POLG (Thermo Fisher Scientific, #PA5-29676), anti-p.MT-CO1 (Thermo Fisher Scientific, #459600), anti-SDHA (Thermo Fisher Scientific, #459200), and anti-Actin (Sigma, #A 2066).

## Cytochrome C oxidase specific activity

Cytochrome c oxidase (CIV) and citrate synthase (CS) specif- ic activities were measured in digitonin solubilized cell sam- ples as described previously [[24](#_bookmark24)].

## Oxygen consumption

Oxygen consumption was analyzed using the high-resolution oxygraph OROBOROS® (Oroboros Instument, Innsbruck, Austria). Exponentially growing cells were collected by trypsinization, counted, and resuspended at 1 × 106 cells/ml in DMEM. Oxygen consumption was monitored at 37 °C, with chamber volumes set at 2 ml and increasing doses of lidocaine hydrochloride (Sigma-Aldrich GmbH) or ceftriax- one disodium salt hemi(heptahydrate) (Sigma-Aldrich GmbH).

## Statistical analysis

The statistical package StatView 6.0 was used for statistical analysis. Data are expressed as mean ± SD (standard devia- tion). The non-parametric Mann-Whitney test was used to evaluate the statistical significance between experimental groups. *p* values lower than 0.05 (\**p* < 0.05) were considered statistically significant.

# Results

## Clinical case

Antecedents A 4-year-old girl born from healthy non- consanguineous Russian parents was visited in our hospital. Pregnancy and delivery were uneventful. At 19 months of age,

the first epileptic event appeared. At 3 years of age, the patient presented severe epilepsy (more than 10 episodes per day) in the context of an infectious process. At this time, she started treatment with valproate with good seizure control. IgM anti- Borrellia titers were high, and in the physical examination, insect bite was observed. Intramuscular treatment with ceftriaxone/lidocaine was indicated, and after that, she pre- sented an episode of secondarily generalized focal crisis, hy- pertonia, and myoclonus of the 4 limbs. After 4 days, she was discharged from the hospital maintaining an adequate level of consciousness and motor skills. After the second ceftriaxone/ lidocaine injection, she presented again a new secondarily generalized focal crisis refractory to treatment associating consciousness level and motor deterioration. A single high blood lactate value was reported (4.9 mmol/l: reference values (RV), < 2.0 mmol/l). Neuroimaging studies disclosed an in- crease of the subarachnoid space at the frontoparietal level and an atrophy of the left hemisphere. Pale eye fundus was also observed. Treatment with phenobarbital, carbamazepine, and leviracetam led to seizure control. She occasionally presented motor focal seizures and upper extremity myoclonus. At 4 years of age, she was admitted to the neuropediatric depart- ment of our hospital and she presented with encephalopathy and motor disturbances including inexpressive facies, facial hypomimia, axial hypotonia, hypo/areflexia, distal limb in- creased tone, and right tetraparesis. She had lost deambulation and disclosed difficulties in handling objects. Drooling was also observed. Electroencephalogram disclosed spontaneous- ly slow and poorly organized trace when compared with age- matched controls. Frequent paroxysmal outbreaks of high- voltage delta waves in bioccipital regions with maximums and right predominance activated during sleep were observed. Mitochondrial biomarkers such as serum/plasma FGF21, ami- no acids, and lactate disclosed normal results. In cerebrospinal fluid, lactate was normal, but high total protein values (190 mg/dl: RV, 15–50) and deep cerebral folate deficiency 3 nmol/ l (RV, 35–124) was observed, supporting the diagnosis of a mitochondrial disorder [[25](#_bookmark25)].

