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POLG Heterozygosity Manifests Clinically in Numerous Pediatric Cases

Anna Betler, Savannah Battleson, Molly Sawyer, Jack Kloecker, Iradukunda Esperance, Azhar Rahama, Hayden Divers, Ojus Dalvi, Nicholas Farrell, and Gary Vanderlaan*

Biology Department, Gannon University, Erie, PA, USA

***Corresponding author:** Gary Vanderlaan, Gannon University, Biology Dept. 109 University Square, Zurn 208, Erie PA 16541. Tel: +1-814-871-7634; Email: vanderla002@gannon.edu

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Abstract

Mitochondrial DNA (mtDNA) depletion syndromes (MDDS) present across a wide range of clinical manifestations. Defects in mtDNA can arise from mutations afflicting genes involved in nucleotide import from the cytosol to the mitochondrial matrix, nucleotide anabolism within the mitochondrial matrix itself, or mtDNA replication machinery. The hepatocerebral form of MDDS is driven by null mutations at the nuclear-encoded POLG locus which specifies the catalytic subunit of the trimeric mtDNA polymerase complex. POLG-related disorders are typified by clinical manifestations that target the central nervous system, including ataxia, epilepsy, seizures, and neuropathy accompanied by metabolic lactic acidosis and acute liver failure in many pediatric cases. Germline transmission or somatic inactivation yielding two loss-of-function POLG alleles are the typical genetic bases underlying POLG-related disorders. However, carriers of a single pathogenic variant of POLG are prone to clinical manifestations that track POLG$^{-/-}$ genotypes. To examine the extent by which POLG heterozygosity contributes to clinical manifestations that recapitulate the hepatocerebral form of MDDS, we performed an exhaustive analysis of all peer-reviewed, clinical literature documenting POLG-related disorders in the last two decades. Our findings indicate that POLG heterozygotes are not without clinical manifestations, presenting with a common permutation of signs and symptoms that fully tracks POLG$^{-/-}$ patients. Thus, POLG heterozygotes exhibit POLG-related disorder trait (PRDT), a genotypic condition likely prone to haploinsufficiency on account of either genotoxicity or mitochondrial toxicity.

Keywords: *Alpers-Huttenlocher syndrome (AHS); Ataxia neuropathy spectrum (ANS); Haploinsufficiency; Mitochondrial DNA (mtDNA); Mitochondrial DNA depletion syndrome (MDDS); Mitochondrial encephalomyopathy; Mitochondrial hepatocerebral syndrome; Mitochondrial myopathy; Mitochondrial neurogastrointestinal (MNGIE) disease; Myocerebrohepatopathy spectrum (MCHS); Myoclonic epilepsy myopathy sensory ataxia (MEMSA); POLG-related disorders; POLG-related disorder trait (PRDT); Progressive external ophthalmoplegia (PEO).*

Introduction

Approximately 2.7 billion years ago, an ancestral archaebacteria partially endocytosed an ancestral proteobacteria giving rise to the first eukaryotic cell [1,2]. In lieu of being digested, the proteobacteria became an endosymbiont, persisting as mitochondrial organelles in most eukaryotic cells today [2,3]. In fact, extant mitochondria still retain vestiges of their bacterial past in the form of circular mitochondrial chromosomes as well as intact mitochondrial protein translation machinery highly reminiscent of prokaryotic 70S ribosomes [1,3,4]. In human cells, the mitochondrial DNA (mtDNA) is found in the mitochondrial matrix and each circular mitochondrial chromosome contains 37 essential mitochondrial genes which uniquely encode 13 mRNAs, 22 tRNAs, and 2 rRNAs [5]. All 13 protein-encoding mitochondrial genes are essential for oxidative phosphorylation (OXPHOS) which is how most eukaryotic cells derive the majority of ATP production [6]. Across endosymbiotic time, approximately 1,136 genes relocated from the mtDNA into the human nucleus, and these genetic loci are collectively known as the nuclear-encoded mitochondrial genes [7,8]. As such, the extant mitochondrial organelles are highly reliant on the human host cell for numerous molecular services [9,10]. For example, mitochondria leverage transporter proteins to ferry, from the cytosol to the mitochondrial matrix, the various constituents of deoxyribonucleotides, including nitrogenous bases, nucleosides, dNMPs, dNDPs, and dNTPs [11-13]. Once a suite of chemical building blocks is imported into the matrix, nucleotide anabolism ensues followed by mtDNA replication, both of which also leverage nuclear-encoded mitochondrial enzymes [14,15].

Depletion of mtDNA in mitochondrial organelles can therefore arise from loss-of-function (LOF) mutations in the human genes that play a role in the mitochondrial matrix for either nucleotide anabolism (i.e., monomer synthesis) or nucleic acid synthesis (i.e., mtDNA polymer replication) [13,16]. Mitochondrial DNA depletion syndromes (MDDS) are thus characterized by a wide berth of genetic etiologies presenting across a large clinical spectrum [14]. MDDS typically presents in stereotyped tissues and organs in any 1 of 4 forms: a neurogastrointestinal form associated with LOF mutations in the *TYMP* gene, a myopathic form driven by lesions at the *TK2* locus, an encephalomyopathic form involving mutations in the *SUCLG1*, *SUCLG2*, and *RRM2B* genes, and a hepatocerebral form that is associated with LOF mutations in the *DGUOK*, *MPV17*, *TWNK*, and *POLG1* loci [11,14]. Indeed, one of the most common drivers of MDDS involves lesions at the *POLG1* locus (aka *POLG*), which encodes the catalytic subunit of the trimeric mtDNA polymerase complex [16]. Patients with LOF mutant alleles of *POLG1* are

collectively diagnosed with *POLG*-related disorders, as a hepatocerebral form of MDDS [17].

Clinically, the manifestation spectrum of *POLG*-related disorders spans six subtypes: Alpers-Huttenlocher Syndrome (AHS), Myocerebrohepatopathy Spectrum (MCHS), Myoclonus Epilepsy Myopathy Sensory Ataxia (MEMSA), Ataxia Neuropathy Spectrum (ANS), autosomal recessive Progressive External Ophthalmoplegia (arPEO), and autosomal dominant Progressive External Ophthalmoplegia (adPEO) [17]. PEO is typically seen in adults, while pediatric cases of *POLG*-related disorders manifest varying permutations of AHS, MCHS, MEMSA, or ANS [18]. Although inherited *POLG*-related disorders are transmitted genetically as autosomal recessive conditions, the heterozygous (aka carrier) state may encounter clinical manifestations under specific conditions, including haploinsufficiency due to excessive mitochondrial toxicity leveraged by an infectious agent or contraindicated medications. Here we present a thorough review of nearly twenty years of peer-reviewed clinical literature documenting the precise molecular basis of *POLG* heterozygosity alongside clinically observed manifestations of the hepatocerebral form of MDDS. Our analysis reveals numerous examples of *POLG* heterozygous patients, particularly in pediatric cases, that tracked hepatocerebral MDDS, and thus these *POLG* heterozygotes exhibit *POLG*-related disorder trait (PRDT).

Methods

Data Management

A comprehensive search was performed for all peer-reviewed scientific literature for *POLG* missense mutations and accompanying clinical manifestations. Candidate papers were identified via three primary routes: Google Scholar, PubMed, and datamining of the Human DNA Polymerase Gamma Mutation Database, maintained by the National Institute of Environmental Health Sciences (NIEHS) [19]. Custom Python scripts leveraging BeautifulSoup4 (bs4), requests, and pandas were written to scrape the NIEHS database as well as guide search queries in Google Scholar and PubMed in a robust fashion. Collectively, 218 peer-reviewed articles spanning approximately two decades of research were identified using these strategies, and in totality, this body of work described 1,260 different human patients that exhibited some degree of MDDS presentation with a known *POLG* genotype. Of this pool, 259 of 1,260 human patients were identified as heterozygous carriers of a single *POLG* variant, spanning 42 different peer-reviewed articles. A further 79 *POLG* carriers were removed from analysis pipelines due to missing metadata on patient age. Our final dataset thus entails a list of 179 different human patients that simultaneously 1) carry a single *POLG* variant in a heterozygous state, 2) present with at least one clinical manifestation of MDDS, and 3) exhibit either an age of MDDS onset or a described patient age at the time of publication. For each patient, 55 different clinical manifestations of hepatocerebral MDDS were manually curated. A full list of each individual patient of the total cohort of 179 *POLG* heterozygotes with precise molecular *POLG* lesion data and observed clinical manifestations are available as **Supplementary Table 1** as a data matrix of 179 rows and 67 columns.

