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Biallelic Variants in the Ectonucleotidase \textit{ENTPD1} Cause a Complex Neurodevelopmental Disorder with Intellectual Disability, Distinct White Matter Abnormalities, and Spastic Paraplegia

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Objective: Human genomics established that pathogenic variation in diverse genes can underlie a single disorder. For example, hereditary spastic paraplegia is associated with >80 genes, with frequently only few affected individuals 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 (Mendelian Inheritance in Man # 615683).

Methods: Individuals with biallelic ENTPD1 variants were recruited worldwide. Deep phenotyping and molecular characterization were performed.

Results: A total of 27 individuals from 17 unrelated families were studied; additional phenotypic information was collected from published cases. Twelve novel pathogenic ENTPD1 variants are described (NM_001776.6: c.398_399delinsAA; p.(Gly133Glu), c.540delG; p.(Thr181Leufs*18), c.640delG; p.(Gly216Glufs*75), c.185 T > G; p.(Leu62*), c.1531 T > C; p.(*511Glnfs*100), c.967 C > T; p.(Gln323*), c.414-2_414-1del, and c.146 A > G; p.(Tyr49Cys) including 4 recurrent variants c.1109 T > A; p.(Leu370*), c.574-6_574-3del, c.770_771del; p.(Gly257Glufs*18), and c.1041del; p.(Ile348Phefs*19). Shared disease traits include childhood onset, progressive spastic paraplegia, intellectual disability (ID), dysarthria, and white matter abnormalities. In vitro assays demonstrate that ENTPD1 expression and function are impaired and that c.574-6_574-3del causes exon skipping. Global metabolomics demonstrate ENTPD1 deficiency leads to impaired nucleotide, lipid, and energy metabolism.

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 (1) expands previously described features of ENTPD1-related neurological disease, (2) highlights the importance of genotype-driven deep phenotyping, (3) documents the need for global collaborative efforts to characterize rare autosomal recessive disease traits, and (4) provides insights into disease trait neurobiology.

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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 single families of disorders can be caused by pathogenic variation in genes with diverse functions. For example, hereditary spastic paraplegias (HSPs) are a 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 >80 genes/loci involved in mitochondrial functioning, lipid metabolism, vesicle/axonal trafficking, and myelination. With the rapid pace of disease gene discovery, this number is anticipated to expand. An “HСПome” of known HSP disease genes, candidate disease genes, and proximal interactors has implicated almost 600 potential HSP genes.6

HSP is classified into “pure” and “complex/complicated” with unifying features of corticospinal tract nerve axonopathy, progressive gait difficulty, and axonal length-dependent neuropathy.7,8 Complex HSP additionally encompasses developmental delay/intellectual disability (DD/ID), structural brain abnormalities, ataxia, epilepsy, amyotrophy, and visual abnormalities.8 Consequently, complex HSP frequently overlaps with neurodevelopmental disorders (NDDs) including leukodystrophies, cerebellar ataxias, and syndromic DD/ID.9

As with hereditary neuropathies,10 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 (Mendelian Inheritance in Man [MIM] # 182601), accounts for approximately 60% of HSP diagnoses.11-14 The abundance of AD spastic paraplegia 4 and other “common” HSP causes reflects historical population-specific events like founder effect or population bottlenecks, or high-frequency mutational events occurring as a consequence of genomic architecture such as Alu/Alu-mediated rearrangements due to abundance of Alu repetitive elements and genomic instability within SPAST.15

The remaining allelic spectrum of HSP exhibits extensive molecular heterogeneity and is a collection of ultrarare 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, that is, 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.6,16-18 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, for example, due to biallelic ENTPD1 variants, potentially revealing previously undescribed features (e.g., phenotypic expansion and multilocus pathogenic variation), and therefore providing a comprehensive understanding of the disease process and gene-associated traits.

Herein, we describe phenotypic and molecular features of a large (N=27) worldwide cohort of individuals with biallelic variation in ENTPD1 and provide evidence for a complex NDD with progressive spastic paraplegia with previously unrecognized clinical features.

