Biallelic variants in the ectonucleotidase *ENTPD1* cause a complex neurodevelopmental disorder with intellectual disability, distinct white matter abnormalities, and spastic paraplegia

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Running head: *ENTPD1*-related neurodevelopmental disorder

Summary for Social Media

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What is the current knowledge on the topic?

Hereditary spastic paraplegia is a progressive inherited neurological disorder caused by mutations in over 80 different genes. Because of this genetic heterogeneity, frequently only few affected individuals are identified for each genetic subtype, resulting in uncertainty regarding the clinical validity of gene-disease associations and the disease's phenotypic spectrum.

What question did this study address?

ENTPD1 is a gene essential in purine metabolism and has been linked to a poorly characterized hereditary spastic paraplegia (HSP), SPG64, with only a few affected individuals identified throughout the world. We set out to examine a large cohort of patients with ENTPD1-related neurological disorder to fully evaluate the disease spectrum and molecular mechanisms underlying disease pathogenicity.

What does this study add to our knowledge?

Through the identification of 18 previously unreported families with 29 affected individuals and 12 novel *ENTPD1* variants, this study firmly establishes the gene-disease validity of *ENTPD1* as the cause of an autosomal recessive (AR) complex HSP characterized by childhood onset, developmental delay/intellectual disability (DD/ID), dysarthria, progressive spastic paraplegia, dysmorphic features, cerebral white matter abnormalities, and developmental regression. We additionally identify multiple clinical diagnostic biomarkers including 1) T2-hyperintense signal in the posterior limb of the internal capsule (PLIC) on

brain MRI, 2) diminished ENTPD1/CD39 staining on peripheral blood cells by flow cytometry and peripheral nerve biopsies by immunohistochemistry, and 3) diminished ATP and ADP breakdown in a lymphoblast-based cellular assay.

How might this potentially impact the practice of Neurology?

Defining the full genotypic and phenotypic spectrum of rare neurological disease traits like SPG64 is essential for clinical management and patient and family counseling. Expansion of the allelic spectrum and development of new diagnostic biomarkers will facilitate earlier recognition of SPG64, act as a springboard for additional mechanistic studies of the role of purine metabolism in neurodevelopment and neurodegeneration, and aid therapeutic investigations.

Abstract

Objective: The study of human genomics has established that pathogenic variation in genes with diverse molecular functions can underlie a single disorder. For example, hereditary spastic paraplegia (HSP) is a phenotypic trait associated with over 80 genes; frequently only few individuals are described for each gene. Herein, we characterize a large cohort of individuals with biallelic variation in *ENTPD1*, a gene previously linked to spastic paraplegia 64 (MIM# 615683).

Methods: Affected individuals with biallelic *ENTPD1* variants were recruited worldwide. Deep phenotyping and molecular characterizations were performed.

Results: A total of 29 undescribed individuals from 18 unrelated families were studied, and additional phenotypic information was collected from published cases. Twelve novel pathogenic *ENTPD1* variants are described: c.398_399delinsAA; p.(Gly133Glu), c.540del; p.(Thr181Leufs*18), c.640del; p.(Gly216Glufs*75), c.185T>G; p.(Leu62*), c.1531T>C; p.(*511Glnext*100), c.967C>T; p.(Gln323*), c.414-2_414-1del, c.520G>T; p.(Glu174*), and c.146 A>G; p.(Tyr49Cys) including four recurrent variants c.1109T>A; p.(Leu370*), c.574-6_574-3del, c.770_771del; p.(Gly257Glufs*18), and c.1041del; p.(Ile348Phefs*19). Common shared disease features include: early childhood onset, progressive spastic paraplegia, intellectual disability (ID), dysarthria, dysmorphisms, and white matter abnormalities. *In vitro* assays demonstrate that *ENTPD1* expression and ATP hydrolysis are impaired and that the recurrent variant c.574-6_574-3del causes exon skipping.

Interpretation: The *ENTPD1* locus trait consists of childhood disease onset, ID, progressive spastic paraparesis, dysarthria, dysmorphisms, and white matter abnormalities with some individuals showing neurocognitive regression. Investigation of an allelic series of *ENTPD1*: i) expands previously described features of *ENTPD1*-related neurological disease, ii) highlights

the importance of genotype-driven deep phenotyping, iii) documents the utility of worldwide families for the study of rare AR disease traits, and iv) provides insights into the disease trait neurobiology.

Introduction

Genome sequencing and clinical genomics have markedly improved molecular diagnostic rates in rare Mendelian disorders by accelerating novel disease gene and variant allele discovery and expanding the phenotypic spectrum associated with known disease genes[1-3]. This progress has resulted in the understanding that a single family of disorders can be caused by pathogenic variation in genes with diverse functions. For example, hereditary spastic paraplegias (HSP) are a large group of neurological disorders affecting 1.8 in 100,000 individuals globally[4]. Inheritance patterns for HSP disease traits are variable, including autosomal dominant (AD), autosomal recessive (AR), X-linked, *de novo*, and mitochondrial inheritance[5]. Despite shared features, HSP results from pathogenic variation in over 80 genes/loci involved in mitochondrial functioning, lipid metabolism, vesicle/axonal trafficking, and myelination[6]. With the rapid pace of disease gene discovery[7], this number will likely continue to expand. In fact, an "HSPome" of known HSP disease genes, candidate disease genes, and proximal interactors has implicated almost 600 potential HSP genes[8].