## Genetic analysis

The presence of mtDNA pathogenic point mutations and de- letions were excluded. Next-generation sequencing analysis identified in the patient two missense changes in *POLG* gene (NM\_002693.2) involving evolutionarily conserved amino acid residues of the polymerase domain (Fig. [1a](#_bookmark0)). The first one was a recessive pathogenic mutation (c.2591A>G; p.Asn864Ser) previously reported in heterozygosis in two cases [[26](#_bookmark26)], and classified as variant of unknown significance according to the ACMG guidelines [[27](#_bookmark27)]. The second one was an heterozygous change (c.3649G>C; p.Ala1217Pro) classi- fied as variant of unknown significance [[27](#_bookmark27)], SNP (rs569063066), with MAF (minor allele frequency) of

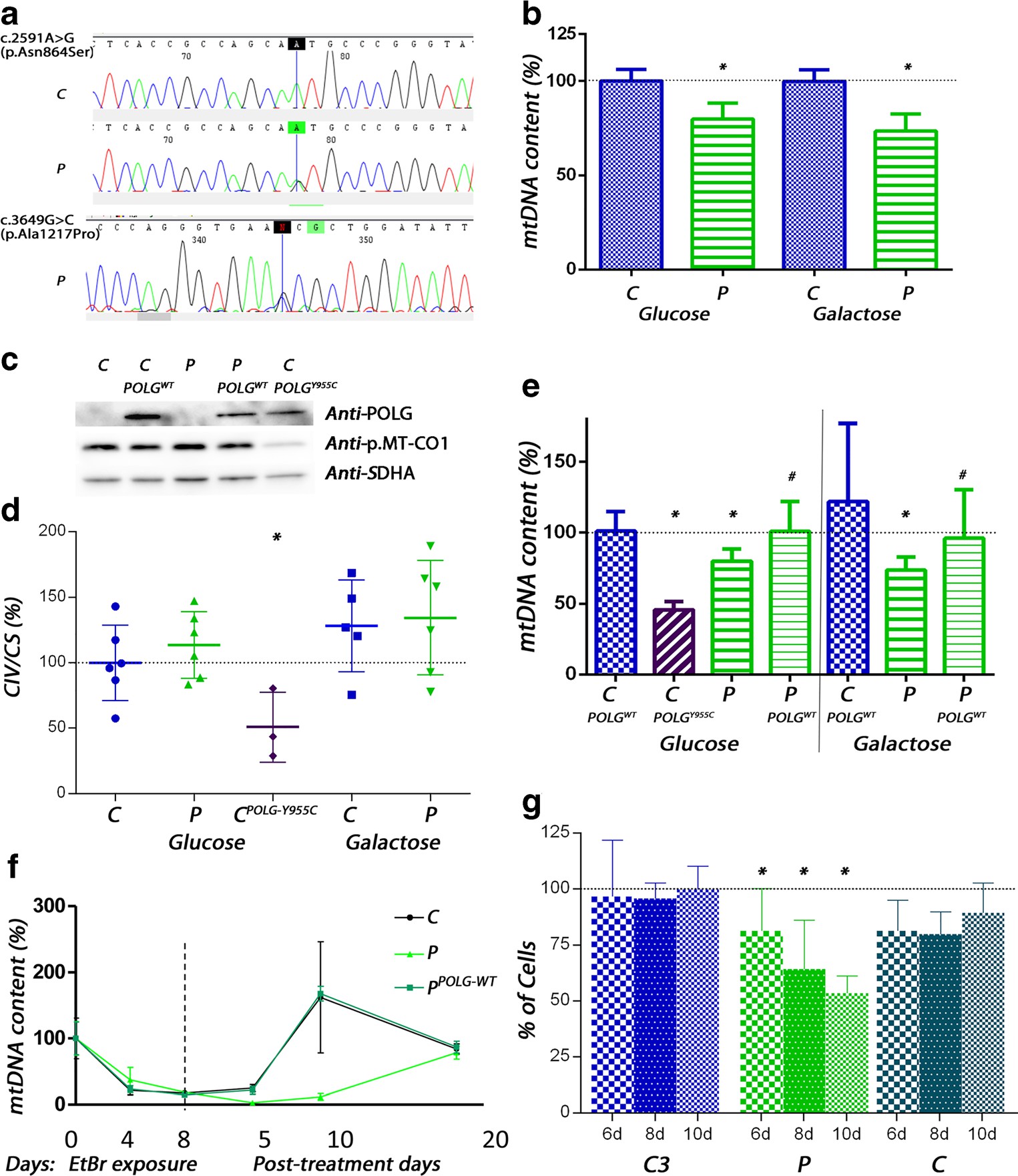


Fig. 1 mtDNA copy number and turnover and POLG complementation assays. a Sanger confirmation of the missense mutations in the *POLG* gene. b Quantification of mtDNA copy number of patient (P) fibroblasts and control (C) fibroblasts. Cells were grown in glucose or in galactose- containing medium for 3 days. The bars represent percentage of mtDNA normalized to nuclear DNA values relative to the mean value of control cells grown in glucose (dotted line, 100%). Four independently isolated DNA samples (*n* = 4) were measured in triplicate. \**:p* < 0.05, compared with C cells grown in the same medium. c Effect of POLG on p.MT-CO1 levels. WB immunodetection of SDS-PAGE separated total cell lysate isolated from patient (P) and control (C) fibroblasts and those transduced with *POLG*-WT or *POLG*-Y955C expressing constructs, using the indicated antibodies. d Complex IV activity normalized by citrate synthase (CS) activity of cell lines grown in glucose or galactose-containing medium for 3 days. The bars represent relative activity values, compared with the mean value of controls (dotted line, 100%). Three independently isolated samples (*n* = 3) in glucose and six (*n*

= 6), in galactose were measured. \*: *p* < 0.05, compared with C cells grown in the same medium. e Quantification of mtDNA copy number of patient (P) or control (C) fibroblasts transduced with the canonical