Patients and Methods
Patient Identification and Recruitment
This study was approved by the institutional review board (IRB) at Baylor College of Medicine (BCM; Protocol H-29697) or through other collaborative local IRBs. Additional affected participants were identified through GeneMatcher19 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.20 Brain magnetic resonance imaging (MRI) was retrospectively reviewed and analyzed by a single observer, a board certified neuroradiologist (J.V.H.).

Exome Sequencing
For Family 1, trio exome sequencing (ES) was performed at the Human Genome Sequencing Center at BCM through the Baylor-Hopkins Center for Mendelian Genomics (BHC MG) initiative as previously described.21 For all other identified families, ES was performed by local institutions or commercial clinical molecular diagnostic laboratories via previously established protocols.22

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 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.

**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 complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) with poly-dT (20) primers according to the manufacturer’s protocol. Amplicons were generated from control, proband, and parental cDNA using HotStartTaq DNA polymerase (Qiagen) according to the manufacturer’s protocol. DNA bands at various sizes were excised and purified via the PureLink polymerase chain reaction (PCR) purification and gel extraction kit (Invitrogen, Carlsbad, CA), and Sanger dideoxy DNA sequencing was 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, Ontario, Canada). Total RNA was obtained from affected and control lymphoblast cell lines with the RNaseasy Minikit (Qiagen) and reverse transcribed into cDNA with iScript kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s protocol. cDNA was amplified with gene-specific primers and iQ SYBR Green Supermix (Bio-Rad Laboratories) 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 were 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 10mg/ml each in the composite solution (Sigma-Aldrich, St Louis, MO), and concentrations were determined by Bradford assay (Bio-Rad Laboratories). Protein samples were resolved by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and incubated in blocking buffer, followed by overnight incubation with primary antibody (ENTPD1, ab108248; Abcam, Cambridge, MA). Membranes were washed and incubated with secondary antibody (horseradish peroxidase-conjugated antirabbit, Bio-Rad Laboratories). Blots were visualized by chemiluminescence using the Clarity Western ECL substrate (Bio-Rad Laboratories). Control protein was extracted from healthy, unrelated, age-matched control cell lines.

**ATPase and Adenosine Diphosphatase Assay**
A total of 250,000 lymphoblasts were harvested per technical replicate from each cell line. The cells were washed, and each replicate was plated in a single well of a round-bottom 96-well plate. Cells were then incubated with either 10mM adenosine diphosphate (ADP) or ATP, or left untreated, for 30 minutes 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) 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 3ml ethylenediaminetetraacetic acid (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 a Beckman Coulter (Brea, CA) dual Laser Navios Flow Cytometer equipped with a 488nm argon and a 635nm diode laser, allowing 6-color fluorescence data acquisition.

**Sural Nerve Biopsy**
Paraffin-embedded sural nerve biopsy sections of the patient and a control sample were stained immunohistochemically for CD39 (Leica, Wetzlar, Germany/Novocastra, Newcastle, UK; 1cc, clone NCL-CD39, LOT-6017994, 1:50), according to the manufacturer’s instructions and standard staining protocols.

**Untargeted Metabolomic Analyses**
Clinical untargeted metabolomics was performed under an IRB-approved research protocol (H-35388) by Baylor Genetics and Metabolon, as previously described. EDTA plasma samples were collected from Family 6 (P8, P9) and Family 9 (P15), frozen, and shipped on dry ice overnight and kept at −80°C until analyzed, as previously described. Metabolites were identified and z scores were generated relative to the reference population. An average of 798 ± 7 metabolites were detected in the
plasma of each of the 3 patient samples analyzed. Due to the age range of the subjects in this study, androgenic, pregnenolone, and progestin steroids were excluded from the analysis. Also excluded were known prescription medications, over the counter drugs and/or supplements, and partially characterized molecules, leaving approximately 773 molecules for further assessment. These metabolomics datasets for each patient were collated, and samples were filtered to identify metabolites that were altered in patient biofluids with z score > +1.5 or < −1.5 and in the top 10% or bottom 10%, i.e. phenotypic extremes, of all cases in the laboratory clinical testing database. Molecules absent in the patient sample but detected in the batch analysis and typically detected in >90% of patient samples were reviewed for possible contribution to the biochemical phenotype. Specific molecules altered in at least 2 of the 3 patient samples were further analyzed to highlight key metabolic features. Metabolite enrichment and network analyses were performed by using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) using the integrated gene–metabolic pathways network analysis with the metabolite–Kyoto Encyclopedia of Genes and Genomes interaction network.