HSP is classified into "pure" and "complex/complicated" with unifying features of corticospinal tract nerve axonopathy, progressive gait difficulty, and axonal length-dependent neuropathy[9,10]. Complex HSP additionally encompasses developmental delay/intellectual disability (DD/ID), structural brain abnormalities, ataxia, epilepsy, amyotrophy, and visual abnormalities[10]. Consequently, complex HSP frequently overlaps with neurodevelopmental disorders (NDD); e.g. leukodystrophies, cerebellar ataxias, and syndromic DD/ID[11].

As with hereditary neuropathies[12], the allelic spectrum of HSP is unevenly distributed across known disease genes. For example, pathogenic variation in *SPAST*, the cause of AD spastic paraplegia 4 (MIM# 182601), accounts for approximately 60% of HSP diagnoses[13-16]. The abundance of AD spastic paraplegia 4 and other "common" HSP causes reflects historical population-specific events, e.g. founder effect or population bottlenecks, or high frequency

mutational events occurring as a consequence of genomic architecture, e.g. *Alu/Alu*-mediated rearrangements (AAMRs) due to abundance of *Alu* repetitive elements and genomic instability within *SPAST*[17].

The remaining allelic spectrum of HSP exhibits extensive molecular heterogeneity and is a collection of ultra-rare diseases, often with only a few individuals described for each gene locus. Studies investigating the phenotypic spectrum from different families and ethnicities worldwide and diverse pathogenic variant alleles, i.e., an allelic series, are often lacking. For example, AR spastic paraplegia type 64 (SPG64, MIM #615683) due to biallelic pathogenic variation in *ENTPD1*, the gene encoding the ectonucleosidase ENTPD1 involved in adenosine triphosphate (ATP) hydrolysis, has been described in only a few individuals with limited and seemingly dissimilar phenotypic characterization[8,18-20]. Affected individuals have shared features of childhood-onset disease with progressive spastic paraplegia, DD/ID, and variable findings of brain abnormalities and abnormal reflexes ranging from areflexia to hypo-and hyperreflexia. It is therefore critical to deeply phenotype large cohorts of individuals with rare diseases, e.g. due to biallelic *ENTPD1* variants, potentially revealing previously undescribed features (e.g. phenotypic expansion and multilocus pathogenic variation, MPV), and therefore providing a comprehensive understanding of the disease process and gene-associated trait.

Herein, we describe phenotypic and molecular features of a large cohort of individuals with biallelic variants in *ENTPD1* and provide evidence for a complex neurodevelopmental disorder (NDD) with progressive spastic paraplegia with previously unrecognized clinical features.

Materials and methods

Patient identification and recruitment

This study was approved by the Institutional Review Board (IRB) at Baylor College of Medicine (Protocol H-29697) or through other collaborative local IRBs. Additional affected participants were identified through GeneMatcher[21] or personal communication. Written consent, including consent for publication of photographs, was obtained for all participants. Study participants were examined by a clinical geneticist and/or neurologist and phenotypic features were described using Human Phenotype Ontology (HPO) terms[22]. Brain magnetic resonance images (MRIs) were retrospectively reviewed and analyzed by a single observer; board certified neuroradiologist (JVH).

Exome sequencing

For family 1, trio exome sequencing (ES) was performed at the Human Genome Sequencing Center (HGSC) at Baylor College of Medicine (BCM) through the Baylor-Hopkins Center for Mendelian Genomics (BHCMG) initiative as previously described[23]. For all other identified families, ES was performed by local institutions or commercial clinical molecular diagnostic laboratories via previously established protocols[24].

Absence of heterozygosity

BafCalculator (https://github.com/BCM-Lupskilab/BafCalculator)[2], an in-house developed bioinformatic tool that extracts the calculated B-allele frequency (ratio of variant reads/total reads) from unphased exome data, was used to calculate genomic intervals and total genomic content of absence of heterozygosity (AOH) intervals as a surrogate measure for runs of homozygosity (ROH) likely representing identity-by-descent (IBD) genomic intervals. B-allele frequency was transformed by subtracting 0.5 and taking the absolute value for each data point before being processed by circular binary segmentation using the DNAcopy R Bioconductor package. The estimated coefficient of inbreeding values from ROH were

calculated as the fraction of the sum of AOH genomic intervals >1.5 Mb in size to the length of the autosomal genome (3,100 Mb).

Confirmation of alternative splicing

Whole blood RNA from family 5 was extracted using the PAXgene Blood RNA kit (Qiagen, Germantown, MD) according to the manufacturer's instructions and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) with poly-dT (20) primers according to manufacturer's protocol. Amplicons were generated from control, proband, and parental cDNA using HotStartTaq DNA polymerase (Qiagen, Germantown, MD) according to manufacturer's protocol. DNA bands at various sizes were excised, purified via the PureLink PCR purification and gel extraction kit (Invitrogen, Carlsbad CA), and Sanger dideoxy DNA sequencing implemented at the BCM sequencing core facility.