version of *POLG* (PPOLG-WT and CPOLG-WT) or with *POLG* with the mutation Y955C (CPOLG-Y955C) expressing constructs. Cells were grown in glucose medium, or in galactose-containing medium for 3 days. The bars represent percentage of mtDNA normalized to nuclear DNA values relative to the mean value of untransfected control cells grown in glucose (dotted line, 100%). Four independently isolated DNA samples (*n* = 4) were measured in triplicate. \*:*p* < 0.05, compared with C cells grown in the same medium. #*:p* < 0.05, compared with P cells grown in the same medium. f mtDNA depletion and repopulation kinetics of control (C), patient fibroblasts (P), and patient overexpressing the canonical version of *POLG* (PPOLG-WT). Cells were treated for 8 days with 50 ng/ml of ethidium bromide and allowed to recover during 12 days. Three indepen- dently isolated samples (*n* = 3) were analyzed. g Relative effect of AZT 10 μM on cell growth after 6, 8, and 10 days of exposure. The dotted line (100%) indicated mean value of untreated cells. Similar C, C3, and P cellular passages were compared. 2 to 4 biological replicates (*n* = 2 in C and P cell lines and *n* = 4 in C3 cell line) were analyzed with 2 technical replicates. \*:*p* < 0.05 in the presence of AZT 10 μM compared with untreated cells.

2.165e−5 in the general population in gnomAD (Genome Aggregation Database). The familiar study confirmed that both mutations were present in different alleles in the patient. Suitable prediction software packages (Polyphen 2, Pmut, Mutation Taster) assigned high scores for pathogenicity to both mutations. We aligned 210 POLG sequences from dif- ferent animals and observed that the asparagine at amino acid position 864 was conserved in 100% of the sequences and the alanine at position 1217 in 98.6%. The mean conservation index (CI) of 136 previously reported pathological mutations

[[17](#_bookmark17)] was 91.2 ± 19.6%, whereas the CI of 24 previously re-

ported SNPs [[17](#_bookmark17)] was 57.1 ± 33.9%.