Results

Index Proband

The index patient (P1, Family 1; Fig 1A) was an 8-year-old girl referred to BHCMG for DD/ID, spastic paraplegia, and progressive gait impairment. Concerns about the proband’s development arose due to delayed walking after 2 years of age. At 3 years, she developed spastic paraplegia. Her neurological examination at 8 years showed dystarthritis, 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) (see Fig 1A). The variant is absent from gnomAD and 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. 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 (see Fig 1B).

Recruitment of Additional Families with Biallelic ENTPD1 Variation

Given limited phenotypic characterization of ENTPD1-related neurological disease, we identified a total of 17 unrelated families with 27 affected individuals through GeneMatcher (https://genematcher.org/) and communication with neurogenetic research laboratories from around the globe (see Fig 1C, Table S1). Families were from diverse countries, including Turkey (Family 2), Brazil (Families 3–5), Puerto Rico (Family 6), Poland (Family 7), Israel (Family 8), Portugal (Family 9), Iran (Families 10–12, 15–17), Egypt (family 13), and the Dominican Republic (Family 14). Review of the literature revealed an additional 5 families with 9 affected individuals (see Table S1).6,16–18

Phenotypic Spectrum of ENTPD1-Related Neurological Disease

Comparison of phenotypic features using HPO terminology22 among all affected individuals revealed major similarities as well as differences, suggestive of a phenotypic spectrum with a "clinical synopsis" of shared commonalities (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 (see Table 1C). We observed a broad phenotypic spectrum, with the most common features being developmental delay/intellectual disability, gait impairment, weakness, and dysarthria. Other features, such as neuropathy, spasticity, and cerebral white matter abnormalities, were present in a minority of cases (see Table 1C). The frequency of each feature is presented in Table 1C.

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>This Cohort</th>
<th>Prior Publications</th>
<th>All Affected Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early childhood age of onset (HP:0011463)</td>
<td>27/27</td>
<td>9/9</td>
<td>36/36</td>
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<td>Developmental delay/intellectual disability (HP:0012758)</td>
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<td>9/9</td>
<td>36/36</td>
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<tr>
<td>Progressive spastic paraplegia (HP:0007020)</td>
<td>27/27</td>
<td>9/9</td>
<td>36/36</td>
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<tr>
<td>Gait impairment (HP:0002355)</td>
<td>27/27</td>
<td>9/9</td>
<td>36/36</td>
</tr>
<tr>
<td>Weakness (HP:0001324)</td>
<td>23/27</td>
<td>5/9</td>
<td>28/36</td>
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<tr>
<td>Dysarthria (HP:0001260)</td>
<td>20/27</td>
<td>7/9</td>
<td>27/36</td>
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<td>Abnormal reflexes (HP:0031826)</td>
<td>18/27</td>
<td>9/9</td>
<td>27/36</td>
</tr>
<tr>
<td>Neurocognitive regression (HP:0002376)</td>
<td>18/27</td>
<td>3/9</td>
<td>21/36</td>
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<td>Behavioral abnormalities (HP:0000708)</td>
<td>17/27</td>
<td>7/9</td>
<td>22/36</td>
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<td>15/27</td>
<td>4/9</td>
<td>19/36</td>
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<td>Neuropathy (HP:0009830)</td>
<td>14/27</td>
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<td>Spasticity (HP:0001257)</td>
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<td>7/9</td>
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<td>13/36</td>
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<td>Cerebral white matter abnormalities (HP:0002500)</td>
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<td>Hypotonia (HP:0001252)</td>
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<td>2/22</td>
<td>0/6</td>
<td>2/28*</td>
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</table>

*Magnetic resonance imaging brain images and/or reports only available for 28/36 individuals. HP = Human Phenotype Ontology terms.
childhood, DD/ID, and progressive spastic paraplegia with impaired ambulation. Behavioral abnormalities, including attention-deficit/hyperactivity disorder, aggression, and stereotypies, were common, as was neurocognitive and language regression. 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.