Lesion Mapping

For most of the 179 *POLG* carriers, the precise molecular nature at both the DNA and protein levels was previously fully characterized in the published literature. However, in a few cases, only POLG peptide consequences were provided. To intuit precise point mutations, we leveraged Ensembl for genomic DNA coordinates, and NCBI CCDS for mature mRNA position information and exon-only sequence data [20-22]. As *POLG* is a nuclear-encoded gene, a human translation table was referenced using Expasy Translate [23]. In this way, three previously characterized *POLG* alleles were unambiguously mapped with respect to their molecular basis underlying each point mutation type. We determined the *POLG Y955H* missense allele to arise from a 2863 T>C point mutation resulting in a UAU (Tyr) to CAU (His) codon replacement [24]. Likewise, the *POLG A1105T* missense mutation yields a 3313 G>A point mutation that alters the normal GCU (Ala) codon to an ACU (Thr) codon [25]. Lastly, a C>U point mutation at nucleotide 3689 causes the *POLG S1230F* substitution by altering the UCC (Ser) codon into a UUC (Phe) codon [26]. All mapped lesions alongside previously documented *POLG* carrier alleles are fully cataloged in **Supplementary Table 1**.

Results

Peer-reviewed clinical literature documents *POLG* heterozygosity

To establish the clinical extent of *POLG* heterozygosity, approximately 1,260 *POLG* patients were systematically analysed across nearly two decades of scientific discourse. Many patients exhibited a double LOF condition ($POLG^{-/-}$) in the form of homozygosity, or some form of compound heterozygosity, typically in the trans configuration (i.e., LOF lesions that are found across both alleles at the *POLG* locus on each human chromosome 15). Nearly 260 different *POLG* carriers of known molecular etiology were thus identified of whom presented with at least one clinical sign or symptom of MDDS, stemming from a total of 42 peer-reviewed scientific bodies of work (**Fig 1**). Most publications described just a single case of *POLG* heterozygosity as approximately 200 of 260 *POLG* heterozygotes were examined in just the ten most prolific journals (**Fig 1**).

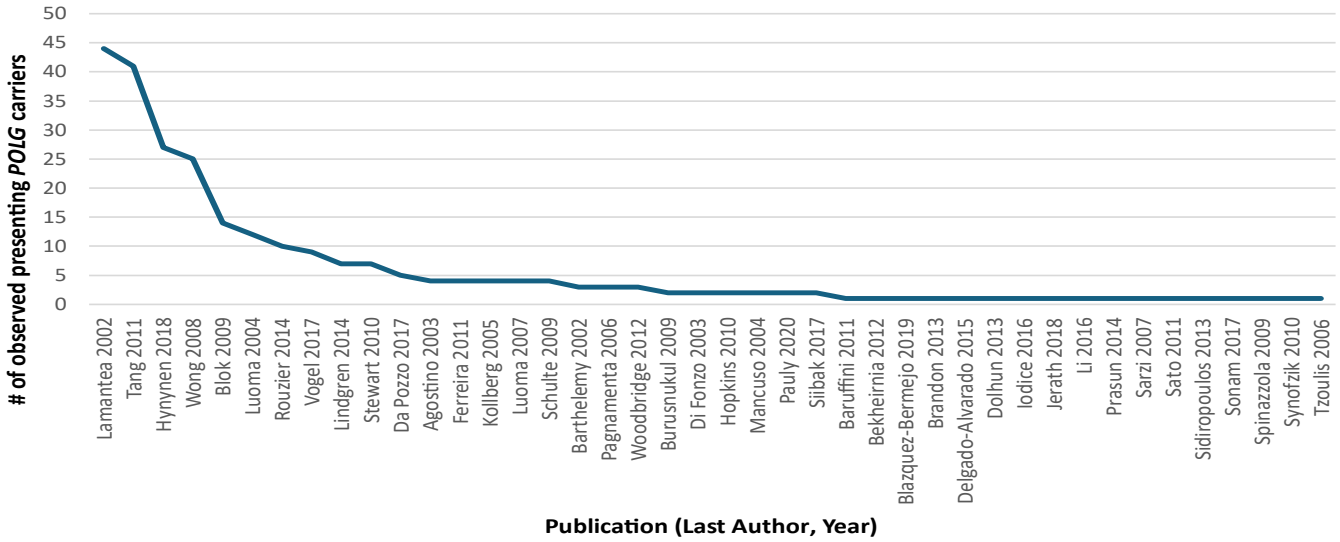

Figure 1: Scientific literature containing clinical descriptions and examinations of heterozygous *POLG* patients.

Most peer-reviewed articles are clinical case studies that examine a small patient cohort diagnosed with *POLG*-related disorders. A few articles however systematically examined double *POLG* LOF conditions as well as *POLG* carriers. In total, 179 different patients with published age (or age at disease onset) and the precise *POLG* heterozygous genotype is available from nearly two decades of research, spanning 2002-2020.

The rate of *POLG* heterozygosity manifestation in the clinic varied across nearly two decades, with spikes of clinical observations in 2002 and 2011 (**Fig 2**). Of these, we removed 80 *POLG* carriers due to lack of metadata regarding patient age or age at disease onset. The results showcase that a sizeable cohort of *POLG* heterozygotes have been previously characterized in clinical studies, and thus the heterozygous condition likely harbors the *POLG*-related disorder trait (PRDT), a clinically-measurable phenotype (**Table 1**).

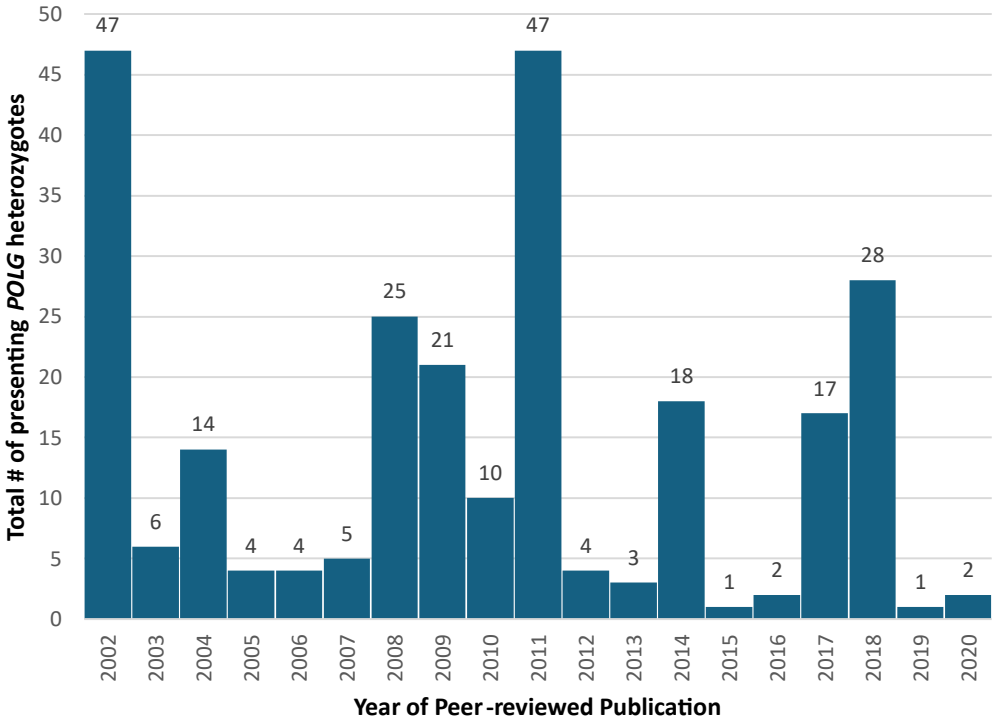

Figure 2: *POLG* heterozygosity cases within clinical manifestations are described in sporadic fashion.

Across nearly two decades of peer-reviewed, clinical literature documenting *POLG*-related disorders, a sizeable cohort of *POLG* carriers can be visualized. The clinical characterization of *POLG* heterozygous patients was unevenly reported in a sporadic fashion from 2002-2020, with the most abundant descriptions made in 2002 and 2011.

Table 1: Manifest of *POLG* heterozygotes that presented with at least one clinical manifestation of hepatocerebral MDDS. A total of 42 unique publications document 259 different *POLG* carriers of missense alleles examined across approximately two decades of peer-reviewed scientific literature.