## mtDNA turnover is compromised in patient fibroblasts

To analyze the effect of the *POLG* mutations on mtDNA levels in patient tissues, mtDNA related to nDNA was quan- titated in a muscle biopsy of the patient and compared with 4 controls. The amount of mtDNA detected in the patient was close to that in the controls. In patient-derived fibroblasts, a mild reduction in mtDNA content (18%) compared with con- trol fibroblasts was detected. When cells were grown in mito- chondrial biogenesis–inducing conditions (galactose-contain- ing medium [[28](#_bookmark28)]), the reduction became more apparent (26%) (Fig. [1b](#_bookmark0)).

To analyze genetic complementation of the *POLG* muta- tions, we generated patient (P) and control (C) fibroblast cell lines expressing the canonical version of *POLG* (PPOLG-WT and CPOLG-WT respectively). A control cell line overexpress- ing *POLG* with the dominant mutation c.2864A>G, p.Y955C in the polymerase domain was also generated (CPOLG-Y955C). This dominant mutation has been described to induce severe mtDNA depletion [[29](#_bookmark29)]. Overexpression was confirmed by SDS-PAGE separation and immunodetection of total protein lysates. A band of approximately 140 kDa corresponding to POLG protein was observed in all overexpressing cell lines (Fig. [1c](#_bookmark0)).

mtDNA related to nDNA was next examined in these cell lines. The overexpression of the POLG p.Y955C variant in control fibroblasts induced severe mtDNA depletion (54%), as expected, leading to a strong reduction in the mitochondrial encoded CIV subunit I (p.MT-CO1) (Fig. [1c](#_bookmark0)) and to a signif- icant decrease of CIV activity (Fig. [1d](#_bookmark0)). On the other side, mtDNA content was not different in CPOLG-WT fibroblasts compared with C fibroblasts, whereas a significant increase in mtDNA copy number was observed in patient overexpresing cell line, PPOLG-WT, related to P fibroblasts both in glucose medium and in galactose-containing medium (Fig. [1e](#_bookmark0)), suggesting that the mtDNA reduction in the patient is complemented by overexpression of the canonical version of *POLG*. p.MT-CO1 subunit amount as well as CIVactivity was not altered in patient fibroblasts compared with control (Fig

1. c, d), indicating that the combination of the two recessive variations present in the patient has a milder effect than the dominant p.Y955C variant.