The neurological examination revealed dysarthria (27/36), axial hypotonia (7/36), ataxia (9/36), and weakness (28/36). Abnormal reflexes were common and included hyperreflexia (8/36), hyporeflexia (5/36), and areflexia (3/36). A total of 20 individuals had both hyperreflexia and hypo-/areflexia, consistent with mixed upper and lower motor neuron dysfunction. Additionally, a total of 12 individuals had electromyography/nerve conduction studies (EMG/NCS). Two cases with areflexia showed findings consistent with motor axonal neuropathy (P12 and P13), 1 case with hyporeflexia showed myopathic findings (P16), and 2 cases with hyporeflexia showed findings consistent with polyradiculopathy and motor axonal neuropathy, respectively (P21 and P32). Of the 7 cases with normal electrodiagnostic studies, 2 were hyperreflexic, 3 cases showed a mixed picture of upper and lower motor neuron signs, and 2 individuals did not have reflexes documented (see 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 neuropsychiatric pain. Dysmorphic facies were common (13/36) and included low anterior hairline, synophrys, low-set ears and fleshy lobes, prominent philtrum, and mild micrognathia (Fig 2). Centripetal obesity, scoliosis, and genu valgus were observed. Additional musculoskeletal abnormalities included camptodactyly, spatulated and broad toes, pes cavus, and pes planus. Hip X-rays were generally unremarkable, with normal bone structure.

**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 2 of 9 affected individuals,6 we sought to better characterize neuroimaging features of the ENTPD1-related disease trait. Brain MRI was available for 10 individuals and was reviewed by a board certified pediatric neuroradiologist (Fig 3). For 18 individuals, an MRI report was provided, and 8 individuals did not have any neuroimaging. MRI abnormalities included white matter and corpus callosus findings, cerebellar atrophy, and abnormal signal in the posterior limb of the internal capsule.

Thinning of the corpus callosum was present in 3 individuals (P4, P9, P21) and predominantly affected the isthmus. White matter abnormalities were observed in 15 of 28 patients for whom brain MRI was performed. Of these, 12 individuals had persistent T2 signal abnormalities in the posterior limb of the internal capsule (PLIC). On review, a previously published individual (P34)17 not initially reported to have brain MRI abnormalities was subsequently found to have abnormal T2 signal hyperintensity in the PLIC at 15 years of age (see Fig 3G). Cerebellar atrophy was observed in 3 individuals (P19, P20, P23).

**Biallelic Pathogenic ENTPD1 Variants Identified in This Cohort**

**ENTPD1** is located on chromosome 10 and contains 10 exons; the major annotated transcript is NM_001776.5 (ENST00000371205.5). Previous reports identified 5 distinct ENTPD1 variants with 2 missense and 3 predicted loss-of-function (LoF) variant alleles: c.628G > A; p.(Gly210Arg), c.520G > T; p.(Glu174*), c.401 T > G; p.(Met134Arg), c.970C > T; p.(Gln324*), and c.978del; p.(Gln327_Lysfs*40).6,16–18 We describe 12 novel variants (Fig 4), of which 10 are LoF: c.540del; p.(Thr181Leufs*18), c.640del; p.(Gly216Glufs*75), c.1109 T > A; p.(Leu370*), c.185 T > G; p.(Leu62*), c.1531 T > C; p.(*511Glnext*100), c.967C > T; p.(Gln323*), c.770_771del; p.(Gly257Glufs*18), c.1041del p.(Ile348Phefs*19), c.414-2_414-1del, and c.574-6_574-3del. c.1531 T > C; p.(*511Glnext*100) is a stop-loss variant, predicted to result in a 100-amino acid extension on the 522-amino acid ENTPD1 protein. The ENTPD1 protein has 2 transmembrane domains on the N and C terminus,28 and this large extension is likely to disrupt the C terminus transmembrane domain, resulting in impaired catalytic activity. Additionally, one missense variant c.146C>G; p.(Tyr49Cys) and one multinucleotide variant resulting in a single amino acid substitution, c.398_399delinsAA; p.(Gly133Glu), were identified (see Fig 4).