# of *POLG* carriers	Assessed *POLG* carrier alleles (# patients)	Ref
44	G923D (1), R943H (3), Y955C (27), A957S (9), S1176L (4)	[54]
41	K8N (1), Q144R (1), A154T (1), A212V (1), I224T (1), S240L (1), R275Q (1), H277L (2), R443C (1), A467T (5), H613Y (1), R617H (1), P625L (1), R627Q (1), G647D (1), A676V (1), K704T (1), R722L (1), G737R (4), W478S (1), R790H (1), S863R (1), Y951N (1), Y955C (2), R964C (3), R972Q (1), R1026H (1), A1033V (1), V1044A (1), I1223V (1)	[55]
27	E1143G (3), Q1236H (24)	[56]
25	G11D (1), G1205A (1), G517V (5), G737R (2), H110Y (1), I1079L (1), L392V (1), P587L (1), R617C (1), R964C (1), S1095R (1), S305R (1), T251I (1), T914P (1), V855A (1), Y831C (4), Y955C (1)	[57]
14	G268A (3), G426S (1), A467T (2), N468D (1), G517V (4), A804T (1), R869Q (1), R943H (1)	[38]
12	Y955C (11), A1105T (1)	[25]
10	R232H (3), A467T (1), W748S (1), Q975X (1), P1073L (1), D1184N (2), R1187W (1)	[58]
9	A467T (3), R627Q (1) W748S (2), Y955C (3)	[59]
7	A194V (1) E1143G (1) Q1236H (5)	[60]
7	E1143G (2), Q1236H (5)	[44]
5	P412L (1), H613Y (1), Y831C (2), Y955C (1)	[61]
4	Q43R (2), R722H (1), S1230F (1)	[26]
4	W312R (1), R1047Q (1), R1096C (1), S1104C (1)	[62]
4	N468D (3), G517V (1)	[63]
4	G517V (2), R807C (1), R1081Q (1)	[64]
4	Y955C (4)	[65]
3	G517V (1), Y831C (2)	[32]
3	Y831C (3)	[66]
3	Y955C (3)	[39]
2	T251I (1), R562Q (1)	[67]
2	Y282D (2)	[68]
2	G517V (2)	[69]
2	G517V (2)	[30]
2	Y831C (2)	[70]
2	Y955C (1), Y955H (1)	[24]
1	H110Y (1)	[71]
1	A143V (1)	[72]
1	R386H (1)	[73]
1	R457Q (1)	[74]
1	K512M (1)	[75]
1	W748S (1)	[76]
1	D890A (1)	[77]
1	E895G (1)	[78]
1	R943H (1)	[79]
1	R943C (1)	[80]
1	H945L (1)	[81]
1	Y951N (1)	[82]
1	R953C (1)	[83]
1	Y955C (1)	[84]
1	Y955C (1)	[85]
1	S1080T (1)	[86]
1	N1098K (1)	[87]

Carriers of *POLG* lesions exhibit missense mutation hotspots

The *POLG* heterozygote cohort (n = 179) were carefully curated for each specific patient's precise molecular lesion. The *POLG* gene encodes a bifunctional enzyme that comprises the catalytic component of the trimeric mtDNA polymerase complex. At the POLG protein level, the amino terminal domain (NTD) carries an exonuclease domain essential for proofreading activities while the active site for transesterification reactions resides in the polymerase domain found in the POLG carboxyl terminal domain (CTD) [17]. Generally-speaking, mutations afflicting the POLG NTD typically result in enhanced mutation rates in replicated mtDNA chromosomes and when approximately 70% heteroplasmy is reached, clinical manifestations begin to exhibit dysfunctional mtDNA activities [18]. LOF lesions at the POLG CTD typically abrogate mtDNA replication activities, and thus directly deplete the cell of the requisite mtDNA levels needed to sustain normal mitochondrial activities, including OXPHOS [17,27]. Carrier mutations at the *POLG* locus were not equally distributed across the full-length (i.e., 1,239 amino acids) primary sequence of the POLG protein (**Fig 3**). The most frequent alleles observed in *POLG* heterozygotes reflected common missense polymorphisms extensively studied in the *POLG* homozygous recessive or *POLG* compound heterozygous genotypic backgrounds. *POLG* heterozygotes most frequently carried the *POLG* missense alleles of *A467T*, *G517V*, *Y831C*, and *Y955C* (**Fig 3**). Extensive clinical work has been done on these various alleles in the double LOF state at the *POLG* locus. For example, *POLG A467T* has been linked to hepatocerebral forms of MDDS [28,29]. Likewise, *POLG G517V* has been assessed for various deficits including infantile epilepsy, cerebellar ataxia, neuropathy and ophthalmoplegia [30]. *POLG Y831C* has been described in studies examining seizures, abnormal electroencephalograms (EEGs), neuropathy, ataxia, and liver failure [26,31,32]. Lastly, the most common missense substitution found in *POLG* heterozygotes is *POLG Y955C*, an allele that has been extensively studied for numerous hepatocerebral MDDS presentations, including PEO, mtDNA depletions and deletions, epilepsy, seizure, hypotonia, and

neuropathy [16,24,33-35]. Thus, *POLG* heterozygotes phenotypically present while carrying the most common allelic forms of the *POLG* locus in a fashion not atypical of the homozygous recessive or compound heterozygous *POLG* genotypes underlying *POLG*-related disorders.

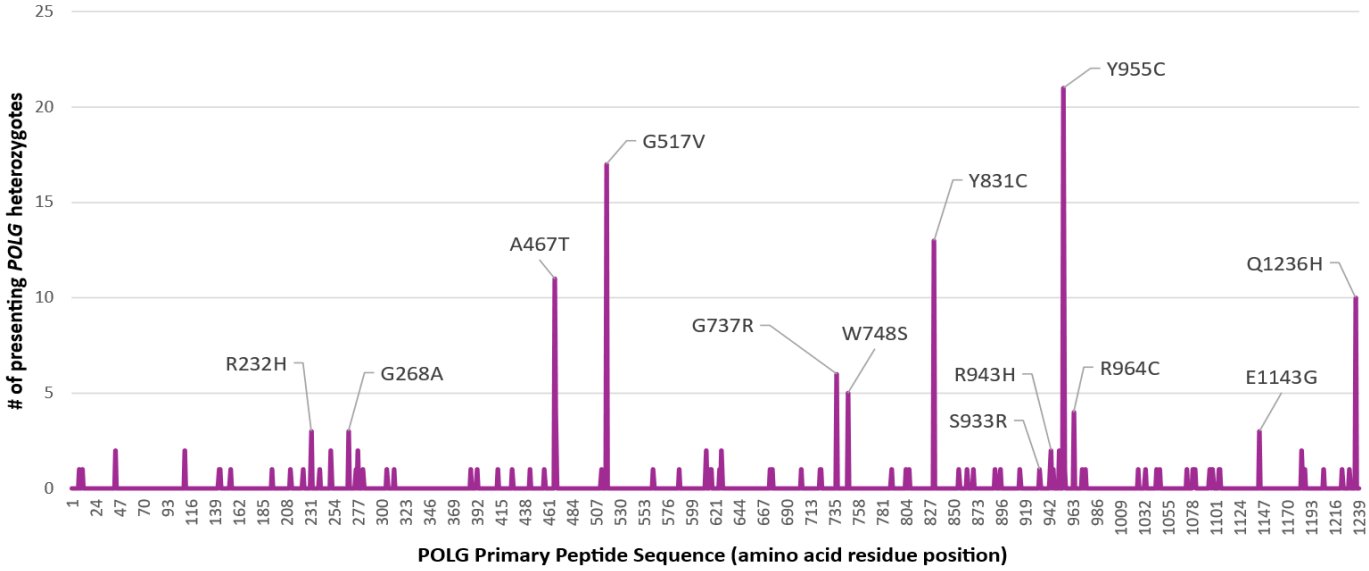

Figure 3: Frequency of carriers of *POLG* missense alleles as described in the clinical literature.

The *POLG* gene encodes an enzyme comprising 1,239 amino acid residues. The POLG enzyme contains two active sites, an exonuclease domain found in the amino terminal domain (NTD) and a polymerase domain near the carboxyl terminal domain (CTD). *POLG* heterozygotes harbor missense alleles unevenly across the primary peptide sequence, exhibiting mutational hotspots, with the *POLG Y955C* and *POLG G517V* substitutions as the most frequently carried alleles.

***POLG* heterozygotes present across a wide spectrum of physiological systems and clinical manifestations**

To determine the extent by which *POLG* heterozygotes exhibited *POLG*-related disorder trait (PRDT), we carefully evaluated each of the 179 different *POLG* carriers using a panel of 55 clinical manifestations characteristic of hepatocerebral MDDS [14,18,36]. Across all 179 patients, we recorded a total of 793 medically-documented clinical manifestations in the nearly two decades of clinical literature (i.e., 42 unique publications). Each of the 55 clinical manifestations of *POLG*-related disorders was then classified into 1 of 9 afflicted, physiological systems to map out the general frequency of clinically-relevant descriptions of manifesting signs and symptoms regarding mitochondrial disease (**Fig 4**). The most common clinically-described manifestations in *POLG* carriers afflicted neurologic (38.4%), followed by ophthalmologic (16.6%), and neuromuscular (15.6%) systems (**Fig 4**). A sizeable amount (11.7%) of clinical literature directly assessed mitochondrial function and/or morphology in *POLG* heterozygotes across all manifestations (**Fig 4**).