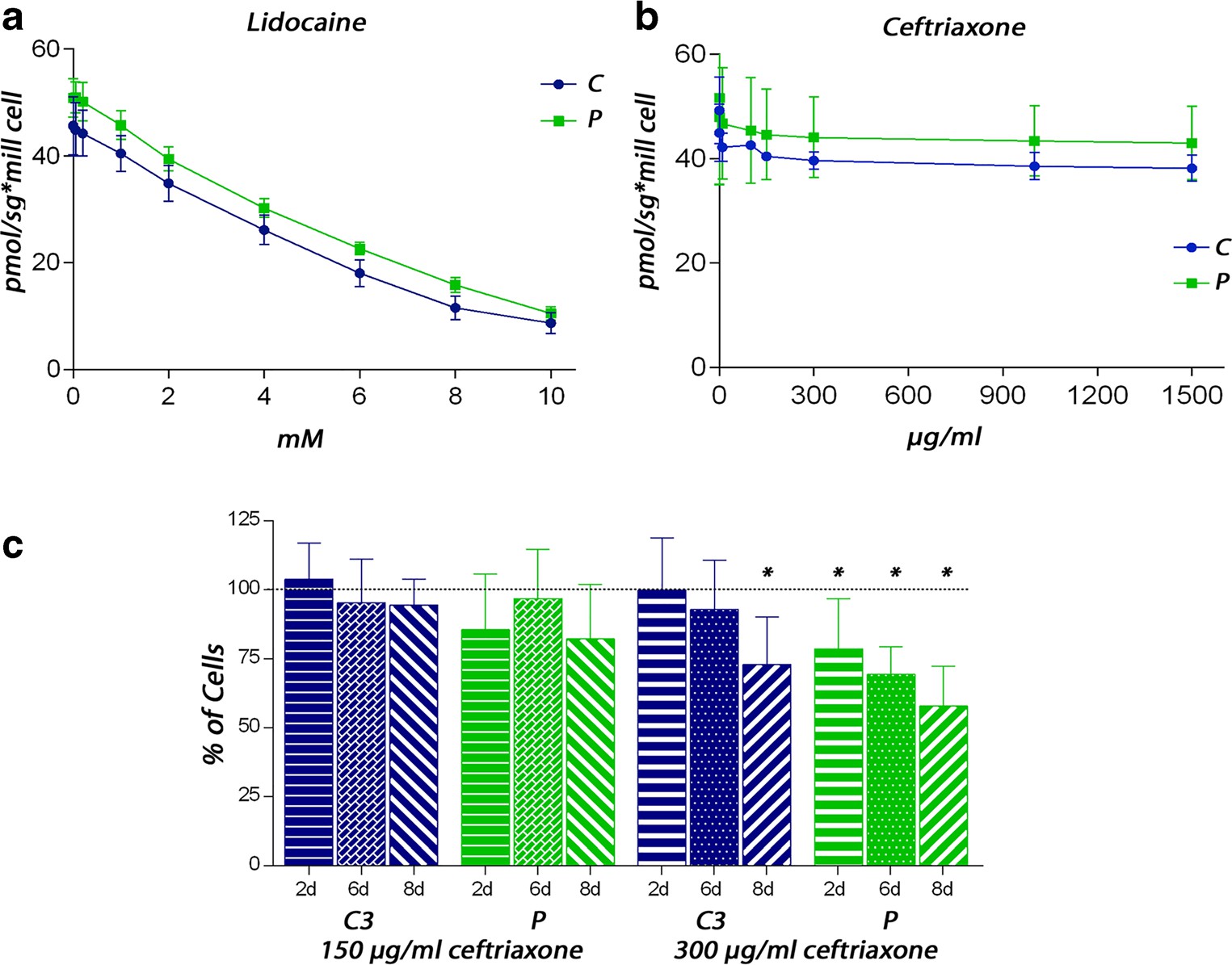
To determine the rate of depletion and repopulation of mtDNA in the fibroblasts, cells were exposed to EtBr during 8 days and let recover for 12 days (Fig. [1f](#_bookmark0)). EtBr-induced mtDNA depletion occurred at the same rate in all cell lines, C, P, and PPOLG-WT. By contrast, the restoration of mtDNA levels was markedly delayed in P cells, whereas PPOLG-WT showed the same repopulation profile than C cells. These re- sults strongly suggested that the capacity for adequate mtDNA turnover in patient fibroblasts is compromised by the combi- nation of the two recessive *POLG* mutations. Moreover, they indicate that the treatment with a mitochondrial stressor (EtBr) can unmask the POLG deficient phenotype of the patient.

The effect of the nucleoside analog AZTon the growth of C and P cell lines was next tested. The presence of 10 μM AZT did not have any significant effect on the growth of control cells (C), but significantly reduced the growth of patient fibro- blasts after 6, 8, and 10 days in the presence of the drug (Fig. [1g](#_bookmark0)). A second age- and passage-matched control fibroblast cell line (C3) did not show reduced growth either, when ex- posed to 10 μM AZT (Fig. [1g](#_bookmark0)). This result shows that *POLG* mutations encountered in the patient make cells more suscep- tible to the presence of POLG stressors, reinforcing their path- ogenic nature.

## Ceftriaxone exposure induces toxicity in fibroblasts

When C and P cells were exposed to the anesthetic lidocaine, the cellular respiration (O2 consumption) showed the same dose-dependent inhibition in both cell lines (50% inhibition at ~ 4.5 mM) (Fig. [2a](#_bookmark1)). The values were similar to those reported in isolated pig mitochondria [[30](#_bookmark30)]. Since we did not detect in vitro inhibition of cellular respiration below 100 μM, and the published concentration of lidocaine with effect on human fibroblast proliferation and viability is above 300 μg/ml [[31](#_bookmark31)], much higher than plasma doses [[32](#_bookmark32)], a severe impact of this drug in the patient disease progression was excluded.