All variants are ultrarare29 and absent from gnomAD. The exception, c.1109 T > A; p.(Leu370*), is found in 2 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 (see Table).

**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 or result in a truncated protein (see Fig 4B). 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,
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 P22 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 fourth 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 P34 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 abnormalities, thinning of the corpus callosum, cerebellar atrophy, and signal abnormalities in the posterior limb of the internal capsule. Representative magnetic resonance imaging of the brain of affected individuals from different families at different ages is shown. Arrows in the axial images highlight abnormal signal hyperintensity of the posterior limb of the internal capsule. (A) Sagittal T1-weighted imaging (1) and axial T2-fluid-attenuated inversion recovery (FLAIR) (2) of P7 at 17 years of age. (B, C) Sagittal T1-weighted imaging (1) and axial T2-FLAIR (2) of P8 at 3 and 4 years, respectively. (D) Sagittal T1-weighted imaging (1) and axial T2-FLAIR (2) of P9 at 3 years. (E) Sagittal T2-weighted imaging (1) and axial T2-FLAIR (2) 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-weighted imaging of P34 at 15 years.
cDNA was synthesized from control, homozygous proband (P15), and heterozygous carrier parents, and amplified using separate primer pairs for exons 4 to 7 and exons 6 to 10 (Fig 5A). Amplification of exons 4 to 7 showed an 818 bp product in wild-type control and heterozygous parents but not the homozygous affected proband (see 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, and 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 (see Fig 5C). In the control and parental samples, an 861 bp
PCR product was detected with Sanger sequencing confirming the 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.

**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 (F = forward; R = reverse). (B) Agarose gel electrophoresis image of ENTPD1 cDNA exon 4F and 7R amplification and schematic of resultant splicing products. Unaffected wild-type control cDNA and heterozygous parental cDNA show amplification of a band 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 controls and unaffected parents. 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 3 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 wild-type 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.

**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 adenosine...
(Figure legend continues on next page.)
monophosphate, we next tested the effect of the homozygous ENTPD1 missense variant c. 401 T > G; p. (Met134Arg) on ATP/ADP metabolism. Patient lymphoblasts were obtained from Family 20 (P32 and P33; Fig 6). Quantitative PCR of control and proband samples revealed significantly decreased mRNA levels in both affected individuals compared to control, with approximately 50% reduction. Western blot analysis of ENTPD1 protein showed a substantial reduction in the predominant, higher molecular weight/relative molecular mass 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.

To test the functional effect of altered ENTPD1 protein expression on ATP and ADP hydrolysis, ATPase and ADPase activity of proband samples was measured using normalized phosphate production as a readout. This experiment showed significantly decreased phosphate production in lymphoblasts obtained from P32 and P33, consistent with impaired ATP/ADP hydrolysis (see Fig 6E). Given that ENTPD1 is highly expressed in lymphocytes and polymorphonuclear leukocytes, flow cytometry was performed on whole blood from P12 and P13, which showed complete absence of ENTPD1+ cells (see Fig 6F). Furthermore, immunohistochemistry for ENTPD1 on paraffin sections of sural nerve from P12 showed complete absence of endothelial and epineural vascular staining, indicating lack of ENTPD1 expression (see Fig 6G).

Untargeted Metabolomics Identifies Perturbations in Nucleotide, Lipid, and Energy Metabolism

Purinergic signaling is an important regulator of cellular metabolism and inflammation. Ectonucleotidase deficiency in mouse models causes hepatocellular dysfunction, impaired glucose homeostasis, intestinal inflammation, and microbiome alterations. Therefore, we explored the impact of ENTPD1 deficiency on metabolic homeostasis using untargeted metabolomics. Integrated gene–metabolic pathway network analysis (Fig 7) revealed consistent patterns of metabolic abnormalities in plasma samples from 3 subjects. Metabolic alterations revealed multiple alterations in nucleotide, carbohydrate, and lipid metabolism, with 37 molecules altered in at least 2 of 3 patient samples. When considering broader biochemical pathways, ~140 molecules were perturbed within the same metabolic networks (see Fig 7). These data point to significant roles for ENTPD1 and purinergic signaling in metabolic homeostasis. In each sample, multiple endocannabinoids and related metabolites were altered, perhaps indicating an elevated inflammatory state. Many alterations involved metabolites associated with liver dysfunction, such as bilirubin, multiple altered long-chain fatty acids, and glycerophospholipids. Furthermore, several reported biomarkers associated with obesity, insulin resistance, and metabolic syndrome were identified in all patient samples, including elevations of 3-ketosphinganine, glucose, and N-acetylated amino acids, as well as low fructose.