Real-time PCR

Immortalized lymphoblast cell lines from affected individuals were established from blood samples at The Centre for Applied Genomics (Toronto, Canada). Total RNA was obtained from affected and control lymphoblast cell lines with the RNeasyMinikit (Qiagen, Germantown, MD) and reverse transcribed into complementary DNA (cDNA) with iScript kit (BioRad Laboratories, Hercules, CA) according to manufacturer's protocol. cDNA was amplified with gene-specific primers and iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA) and read on a CFX96 Touch Real-time PCR Detection System. Gene expression was quantified using the standard Ct method with CFX software, and all data corrected against *GAPDH* as an internal control.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay buffer containing aprotinin, phenylmethanesulfonyl fluoride, and leupeptin at a final concentration of 10 mg/ml each in the composite solution (Sigma-Aldrich, St. Louis, MO) and concentrations were determined by Bradford assay (BioRad Laboratories, Hercules, CA). Protein samples were resolved by standard SDS-PAGE, transferred onto nitrocellulose membrane, incubated in blocking, followed by overnight incubation with primary antibody (ENTPD1, Abcam ab108248). Membranes were washed and incubated with secondary antibody (HRP conjugated anti-rabbit; BioRad Laboratories). Blots were visualized by chemiluminescence using the Clarity Western ECL substrate (BioRad Laboratories). Control protein was extracted from healthy, unrelated, age-matched control cell lines.

ATPase and ADPase assay

A total of 250,000 lymphoblasts were harvested per technical replicate from each cell line. The cells were washed and each replicate plated in a single well of a round bottom 96-well plate. Cells were then incubated with either 10 mM ADP or 10 mM ATP, or left untreated, for 30 min at 37°C. The supernatant was transferred to a new, flat bottom 96-well plate and phosphate concentration was measured using the Malachite Green Phosphate Assay kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The normalized phosphate production reported is fold change relative to untreated samples.

Flow Cytometry

Blood samples were collected in 3 ml EDTA tubes and analyzed for immune cell subsets using the following surface markers: CD16, CD56, CD3, CD4, CD8, CD2, CD15, CD19, CD20, HLA-DR, CD39 and CD73. All samples were analyzed using Beckman Coulter dual Laser Navios Flow Cytometer equipped with 488nm Argon and a 635 nm-diode laser, allowing six color fluorescence data acquisition (Beckman Coulter, Inc., 250 S., Kraemer Blvd, Brea, CA).

Sural nerve biopsy

Paraffin embedded sural nerve biopsy sections of the patient and a control sample were stained immunohistochemically for CD39 (Leica/Novocastra, 1cc, clone NCL-CD39, LOT-6017994, 1:50), according to the manufacturer's instructions and standard staining protocols.

Results

Index proband

The index patient (P1, family 1; **Fig. 1A**) is an eight-year-old girl referred to the Baylor-Hopkins Center for Mendelian Genomics (BHCMG) for DD/ID, spastic paraplegia and progressive gait impairment. She was born at term to first cousin Iraqi parents and has three healthy living siblings and three unaffected deceased siblings (age of death at five years, 13 years, and five years from leukemia, unknown etiology, and febrile illness, respectively). Her prenatal course was complicated by intrauterine growth restriction of unknown etiology. Concerns about the proband's development arose at one year of age due to lack of independent ambulation. First steps did not occur until after two years of age. At three years of age, the ability to ambulate independently deteriorated and she developed spastic paraplegia. Her neurological examination at 8 years showed dysarthria, muscle weakness with amyotrophy, and hyperreflexia in the upper extremities with areflexia in the lower extremities (**Table S1**). Trio ES revealed a novel homozygous variant in *ENTPD1*,

NM_001776.6: c.398_399delinsAA; p.(Gly133Glu). Sanger sequencing confirmed the multinucleotide variant allele in the homozygous state in the proband and in the heterozygous state in her unaffected parents (**Fig. 1A**). The variant is absent from gnomAD, is only found in this family in our research database of >13,000 exomes, and the affected amino acid residue is fully conserved across species (**Fig. S1**, **Table 1**). *ENTPD1*: c.398_399delinsAA is located in an 11.1 Mb AOH block and the total AOH size of the proband is 310 Mb, consistent with the offspring of a first cousin mating (**Fig. 1B**).

Recruitment of additional families with biallelic *ENTPD1* variation

Given limited phenotypic characterization of *ENTPD1*-related neurological disease, we identified additional cases through GeneMatcher (https://genematcher.org/)[21] and communication with neurogenetic research laboratories from around the globe. These efforts resulted in a total of 18 unrelated families with 29 affected individuals (**Fig. 1C, Table 1, Table S1**). Additional recruited families were from diverse countries and backgrounds, including Turkey (Family 2), Brazil (Families 3, 4, and 5), Puerto Rico (Family 6), Poland (Family 7), Israel (Family 8), Portugal (Family 9), Persia (Families 10, 11, 12, 15, 16, 17, 18), Egypt (family 13), and the Dominican Republic (Family 14). All affected individuals were born to consanguineous parents except families 6, 14, and 16. Review of the literature revealed an additional five families with nine affected individuals for whom further phenotypic data were obtained (**Table S1**)[8,18-20].

Phenotypic spectrum of *ENTPD1*-related neurological disease

Comparison of phenotypic features using HPO terminology, a structured ontology[22] among all affected individuals revealed both major similarities as well as differences,

suggestive of a phenotypic spectrum with a 'clinical synopsis' of shared commonalities in individuals with an *ENTPD1*-related pleiotropic neurological disease trait (**Table 2, Table S1**). The average age of the cohort at last examination was 16 years (range 3-32 years, median 15 years). All affected individuals had symptom onset in early childhood, DD/ID, and progressive spastic paraplegia with impaired ambulation. Behavioral abnormalities, including attention-deficit hyperactivity disorder (ADHD), aggression, and stereotypies were common and occurred in 18/38 individuals. A total of 23/38 individuals had neurocognitive regression in addition to progressive spastic paraplegia. DD/ID was diagnosed in clinical or education settings due to deficits in intellectual and adaptive functioning. Neurocognitive regression was determined by a progressive decline in functioning not attributable to progressive spastic paraplegia. Language regression was also common in these individuals.