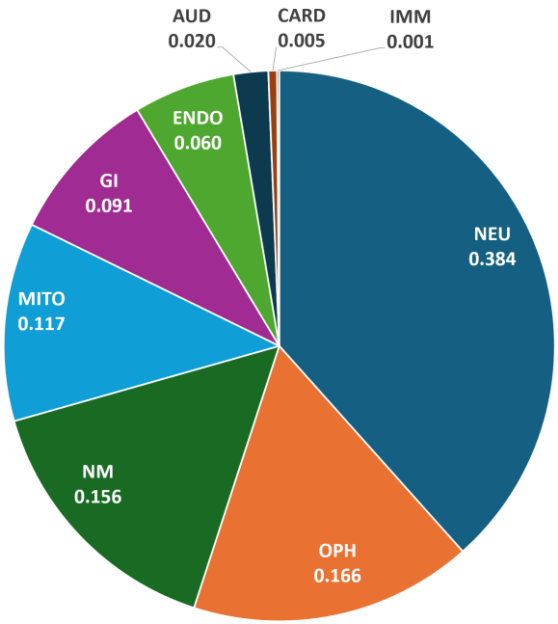

Figure 4: *POLG* heterozygotes present across a wide spectrum of afflicted, physiological systems.

A total of 55 different clinical manifestations of *POLG*-related disorders were manually extracted from nearly two decades of research articles documenting 179 *POLG* carriers. Each clinical manifestation was then categorized into 1 of 9 categories based on afflicted, physiological or molecular system: AUD, Audiologic; CARD, Cardiologic; ENDO, Endocrine; GI, Gastrointestinal; IMM, Immunologic; MITO, Mitochondrial abnormality; NEU, Neurologic; NM, Neuromuscular / myopathy; OPH, Ophthalmologic. *POLG* heterozygotes present in a stereotyped fashion, with approximately 80% of observed clinical manifestations afflicting neurologic, ophthalmologic, and neuromuscular systems.

Precise clinical descriptors of mitochondrial disease manifestations were highly representative across a wide spectrum as well (**Fig 5**). The most frequently described clinical manifestation occurring in all 179 *POLG* heterozygous patients in the clinical literature was epilepsy and seizures (8%), followed by myopathy (7%), PEO (7%), cerebellar ataxia (6%), failure to thrive and developmental delay (6%), ptosis (6%), weakness (5%), neuropathy (5%), mitochondrial DNA depletion (4%), and COX-deficient fibers (3%) which is a measure of mitochondrial dysfunction (**Fig 5**). Across all manifestations in *POLG* carriers, a ~2% frequency was seen for lactic acidosis, hypotonia, OXPHOS deficiency, Parkinsonism, hearing loss, acute liver failure, ragged-red fibers (i.e., a measure of mitochondrial integrity loss), cataracts, abnormal magnetic resonance imaging (MRIs), and gait abnormality (**Fig 5**). Collectively, these data reveal that PRDT is quite visible in the *POLG* carrier population at varying degrees of clinical manifestations, and PRDT phenotypes mirror known hepatocerebral forms of MDDS [14].

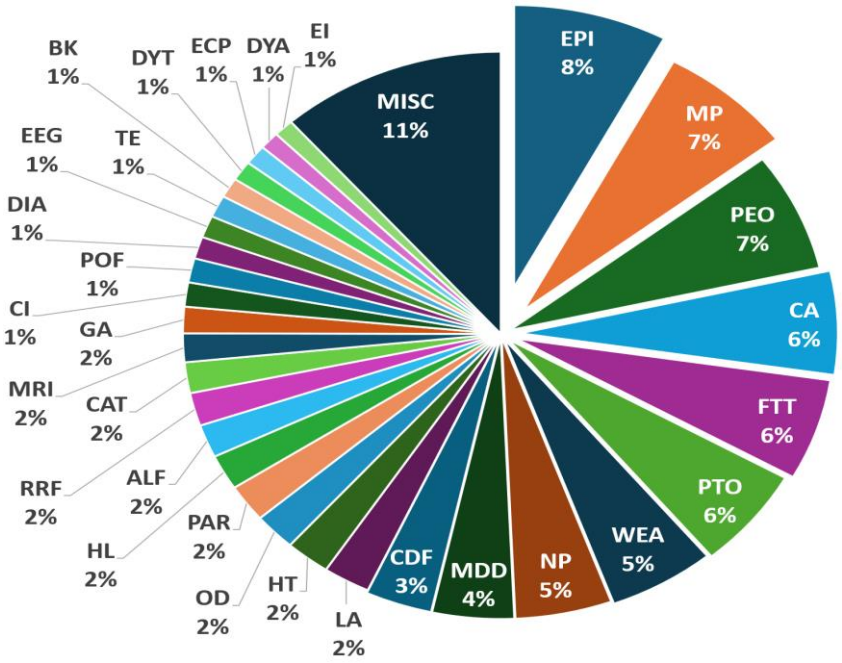

Figure 5: *POLG* heterozygotes present across a wide spectrum of clinical manifestations.

POLG-related disorders occupy a wide spectrum of clinical manifestations. A total of 55 different clinical manifestations of *POLG*-related disorders were manually extracted from nearly two decades of research articles documenting 179 *POLG* carriers. Each clinical manifestation tracks a facet of hepatocerebral forms of MDDS. **Abbreviations**: ALF, acute liver failure / hepatopathy; BK, bradykinesia; CA, cerebellar ataxia; CAT, cataracts; CDF, COX-deficient fibers; CI, cognitive impairment; DIA, diabetes; DYA, dysarthria; DYT, dystonia; ECP, encephalopathy; EEG, abnormal electroencephalogram; EI, exercise intolerance; EPI, epilepsy / seizures; FTT, failure to thrive / developmental delay; GA, gait abnormality; HL, hearing loss; HT, hypotonia; LA, lactic acidosis; MDD, mtDNA deletion / depletion; MISC, miscellaneous (25); MP, myopathy; MRI, abnormal magnetic resonance imaging; NP, neuropathy; OD, oxidative phosphorylation deficiency; PAR, Parkinsonism; PEO, progressive external ophthalmoplegia; POF, premature ovarian failure; PTO, ptosis; RRF, ragged-red fibers; TE, elevated transaminases; WEA, weakness. The MISC category contains 25 collated manifestations described in *POLG* heterozygotes: adrenal insufficiency, Alpers-like syndrome, camptocormia, cardiomyopathy, chorea, dementia, depression, diplopia, dysphagia, goiter / hyperthyroidism, hepatocerebral syndrome, hypergammaglobulinemia, hypnic myoclonia, hypoacusia, hypophonia, inclusion body myositis, mental retardation, microcephaly, nystagmus, optic atrophy, presbycusis, psychosis, retinitis pigmentosa, strabismus, and vision loss.

Clinical presentation of *POLG* heterozygotes is enriched in pediatric cases

POLG heterozygosity and clinical presentation of at least 1 of 55 manifestations of the hepatocerebral form of MDDS is especially important in pediatric cases. Across all 179 *POLG* heterozygotes, the largest age group that presents with mitochondrial disease signs and symptoms is the pediatric age group spanning 0-5 years of age (**Fig 6**).

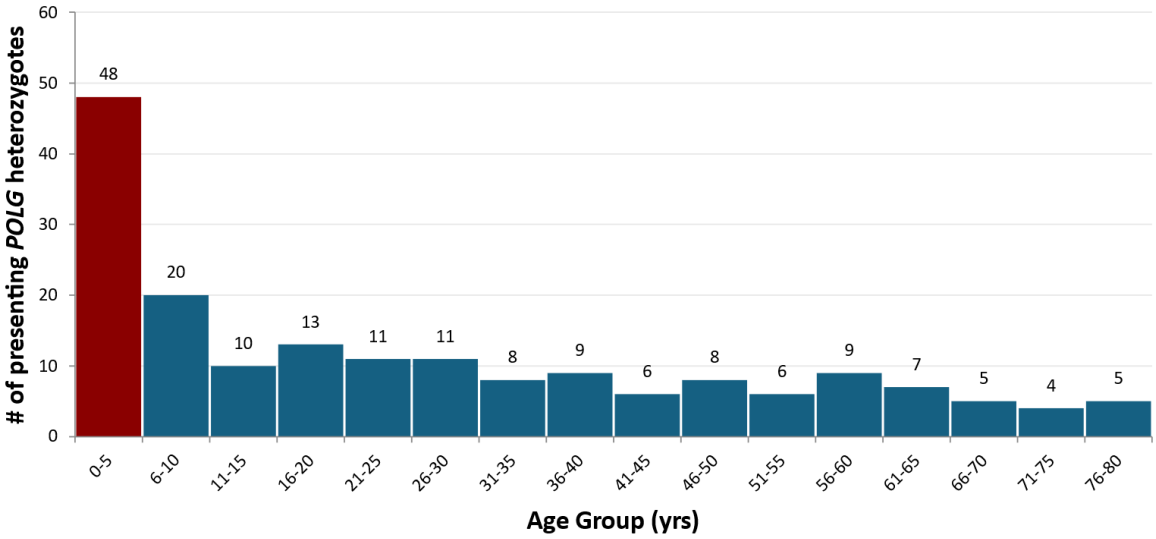

Figure 6: *POLG* heterozygosity presents with clinical manifestations of hepatocerebral MDDS across decades with enrichment in pediatric age groups.