Discussion

ENTPD1 was first identified as a candidate disease gene for AR DD/ID and subsequently linked to SPG64 (MIM # 615683). These individuals had overlapping features of spastic paraplegia and DD/ID, but were highly variable in other neurological symptoms (see 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 (see 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 involvement, including hyporeflexia, muscle atrophy, fasciculations, distal wasting, and typical positive sharpwave responses. Imaging studies and molecular laboratory evaluations were consistent with the diagnosis of SPG64.
lower motor neuron dysfunction. 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 25 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, SPG7, SPG30, SPG31), and some have neuropathy as a major clinical feature (e.g., SPG15, SPG20, SPG26, SPG35, SPG39).

Although EMG/NCS was only performed in a subset of cases, 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 central nervous system (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, because the individual in question originates from a consanguineous family, she may have a dual molecular diagnosis resulting from multilocus pathogenic variation and a blended myopathy–HSP phenotype, a finding seen in ~20 to 30% of consanguineous families.

**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. Persistent signal abnormalities in the PLIC have previously been reported in a specific disorder of purine metabolism...
due to biallelic variants in *ITPA*. This is especially intriguing given that *ENTPD1* is intrinsically involved in purine metabolism as an ectonucleotidase, and the neuroimaging findings observed in many of our patients are strikingly similar to that observed in association with *ITPA* variant alleles, i.e. a phenocopy. 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 to be likely damaging and to cause LoF. Additionally, one missense variant c.146 C > G; (p.Tyr49Cys) and one multinucleotide variant c.398_399delinsAA; p.(Gly133Glu) causing a single amino acid substitution were uncovered. Double missense variants, a type of multinucleotide variants, are rare but occur due to replication error introduced by DNA polymerase zeta during DNA damage repair and translesional DNA synthesis. 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); and c.1109 T > A in Family 7 (Poland, homozygous) and Families 10, 12, and 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. 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 Iran, whereas the stop gain variant c.1109 T > A; p. (Leu370*) was found in 4 unrelated families from Iran 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 occurs in a proband harboring this variant in the homozygous state (see Fig 5). *ENTPD1* has 13 recognized splice variants, of which 4 are protein-coding. The aberrant splice product observed in our studies has not been reported to occur in normal tissue. 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 and/or small interfering RNA molecules.

**Function of *ENTPD1* in Health and Disease**

*ENTPD1* (ectonucleoside triphosphate diphosphohydrolase 1; MIM * 601752) is the prototype ectonucleoside triphosphate diphosphohydrolase of the CD39 family involved in extracellular ATP and ADP phosphohydrolysis. Given its modulatory roles in purine metabolism and purinergic signaling, *ENTPD1* expression by glial cells is important in the CNS, where it plays an essential role in regulating neuronal activity. ATP is stored in neuronal synaptic vesicles and glial cells together with classic neurotransmitters (e.g., γ-aminobutyric acid and glutamate), and is released by exocytosis upon neuronal stimulation. 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 IA.

*ENTPD1* plays an important role in the cell-surface catabolism of ATP (see Fig 6A). A previous study using nuclear magnetic resonance spectroscopy reported that the LoF variant c.185 T > G; p.(Leu62*) found in Family 8 in this report affects *ENTPD1* enzymatic function with impaired ATP and ADP hydrolysis. 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.185 T > G is likely a consequence of the complete absence of *ENTPD1* protein *in vivo* (see Fig 6E, F).