The neurological examination revealed dysarthria (26/38), axial hypotonia (6/38), amyotrophy (8/38), and weakness (26/38). Abnormal reflexes were common and included hyperreflexia (8/36), hyporeflexia (5/36), and areflexia (3/36). A total of 22 individuals had both hyperreflexia and hypo/areflexia in individually tested reflexes, consistent with mixed upper and lower motor neuron dysfunction. Additionally, a total of ten individuals had electromyography/nerve conduction studies (EMG/NCS). Two cases with areflexia showed findings consistent with motor axonal neuropathy (P12 and 13), one case with hyporeflexia showed myopathic findings (P16), and two cases with hyporeflexia showed findings consistent with polyradiculopathy and motor axonal neuropathy, respectively (P 21 and 32). In the five cases with normal electrodiagnostic studies, two were hyperreflexic and the remaining three cases showed a mixed picture of upper and lower motor neuron signs (**Table S1**). For individuals who did not have EMG/NCS performed, neuropathy was determined by clinical signs and symptoms including absent or decreased reflexes, impaired sensation, and neuropathic pain. Dysmorphic facies were common (13/38) and included low anterior hairline,

synophrys, low-set ears with fleshy lobes, prominent philtrum, and mild micrognathia (**Fig. 2A**). Centripetal obesity, scoliosis, and genu valgus were observed (**Fig. 2B**). Additional musculoskeletal abnormalities included camptodactyly, spatulated and tapered fingers, broad toes, and *pes cavus* with progressive worsening of camptodactyly with age (**Fig. 2C-H**). Spine radiographs confirmed scoliosis (**Fig. 2I**). Hip x-rays performed due to progressive gait difficulties were generally unremarkable with normal bone structure (**Fig. 2J**).

ENTPD1-related neurological disease causes MRI abnormalities

As previous reports of affected individuals with biallelic pathogenic *ENTPD1* variants described brain white matter abnormalities in only two of nine affected individuals[8], we sought to better characterize neuroimaging features of the *ENTPD1*-related disease trait. Brain MRI images were available for ten individuals and were reviewed by a board certified pediatric neuroradiologist (**Fig. 3A-G**). For an additional 18 other individuals, the referring clinicians provided an MRI interpretation. Finally, for ten individuals, no neuroimaging was obtained. Abnormalities of the MRI included white matter and corpus callosum findings, cerebellar atrophy, and abnormal signal in the posterior limb of the internal capsule

Thinning of the corpus callosum was present in three individuals (P4, 9, 21). The thinning affected predominantly the isthmus of the corpus callosum (Fig. 4B-E). White matter abnormalities were observed in 16/28 patients for whom a brain MRI was performed and in nine of ten cases for which MRIs were available for review. Of these, 12 individuals had persistent T2 signal abnormalities in the posterior limb of the internal capsule. Review of a previously published individual (P34)[19] not initially reported to have brain MRI abnormalities was subsequently found to have abnormal T2 signal hyperintensity in the posterior limb of the

internal capsule at 15 years of age (**Fig. 3G**). Cerebellar atrophy was observed in 3 individuals (P19, 20, 23).

Biallelic pathogenic *ENTPD1* variants identified in this cohort

The cohort of individuals with biallelic *ENTPD1*-related neurological disease is diverse with 18 newly identified families originating from 10 different countries (**Fig. 1C**). *ENTPD1* is located on chromosome 10 and contains 10 exons; the major annotated canonical transcript is NM_001776.6 (ENST00000371205.5). Previous reports identified five distinct *ENTPD1* variants with two missense and three predicted loss-of function (LoF) variant alleles: c.628G>A; p.(Gly210Arg), c.520G>T; p.(Glu174*), c.401T>G; p.(Met134Arg), c.970C>T; p.(Gln324*), and c.978del; p.(Gly327Lysfs*40)[8,18-20]. We describe 12 novel variants (**Fig. 4A, B**) of which 11 are LoF: c.540del; p.(Thr181Leufs*18), c.640del; p.(Gly216Glufs*75), c.1109T>A; p.(Leu370*), c.185T>G; p.(Leu62*), c.1531T>C; p.(*511Glnext*100), c.967C>T; p.(Gln323*), c.770_771del; p.(Gly257Glufs*18), c.520G>T; p.(Glu174*), c.1041del p.(Ile348Phefs*19), c.414-2_414-1del, and c.574-6_574-3del. Additionally, one missense variant c.146 C>G; (p.Tyr49Cys) and one multinucleotide variant resulting in a single amino acid substitution, c.398_399delinsAA; p.(Gly133Glu) were identified (**Fig. 4A, B**).

All variants are ultra-rare[25] and absent from gnomAD in both the heterozygous and homozygous state. The exception, c.1109T>A; p.(Leu370*), is found in two heterozygotes of European non-Finnish descent; but it is important to note the bias of European descent genomes in the gnomAD database. All variants are predicted damaging by *in silico* analysis and have high CADD scores when available (**Table 1**). The most common variants observed in this cohort were the splicing variant c.574-6_574-3del found in families 5 (Brazilian), 6 (Puerto

Rican), and 9 (Portuguese) and c.1109T>A; p.(Leu370*) found in families 7, 10, 12, and 17 (Polish and Persian).