The addition of ceftriaxone did not inhibited P or C cellular respiration when added to the polarography chamber up to a concentration of 1500 μg/ml (ten times higher than the max- imum patient plasma concentration, 141 μg/ml) [[32](#_bookmark32)] (Fig. [2b](#_bookmark1)). This excludes a direct effect of the antibiotic on respiratory function. To analyze a longer-term impact, we tested the effect of ceftriaxone on cell growth over 8-day period. Cells were cultured in galactose-based medium to facilitate the detection of defects in oxidative phosphorylation [[28](#_bookmark28)]. The growth of patient cells was reduced in the presence of 150 μg/ml ceftri- axone, after 2, 6, or 8 days of treatment when compared with untreated cells. Although this reduction was not statistically significant, it was higher than that for the age- and passage-

Fig. 2 Ceftriaxone and lidocaine toxicity assays. a Oxygen uptake of control (C) and patient (P) fi- broblasts in the presence of in- creasing doses of lidocaine. Three assays were performed (*n* = 3). The values are expressed in pico- moles of O2 per second per mil- lion cells. b Oxygen uptake of control (C) and patient (P) fibro- blasts in the presence of increas- ing doses of ceftriaxone. Two as- says were performed (*n* = 2). The values are expressed in picomoles of O2 per second per million cells. c Relative effect of ceftriaxone on cell growth after 2, 6, and 8 days of exposure. The dotted line (100%) indicated mean value of untreated cells. Similar C3 and P cellular passages are compared. 4 biological replicates (*n* = 4) were analyzed with 2 technical repli- cates. \**: p* < 0.05 in the presence of ceftriaxone compared with un- treated cells.

matched control cell line (C3) (18% vs. 6% at 8 days) (Fig. [2c](#_bookmark1)). Higher ceftriaxone concentration, 300 μg/ml, caused a higher reduction (42% at 8 days) of P cells, indicating a dose-dependent effect of the drug (Fig. [2c](#_bookmark1)). The number of P cells was significantly reduced when compared with the untreated cells after 2, 6, or 8 days in the presence of 300 μg/ml ceftriaxone, whereas C3 cells were significantly re- duced only after 8 days (Fig. [2c](#_bookmark1)). These results indicated higher sensibility of the patient to the drug.

# Discussion

In the case presented here, a 3-year-old girl experienced severe consciousness level and motor manifestations during the treat- ment of an infection with *Borrelia* sp. and was found to have an underlying POLG-related mitochondrial disease. The c.2591A>G; p.Asn864Ser variant was previously reported in heterozygosis in two 15-year-old sisters with mitochondrial disease [ [26](#_bookmark26) ]. The second variant ( c. 3649 G>C; p.Ala1217Pro) has not been previously associated with dis- ease. Both variants involve highly conserved amino acid res- idues, which suggest having important functional roles. Proving the pathogenic nature of novel POLG mutations is a major challenge because the *POLG* locus is highly polymor- phic and the disease manifestation is often dependent on epi- genetic and environmental factors [[5](#_bookmark6), [7](#_bookmark8)]. POLG-related

diseases are expected to share mtDNA depletion as a common pathogenetic feature, possibly in combination with mtDNA damage including multiple deletions and increased burden of point mutations. However, mtDNA depletion assays can be misleading in the diagnosis because mtDNA copy number is highly variable among different tissues [[33](#_bookmark33)] and mtDNA de- pletion is not always present, especially in the early stages of the disease [[34](#_bookmark34)]. Furthermore, in POLG-related epilepsy, my- opathy is not a common clinical feature and normal muscle and fibroblast studies do not exclude the diagnosis of POLG- related mitochondrial disease [[35](#_bookmark35)]. Therefore, although quan- titative mtDNA depletion was not detected in patient muscle at the time when the clinical symptoms appear, we further evaluated patient POLG performance in cell culture [[7](#_bookmark8)]. Patient skin fibroblasts showed delayed rate of mtDNA repop- ulation after induced depletion, when compared with canoni- cal *POLG* overexpressing patient cells or with control fibro- blasts, an established criterion of pathogenicity [[7](#_bookmark8)]. These re- sults indicated that the combination of the two recessive pa- tient variations in POLG polymerase domain is pathogenic. CIV activity reduction was not detected in patient fibroblasts, probably because it lowers only with severe depletion.