Of 179 *POLG* heterozygotes documented across two decades of clinical research articles, presentation of hepatocerebral MDDS occurs most frequently in pediatric cases aged 0-5 years, followed by juvenile cases, and then adult cases.

This pediatric age group, consisting of *POLG* heterozygotes, is enriched for AHC, MCHS, MEMSA and ANS categories of *POLG*-related disorders and typically without presentation of either arPEO or adPEO. Careful examination of *POLG* carriers for neurologically-relevant manifestations, such as cerebellar ataxia, hypotonia, abnormal EEG, abnormal MRI, epilepsy or seizure, and neuropathy reveals a PDRT presentation that is enriched in pediatric cases (**Fig 7A-7E**).

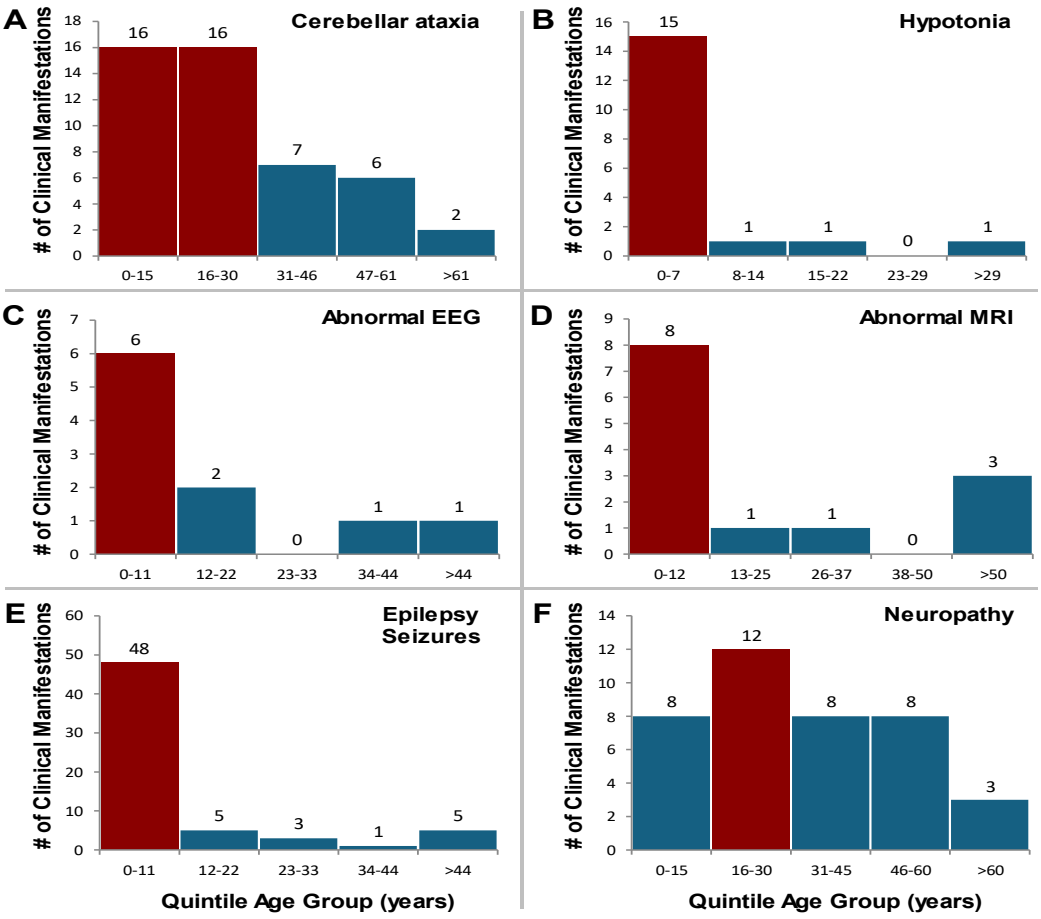

Figure 7: Neurological manifestations in *POLG* carriers are enriched in pediatric hepatocerebral MDDS cases.

The hallmarks of hepatocerebral MDDS involving lesions at the *POLG* locus are typified by A) cerebellar ataxia, B) hypotonia, C) abnormal EEGs, D) abnormal MRIs, E) epilepsy, and F) neuropathy. Quintile histogram analyses reveal that *POLG* heterozygotes present most of these clinical manifestations in the earliest age group (i.e., the youngest 20% of the population).

Common clinical manifestations in *POLG*-related disorders also includes failure to thrive, developmental delay, myopathy, metabolic lactic acidosis, weakness, liver failure, hepatopathy, and PEO. In each of these metrics, the top quintile (i.e., the youngest 20% of observed patients) of *POLG* heterozygotes typically exhibited higher rates of clinical metrics of MDDS (**Fig 8A-8F**). Thus, *POLG* heterozygotes recapitulate phenotypes commonly described in the clinical manifestations *POLG*-related disorders, traditionally relegated to double LOF genotypic backgrounds. *POLG* carriers (i.e., heterozygous *POLG* patients) thus exhibit varying degrees of clinical manifestations that track fully $POLG^{-/-}$ genotypes that stem from either LOF homozygosity or compound LOF heterozygosity in the *trans* configuration.

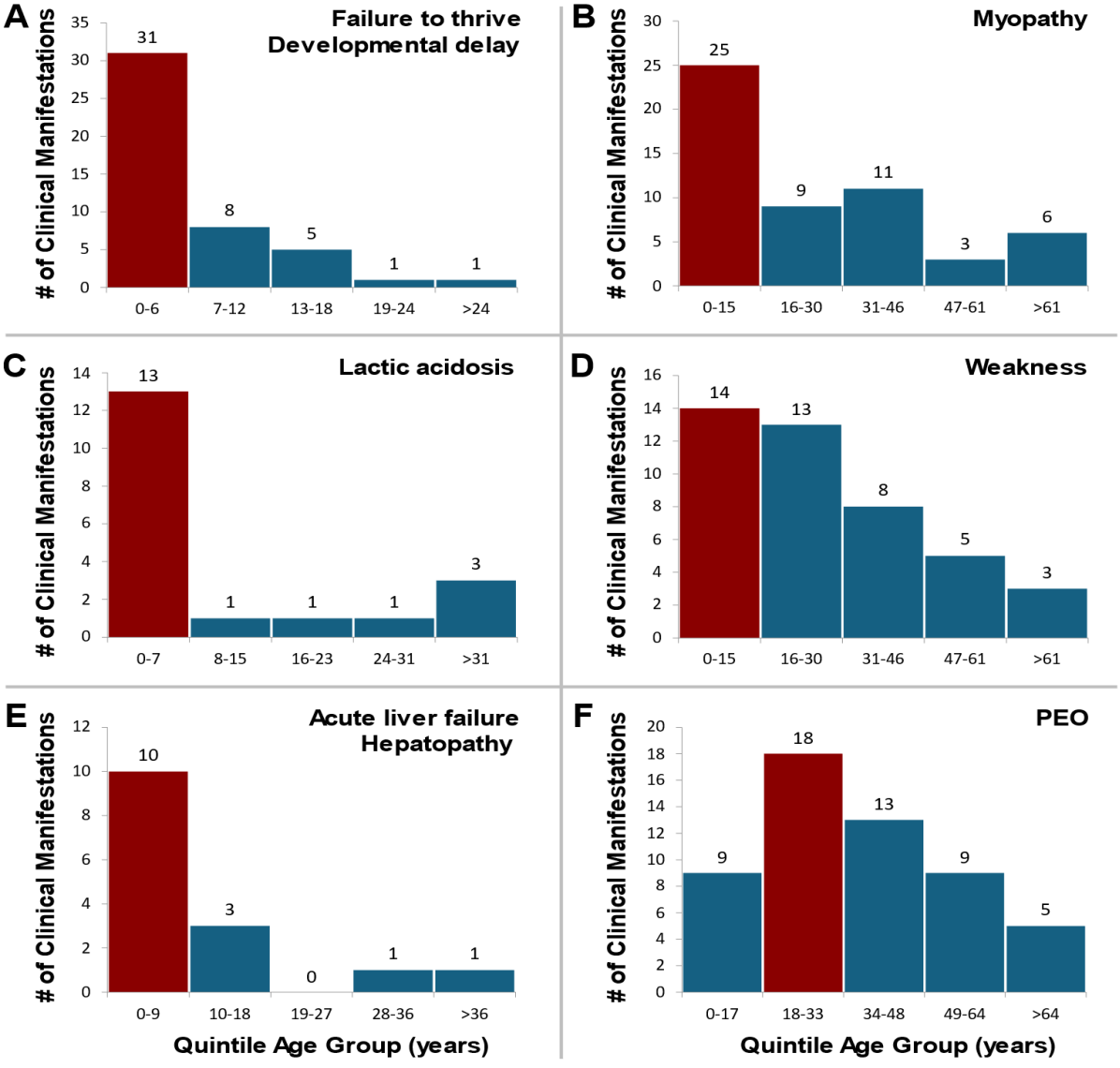

Figure 8: Pediatric *POLG* heterozygotes exhibit stereotypical clinical manifestations of hepatocerebral MDDS.