Intronic splicing variant results in alternative splicing and exon skipping

The majority of *ENTPD1* variant alleles identified in this study are LoF variants predicted to undergo nonsense mediated decay (NMD) or result in a truncated protein (**Fig. 4B**)[26]. However, the impact on gene function of the intronic variant c.574-6_574-3del was unclear. As the variant falls within intron 5, we hypothesized it causes aberrant splicing via either exon skipping, intron inclusion or a combination of both. To test this hypothesis, cDNA was synthesized from control, homozygous proband (P15), and heterozygous carrier parents from family 9 and amplified using separate primer pairs for exons 4-7 and exons 6-10 (**Fig. 5A**). Amplification of exons 4-7 showed an 818 bp product in wildtype control and heterozygous parent but not the homozygous affected proband (**Fig. 5B**). Sanger dideoxynucleotide DNA sequencing confirmed that this product contains exons 4, 5, 6, and 7. Furthermore, the proband showed a 572 bp product absent from the control and parental samples. Sanger sequencing of the 572 bp product showed exons 4, 5, 7 and complete absence of exon 6, evidence confirming exon skipping in the affected proband. As a second confirmatory step of exon 6 skipping in the proband, primers for exon 6 and exon 10 were used for amplification (**Fig. 5C**). In the control and parental samples, an 861 bp PCR product was detected with Sanger sequencing confirming presence of exons 6, 7, 8, 9, and 10. No amplification was present in the proband P7, consistent with absence of cDNA transcript including exon 6.

Biallelic *ENTPD1* variants impair ATP hydrolysis and ENTPD1 expression

Given that ENTPD1 is an essential enzyme in the hydrolysis of ATP to ADP and ADP to AMP (**Fig. 6A**), we next tested the effect of the homozygous *ENTPD1* missense variant c. 401T>G; p.(Met134Arg) on ATP/ADP metabolism. Patient lymphoblasts were obtained from family 21 (P34 and P35) (**Fig. 6B**). Quantitative PCR of control and proband samples revealed significantly decreased mRNA levels in both affected individuals compared to control with approximately 50% reduction (**Fig. 6C**). Western blot analysis of ENTPD1 protein from control and affected probands showed a substantial reduction in the predominant, higher molecular weight/relative molecular mass (M_r) band compared to control individuals with concurrent increase in the intensity of the lower weight band. However, overall ENTPD1 protein levels were still markedly decreased in the affected individuals compared to controls (**Fig. 6D**).

To test the functional effect of altered ENTPD1 protein expression on ATP and ADP hydrolysis, ATPase and ADPase activity of proband samples were measured using normalized phosphate production as a readout. This experiment showed significantly decreased phosphate production in lymphoblasts obtained from P34 and P35 compared to control, consistent with impaired ATP/ADP hydrolysis due to the homozygous missense variant *ENTPD1*:c.401T>G (**Fig. 6E**). Given that ENTPD1 is highly expressed in lymphocytes and polymorphonuclear leukocytes (PMNs), flow cytometry was performed on whole blood from P12, P13, and heterozygous parental sample with *ENTPD1*:c.185T>G; p.(Leu62*), which showed complete absence of ENTPD1+ cells in homozygous individuals compared to parental and wildtype control (**Fig. 6F**). Furthermore, immunohistochemistry for ENTPD1 on paraffin sections of sural nerve from P12 showed complete absence of endo- and epineural vascular staining compared to control sample (**Fig. 6G**).

Discussion

ENTPD1 was first identified as a candidate disease gene for AR DD/ID[27] and subsequently linked to SPG64 (MIM# 615683) with only few affected individuals described to date[8,18-20]. These individuals had overlapping features of spastic paraplegia and DD/ID, but were highly variable in other clinical neurologic characteristics, including reflexes, neuropathic findings, and brain white matter abnormalities (**Table S1**). Deep phenotyping of all identified *ENTPD1* patients herein delineated a clinical synopsis of: childhood disease onset, DD/ID, spastic paraplegia, dysarthria, neurocognitive regression, dysmorphic facies, and white matter abnormalities (**Table 2, Table S1**). Given the progressive nature and potential neurodegenerative process accompanying *ENTPD1*-related disease, natural history studies and longitudinal follow up will be required to better understand this disease trait.

Mixed upper and lower motor neuron findings in *ENTPD1*-related neurologic disorder

Several cases of SPG64 had clinical features and electrodiagnostic findings suggestive of combined upper and lower motor neuron dysfunction[,18]. Here, we expand upon these preliminary observations, demonstrating additional cases with findings consistent with both upper and lower motor neuron dysfunction and neuropathy. Of the twenty-five cases for which details of reflex examination are available, 40% show a mixed picture, 36% hyporeflexia/areflexia, and 24% hyperreflexia. Areflexia/hyporeflexia is not typical of 'classic' HSP as upper motor neuron degeneration results in loss of inhibitory descending pathways with resultant exaggeration of the stretch reflex. However, several HSP subtypes exhibit features of mixed upper and lower motor neuron dysfunction (e.g. SPG3, 7, 30, 31), and some have neuropathy as a major clinical feature (e.g. SPG 15, 20, 26, 35, 39).