There are a few well-established examples of drug-induced mitochondrial disorders. Patients treated with antiviral nucle- oside analogs (NRTIs), such as AZT, developed a mitochon- drial myopathy characterized by the appearance of ragged-red fibers and cytochrome oxidase–negative fibers [[12](#_bookmark12)]. Although

the NRTIs, like AZT, are designed to inhibit HIV reverse transcriptase, its incorporation inhibits mitochondrial pol γ replication inducing mitochondrial toxicity as a major adverse effect. In our in vitro assays, AZT 10 μM had little or no effect on control skin fibroblast proliferation, as published previous- ly [[36](#_bookmark36)]. However, the patient POLG mutations facilitated the AZT-induced toxicity and increased 11% the doubling time of the cells. This effect that has been previously described in a cell line with other *POLG* mutations [[13](#_bookmark13), [14](#_bookmark14)] corroborates the deleterious nature of our patient mutations.

Progression of symptoms and deterioration of mitochondri- al disorders in association with an intercurrent illness have been well described [[5](#_bookmark6)]. In our patient, IgM anti-Borrelia titers were high suggesting that infection with *Borrelia* sp. was an important event in the onset of the symptoms of mitochondrial pathology. Interestingly, *Borrelia* spp. are dependent on pu- rine salvage pathway from the host environment for survival

[[37](#_bookmark37)] and therefore could be an additional stress factor for a patient with compromised mtDNA synthesis. In a previous case, the onset of Leber’s hereditary optic neuropathy (LHON) in a young patient appeared in association with *Borrelia* infection [[38](#_bookmark38)]. In other case, a woman with antibod- ies against *Borrelia* sp. in serum developed dropped head syndrome (DHS) that was attributed to an underlying multi- organ mitochondriopathy [[39](#_bookmark39)]. Remarkably, our patient pre- sented an episode of secondarily generalized focal crisis, hy- pertonia, and myoclonus of the 4 limbs and *Borrelia* sp. in- fection has been frequently associated with myoclonus [[40](#_bookmark40)–[42](#_bookmark41)].

During her hospital admission, our patient suddenly wors- ened with the administration of ceftriaxone/lidocaine. Cefalosporin antibiotics, as well as the anesthetic lidocaine, have being previously associated with mitochondrial toxicity. Lidocain inhibits the electron transport chain, although the exact mechanism involved in its cytotoxicity is not well established [[30](#_bookmark30), [43](#_bookmark42), [44](#_bookmark43)]. The in vitro exposure of patient fi- broblasts to lidocaine excluded a severe impact of this drug at the therapeutic dose. The antibiotic treatment, on the contrary, could have been a key environmental factor that worsened the underlying mitochondrial disease of the patient. In our in vitro assays, patient skin fibroblasts were more susceptible to long- term culture in the presence of the drug than controls. More importantly, ceftriaxone and other cephalosporin antibiotics have been associated with serious central nervous system side effects including mainly encephalopathy, convulsion, and myoclonia [[45](#_bookmark44)–[48](#_bookmark45)].

In summary, we report a clinical case of POLG-related mitochondrial disease hastened by environmental stress, infectious, and therapeutic. This case highlights the impor- tance of early genetic diagnoses of the patients and the necessity of consideration of risks and benefits in the se- lection of pharmacological treatment for patients with suspected mitochondrial disorders.

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## Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the ethics committee of the Government of Aragón (CEICA CP- 12/2014) and was performed in accordance with the ethical standards laid down in the Declaration of Helsinki.

Informed consent Written informed consent for participation in the study was obtained from the patient guardian prior to sample collection.

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