Pediatric MDDS cases involving *POLG* heterozygosity exhibit reproducible clinical manifestations that are characterized by A) failure to thrive & developmental delay, B) myopathy, C) metabolic lactic acidosis, D) weakness, E) acute liver failure, liver damage, & hepatopathy, and F) both forms of progressive external ophthalmoplegia (arPEO and adPEO). Quintile histogram analyses reveal that *POLG* heterozygotes present most of these clinical manifestations in the earliest age group (i.e., the youngest 20% of the population) except for weakness and PEO facets.

Discussion

POLG heterozygosity and *POLG*-related disorders trait (PRDT)

Taken together, *POLG* heterozygosity is not completely replete without mitochondrial disease manifestations. Carriers of at least one pathogenic (i.e., LOF allele) variant of the *POLG* locus are thus still capable of clinical manifestations. There are many examples of established precedent for heterozygosity and clinical presentation. Perhaps the most commonly studied example is that of sickle cell anemia (SCA) which is a homozygous recessive genetic disorder in which SCA patients

carry two inactivating alleles at the *HBB* (i.e., beta-globin) locus [37]. SCA patients exhibit dramatic reductions in their lifespans due to compromised hemoglobin formation and perturbed oxygen delivery to respiring cells and tissues [37]. In $HBB^{+/-}$ heterozygotes (aka sickle-cell trait, or SCT), clinical manifestations in delivered oxygen do not become apparent unless at high altitudes whereby reduced partial pressure of oxygen gas requires an efficiency of oxygen delivery by hemoglobin that is insufficiently met in the heterozygous SCT state [37]. Heterozygosity under certain conditions can thus reveal haploinsufficiency. For SCT, that haploinsufficiency trigger is high-altitude but for PRDT patients, it can range across any agent or drug that might harm mitochondrial integrity and function. Damaged mitochondria in turn require organellar renewal which necessitates mtDNA replication services contingent on the nuclear-encoded *POLG* locus. Excessive mitochondrial damage may overwhelm PRDT patients (i.e., *POLG* heterozygotes) revealing clinical manifestations otherwise relegated to double *POLG* LOF conditions.

PRDT may predispose patients to contraindications or pathogens that exhibit mitochondrial toxicity

PRDT is thus the heterozygous trait associated with *POLG* heterozygosity with accompanying manifestation onset resembling that of *POLG*-related disorders. The significance of PRDT per each individual *POLG* carrier stems from several, overlapping criteria. Firstly, each *POLG* allele may exhibit varying degrees of penetrance, from hypomorphic or partial loss-of-function manifestations to null or complete loss-of-function alleles. Further, in some *POLG* heterozygotes, some *POLG* alleles may act at the molecular level via some degree of dominant-negative behavior, whereby mutant POLG1 peptide subunits sequester POLG2 subunits from interacting with wild-type (i.e., normal) POLG1 subunits. This might partially explain the autosomal dominant forms of PEO seen in adult forms of *POLG*-related disorders [30,38,39]. In any case, PRDT (i.e., *POLG* heterozygosity) is clinically visible and presents with stereotyped clinical manifestations reminiscent of *POLG*-related disorders in *POLG* homozygotes or *POLG* compound heterozygotes. Due to clinical manifestations in the PRDT population, care should be taken when prescribing medications that are contraindicated for either mitochondrial toxicity or *POLG*-related disorders [36,40,41]. For example, pediatric PRDT patients exhibit a sizeable share of epilepsy and seizure manifestations (**Fig 7E**), and antileptics that are contraindicated for their mitochondrial toxicity should thus be avoided [42-44]. These include valproate sodium and valproic acid due to their potential to drive fatality due to acute liver failure in patients diagnosed with *POLG*-related disorders [44,45]. Similarly, other antileptics such as phenobarbital and oxcarbazepine should be avoided in PRDT cases due to evidence of mitochondrial damage [46,47]. In such PRDT cases, excessive mitochondrial toxicity necessitates *de novo* renewal and biogenesis of nascent mitochondria [48]. As each mitochondrial organelle requires a copy number of 2-10 mtDNA molecules, and each human cell can house upwards of 1,000 total mitochondria, a sizeable molecular burden for sustaining all mtDNA replication activities is placed on the sole functioning WT *POLG* gene locus found in a PRDT patient exhibiting *POLG* heterozygosity [49-51]. It is not beyond reason that with enough exposure to contraindicated medications that are toxic to mitochondrial function and integrity that a *POLG* haploinsufficiency event might reveal itself in due course within a *POLG* carrier. Other contraindications include aminoglycoside antibiotics and antiretrovirals for their known ability to either block mitochondrial protein translation by mitochondrial ribosomes or by direct interference of the trimeric mtDNA polymerase complex, respectively [52,53].

Heterozygosity across all genetic loci underlying MDDS likely exhibits clinical manifestations

Mitochondrial DNA depletion syndromes (MDSS) occur in four major forms: a neurogastrointestinal form, a myopathic form, an encephalomyopathic form, and lastly a hepatocerebral form [14]. The hepatocerebral form of MDDS is typically characterized by lesions at the *POLG* locus which encodes the catalytic component of the trimeric mtDNA polymerase complex. *POLG*-related disorders in turn are classified in 6 different ways: Alpers-Huttenlocher Syndrome (AHS), Myocerebrohepatopathy Spectrum (MCHS), Myoclonus Epilepsy Myopathy Sensory Ataxia (MEMSA), Ataxia Neuropathy Spectrum (ANS), autosomal recessive Progressive External Ophthalmoplegia (arPEO), and autosomal dominant Progressive External Ophthalmoplegia (adPEO) [17]. A graphical summary of *POLG* heterozygosity (i.e., PRDT) presentation of clinical manifestations of hepatocerebral MDDS is illustrated in **Fig 9**. Pediatric cases typically present with AHS, MCHS, MEMSA, and ANS while adult cases harbor PEO manifestations. Afflicted physiological systems for pediatric PRDT presentation tracks deficits in central nervous system activity and neuromuscular function (**Fig 9**). In many pediatric cases, acute liver failure, hepatopathy or liver dysfunction (i.e., as measured via elevated serum transaminases) is typical of pediatric PRDT manifestations (**Fig 9**). Pediatric *POLG* carriers also exhibited failure to thrive and short stature, as well as metabolic lactic acidosis (**Fig 9**).