While EMG/NCS was only performed in a subset of cases (10/36), 30% had evidence of motor axonal neuropathy, confirming involvement of the peripheral nervous system in SPG64. One potential explanation is ATP/ADP accumulation due to impaired hydrolysis triggers excitotoxicity within the central and peripheral nervous system with greater impact on the CNS, resulting in an upper motor neuron syndrome with variable lower motor neuron dysfunction. The observation of myopathic findings in a single case is also curious and may reflect an impact of ENTPD1 deficiency on both the nervous system and skeletal muscle. Alternatively, since the individual in question comes from a consanguineous family, it is possible she may have a dual molecular diagnosis resulting from multilocus pathogenic variation and a blended myopathy-HSP phenotype, a finding seen in ~20-30% of consanguineous families[3].

White matter abnormalities in *ENTPD1*-related neurological disease

A remarkable feature of *ENTPD1*-related neurological disease is the unique pattern of white matter abnormalities and persistent T2 signal hyperintensities in the PLIC observed in 17 affected individuals. Persistent signal abnormalities in the PLIC have previously been reported in a specific disorders of purine metabolism due to biallelic variants in *ITPA* [28]. This is especially intriguing given that *ENTPD1* is intricately involved in purine metabolism as an ectonucleotidase and the neuroimaging findings observed in many of our patients are strikingly like those seen with *ITPA*. It is therefore possible that PLIC signal abnormality constitutes a pathognomonic finding of diseases affecting purine metabolism.

Spectrum of pathogenic biallelic *ENTPD1* variants

We identified 12 previously unpublished variants, the majority of which are predicted likely damaging and to cause LoF. Additionally, one missense variant c.146 C>G; (p.Tyr49Cys) and one multinucleotide variant causing a single amino acid substitution were uncovered. Double missense variants, a type of multi-nucleotide variant (MNV), are rare but occur due to replication error introduced by DNA polymerase zeta (pol-zeta) during DNA damage repair and translesion DNA synthesis[29]. Four recurrent variants were identified including c.574-6_574-3del found in families 5 (Brazilian, homozygous), 6 (Puerto Rican, compound heterozygous), and 9 (Portuguese, homozygous); c.770_771del in families 3 and 4 (both Brazilian, homozygous); c.1041del in families 15 and 16 (both Persian, homozygous); c.1109T>A in families 7 (Poland, homozygous) and families 10, 12, 17 (Persian, homozygous). The observation that c.574-6_574-3del and c.770_771del were found in the homozygous state in unrelated consanguineous families from countries with substantial Portuguese ancestry (Brazil and Portugal) may suggest these variants represent founder alleles from the Iberian peninsula homozygosed through clan genomics IBD or population/geographic isolation[30,31]. Alternatively, the *de novo* variant allele may be a recurrently derived new mutation in antecedent generations of each clan. Similarly, the LoF variant c.1041del was found in families 16 and 17 may represent a founder allele in Persia, whereas the stop gain variant c.1109T>A; p.(Leu370*) found in four unrelated families from Persia and Poland, consistent with a recurrent mutation.

Aberrant splicing in neurological disease

Given that the splicing variant c.574-6_574-3del was identified in multiple unrelated families, we hypothesized pathogenicity based on aberrant splicing and found that exon 6 skipping indeed occurs in a proband harboring this variant in the homozygous state (**Fig. 5**).

ENTPD1 has 13 recognized splice variants of which four are protein coding[32]. The aberrant splice product observed in our studies has not been reported to occur in normal tissue[32]. Identification of the splicing defect caused by the recurrent variant c.574-6_574-3del provides an opportunity for nucleic acid based-molecular therapeutic intervention using antisense oligonucleotides (ASOs) and/or short hairpin RNA (shRNA) molecules[33].

Function of ENTPD1 in health and disease

ENTPD1, ectonucleoside triphosphate diphosphohydrolase 1 (MIM*601752) is part of a group of nucleotide triphosphate dihydrolases involved in ATP hydrolysis and purine metabolism and signaling and is important in the central nervous system where it plays an essential role in neuronal activity[34]. ATP is stored in neuronal synaptic vesicles and glial cells together with classic neurotransmitters, e.g. GABA and glutamate, and is released by exocytosis upon neuronal stimulation[35]. High concentrations of ATP trigger neurotoxicity through the purinergic receptor P2X7 and are implicated as potential therapeutic targets in motor neuron diseases and Charcot-Marie-Tooth disease type 1A[36,37].

ENTPD1 plays an important role in the cell-surface catabolism of ATP (**Fig. 6A**). A previous study using nuclear magnetic resonance spectroscopy (NMR) reported that the LoF variant c.185T>G; p.(Leu62*) found in family 8 in this report affects ENTPD1 enzymatic function with impaired ATP and ADP hydrolysis, although no specific clinical details about the family were provided at the time[38]. Here, we provide evidence from flow cytometry and immunohistochemistry, two clinically accessible tests, that the previously reported impairment of ATP/ADP metabolism caused by the *ENTPD1* variant c.185T>G is likely a consequence of the complete absence of ENTPD1 protein *in vivo* (**Fig. 6E, F**).