*ENTPD1* is a highly glycosylated protein, and alterations in glycosylation affect electrophoretic mobility, stability, and enzymatic activity. Therefore, it is likely the overall reduction in *ENTPD1* protein levels as well as the relative increase in the lower molecular weight species may reduce *ENTPD1* stability and/or impair ATPase/ADPase activity, disturbing purinergic neurotransmission and/or causing neurotoxicity as a potential disease mechanism. Similarly, the LoF variant c.185 T > C; p.(Leu62*) resulted in absence of *ENTPD1* in the vasculature of the epi-/perineurium, with possible implications for peripheral nerve health and function (see Fig 6G).

Furthermore, an *in vitro* study using a cellular model of sympathetic neurons demonstrated that *ENTPD1* modulates exocytotic and ischemic neurotransmitter release, and targeted LoF Entpd1−/− mouse models exhibit a proepileptogenic phenotype. Given the important cellular function of *ENTPD1* and its ubiquitous expression, it is possible that impaired *ENTPD1* function...
could have extra-CNS manifestations. For example, LoF Entpd1 mouse models exhibit impaired hemostasis and thromboregulation due to platelet dysfunction and hepatic insulin resistance.\textsuperscript{48–50} Although platelet dysfunction or glucose intolerance was not identified in our cohort, untargeted plasma metabolomic analysis in 3 affected individuals demonstrated consistent patterns of metabolite abnormalities, indicating dysfunctional nucleotide, lipid, and energy metabolism. Several alterations were suggestive of subclinical liver dysfunction, an elevated inflammatory state, and abnormal glucose metabolism and may warrant clinical surveillance. Finally, these abnormalities may represent a “metabolomic signature” of ENTPD1 deficiency, which could prove a useful diagnostic or therapeutic biomarker. The full extent of these perturbations across the lifespan and clinical significance remain to be determined.

**Treatment and Development of Therapeutics**

The HSPs constitute a spectrum of progressive neurological disorders with only supportive therapies to ameliorate or mitigate disease.\textsuperscript{5} A major challenge in therapeutic development stems from the diverse molecular pathways, with >80 HSP disease genes identified to date. Another challenge in the evaluation of potential therapeutics is the insidious and progressive nature of the disease, leading to challenges in therapeutic endpoints and efficacy assessments. Nevertheless, with current advances in genome medicine and evolving understanding of molecular disease etiology, therapeutic development targeting diverse molecular disease mechanisms is 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 suggest antagonism of the purinergic receptor P2X7 may be a potential efficacious therapeutic target for 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, which could help gauge longitudinal disease severity and progression.\textsuperscript{51} Either formal neuropsychiatric testing was not performed or results were unavailable, limiting more precise characterization of the degree of ID. Similarly, only a subset of the cohort underwent electrodiagnostic evaluation or had MRI available for retrospective review. Finally, replication of our ADPase/ATPase assay, ENTPD1 immunostaining, and global metabolomics analysis in additional patients would help strengthen validity of these tests as diagnostic biomarkers.

In conclusion, we establish ENTPD1 as the etiology of a complex NDD in the HSP spectrum characterized by ID, white matter abnormalities, and progressive spastic paraplegia. Allelic series and detailed phenotyping in rare neurological disease 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 therapeutic options for rare neurological disorders based on shared molecular features. Future endeavors should involve the development of diagnostic biomarkers for early disease screening, as a readout of disease activity, and as a measure of treatment response for SPG64.

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Author Contributions
D.G.C., I.H., and J.R.L. contributed to the conception and design of the study; all authors contributed to the acquisition and analysis of the data; D.G.C. and I.H. contributed to drafting of the text and preparing the figures; all authors approved the final manuscript.

Potential conflicts of interests
J.R.L. has stock ownership in 23 and Me, is a paid consultant for Regeneron Genetics Center, and is a co-inventor on multiple US and European patients related to molecular diagnostics for inherited neuropathies, eye diseases, macular dystrophies and degeneration, genomic disorders, and bacterial genomic fingerprinting. The Department of Molecular and Human Genetics at Baylor College of Medicine receives revenue from genetic and genomic testing conducted at Baylor Genetics (BG); JRL serves on the scientific advisory board (SAB) for BG. S.C.R. is a scientific founder of Purinomia Biotech Inc and consults for eGenesis, AbbVie and SynLogic Inc; his interests are reviewed and managed by HMFP at Beth Israel Deaconess Medical Center in accordance with the conflict of interest policies.

References