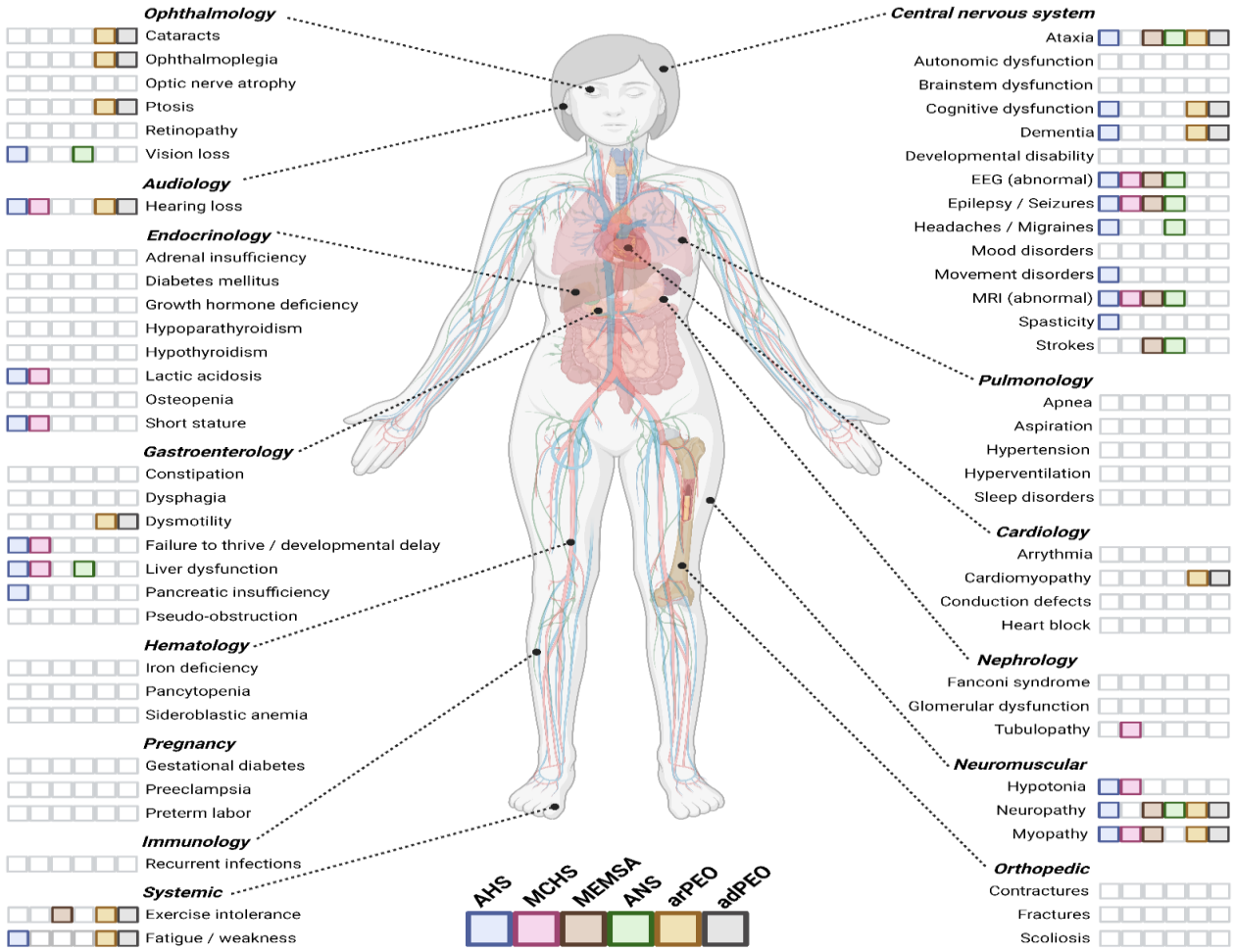

Figure 9: *POLG*-related disorders exhibit hepatocerebral-specific manifestations across the MDDS spectrum.

The spectrum of MDDS is vast, clinically afflicting a wide range of host processes and physiological systems. In the case of *POLG*-related disorders, hepatic and cerebral tissues are most commonly afflicted. *POLG* heterozygotes exhibited various facets of the six types of *POLG*-related disorders: AHS, MCHS, MEMSA, ANS, arPEO, and adPEO. Pediatric *POLG* carriers typically present a permutation of AHS, MCHS, MEMSA, and ANS, while PEO is enriched in elderly patients of *POLG* heterozygosity. **Abbreviations**: adPEO, autosomal dominant progressive external ophthalmoplegia; AHS, Alpers-Huttenlocher Syndrome; ANS, Ataxia Neuropathy Spectrum; arPEO, autosomal recessive progressive external ophthalmoplegia; MCHS, Myocerebrohepatopathy Spectrum; MEMSA, Myoclonus Epilepsy Myopathy Sensory Ataxia. Modified from Parikh *et al.*, 2017 [36]. Created via BioRender.

Heterozygosity at the *POLG* locus, effectively conferring PRDT, is thus one of many heterozygous forms that mechanistically can drive MDDS. Other nuclear-encoded mitochondrial genes that contribute to MDDS include *TYMP* in neurogastrointestinal MDDS, the *TK2* locus in myopathic MDDS, and lesions at either the *RRM2B*, *SUCLG1*, or *SUCLG2* locus for encephalomyopathic MDDS (**Fig 10**). Additionally, other forms of hepatocerebral MDDS are possible with LOF lesions arising from *POLG2*, *TWNK*, *MPV17* and *DGUOK* genetic loci. Future work would benefit from a careful heterozygosity analysis at each of these 9 additional genetic loci outside of the *POLG* locus to determine the extent of clinical manifestations in the heterozygous state, in a fashion resembling PRDT (**Fig 10**).

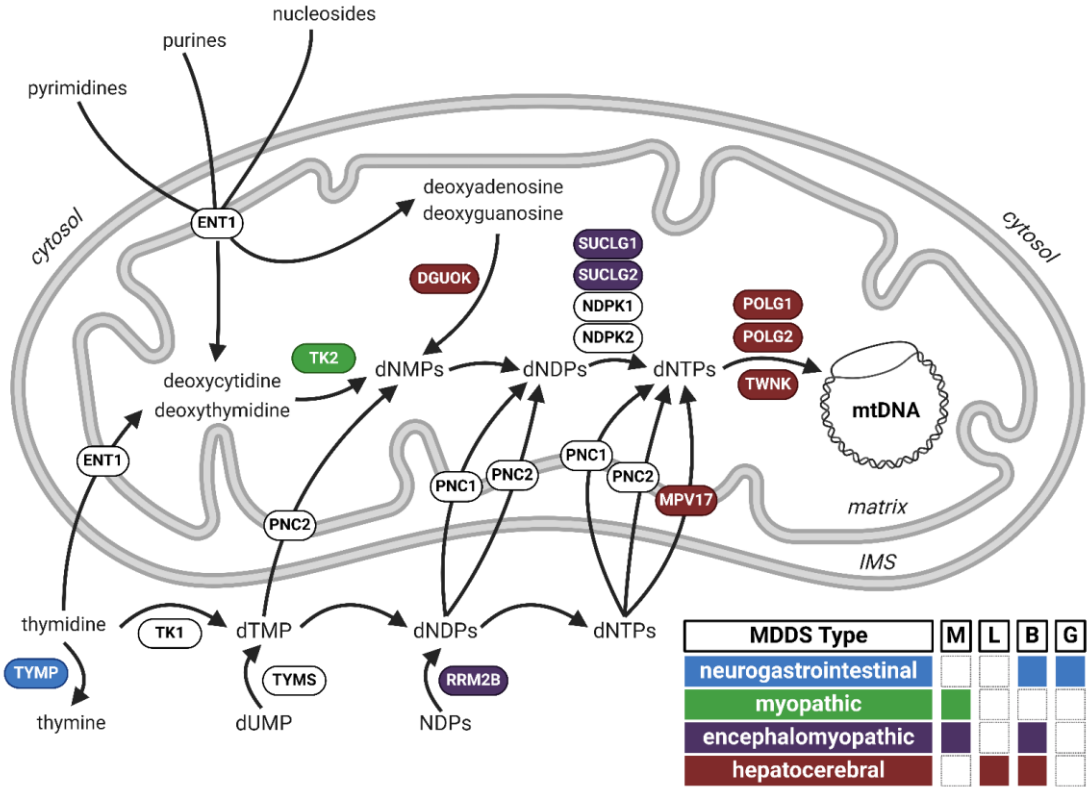

Figure 10: *POLG*-related disorders disrupt hepatocerebral tissues as one of four types of MDDS.

Mitochondrial DNA depletion syndromes (MDDS) typically present in stereotyped manifestations in the clinic. (BLUE): Mutations in the *TYMP* gene disrupt thymidine phosphorylase enzyme activity in the cytosol which causes mitochondrial neurogastrointestinal encephalopathy (MNGIE) syndrome. (GREEN): LOF lesions at the *TK2* locus are deleterious to mitochondrial thymidine kinase 2 enzyme activity. TK2 functions in the mitochondrial matrix to generate deoxyribonucleoside monophosphates (dNMPs) involving deoxycytidine and deoxythymidine molecules. TK2 mutations are associated clinically with the myopathic form of MDDS. (PURPLE): Null mutations at either *RRM2B*, *SUCLG1*, or *SUCLG2* block mitochondrial access to either deoxyribonucleoside diphosphates (dNDPs) or deoxyribonucleoside triphosphates (dNTPs). *RRM2B* encodes the p53-inducible subunit (p53R2) of the ribonucleotide reductase complex that is essential for conversion of NDPs of the RNA world into dNDPs of the DNA world. *SUCLG1* and *SUCLG2* genes encode the alpha and beta subunits, respectively, of the succinyl coenzyme A ligase, a key enzyme complex that produces dNTPs in the mitochondrial matrix. LOF lesions at either the *RRM2B*, *SUCLG1*, or *SUCLG2* loci result in encephalomyopathic forms of MDDS. (RED): Disrupting mutations at either the *DGUOK*, *MPV17*, *POLG1*, *POLG2*, and *TWNK* loci yield the hepatocerebral manifestation form of MDDS. The *DGUOK* gene encodes the mitochondrial deoxyguanosine kinase which converts deoxyadenosines and deoxyguanosines in the mitochondrial matrix into concomitant dNMPs. *MPV17* encodes a dNTP transporter protein localized to the innermost mitochondrial membrane (i.e., cristae membrane). The mtDNA helicase enzyme is encoded by the *TWNK* gene while the trimeric mtDNA polymerase complex is comprised of one subunit of the *POLG1* gene product, and two protein subunits derived from *POLG2* expression. **Abbreviations**: DGUOK, deoxyguanosine kinase; dNDP, deoxyribonucleoside diphosphate; dNMP, deoxyribonucleoside monophosphate; dNTP, deoxyribonucleoside triphosphate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; ENT1, equilibrative nucleoside transporter 1; IMS, intermembrane space; MPV17, mitochondrial inner membrane protein MPV17; mtDNA, mitochondrial DNA; MDDS, mitochondrial DNA depletion syndrome; NDP, ribonucleoside diphosphate; NDPK1, nucleoside diphosphate kinase 1; NDPK2, nucleoside diphosphate kinase 2; PNC1, pyrimidine nucleotide carrier 1; PNC2, pyrimidine nucleotide carrier 2; POLG1, catalytic subunit of mtDNA polymerase complex; POLG2, scaffold subunit of mtDNA polymerase complex; RRM2B, regulatory TP53-inducible subunit M2B of the ribonucleotide reductase complex; SUCLG1, alpha subunit of the succinyl CoA ligase complex; SUCLG2, beta subunit of the succinyl CoA ligase complex; TK1, cytosolic thymidine kinase 1; TK2, mitochondrial thymidine kinase 1; TWNK, mtDNA helicase; TYMP, thymidine phosphorylase; TYMS, thymidylate synthetase. Modified from Chan and Copeland, 2009 [11]. Created via BioRender.