ENTPD1 is a highly glycosylated protein and alterations in glycosylation affect the protein's electrophoretic mobility, stability and enzymatic activity[39]. Therefore, it is likely the overall reduction in ENTPD1 protein levels as well as the relative increase in the lower molecular weight species reflecting defective glycosylation in individuals P27 and P28 harboring the homozygous c.401T>G; p.(Met134Arg) variant that may reduce ENTPD1 stability and/or impair ATPase and ADPase activity. This impairment leads to an imbalance of extracellular ATP and adenosine and presumably disturbs purinergic neurotransmission and/or causes neurotoxicity as potential disease mechanism. Similarly, the LoF variant c.185T>C; p.(Leu62*) resulted in absence of ENTPD1 in the vasculature of the epi- and perineurium with possible implications for peripheral nerve health and function (**Fig. 6G**).

Furthermore, an *in vitro* study using a cellular model of sympathetic neurons demonstrated that ENTPD1 modulates exocytotic and ischemic neurotransmitter release[40] and targeted 'knockout' LoF *Entpd1*$^{-/-}$ mouse models exhibit a pro-epileptogenic phenotype[41], although epilepsy was only seen in a small fraction of *ENTPD1*-related neurological disease cohort (4/38) to date and consisted of a generalized epilepsy. Given the important cellular function of ENTPD1 and its ubiquitous expression it is possible that impaired ENTPD1 function could have additional extra-CNS manifestations. In fact, flow cytometry from individuals with the LoF allele c.185T>C; p.(Leu62*) showed absence of ENTPD1 expression in lymphocytes and PMNs (**Fig. 6F**). While LoF *Entpd1* mouse models exhibit impaired hemostasis and thromboregulation due to platelet dysfunction and hepatic insulin resistance[42-45], these features were not observed within this cohort. It remains to be determined if individuals with *ENTPD1*-related neurological disease develop additional extra-CNS disease manifestations over time.

Treatment and development of therapeutics

The HSPs constitute a spectrum of progressive neurological disorders with supportive therapies, including physical therapy and stretching, but no molecular interventions to ameliorate or mitigate disease[5]. Pharmacologic intervention using baclofen and benzodiazepines can relieve spasticity and muscle spasms, respectively. A major challenge in therapeutic development stems from the diverse molecular pathways resulting from over 80 disease genes. Another challenge in the evaluation of potential therapeutics is the insidious, slow and progressive nature of the disease, which makes therapeutic endpoints and efficacy assessment challenging. Nevertheless, with current advances in genome medicine and evolving understanding of molecular disease etiology, therapeutic development targeting diverse molecular disease mechanisms are now feasible. Accurate and timely molecular diagnosis and natural history studies will greatly facilitate clinical trials. The known role of ENTPD1 in ATP breakdown and our experimental evidence of impaired ATP/ADP hydrolysis in patients with *ENTPD1*-related neurological disease suggests antagonism of the purinergic receptor P2X7 may be a worthwhile target for therapeutic intervention.

Limitations of this study include its reliance on retrospective chart review and diverse clinical evaluations precluding use of HSP specific metrics such as the Spastic Paraplegia Rating Scale (SPRS) which could help gauge disease severity and progression over time[46]. Formal neuropsychiatric testing was either not performed or results were unavailable, limiting more precise characterization of the degree of intellectual disability. Similarly, only a subset of the cohort underwent electrodiagnostic evaluation or had MRIs available for retrospective review. Finally, replication of our ADPase/ATPase assay and ENTPD1 immunostaining in additional patients and variant types would help strengthen validity of these tests as diagnostic biomarkers. In conclusion, we establish *ENTPD1* as the etiology of a complex neurodevelopmental disorder in the HSP spectrum characterized by intellectual disability, hypomyelination, and progressive spastic paraplegia. Allelic series studies and detailed

phenotyping in rare neurological disease research can capture a more comprehensive spectrum of disease and define disease traits. Moreover, such information provides recurrence risk and prognostic information for family counseling, establishes pathophysiological mechanisms, provides neurobiological insights, and may ultimately lead to the development of novel interventional options for rare neurological disorders based on shared molecular features. Future endeavors should involve the development of biomarkers with the goals of early disease screening, as a readout of disease activity, and as a measure of treatment response for SPG64.

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Author Contributions

DGC, IH, and JRL contributed to the conception and design of the study; DGC, IH, AEM, RM, KCD, JMF, TM, HD, CMG, SS, SB, YAI, CR, JVH, VRS, LTE, KMB, AL, YF, EP, YK, VM, MS, CR, KM, NWS, MSZ, AB, TSB, DLS, MO, TOS, HH, DM, EGK, YJ, AGJ, HT, SCJ, MRA, REV, SH, SA, AB, EZ, MMS, SR, ZCA, LT, FC, SNJ, RAG, JEP, MCK, KDK, JAMS, AV, DP, DM, JRL contributed to the acquisition and analysis of the data; DGC, IH contributed to drafting of the text and preparing the figures. All authors approved the final manuscript.

Potential conflicts of interests

Nothing to report.

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Figure legends

Figure 1 Pedigrees and variant information for families with *ENTPD1*-related neurological disease. (**A**) Pedigree and Sanger sequencing results of index family 1 with the homozygous variant NM_001766.6: c.398_399delinsAA; p.(Gly133Glu). (**B**) Absence of heterozygosity (AOH) plot of P1 showing a total AOH size of 310 Mb and 11.1 Mb of AOH around *ENTPD1*: c.398_399delinsAA (red line). (**C**) Pedigrees and variant information of newly identified families with biallelic *ENTPD1* variants and countries of origin.

Figure 2 Representative photographs and radiographs of individuals with *ENTPD1*-related neurological disease. (**A**) Facial pictures of affected individuals showing low anterior hairline, synophrys, low-set ears with fleshy lobes, prominent philtrum, and micrognathia. (**B**) Pictures of P1 at 8 years of age and P21 at 8 years showing centripetal obesity, thoracic kyphosis, decreased lumbar lordosis, genu valgus, and cubitus valgus. (**C**) Representative hand images of P1, P4, P8, and P24 showing broad fingers with camptodactyly and spatulated finger tips (**D**) Representative foot images of P1, P4, and P8 at 8, 15, and 3 years, respectively,

showing broad toes with camptodactyly (P1), *pes cavus* with camptodactyly (P4), and broad great toes bilaterally and broad right 4th toe with camptodactyly (P8). (**E**) Hand radiographs of P1 at 8 years of age showing severe camptodactyly. (**F**) Foot radiographs of P1 at 8 years showing camptodactyly. (**G**) Foot radiographs of P5 at 15 years showing *pes cavus* and camptodactyly. (**H**) Foot radiographs of P36 at 19 years of age showing severe camptodactyly. (**I**) Sagittal spine radiograph of P1 at 8 years showing lumbar lordosis. (**J**) Hip radiograph of P8 at 3 years showing no gross abnormalities.

Figure 3 Individuals with biallelic pathogenic *ENTPD1* variants show white matter abnormalties, thinning of the corpus callosum, cerebellar atrophy, and signal abnormalities in posterior limb of the internal capsule. Representative magnetic resonance imaging (MRI) of the brain of affected individuals from different families at different ages. Arrows in the axial images are highlighting abnormal signal hyperintensity of the posterior limb of the internal capsule. (**A**) Sagittal T1-weighted imaging (1) and axial T2-FLAIR of P7 at 17 years of age. (**B**) and (**C**) Sagittal T1-weighted imaging (1) and axial T2-FLAIR of P8 at 3 and 4 years, respectively. (**D**) Sagittal T1-weighted imaging (1) and axial T2-FLAIR of P9 at 3 years. (**E**) Sagittal T2-weighted imaging (1) and axial T2-FLAIR of P16 at 7 years. (F) Sagittal (1) and axial (2) T2-weighted imaging of P23 at 8 years, G) Sagittal T1-weighted imaging (1) and axial T2 of P36 at 15 years.

Figure 4 Biallelic pathogenic *ENTPD1* variants identified in this cohort. (**A**) Schematic showing chromosomal location and gene structure of *ENTPD1*. Previously unreported variants are labeled in red and previously published variants in black. (B) Linear amino acid structure of ENTPD1 and location of previously unreported (red) and published variant alleles (black).

Figure 5 Alternative splicing due to *ENTPD1*:c.574-6_574-3del results in skipping of exon 6. (**A**) Schematic of *ENTPD1* NM_001776.6, the most widely expressed transcript, showing 10 different exons. *ENTPD1*: c.574-6_574-3del is located in intron 5 (red asterisk). Arrows show location of primers for cDNA amplification. (**B**) Agarose gel electrophoresis image of *ENTPD1* cDNA exon 4F and 7R amplification and schematic of resultant splicing products. Unaffected wildtype control cDNA and heterozygous parental cDNA show amplification of a bands at 818 bp not found in the affected proband sample (P15). Sanger sequencing confirmed that the 818 bp band contains exons 4, 5, 6, and 7 in control and unaffected parent. By contrast, the proband P15 who carries the homozygous *ENTPD1*: c.574-6_574-3del variant contains an alternative 572 bp product including exons 4, 5, and 7 only and thus skipping exon 6 completely. All three samples additionally contain a smaller product at 412 bp only containing exons 4 and 7. (**C**) Agarose gel electrophoresis image of *ENTPD1* cDNA exon 6F and 10R amplification. Unaffected wildtype control cDNA and heterozygous parental cDNA show amplification of an 861 bp band containing exons 6, 7, 8, 9, and 10. No amplification is present in the homozygous proband sample.

Figure 6 Biallelic *ENTPD1* variants impair ATP hydrolysis and ENTPD1 expression. (**A**) ATP metabolic pathway showing the role of ENTPD1 in hydrolysis of ATP to ADP and ADP to AMP. (**B**) Lymphoblasts were derived from family 16 with two affected siblings (P34 and P35 with homozygous *ENTPD1*:c.401T>G; p.(Met134Arg) variant. (**C**) Reverse transcription-quantitative PCR of ENTPD1 mRNA levels, using primers spanning both exons 1-2 and exons 9-10 showing significantly decreased ENTPD1 mRNA levels in lymphoblasts from individuals with homozygous *ENTPD1*:c.401T>G variant. (**D**) Western blot of ENTPD1 p.(Met134Arg)

showing deceased protein levels in patient lymphoblasts. The stain-free gel serves as the loading control. (**E**) Measured ATPase and ADPase activity using normalized phosphate production after incubation with either ATP or ADP at a final concentration of 10 mM for 30 min. * $p<0.05$. (**F**) Flow cytometry of ENTPD1+ lymphocytes and polymorphonuclear leukocytes (PMNs) in blood samples from P12 and P13 with homozygous *ENTPD1*:c.185T>G; p.(Leu62*) variant compared to control and heterozygous parental samples. (**G**) Immunohistochemical staining for ENTPD1 performed on paraffin sections of sural nerve biopsy from P12 with variant *ENTPD1*:c.185T>G shows complete absence of endo- and epineural vascular staining compared to control sample.