The rationale behind why nuclear-encoded mitochondrial enzymes play such an outsized role in mitochondrial diseases stems from the enormous relocation of mitochondrial genes from the mtDNA chromosome towards the nucleus integrating within human chromosomes, a process that occurred across endosymbiotic time [7,8]. Movement of 1,136 mitochondrial genes into the human nucleus allowed the mitochondrial organelle to become reliant on the host nucleus for encoding essential services, including transport of nitrogenous bases from the cytosol to the matrix, nucleotide anabolism, and nucleic acid synthesis (i.e., mtDNA replication) (**Fig 11**). LOF mutations that arise either in the germline nuclear DNA or via somatic mutation in the adult condition can thus alter either the molecular assembly of nucleotide monomers or nucleic acid polymers

within the mitochondrial matrix (**Fig 11**). Here we systematically show that *POLG* heterozygosity is sufficient to present clinically in numerous cases, particularly in pediatric settings, and thus *POLG* carriers exhibit *POLG*-related disorder trait (PRDT) and are not completely normal. Care must be taken regarding exposure to either genotoxicity (i.e., DNA mutagens) or mitochondrial toxicity (i.e., contraindicated medications) in all PRDT cases, particularly those in pediatric age groups, to reduce the risk of haploinsufficiency induction at the *POLG* locus.

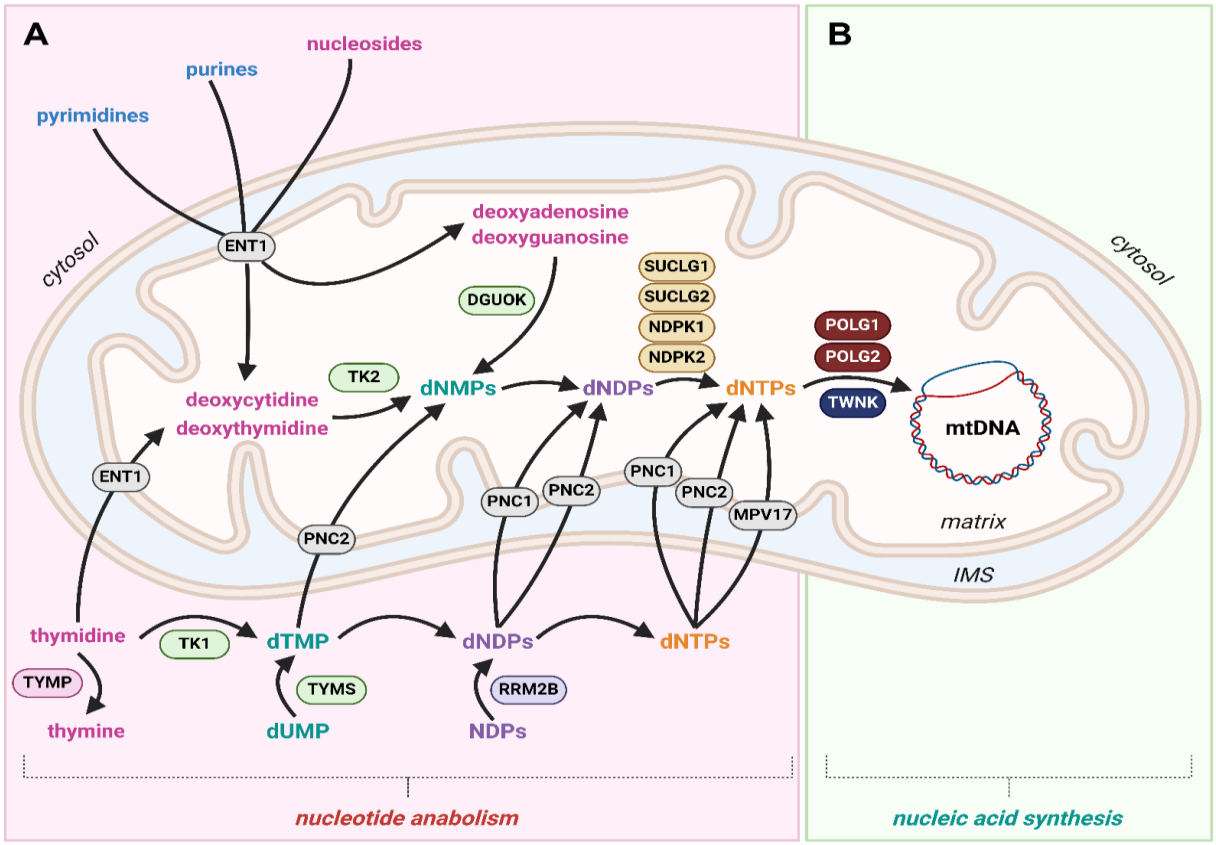

Figure 11: Disruption of mitochondrial nucleotide import, nucleotide anabolism, or mtDNA replication drives MDDS clinically.

Mitochondrial DNA depletion syndromes (MDDS) typically present in stereotyped manifestations in the clinic. (LEFT): Depletion of mtDNA occurs due to deficits in nucleotide anabolism or nucleotide component import from the host cytosol to the mitochondrial matrix. The mitochondrial inner membrane employs numerous protein transporter systems (ENT1, PNC1, PNC2, and MPV17) to import, from the cytosol into the mitochondrial matrix, key ingredients involved in the anabolic synthesis of deoxyribonucleotides. Such metabolites include various nitrogenous bases, deoxyribonucleosides, dNMPs, dNDPs, and dNTPs for the express purpose of sustaining the requisite matrix levels of deoxyribonucleotide monomer pools needed to sustain mtDNA polymer formation. (RIGHT): Depletion of mtDNA can also occur due to defective nucleic acid synthesis by virtue of a disrupted helicase (TWNK) or lesioned mtDNA polymerase complexes (POLG1 and POLG2). **Abbreviations**: DGUOK, deoxyguanosine kinase; dNDP, deoxyribonucleoside diphosphate; dNMP, deoxyribonucleoside monophosphate; dNTP, deoxyribonucleoside triphosphate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; ENT1, equilibrative nucleoside transporter 1; IMS, intermembrane space; MPV17, mitochondrial inner membrane protein MPV17; mtDNA, mitochondrial DNA; MDDS, mitochondrial DNA depletion syndrome; NDP, ribonucleoside diphosphate; NDPK1, nucleoside diphosphate kinase 1; NDPK2, nucleoside diphosphate kinase 2; PNC1, pyrimidine nucleotide carrier 1; PNC2, pyrimidine nucleotide carrier 2; POLG1, catalytic subunit of mtDNA polymerase complex; POLG2, scaffold subunit of mtDNA polymerase complex; RRM2B, regulatory TP53-inducible subunit M2B of the ribonucleotide reductase complex; SUCLG1, alpha subunit of the succinyl CoA ligase complex; SUCLG2, beta subunit of the succinyl CoA ligase complex; TK1, cytosolic thymidine kinase 1; TK2, mitochondrial thymidine kinase 1; TWNK, mtDNA helicase; TYMP, thymidine phosphorylase; TYMS, thymidylate synthetase. Modified from Chan and Copeland, 2009 [11]. Created via BioRender.

Author Contributions

Conceptualization, AB, SB, MS, JK, HD, GV; Methodology, GV; Software, GV; Validation, IE, AR, HD, OD, NF, GV; Formal Analysis, AB, SB, MS, JK, GV; Investigation, AB, SB, MS, JK, GV; Resources, AB, SB, MS, JK, GV; Data Curation, GV; Writing — original draft preparation, AB, SB, MS, JK, GV; Writing — review and editing, IE, AR, HD, OD, NF, GV; Visualization, AB, SB, MS, JK, GV; Supervision, GV; Project Administration, GV.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Data

Supplementary Table 1. Complete data matrix of all *POLG* heterozygotes and curated, clinical manifestations of *POLG*-related disorders. (Please see CSV file entitled: "Betler 2024 Supplementary Table 1.csv")

Betler_2024_Supplementary_Table_1.csv